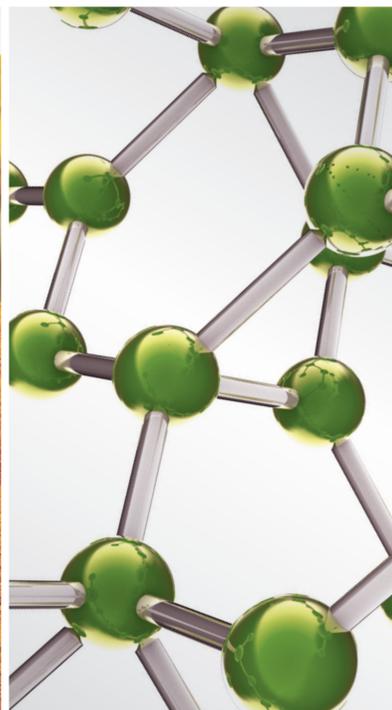
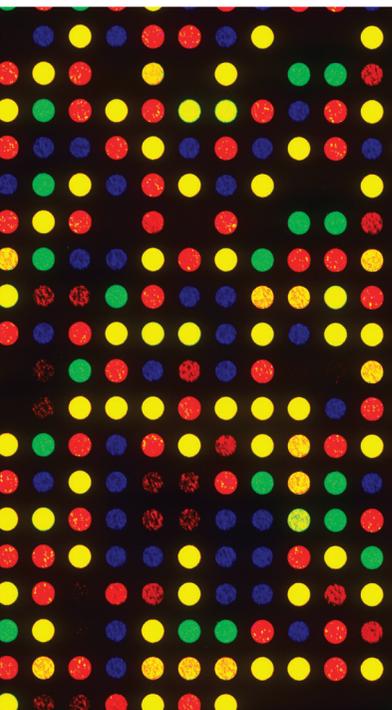


# COMPLEMENTARY/ALTERNATIVE MEDICINE IN CARDIOVASCULAR DISEASES 2013

GUEST EDITORS: KE-JI CHEN, KA KIT HUI, MYEONG SOO LEE, AND HAO XU





---

**Complementary/Alternative Medicine in  
Cardiovascular Diseases 2013**

Evidence-Based Complementary  
and Alternative Medicine

---

**Complementary/Alternative Medicine in  
Cardiovascular Diseases 2013**

Guest Editors: Ke-ji Chen, Ka Kit Hui, Myeong Soo Lee,  
and Hao Xu



---

Copyright © 2013 Hindawi Publishing Corporation. All rights reserved.

This is a special issue published in "Evidence-Based Complementary and Alternative Medicine." All articles are open access articles distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

## Editorial Board

Mahmood Abdulla, Malaysia  
Jon Adams, Australia  
Zuraini Ahmad, Malaysia  
Ulysses Albuquerque, Brazil  
Gianni Allais, Italy  
Terje Alraek, Norway  
Souliman Amrani, Morocco  
Akshay Anand, India  
Shrikant Anant, USA  
Manuel Arroyo-Morales, Spain  
Syed Asdaq, Saudi Arabia  
Seddigheh Asgary, Iran  
Hyunsu Bae, Republic of Korea  
Lijun Bai, China  
Sandip K. Bandyopadhyay, India  
Sarang Bani, India  
Vassya Bankova, Bulgaria  
Winfried Banzer, Germany  
Vernon A. Barnes, USA  
Samra Bashir, Pakistan  
Jairo Kenupp Bastos, Brazil  
Sujit Basu, USA  
David Baxter, New Zealand  
Andre-Michael Beer, Germany  
Alvin J. Beitz, USA  
Yong Boo, Republic of Korea  
Francesca Borrelli, Italy  
Gloria Brusotti, Italy  
Ishfaq A. Bukhari, Pakistan  
Arndt Büssing, Germany  
Rainer W. Bussmann, USA  
Raffaele Capasso, Italy  
Opher Caspi, Israel  
Han Chae, Korea  
Shun-Wan Chan, Hong Kong  
Il-Moo Chang, Republic of Korea  
Rajnish Chaturvedi, India  
Chun Tao Che, USA  
Hubiao Chen, Hong Kong  
Jian-Guo Chen, China  
Kevin Chen, USA  
Tzeng-Ji Chen, Taiwan  
Yunfei Chen, China  
Juei-Tang Cheng, Taiwan  
Evan Paul Cherniack, USA

Jen-Hwey Chiu, Taiwan  
William C. S. Cho, Hong Kong  
Jae Youl Cho, Korea  
Seung-Hun Cho, Republic of Korea  
Chee Yan Choo, Malaysia  
Ryowon Choue, Republic of Korea  
Shuang-En Chuang, Taiwan  
Joo-Ho Chung, Republic of Korea  
Edwin L. Cooper, USA  
Gregory D. Cramer, USA  
Meng Cui, China  
Roberto Cuman, Brazil  
Vincenzo De Feo, Italy  
Rocío Vázquez, Spain  
Martin Descarreaux, USA  
Alexandra Deters, Germany  
Siva Durairajan, Hong Kong  
Mohamed Eddouks, Morocco  
Thomas Efferth, Germany  
Tobias Esch, Germany  
Saeed Esmaeili-Mahani, Iran  
Nianping Feng, China  
Yibin Feng, Hong Kong  
Josue Fernandez-Carnero, Spain  
Juliano Ferreira, Brazil  
Fabio Firenzuoli, Italy  
Peter Fisher, UK  
W. F. Fong, Hong Kong  
Romain Forestier, France  
Joel J. Gagnier, Canada  
Jian-Li Gao, China  
Gabino Garrido, Chile  
Muhammad Ghayur, Pakistan  
Anwarul Hassan Gilani, Pakistan  
Michael Goldstein, USA  
Mahabir P. Gupta, Panama  
Mitchell Haas, USA  
Svein Haavik, Norway  
Abid Hamid, India  
N. Hanazaki, Brazil  
K. B. Harikumar, India  
Cory S. Harris, Canada  
Thierry Hennebelle, France  
Seung-Heon Hong, Korea  
Markus Horneber, Germany

Ching-Liang Hsieh, Taiwan  
Jing Hu, China  
Gan Siew Hua, Malaysia  
Sheng-Teng Huang, Taiwan  
Benny Tan Kwong Huat, Singapore  
Roman Huber, Germany  
Angelo Antonio Izzo, Italy  
Kong J., USA  
Suresh Jadhav, India  
Kanokwan Jarukamjorn, Thailand  
Yong Jiang, China  
Zheng L. Jiang, China  
Stefanie Joos, Germany  
Sirajudeen K.N.S., Malaysia  
Z. Kain, USA  
Osamu Kanauchi, Japan  
Wenyi Kang, China  
Dae Gill Kang, Republic of Korea  
Shao-Hsuan Kao, Taiwan  
Krishna Kaphle, Nepal  
Kenji Kawakita, Japan  
Jong Yeol Kim, Republic of Korea  
Cheorl-Ho Kim, Republic of Korea  
Youn Chul Kim, Republic of Korea  
Yoshiyuki Kimura, Japan  
Joshua K. Ko, China  
Toshiaki Kogure, Japan  
Nandakumar Krishnadas, India  
Yiu Wa Kwan, Hong Kong  
Kuang Chi Lai, Taiwan  
Ching Lan, Taiwan  
Alfred Längler, Germany  
Lixing Lao, Hong Kong  
Clara Bik-San Lau, Hong Kong  
Jang-Hern Lee, Republic of Korea  
Tat leang Lee, Singapore  
Myeong S. Lee, UK  
Christian Lehmann, Canada  
Marco Leonti, Italy  
Ping-Chung Leung, Hong Kong  
Lawrence Leung, Canada  
Kwok Nam Leung, Hong Kong  
Ping Li, China  
Min Li, China  
Man Li, China

ChunGuang Li, Australia  
Xiu-Min Li, USA  
Shao Li, China  
Yong Hong Liao, China  
Sabina Lim, Korea  
Bi-Fong Lin, Taiwan  
Wen Chuan Lin, China  
Christopher G. Lis, USA  
Gerhard Litscher, Austria  
Ke Liu, China  
I-Min Liu, Taiwan  
Gaofeng Liu, China  
Yijun Liu, USA  
Cun-Zhi Liu, China  
Gail B. Mahady, USA  
Juraj Majtan, Slovakia  
Subhash C. Mandal, India  
Jeanine Marnewick, South Africa  
Virginia S. Martino, Argentina  
James H. McAuley, Australia  
Karin Meissner, USA  
Andreas Michalsen, Germany  
David Mischoulon, USA  
Syam Mohan, Malaysia  
J. Molnar, Hungary  
Valério Monteiro-Neto, Brazil  
H.-I. Moon, Republic of Korea  
Albert Moraska, USA  
Mark Moss, UK  
Yoshiharu Motoo, Japan  
Frauke Musial, Germany  
MinKyun Na, Republic of Korea  
Richard L. Nahin, USA  
Vitaly Napadow, USA  
F. R. F. Nascimento, Brazil  
S. Nayak, Trinidad And Tobago  
Isabella Neri, Italy  
Télesphore Nguelefack, Cameroon  
Martin Offenbacher, Germany  
Ki-Wan Oh, Republic of Korea  
Y. Ohta, Japan  
Olumayokun A. Olajide, UK  
Thomas Ostermann, Germany  
Stacey A. Page, Canada  
Tai-Long Pan, Taiwan  
Bhushan Patwardhan, India  
Berit Smestad Paulsen, Norway  
Andrea Pieroni, Italy  
Richard Pietras, USA  
Waris Qidwai, Pakistan  
Xianqin Qu, Australia  
Cassandra L. Quave, USA  
Roja Rahimi, Iran  
Khalid Rahman, UK  
Cheppail Ramachandran, USA  
Gamal Ramadan, Egypt  
Ke Ren, USA  
Man Hee Rhee, Republic of Korea  
Mee-Ra Rhyu, Republic of Korea  
José Luis Ríos, Spain  
Paolo Roberti di Sarsina, Italy  
Bashar Saad, Palestinian Authority  
Sumaira Sahreen, Pakistan  
Omar Said, Israel  
Luis A. Salazar-Olivo, Mexico  
Mohd. Zaki Salleh, Malaysia  
Andreas Sandner-Kiesling, Austria  
Adair Santos, Brazil  
G. Schmeda-Hirschmann, Chile  
Andrew Scholey, Australia  
Veronique Seidel, UK  
Senthamil R. Selvan, USA  
Tuhinadri Sen, India  
Hongcai Shang, China  
Karen J. Sherman, USA  
Ronald Sherman, USA  
Kuniyoshi Shimizu, Japan  
Kan Shimpo, Japan  
Byung-Cheul Shin, Korea  
Yukihiro Shoyama, Japan  
Chang Gue Son, Korea  
Rachid Soulimani, France  
Didier Stien, France  
Shan-Yu Su, Taiwan  
Mohd Roslan Sulaiman, Malaysia  
Venil N. Sumantran, India  
John R. S. Tabuti, Uganda  
Toku Takahashi, USA  
Rabih Talhouk, Lebanon  
Wen-Fu Tang, China  
Yuping Tang, China  
Lay Kek Teh, Malaysia  
Mayank Thakur, India  
Menaka C. Thounaojam, India  
Mei Tian, China  
Evelin Tiralongo, Australia  
S. C. Tjen-A-Looi, USA  
MichaThl Tomczyk, Poland  
Yao Tong, Hong Kong  
K. V. Trinh, Canada  
Karl Wah-Keung Tsim, Hong Kong  
Volkan Tugcu, Turkey  
Yew-Min Tzeng, Taiwan  
Dawn M. Upchurch, USA  
Maryna Van de Venter, South Africa  
Sandy van Vuuren, South Africa  
Alfredo Vannacci, Italy  
Mani Vasudevan, Malaysia  
Carlo Ventura, Italy  
Wagner Vilegas, Brazil  
Pradeep Visen, Canada  
Aristo Vojdani, USA  
Y. Wang, USA  
Shu-Ming Wang, USA  
Chenchen Wang, USA  
Chong-Zhi Wang, USA  
Kenji Watanabe, Japan  
Jintanaporn Wattanathorn, Thailand  
Wolfgang Weidenhammer, Germany  
Jenny M. Wilkinson, Australia  
Darren Williams, Republic of Korea  
Haruki Yamada, Japan  
Nobuo Yamaguchi, Japan  
Yong-Qing Yang, China  
Junqing Yang, China  
Ling Yang, China  
Eun Jin Yang, Republic of Korea  
Xiufen Yang, China  
Ken Yasukawa, Japan  
Min H. Ye, China  
M. Yoon, Republic of Korea  
Jie Yu, China  
Jin-Lan Zhang, China  
Zunjian Zhang, China  
Wei-bo Zhang, China  
Hong Q. Zhang, Hong Kong  
Boli Zhang, China  
Ruixin Zhang, USA  
Hong Zhang, Sweden  
Haibo Zhu, China

## Contents

**Complementary/Alternative Medicine in Cardiovascular Diseases 2013**, Ke-ji Chen, Ka Kit Hui, Myeong Soo Lee, and Hao Xu  
Volume 2013, Article ID 538346, 2 pages

**Autonomic Nervous System Mediates the Hypotensive Effects of Aqueous and Residual Methanolic Extracts of *Syzygium polyanthum* (Wight) Walp. var. *polyanthum* Leaves in Anaesthetized Rats**, A. Ismail, M. Mohamed, S. A. Sulaiman, and W. A. N. Wan Ahmad  
Volume 2013, Article ID 716532, 16 pages

**Ginsenoside Rb1 Reduces Isoproterenol-Induced Cardiomyocytes Apoptosis *In Vitro* and *In Vivo***, Xiu-feng Wang, Xin-jun Liu, Qian-mei Zhou, Jia Du, Tian-ling Zhang, Yi-yu Lu, and Shi-bing Su  
Volume 2013, Article ID 454389, 9 pages

**Tai Chi Chuan Exercise for Patients with Cardiovascular Disease**, Ching Lan, Ssu-Yuan Chen, May-Kuen Wong, and Jin Shin Lai  
Volume 2013, Article ID 983208, 9 pages

**The Protective Role of Resveratrol against Arsenic Trioxide-Induced Cardiotoxicity**, Wei-qian Zhang, Changming Guo, Ruifeng Gao, Ming Ge, Yanzhu Zhu, and Zhigang Zhang  
Volume 2013, Article ID 407839, 8 pages

**Effect of KIOM-79 on Diabetes-Induced Myocardial Fibrosis in Zucker Diabetic Fatty Rats**, Junghyun Kim, Eunjin Sohn, Chan-Sik Kim, Yun Mi Lee, Kyuhyung Jo, and Jin Sook Kim  
Volume 2013, Article ID 547653, 8 pages

**Ten Years' Research on a Cardiovascular Tonic: A Comprehensive Approach—From Quality Control and Mechanisms of Action to Clinical Trial**, Ping-Chung Leung, Chi-Man Koon, Clara Bik-San Lau, Ping Chook, William King-Fai Cheng, Kwok-Pui Fung, Timothy Chi-Yui Kwok, and Kam-Sang Woo  
Volume 2013, Article ID 319703, 6 pages

**Effects of Wenxin Keli on the Action Potential and L-Type Calcium Current in Rats with Transverse Aortic Constriction-Induced Heart Failure**, Yu Chen, Yang Li, Lili Guo, Wen Chen, Mingjing Zhao, Yonghong Gao, Aiming Wu, Lixia Lou, Jie Wang, Xiaoqiu Liu, and Yanwei Xing  
Volume 2013, Article ID 572078, 12 pages

**Astragalus Polysaccharide Suppresses the Expression of Adhesion Molecules through the Regulation of the p38 MAPK Signaling Pathway in Human Cardiac Microvascular Endothelial Cells after Ischemia-Reperfusion Injury**, Zhu Hai-Yan, Gao Yong-Hong, Wang Zhi-Yao, Xu Bing, Wu Ai-Ming, Xing Yan-Wei, Liu Bei, Lou Li-Xia, and Chen Li-Xin  
Volume 2013, Article ID 280493, 8 pages

**Ethanol Extract of *Lepidium apetalum* Seed Elicits Contractile Response and Attenuates Atrial Natriuretic Peptide Secretion in Beating Rabbit Atria**, Seung Ju Kim, Hye Yoom Kim, Yun Jung Lee, Hao Zhen Cui, Ji Yeon Jang, Dae Gill Kang, and Ho Sub Lee  
Volume 2013, Article ID 404713, 12 pages

**Effectiveness of *Panax ginseng* on Acute Myocardial Ischemia Reperfusion Injury Was Abolished by Flutamide via Endogenous Testosterone-Mediated Akt Pathway**, Luo Pei, Hou Shaozhen, Dong Gengting, Chen Tingbo, Liu Liang, and Zhou Hua  
Volume 2013, Article ID 817826, 9 pages

**The Electrophysiological Effects of Qiliqiangxin on Cardiac Ventricular Myocytes of Rats**, Yidong Wei, Xiaoyu Liu, Haidong Wei, Lei Hou, Wenliang Che, Erlinda The, Gang Li, Muktanand Vikash Jhummon, and Wanlin Wei  
Volume 2013, Article ID 213976, 4 pages

**Ginseng Extracts Restore High-Glucose Induced Vascular Dysfunctions by Altering Triglyceride Metabolism and Downregulation of Atherosclerosis-Related Genes**, Gabriel Hoi-huen Chan, Betty Yuen-kwan Law, John Man-tak Chu, Kevin Kin-man Yue, Zhi-hong Jiang, Chi-wai Lau, Yu Huang, Shun-wan Chan, Patrick Ying-kit Yue, and Ricky Ngok-shun Wong  
Volume 2013, Article ID 797310, 13 pages

**Gene Expression Profiling on the Molecular Action of Danshen-Gegen Formula in a Randomized Placebo-Controlled Trial of Postmenopausal Women with Hypercholesterolemia**, Chi-Man Koon, Chun-Hay Ko, Xu-Xu Sun, Sandy Wan-Heng Hoi, Jacqueline Chor-Wing Tam, David Wing-Shing Cheung, King-Fai Cheng, Suet-Yee Pang, Wing-Man Lo, Ping Chook, Clara Bik-San Lau, Wai-Yee Chan, Ping-Chung Leung, Timothy Chi-Yui Kwok, and Kwok-Pui Fung  
Volume 2013, Article ID 703705, 14 pages

**Sang-qi Granula Reduces Blood Pressure and Myocardial Fibrosis by Suppressing Inflammatory Responses Associated with the Peroxisome Proliferator-Activated Receptors and Nuclear Factor  $\kappa$ B Protein in Spontaneously Hypertensive Rats**, Lan-Yu Chen, Chun-Shui Pan, Xiao-Hong Wei, Lin Li, Jing-Yan Han, and Li Huang  
Volume 2013, Article ID 721729, 12 pages

**Anti-Proliferative Effect of an Aqueous Extract of *Prunella vulgaris* in Vascular Smooth Muscle Cells**, Sun Mi Hwang, Yun Jung Lee, Yong Pyo Lee, Jung Joo Yoon, So Min Lee, Jeong Dan Cha, Kyung Min Choi, Dae Gill Kang, and Ho Sub Lee  
Volume 2013, Article ID 936463, 10 pages

**Optimizing Prescription of Chinese Herbal Medicine for Unstable Angina Based on Partially Observable Markov Decision Process**, Yan Feng, Yu Qiu, Xuezhong Zhou, Yixin Wang, Hao Xu, and Baoyan Liu  
Volume 2013, Article ID 532534, 6 pages

**Protective Effect of Qiliqiangxin Capsule on Energy Metabolism and Myocardial Mitochondria in Pressure Overload Heart Failure Rats**, Junfang Zhang, Cong Wei, Hongtao Wang, Siwen Tang, Zhenhua Jia, Lei Wang, Dengfeng Xu, and Yiling Wu  
Volume 2013, Article ID 378298, 9 pages

**Tongguan Capsule Protects against Myocardial Ischemia and Reperfusion Injury in Mice**, Jianyong Qi, Juan Yu, Lei Wang, Liheng Guo, Shiyu Ma, Donghui Huang, Miao Zhou, Jiashin Wu, and Minzhou Zhang  
Volume 2013, Article ID 159237, 8 pages

**Inhibitory Effects of Glycyrrhetic Acid on the Delayed Rectifier Potassium Current in Guinea Pig Ventricular Myocytes and HERG Channel**, Delin Wu, Linqing Jiang, Hongjin Wu, Shengqi Wang, Sidao Zheng, Jiyuan Yang, Yuna Liu, Jianxun Ren, and Xianbing Chen  
Volume 2013, Article ID 481830, 11 pages

**The Roles of Traditional Chinese Medicine: Shen-Fu Injection on the Postresuscitation Care Bundle**, Qian Zhang and Chunsheng Li  
Volume 2013, Article ID 319092, 7 pages

**Doinseunggitang Ameliorates Endothelial Dysfunction in Diabetic Atherosclerosis**, Jung Joo Yoon, Yun Jung Lee, Ok Ju Park, So Min Lee, Yong Pyo Lee, Nam Geun Cho, Dae Gill Kang, and Ho Sub Lee  
Volume 2013, Article ID 783576, 10 pages

**Oral *Panax notoginseng* Preparation for Coronary Heart Disease: A Systematic Review of Randomized Controlled Trials**, Qinghua Shang, Hao Xu, Zhaolan Liu, Keji Chen, and Jianping Liu  
Volume 2013, Article ID 940125, 12 pages

**Integrative Western and Chinese Medicine on Coronary Heart Disease: Where Is the Orientation?,**

Siming Li and Hao Xu

Volume 2013, Article ID 459264, 7 pages

**ITIH4: A New Potential Biomarker of “Toxin Syndrome” in Coronary Heart Disease Patient Identified with Proteomic Method,** Hao Xu, Qinghua Shang, Hao Chen, Jianpeng Du, Jianyan Wen, Geng Li, Dazhuo Shi, and Keji Chen

Volume 2013, Article ID 360149, 11 pages

**Effects of Swedish Massage Therapy on Blood Pressure, Heart Rate, and Inflammatory Markers in Hypertensive Women,** Izreen Supaat, Zaiton Zakaria, Oteh Maskon, Amilia Aminuddin, and Nor Anita Megat Mohd Nordin

Volume 2013, Article ID 171852, 8 pages

**Effect of Wenxin Granule on Ventricular Remodeling and Myocardial Apoptosis in Rats with Myocardial Infarction,** Aiming Wu, Jianying Zhai, Dongmei Zhang, Lixia Lou, Haiyan Zhu, Yonghong Gao, Limin Chai, Yanwei Xing, Xiyang Lv, Lingqun Zhu, Mingjing Zhao, and Shuoren Wang

Volume 2013, Article ID 967986, 10 pages

**The Effect of Sodium Tanshinone IIA Sulfate and Simvastatin on Elevated Serum Levels of Inflammatory Markers in Patients with Coronary Heart Disease: A Study Protocol for a Randomized Controlled Trial,** Qinghua Shang, Hanjay Wang, Siming Li, and Hao Xu

Volume 2013, Article ID 756519, 8 pages

**Analysis on Outcome of 3537 Patients with Coronary Artery Disease: Integrative Medicine for Cardiovascular Events,** Zhu-ye Gao, Yu Qiu, Yang Jiao, Qing-hua Shang, Hao Xu, and Da-zhuo Shi

Volume 2013, Article ID 162501, 6 pages

**Long-Term Exercise and Risk of Metabolic and Cardiac Diseases: The Erlangen Fitness and Prevention Study,** Wolfgang Kemmler, Simon von Stengel, Michael Bebenek, and Willi A. Kalender

Volume 2013, Article ID 768431, 9 pages

**Panax Quinquefolius Saponin of Stem and Leaf Attenuates Intermittent High Glucose-Induced Oxidative Stress Injury in Cultured Human Umbilical Vein Endothelial Cells via PI3K/Akt/GSK-3 $\beta$  Pathway,** Jingshang Wang, Huijun Yin, Ye Huang, Chunyu Guo, Chengdong Xia, Qian Liu, and Lu Zhang

Volume 2013, Article ID 196283, 7 pages

**Qi-Shen-Yi-Qi Dripping Pills for the Secondary Prevention of Myocardial Infarction: A Randomised Clinical Trial,** Hongcai Shang, Junhua Zhang, Chen Yao, Baoyan Liu, Xiumei Gao, Ming Ren, Hongbao Cao, Guohua Dai, Weiliang Weng, Sainan Zhu, Hui Wang, Hongjuan Xu, and Boli Zhang

Volume 2013, Article ID 738391, 9 pages

**Combination of Chinese Herbal Medicines and Conventional Treatment versus Conventional Treatment Alone in Patients with Acute Coronary Syndrome after Percutaneous Coronary Intervention (5C Trial): An Open-Label Randomized Controlled, Multicenter Study,** Shao-Li Wang, Cheng-Long Wang, Pei-Li Wang, Hao Xu, Hong-Ying Liu, Jian-Peng Du, Da-Wu Zhang, Zhu-Ye Gao, Lei Zhang, Chang-Geng Fu, Shu-Zheng Lü, Shi-Jie You, Jun-Bo Ge, Tian-Chang Li, Xian Wang, Guan-Lin Yang, Hong-Xu Liu, Jing-Yuan Mao, Rui-Jie Li, Li-Dian Chen, Shu Lu, Da-Zhuo Shi, and Ke-Ji Chen

Volume 2013, Article ID 741518, 8 pages

**The Expression of CD14<sup>+</sup> CD16<sup>+</sup> Monocyte Subpopulation in Coronary Heart Disease Patients with Blood Stasis Syndrome,** Ye Huang, Jing-Shang Wang, Hui-jun Yin, and Ke-ji Chen

Volume 2013, Article ID 416932, 6 pages

**Trends in the Treatment of Hypertension from the Perspective of Traditional Chinese Medicine,**

Xingjiang Xiong, Xiaochen Yang, Wei Liu, Fuyong Chu, Pengqian Wang, and Jie Wang

Volume 2013, Article ID 275279, 13 pages

**Regulation of DDAH1 as a Potential Therapeutic Target for Treating Cardiovascular Diseases,**

Xiaoyu Liu, John Fassett, Yidong Wei, and Yingjie Chen

Volume 2013, Article ID 619207, 6 pages

**Baicalin's Therapeutic Time Window of Neuroprotection during Transient Focal Cerebral Ischemia and Its Antioxidative Effects *In Vitro* and *In Vivo*,**

Fafeng Cheng, Yi Lu, Xianggen Zhong, Wenting Song, Xueqian Wang, Xiaoguang Sun, Jianguo Qin, Shaoying Guo, and Qingguo Wang

Volume 2013, Article ID 120261, 11 pages

**Chinese Herbal Medicine Qi Ju Di Huang Wan for the Treatment of Essential Hypertension:**

**A Systematic Review of Randomized Controlled Trials,** Jie Wang, Xingjiang Xiong, Guoyan Yang,

Yuqing Zhang, Yongmei Liu, Yun Zhang, Zhenpeng Zhang, Jun Li, and Xiaochen Yang

Volume 2013, Article ID 262685, 10 pages

**Effective Components of *Panax quinquefolius* and *Corydalis tuber* Protect Myocardium through Attenuating Oxidative Stress and Endoplasmic Reticulum Stress,**

Mei Xue, Meilin Liu, Xinyuan Zhu, Lin Yang, Yu Miao, Dazhuo Shi, and Huijun Yin

Volume 2013, Article ID 482318, 7 pages

**Systematic Review of Compound Danshen Dropping Pill: A Chinese Patent Medicine for Acute Myocardial Infarction,**

Jing Luo, Hao Xu, and Keji Chen

Volume 2013, Article ID 808076, 15 pages

**The Effect of Ouhyul Herbal Acupuncture Point Injections on Shoulder Pain after Stroke,**

Yu-Ri Seo, Woo-Sang Jung, Seong-Uk Park, Sang-Kwan Moon, Jung-Mi Park, and Joo-Young Park

Volume 2013, Article ID 504686, 5 pages

**Cardiovascular Effects of Salvianolic Acid B,** Jie Wang, Xingjiang Xiong, and Bo Feng

Volume 2013, Article ID 247948, 16 pages

**Inhibition of NADPH Oxidase Mediates Protective Effect of Cardiotonic Pills against Rat Heart**

**Ischemia/Reperfusion Injury,** Xiao-Yuan Yang, Na Zhao, Yu-Ying Liu, Bai-He Hu, Kai Sun, Xin Chang,

Xiao-Hong Wei, Jing-Yu Fan, and Jing-Yan Han

Volume 2013, Article ID 728020, 15 pages

**Virgin Coconut Oil Prevents Blood Pressure Elevation and Improves Endothelial Functions in Rats Fed with Repeatedly Heated Palm Oil,**

Badlishah Sham Nurul-Iman, Yusof Kamisah, Kamsiah Jaarin, and Hj Mohd Saad Qodriyah

Volume 2013, Article ID 629329, 7 pages

**QSYQ Attenuates Oxidative Stress and Apoptosis Induced Heart Remodeling Rats through Different Subtypes of NADPH-Oxidase,**

Yong Wang, Chun Li, Yuli Ouyang, Tianjiao Shi, Xiaomin Yang, Junda Yu, Qi Qiu, Jing Han, Yan Wu, Binghua Tang, and Wei Wang

Volume 2013, Article ID 824960, 8 pages

**Evidence-Based Chinese Medicine for Hypertension,** Jie Wang and Xingjiang Xiong

Volume 2013, Article ID 978398, 12 pages

**Effectiveness of Yoga for Hypertension: Systematic Review and Meta-Analysis,**

Marshall Hagins, Rebecca States, Terry Selfe, and Kim Innes

Volume 2013, Article ID 649836, 13 pages

**Serum Containing Tao-Hong-Si-Wu Decoction Induces Human Endothelial Cell VEGF Production via PI3K/Akt-eNOS Signaling**, DengKe Yin, ZhuQing Liu, DaiYin Peng, Ye Yang, XiangDong Gao, Fan Xu, and Lan Han

Volume 2013, Article ID 195158, 9 pages

**The Cardioprotective Effects of Citric Acid and L-Malic Acid on Myocardial Ischemia/Reperfusion Injury**, Xilan Tang, Jianxun Liu, Wei Dong, Peng Li, Lei Li, Chengren Lin, Yongqiu Zheng, Jincai Hou, and Dan Li

Volume 2013, Article ID 820695, 11 pages

**A Systems Biology Approach to Characterize Biomarkers for Blood Stasis Syndrome of Unstable Angina Patients by Integrating MicroRNA and Messenger RNA Expression Profiling**, Jie Wang and Gui Yu

Volume 2013, Article ID 510208, 21 pages

**Therapeutic Effects of Water Soluble Danshen Extracts on Atherosclerosis**, Yoon Hee Cho, Cheol Ryong Ku, Zhen-Yu Hong, Ji Hoe Heo, Eun Hee Kim, Dong Hoon Choi, Dongkyu Kim, Ae-Jung Kim, Cheol Soon Lee, Mankil Jung, Hyun Chul Lee, MiRan Seo, and Eun Jig Lee

Volume 2013, Article ID 623639, 11 pages

**Banxia Baizhu Tianma Decoction for Essential Hypertension: A Systematic Review of Randomized Controlled Trials**, Xingjiang Xiong, Xiaochen Yang, Wei Liu, Bo Feng, Jizheng Ma, Xinliang Du, Pengqian Wang, Fuyong Chu, Jun Li, and Jie Wang

Volume 2012, Article ID 271462, 10 pages

## Editorial

# Complementary/Alternative Medicine in Cardiovascular Diseases 2013

Ke-ji Chen,<sup>1</sup> Ka Kit Hui,<sup>2</sup> Myeong Soo Lee,<sup>3</sup> and Hao Xu<sup>1</sup>

<sup>1</sup> China Heart Institute of Chinese Medicine, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>2</sup> Center for East-West Medicine, University of California, Los Angeles, Santa Monica, CA 90404, USA

<sup>3</sup> Medical Research Division, Korea Institute of Oriental Medicine, Daejeon 305-811, Republic of Korea

Correspondence should be addressed to Ke-ji Chen; keji.chen@yahoo.com

Received 12 November 2013; Accepted 12 November 2013

Copyright © 2013 Ke-ji Chen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, and the mortality is likely to further accelerate in many developing countries. In 2008, almost one in three deaths all over the world was attributed to CVDs. It is also estimated that by 2030 over 23 million deaths from CVDs will occur each year. Despite advances in modern management of CVDs, either revascularization or medical therapy, complications related to these procedures and recurrent acute cardiovascular events still afflict patients. Meanwhile, patients with CVDs often suffer from unfavorable quality of life. In recent decades, the potential benefit of complementary/alternative medicine (CAM) therapy in improving CVDs prognosis has drawn more and more attention, and the use of CAM by physicians and patients has also increased markedly. However, the evidence of CAM for CVDs patients and the research on mechanism of actions are still insufficient. Last year, the published special issue named “*The potential benefit of complementary/alternative medicine in cardiovascular diseases*” got a great success, which facilitates the compilation of this special issue 2013. We believe that such a series can have a long-term impact, and in time gather a community around it in much the same way a successful annual conference does.

In this issue, original research papers and reviews from different parts of the world including China, Republic of Korea, USA, Germany, and Malaysia are presented. These papers are focused on the mechanism of action and the clinical application of CAM in treating CVDs. In the clinical trials, the benefits of Chinese medicine and some other CAM

therapies for CVDs patients were demonstrated. A multicenter prospective cohort study showed that heart failure, age  $\geq 65$  years old, and myocardial infarction were associated with an increase in one-year follow-up incidence of major adverse cardiac events (MACEs) in hospitalized coronary heart disease patients, and integrative medicine showed a tendency for reducing the incidence of MACEs. The similar benefit of integrative medicine therapy for patients with acute coronary syndrome after PCI was showed in a multicenter randomized controlled trial (RCT), 5C trial. Another RCT compared the effectiveness of Qi-shen-yi-qi dripping pills (QSYQ) with that of aspirin in the secondary prevention of myocardial infarction. This trial did not show significant difference of primary and secondary outcomes between aspirin and QSYQ, which suggest QSYQ might be an alternative medication for patients' intolerance of aspirin in patients who have had an MI. Specifically, a new potential biomarker of “toxin syndrome” in coronary heart disease patients was proposed in a paper containing two clinical trials. The authors concluded that the new biomarker “inter-alpha-trypsin inhibitor heavy chain H4” might have a potential role in early identifying high-risk coronary heart disease patients in stable period. Most of the experiment researches in this issue were focused on Chinese medicine and Korean medicine. Two researches explored the role and mechanism of Qiliqiangxin capsule (QL), an oral Chinese proprietary medicine, for arrhythmia and heart failure, respectively. A pharmacological research showed that blocking androgen receptor could abolish the ability of Panax ginseng to protect the heart from myocardial

ischemia reperfusion injury. The benefit of Doinseunggitang, a Korean traditional prescription, on the treatment and prevention of diabetic vascular complications was also described in a study. A systematic review suggested that oral Panax notoginseng preparation could relieve angina pectoris related symptoms. Meanwhile, the potential benefit of Qiju Dihuang Wan, a Chinese herbal prescription, on the treatment of essential hypertension was also reviewed. Additionally, yoga was recommended as an effective intervention for reducing blood pressure in a systematic review.

Due to indefinite mechanism of actions and lacking of high quality evidence, traditional Chinese medicine and other traditional medicine worldwide are considered as CAM in Western countries. In this case, original researches on mechanism of actions play an important role in the modernization and internationalization of CAM and should be further strengthened in future researches. In the hierarchy of evidence-based medicine, high quality RCT is still considered as the golden standard for evaluating interventional treatment. Currently, multicenter RCTs and a prospective cohort study have been conducted to evaluate the benefit of CAM for CVDs, and most of these researches showed positive findings. Nevertheless, the effectiveness of CAM on the treatment of CVDs in the real world is still unclear which limits the application of CAM. The reason is that efficacy of intervention in RCT is not equal to its effectiveness in the real world. In the future, in addition to high quality RCTs, more evidence from real world researches is warranted to support the use of CAM including traditional Chinese medicine for CVDs patients.

*Ke-ji Chen*  
*Ka Kit Hui*  
*Myeong Soo Lee*  
*Hao Xu*

## Research Article

# Autonomic Nervous System Mediates the Hypotensive Effects of Aqueous and Residual Methanolic Extracts of *Syzygium polyanthum* (Wight) Walp. var. *polyanthum* Leaves in Anaesthetized Rats

A. Ismail,<sup>1,2</sup> M. Mohamed,<sup>3</sup> S. A. Sulaiman,<sup>4</sup> and W. A. N. Wan Ahmad<sup>1</sup>

<sup>1</sup> School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

<sup>2</sup> Department of Basic Medical Sciences, Kulliyah of Dentistry, Kuantan Campus, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia

<sup>3</sup> Department of Physiology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

<sup>4</sup> Department of Pharmacology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

Correspondence should be addressed to A. Ismail; [azlini.ismail@yahoo.com](mailto:azlini.ismail@yahoo.com)

Received 17 April 2013; Revised 22 October 2013; Accepted 30 October 2013

Academic Editor: Keji Chen

Copyright © 2013 A. Ismail et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

*Syzygium polyanthum* (Wight) Walp. var. *polyanthum* leaves are consumed as a traditional Malay treatment of hypertension. This study investigates hypotensive potential of aqueous (AESP) and residual methanolic (met-AESP) extracts of *S. polyanthum* leaves and possible involvement of autonomic receptors. AESP and met-AESP (20 to 100 mg/kg) were intravenously administered into anaesthetized Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Blood pressure and heart were monitored for 20 min. AESP and met-AESP induced significant dose-dependent hypotension, but only 100 mg/kg AESP caused mild bradycardia ( $n = 5$ ). AESP-induced hypotension was more potent than that of met-AESP in WKY. AESP has a faster onset time than that of met-AESP in both WKY and SHR. However, met-AESP-induced hypotension was more sustained than that of AESP in SHR. Blockages of autonomic ganglion and  $\alpha$ -adrenergic receptors using hexamethonium and phentolamine ( $n = 5$  for each group) partially attenuated AESP-induced hypotension, suggesting involvement of  $\alpha$ -adrenergic receptors. Blockages of autonomic ganglion,  $\beta$ -adrenergic, cholinergic receptors, and nitric oxide production using hexamethonium, propranolol, atropine, and N- $\omega$ -nitro-L arginine methyl ester (L-NAME) ( $n = 5$  for each group) partially attenuated met-AESP-induced hypotension, suggesting involvement of  $\beta$ -adrenergic and cholinergic receptors via nitric oxide production.

## 1. Introduction

*Syzygium polyanthum* (Wight) walp. var. *polyanthum*, or known as “serai kayu” or “salam,” is consumed by Malays as a traditional remedy for hypertension. *S. polyanthum* leaves are well-known as traditional medication for various illnesses such as cataract, diarrhoea, gastritis, hypercholesterolemia, skin diseases, and diabetes mellitus [1]. Besides medicinal usages, the young shoots of *S. polyanthum* were commonly consumed as a fresh salad (*ulam*) whereas the mature

leaves were regularly added as a flavour enhancer in Malays’ cuisines.

With the popular use of *S. polyanthum* leaves, few studies on its biological properties were carried out. Among the findings, *S. polyanthum* leaves’ extracts were proven to possess antibacterial activity against *Staphylococcus aureus* [2], antifungal activities against *Alternaria alternata* and *Colletotrichum capsici* [3], antinematodal activity against the pine wood nematode, *Bursaphelenchus xylophilus* [4], anti-tumor promoting activity [5], and antioxidant activity [6–9].

Besides, *S. polyanthum* leaves extract is also noncytotoxic to normal mammalian cell lines [9].

Despite its known biological effects, the phytochemical constituents in the crude extracts of *S. polyanthum* leaves were only preliminarily studied. The crude ethanolic extracts of the leaves and the fruits of *S. polyanthum* contain terpenoids, phenols, tannins, flavonoids, and alkaloids [8]. Steroids were found in the crude ethanolic extract of the leaves and the ripe fruits. Saponins were found in the unripe fruits, whereas carbohydrates were present in both the ripe and unripe fruits [8]. On the other hand, the chemical constituents of the essential oil from *S. polyanthum* leaves are extensively studied. Dalimartha (2000) in [10] reported eugenol as one of the compounds present in *S. polyanthum* leaves. Eugenol, a phenolic compound abundantly found in *Syzygium* family [11], has reputed ability as a vasorelaxant compound that causes vasodilation *in vitro* [12–14] and reduces blood pressure and heart rate of rats *in vivo* [15]. Other major phytochemical constituents of the essential oil from *S. polyanthum* leaves include cis-4-decenal, octanal,  $\alpha$ -pinene, farnesol,  $\beta$ -ocimene, and nonanal [16]. While using hexane as solvent, the essential oil of *S. polyanthum* leaves contains cis-4-decenal, octanal,  $\alpha$ -pinene, farnesol, nerolidol, and decanal at various percentages. Among the compounds, the presence of  $\alpha$ -pinene which belongs to terpenoid family is notable since it was associated with hypotension in both the nonanaesthetized [17] and the urethane-anaesthetized rats [18]. Although these two compounds might correlate with the proclaimed traditional use of *S. polyanthum* leaves as an anti-hypertensive remedy, but these studied compounds were just sparingly soluble in water. Thus, the alleged claim on anti-hypertensive ability of the decoction of *S. polyanthum* leaves still requires verification.

Therefore, the aim of this study was to elucidate the effects of aqueous and residual methanolic extracts of *S. polyanthum* leaves on mean arterial (MAP), systolic (SBP), and diastolic (DBP) blood pressure and heart rate (HR) of anaesthetized male Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Instead of using noninvasive blood pressure measurement method that requires prior warming and restraining of the rats which significantly increased the baseline blood pressure of SHR due to stress [19], the effects of AESP and met-AESP were elucidated in this study in a more calm, resting anaesthesia condition. Indeed, the invasive measurement of blood pressure under anaesthesia was widely used in determining hypotensive or antihypertensive properties of plant extracts [20–23]. Besides, this study also aims to elucidate the possible involvement of autonomic nervous system (ANS) in mediating the hypotensive effects of the extracts.

## 2. Materials and Methods

**2.1. Reagents.** Dimethylsulfoxide (DMSO) and 95% methanol (v/v) were purchased from Merck, Malaysia. Hexamethonium bromide, isoproterenol hydrochloride, propranolol hydrochloride, acetylcholine chloride, atropine sulphate, phentolamine hydrochloride, methoxamine hydrochloride, and N- $\omega$ -nitro-L arginine methyl ester (L-NAME) were

purchased from Sigma, USA. Sodium pentobarbital (Nembutal) was bought as an injectable solution (60 mg/mL, w/v) from Ceva-Sante Animale, France. Heparin (Heparinol-5000, Malaysia) was bought from Ain Medicare Sdn. Bhd, Malaysia.

**2.2. Animals.** Three- to five-month-old male normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) (280–350 g) were supplied by Animal Research and Service Centre, Health Campus, Universiti Sains Malaysia. The research methodology was approved by the Animal Ethics Committee, Universiti Sains Malaysia (USM/Animal Ethics Approval/2010/(59) (244)). These animals were kept in standard rat cages and allowed to acclimatize for 7 days in a standard environmental condition (25°C with 60–70% humidity) on a 12 hr light-dark cycle. Animals were given standard rat pellet (Chipsi Classic Heimtierbett, Germany) and tap water *ad libitum*.

**2.3. Plant Material.** *S. polyanthum* leaves were collected from the District of Bachok, Kelantan, Malaysia, from March to April 2010. The plant was identified by Dr. Richard Chung from Forest Research Institute Malaysia (FRIM). The herbal specimen (dried leaves) was deposited into FRIM herbarium (sample number: PID-171011-10).

**2.4. Preparation of Extracts and Drugs.** Four kilograms of *S. polyanthum* leaves was weighed using digital weighing balance (A&D HV-60KGL, Columbia), washed with distilled water, and dried in an incubator (Memmert GmbH + Co.KG, Germany) at a preset temperature of 50°C for 3 consecutive days. The dried leaves (1.74 kg) were ground into powder in a laboratory blender (WARING Commercial, USA) and the filtrate was sieved off by mechanical siever (No. 35). For extraction, 1.5 kg of the powdered sample was immersed in 15 L of distilled water and heated on hot plate (Erla EMS-HP-700, Illinois) at 80–90°C with continuous stirring for 30 min. The extract was then filtered through Whatman No. 41 filter paper (Whatman Schleicher and Schuell, Malaysia) and then lyophilized in freeze-dryer (ilShin, Korea). The lyophilized sample (147.15 g) was designated as the aqueous extract of *S. polyanthum* leaves (AESP). To extract the remaining less-polar compounds from the residue of the aqueous extraction, 1.4 kg of the residue was extracted using 14 L of 95% methanol (v/v) in Soxhlet apparatus (Favorit, Thailand) for 2 continuous cycles. The extract was then concentrated via rotary evaporator (Heidolph Rotavac, Germany) and dried in the incubator (Memmert GmbH + Co.KG, Germany) at a preset temperature of 50°C. The extract was then designated as the methanolic extract of *S. polyanthum* leaves (met-AESP) (26.04 g). AESP and met-AESP were finally kept in an air-tight bottle and stored in a refrigerator (National NR-B53FE, Malaysia) at 4°C until use. AESP and met-AESP were dissolved in 0.9% (w/v) normal saline but met-AESP was further added with 5% (v/v) DMSO. AESP and met-AESP were freshly prepared by dissolving the extracts in 1 mL of their respective vehicles. AESP and met-AESP solutions (final dose of 100 mg/kg) were further diluted with their respective

vehicles to achieve the doses of 70, 40, 30, 20, and 10 mg/kg. AESP and met-AESP solutions were then homogenized using a homogenizer (Ultra-Turrax T25 Basic, Malaysia) at 24,000/min for 3 min. All drugs were dissolved in 0.9% normal saline except for phentolamine hydrochloride which was dissolved in 0.9% normal saline plus 5% dimethylsulfoxide (DMSO).

**2.5. Effects of Extracts on MAP, SBP, DBP, and HR of Anaesthetized Rats.** WKY ( $n = 5$ , each for AESP- and met-AESP-treated group) and SHR ( $n = 5$ , each for AESP- and met-AESP-treated group) were anaesthetized with 50mg/kg sodium pentobarbital via intraperitoneal injection according to previous studies [20, 22] before being placed on a thermally controlled heating table ( $37 \pm 1^\circ\text{C}$ ). Anaesthetic condition was assessed by pinching the tail and the toe. The use of 50 mg/kg sodium pentobarbital was reported to not significantly affect the baselines of blood pressure and heart rate of SHR [19], but in WKY, only baseline of blood pressure was not significantly affected while the heart rate was increased [24] in comparison with conscious rats. Indeed, several hypotensive studies using similar anaesthetics scheme reported a nonsignificant

difference for the magnitude of hypotensive and bradycardic effects between both anaesthetized and conscious WKY rats [20–22]. Upon tracheotomy, an endotracheal polyethylene tube was inserted into the incised trachea to prevent airway obstruction. The left jugular vein was cannulated for extracts' injection and the right common carotid artery was cannulated for MAP, SBP, DBP, and HR recordings using MP30 BIOPAC acquisition system (BIOPAC Systems Inc., USA) via pressure transducer (SS13L) and analyzed using BIOPAC Student Lab Pro v3.6.7. After 20 min of equilibration period, 0.2 mL of the respective vehicle was intravenously administered as a negative control followed by 0.2 mL of AESP or met-AESP (10, 20, 30, 40, 70, and 100 mg/kg). In between these doses, an additional 0.2 mL of heparinised (5 IU/mL) normal saline (0.9%, w/v) was flushed intravenously to prevent intravascular blood clotting. MAP, SBP, DBP, and HR responses were observed. From MAP, SBP, DBP, and HR recordings, the changes in MAP ( $\Delta\text{MAP}$ ), SBP ( $\Delta\text{SBP}$ ), ( $\Delta\text{DBP}$ ), and HR ( $\Delta\text{HR}$ ) from baseline values were calculated and expressed in percentage as described by Medeiros and colleagues [25] using the following formula.

$$\frac{(\text{MAP/SBP/DBP/HR}_{\text{baseline}} - \text{MAP/SBP/DBP/HR}_{\text{after extracts administration}})}{\text{MAP/SBP/DBP/HR}_{\text{baseline}}} \times 100. \quad (1)$$

Time-course changes in MAP, SBP, DBP, and HR were also recorded and analyzed on minute-to-minute basis for 20 min. The recording time was set to be 20 min based on our preliminary study that showed that the longest recovery time was 17 min.

**2.6. Effects of Autonomic Ganglion,  $\alpha$ -,  $\beta$ -Adrenergic, and Cholinergic Receptors Blockage in MAP, SBP, DBP, and HR of Anaesthetized WKY.** Pharmacological antagonistic studies [15, 23] using blockers were performed on 5 different sets of experiments. Specific ANS receptor antagonists such as hexamethonium bromide (10 mg/kg), phentolamine hydrochloride (2 mg/kg), propranolol hydrochloride (2 mg/kg), and atropine sulphate (2 mg/kg) were used to block the autonomic ganglion,  $\alpha$ -adrenergic,  $\beta$ -adrenergic, and cholinergic receptors, respectively. To investigate the role of nitric oxide, N- $\omega$ -nitro-l arginine methyl ester, L-NAME (20 mg/kg), was used to block the endothelial nitric oxide synthase (eNOS) enzyme. Specific agonists for  $\alpha$ -adrenergic,  $\beta$ -adrenergic, and cholinergic receptors such as methoxamine hydrochloride (50  $\mu\text{g/kg}$ ), isoproterenol hydrochloride (1.2  $\mu\text{g/kg}$ ), and acetylcholine chloride (5  $\mu\text{g/kg}$ ), respectively, were used as positive controls to ensure sufficient blockages. In each of the experiments, 100 mg/kg AESP or met-AESP was introduced. The dose was chosen based on our preliminary experiments whereby only 100 mg/kg AESP caused significant bradycardia. To check for the involvement of autonomic ganglion and eNOS enzyme blockages, these boluses of injections were administered intravenously according to the following sequence; (i) test dose, (ii) blocker, and (iii) test dose. In order

to check for the involvement of  $\alpha$ -adrenergic,  $\beta$ -adrenergic, and cholinergic receptors, the sequence of treatments was as follows: (i) test dose, (ii) agonist, (iii) blocker, (iv) agonist, and (v) test dose.

**2.7. Statistical Analyses.** MAP, SBP, DBP and HR were expressed as mean  $\pm$  standard deviation (SD).  $\text{ED}_{50}$  values for AESP- and met-AESP-induced reductions of MAP, SBP and DBP were derived from nonlinear regression equation and calculated using GraphPad PRISM version 5.01. Statistical analyses were performed using similar software. One-way ANOVA test was used to determine significant differences between multiple doses, whereas repeated measures 2-way ANOVA test was used to determine the differences between the responses over time. By comparing the averaged readings of MAP, SBP, DBP, and HR upon extracts' administration with the initial baseline value every minute for 20 min, recovery time was then allotted when the averaged readings of MAP, SBP, DBP, and HR were no longer significantly different as compared to the initial baseline value. A post hoc Bonferroni test was performed to compare the effects of the multiple doses over time. For antagonistic study, paired  $t$ -test was carried out. The value of  $P$  less than 0.05 was considered to be significant.

### 3. Results

**3.1. Effects of AESP and Met-AESP on MAP, SBP, DBP, and HR.** Baselines of MAP, SBP, DBP, and HR for WKY before any treatment were  $134.53 \pm 10.72$  mmHg,  $150.20 \pm 13.65$  mmHg,

119.935 ± 9.27 mmHg, and 298.88 ± 42.17 beats/min ( $n = 10$ ). Baselines of MAP, SBP, DBP, and HR in SHR were 194.25 ± 8.75, 218.14 ± 11.15 mmHg, 168.90 ± 11.89 mmHg, and 329.90 ± 30.32 bpm ( $n = 10$ ). Intravenous administrations of 0.2 mL vehicles for AESP (0.9% normal saline) and met-AESP (0.9% normal saline + 5% DMSO) did not cause any significant changes to baseline MAP, SBP, DBP, and HR upon 20 min observation period. As compared to negative control, intravenous administrations of AESP bolus (0.2 mL, 100 mg/kg) caused significant changes to baseline MAP, SBP, DBP, and HR (see Figures 1(a), 1(b), 1(c), and 1(d)) while met-AESP bolus (0.2 mL, 100 mg/kg) caused significant changes to baseline MAP, SBP, and DBP (see Figures 1(e), 1(f), and 1(g)), but not to baseline HR (see Figure 1(h)) upon 20 min observation period.

AESP from 20 to 100 mg/kg induced significant dose-dependent reductions of MAP ( $P < 0.001$ ; see Figure 2(a)), SBP ( $P < 0.01$ ; see Figure 2(b)), and DBP ( $P < 0.05$ ; see Figure 2(c)) with only 100 mg/kg AESP causing a significant reduction of HR ( $P < 0.001$ ; see Figure 4) in both WKY and SHR. AESP-induced reduction of MAP in WKY was significantly higher ( $P < 0.05$ ) than in SHR at 30 mg/kg (see Figure 2(a)). AESP-induced reduction of SBP in WKY was comparable to reduction in SHR (see Figure 2(b)). AESP-induced reductions of DBP in WKY were significantly higher than in SHR at 30 ( $P < 0.01$ ), 70 ( $P < 0.01$ ), and 100 mg/kg ( $P < 0.01$ ) (see Figure 2(c)).

Met-AESP from 40 to 100 mg/kg induced significant ( $P < 0.05$ ) dose-dependent reductions of MAP ( $P < 0.05$ , see Figure 2(c)), SBP ( $P < 0.05$ ; see Figure 2(d)) and DBP ( $P < 0.05$ ; see Figure 2(e)) in WKY; whereas, in SHR, 30 mg/kg to 100 mg/kg met-AESP induced significant reductions of MAP ( $P < 0.001$ ; see Figure 2(d)), SBP ( $P < 0.001$ ; see Figure 2(e)) and DBP ( $P < 0.001$ , see Figure 2(f)). Met-AESP-induced reductions of MAP, SBP and DBP in SHR were significantly higher than in WKY at 40 ( $P < 0.001$ ,  $P < 0.001$ , and  $P < 0.001$ , resp.) and at 70 mg/kg ( $P < 0.001$ ,  $P < 0.01$ , and  $P < 0.01$ , resp.) (see Figures 2(d), 2(e), and 2(f)).

AESP-induced reductions of MAP, SBP, and DBP were significantly higher than those of met-AESP-induced hypotension in WKY at 40 ( $P < 0.01$ ,  $P < 0.001$ , and  $P < 0.01$ , resp.) and at 70 mg/kg ( $P < 0.01$ ,  $P < 0.001$ , and  $P < 0.05$ , resp.) (see Figures 3(a), 3(b), and 3(c)). Met-AESP-induced reduction of MAP was significantly higher than AESP-induced reduction of MAP in SHR at 70 mg/kg ( $P < 0.05$ ) (see Figure 3(d)). Met-AESP-induced reduction of SBP in SHR was comparable to AESP-induced reduction of SBP in SHR (see Figure 3(e)). Met-AESP-induced reductions of DBP were significantly higher than AESP-induced reduction of DBP in SHR at 40 mg/kg ( $P < 0.05$ ) and 70 mg/kg ( $P < 0.01$ ) (see Figure 3(f)).

ED<sub>50</sub> value for AESP-induced reductions of MAP, SBP, and DBP was lower in WKY (25.28 ± 2.33, 21.80 ± 2.34, and 26.67 ± 2.34, resp.) than in SHR (32.28 ± 2.53, 26.32 ± 2.52, and 32.97 ± 2.58, resp.) (see Table 1). ED<sub>50</sub> values for met-AESP-induced reduction of MAP, SBP and DBP were lower in SHR (33.05 ± 2.34, 32.08 ± 2.36, and 32.25 ± 2.34, resp.) than in WKY (54.58 ± 2.41, 63.83 ± 2.37, and 72.14 ± 2.43, resp.). Overall, the ED<sub>50</sub> values for AESP-induced reductions

of MAP, SBP and DBP were lower than those for met-AESP in WKY (see Table 1). The ED<sub>50</sub> values for AESP-induced reductions of MAP and SBP were lower than ED<sub>50</sub> values for met-AESP in SHR but not for DBP reduction (see Table 1).

Only 100 mg/kg AESP caused reduction of HR in both WKY and SHR. However, AESP-induced HR reductions in both WKY and SHR were not significantly different (see Figure 4).

**3.2. Time-Course Changes in MAP, SBP, DBP, and HR by AESP and Met-AESP.** In terms of the onset time of action, the maximal AESP-induced hypotension at all doses was achieved within 0.5 min after injection and for met-AESP was within 1.5 min.

Generally, AESP-induced reductions of MAP, SBP, and DBP recovered within 2–6 min in WKY and within 1–4 min in SHR. The highest dose of AESP (100 mg/kg) caused reduction of HR that recovered within 17 min in WKY and 5 min in SHR (see Table 2). Met-AESP-induced reductions of MAP, SBP and DBP recovered within 4–6 min in WKY and within 3–5 min in SHR (see Table 2). Generally, AESP recovered faster in SHR than in WKY. AESP also recovered faster than met-AESP in SHR.

**3.3. Effects of Autonomic Ganglion Receptors Blockage on Changes in MAP, SBP, DBP and HR of Anaesthetized WKY.** Blockage of autonomic ganglion with 10 mg/kg hexamethonium bromide caused significant attenuation in AESP-induced reductions of MAP, SBP, and DBP by 15.11 ± 10.01% ( $P < 0.05$ ), 34.89 ± 14.77% ( $P < 0.01$ ) and 18.21 ± 10.10% ( $P < 0.01$ ), respectively (see Figures 5(a), 5(b), and 5(c)). Similar blockages have also attenuated the met-AESP-induced reductions of MAP, SBP, and DBP by 70.51 ± 10.84% ( $P < 0.01$ ), 51.79 ± 19.43% ( $P < 0.05$ ) and 47.20 ± 23.90% ( $P < 0.05$ ), respectively (see Figures 5(a), 5(b), and 5(c)). The bradycardic effect by 100 mg/kg AESP was not significantly changed (see Figure 5(d)).

**3.4. Effects of  $\alpha$ -Adrenergic Receptors Blockage on Changes in MAP, SBP, DBP, and HR of Anaesthetized WKY.** Phentolamine hydrochloride (2 mg/kg) that significantly blocked the increments of MAP, SBP, and DBP induced by the positive control drug, methoxamine hydrochloride (50  $\mu$ g/kg), by 95.13 ± 7.17% ( $P < 0.001$ ), 91.18 ± 14.76% ( $P < 0.001$ ) and 87.23 ± 23.77% ( $P < 0.001$ ) caused significant attenuations in AESP-induced reductions of MAP, SBP, DBP, and HR by 40.86 ± 24.34% ( $P < 0.05$ ), 67.09 ± 23.69% ( $P < 0.01$ ), 52.98 ± 31.62% ( $P < 0.05$ ), and 70.35 ± 19.54% ( $P < 0.05$ ), respectively (see Figures 6(a), 6(b), 6(c), and 6(d)). In another set of experiments, phentolamine hydrochloride (2 mg/kg) that significantly abolished the increment of MAP, SBP and DBP induced by the positive control drug, methoxamine hydrochloride (50  $\mu$ g/kg), by 80.80 ± 18.61% ( $P < 0.001$ ), 68.16 ± 27.43% ( $P < 0.001$ ), and 58.44 ± 38.61% ( $P < 0.001$ ) did not significantly affect met-AESP-induced reductions of MAP, SBP, and DBP (see Figures 6(a), 6(b), and 6(c)).

**3.5. Effects of  $\beta$ -Adrenergic Receptors Blockage on Changes in MAP, SBP, DBP, and HR of Anaesthetized WKY.** Propranolol

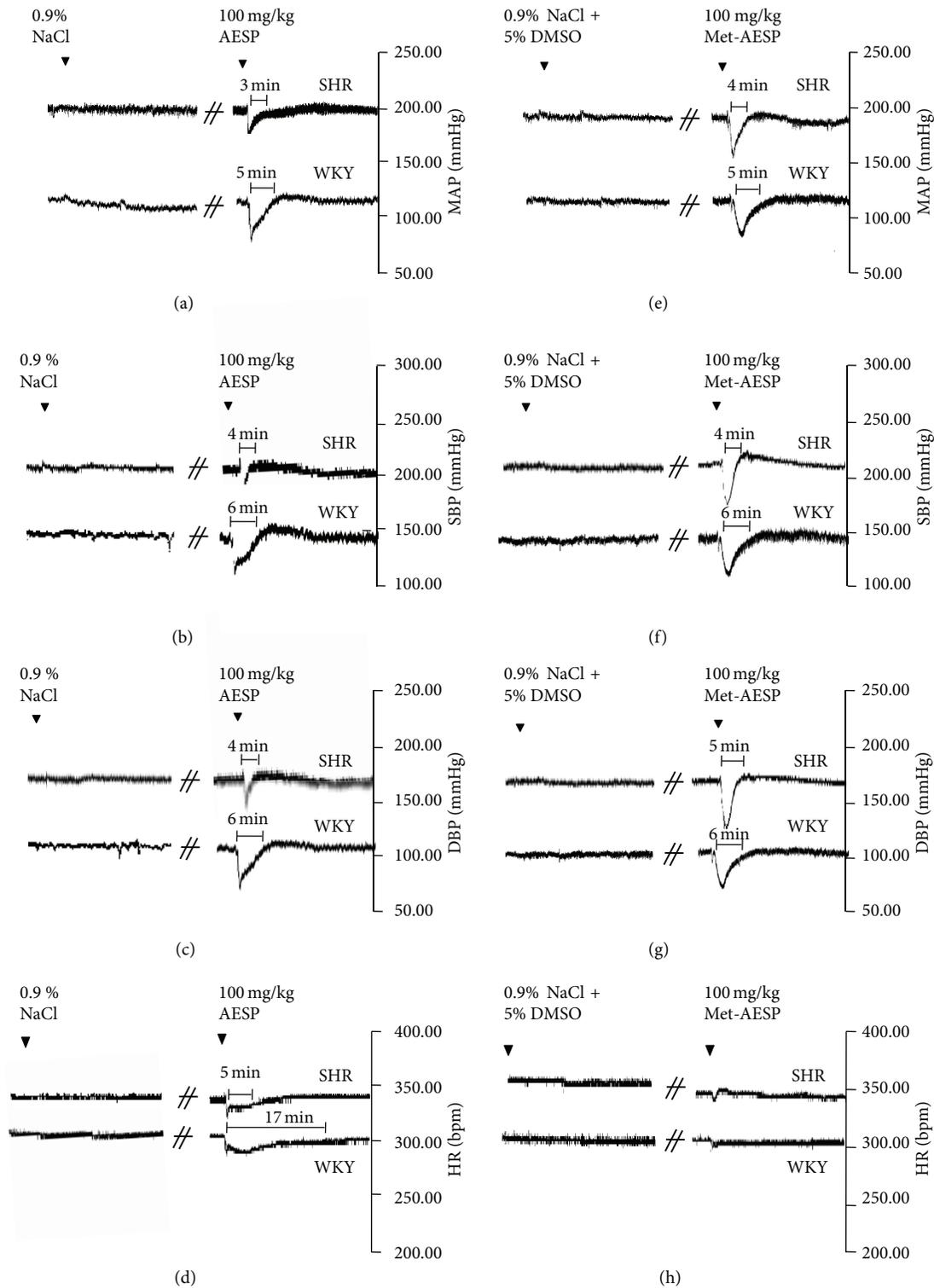


FIGURE 1: Tracings represent the effects of 0.9% normal saline as opposed to 100 mg/kg AESP on (a) MAP, (b) SBP, (c) DBP, and (d) HR and the effects of 0.9% normal saline plus 5% DMSO as opposed to 100 mg/kg met-AESP on (e) MAP, (f) SBP, (g) DBP, and (h) HR. MAP: recorded mean arterial blood pressure in millimetres of mercury, SBP: recorded systolic blood pressure in millimetres of mercury, DBP: recorded diastolic blood pressure in millimetres of mercury, HR: recorded heart rate in beats per minute, AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, SHR: spontaneously hypertensive rats, WKY: Wistar-Kyoto rats, NaCl: sodium chloride, DMSO: dimethylsulfoxide.

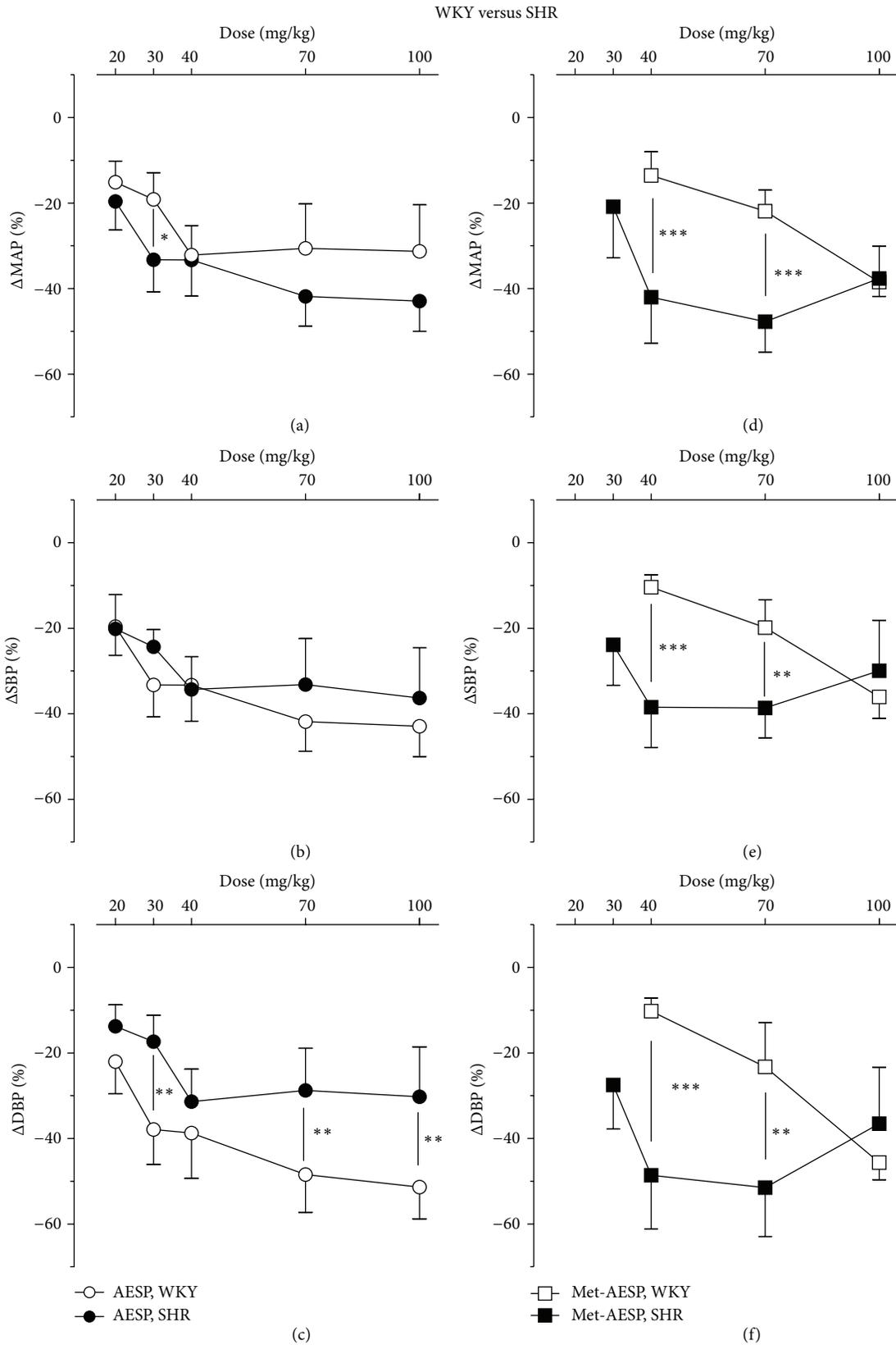


FIGURE 2: Effects of AESP on (a) MAP, (b) SBP, (c) DBP and effects of met-AESP on (d) MAP, (e) SBP, and (f) DBP of both WKY and SHR.  $\Delta$ MAP/SBP/DBP: changes of MAP/SBP/DBP from baseline (expressed as mean percentage  $\pm$  SD,  $n = 5$  animals per group), AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$ .

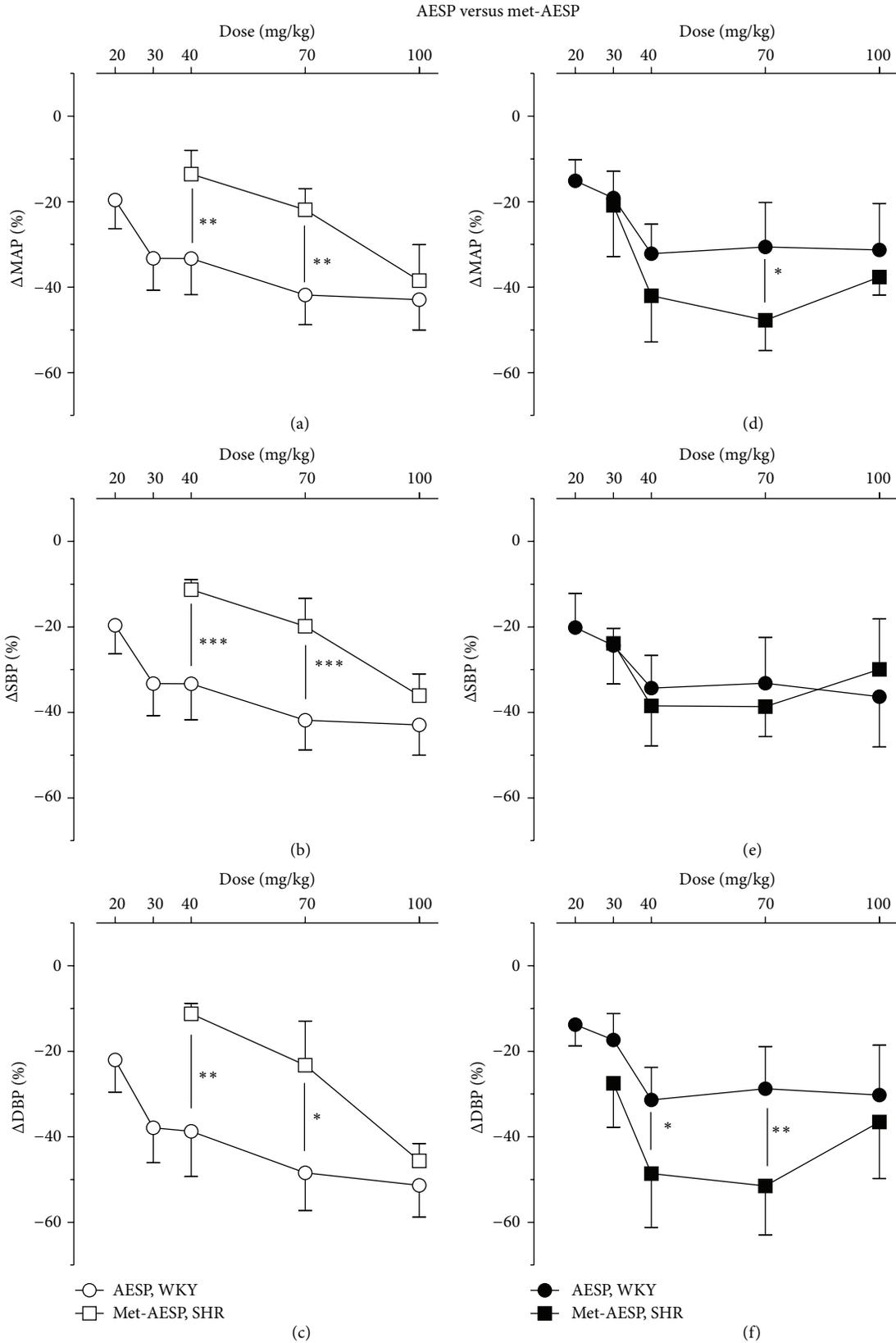


FIGURE 3: Effects of AESP and met-AESP on (a) MAP, (b) SBP, and (c) DBP of WKY and on (d) MAP, (e) SBP, (f) DBP of SHR.  $\Delta$ MAP/SBP/DBP: changes of MAP/SBP/DBP from baseline (expressed as mean percentage  $\pm$  SD,  $n = 5$  animals per group), AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats. \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$ .

TABLE 1: ED<sub>50</sub> values of AESP and met-AESP-induced hypotension.

Model of rats	ED <sub>50</sub> values for the induced hypotension					
	MAP	AESP SBP	DBP	MAP	Met-AESP SBP	DBP
WKY	25.28 ± 2.33	21.80 ± 2.34	26.67 ± 2.34	54.58 ± 2.41	63.83 ± 2.37	72.14 ± 2.43
SHR	32.28 ± 2.53	26.32 ± 2.52	32.97 ± 2.58	33.05 ± 2.34	32.08 ± 2.36	32.25 ± 2.34

ED<sub>50</sub> value: mean of dose ± SD that causes 50% maximal reduction of MAP, SBP, and DBP, calculated by GraphPad PRISM version 5.01 software by using nonlinear regression equation, MAP: mean arterial blood pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats.

TABLE 2: Recovery time (in minutes) for 20, 30, 40, 70, and 100 mg/kg AESP and met-AESP in WKY and SHR.

Dose (mg/kg)		Recovery time (min)							
		MAP	AESP			Met-AESP			
		MAP	SBP	DBP	HR	MAP	SBP	DBP	HR
20	WKY	2	2	2	n.a	n.a	n.a	n.a	n.a
	SHR	1	1	2	n.a	n.a	n.a	n.a	n.a
30	WKY	3	3	3	n.a	n.a	n.a	n.a	n.a
	SHR	1	1	2	n.a	3	3	4	n.a
40	WKY	3	3	3	n.a	4	4	4	n.a
	SHR	2	2	2	n.a	4	4	4	n.a
70	WKY	4	4	4	n.a	4	4	4	n.a
	SHR	2	2	2	n.a	4	5	6	n.a
100	WKY	5	6	6	17	5	6	5	n.a
	SHR	3	4	4	5	4	4	5	n.a

Recovery time: the time when the averaged readings of MAP, SBP, DBP, and HR were no longer significantly different as compared to the initial baseline values. MAP: recorded mean arterial blood pressure in millimetres of mercury, HR: recorded heart rate in beats per minute, AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats, n.a: there were no changes observed in the baselines of MAP, SBP, DBP, and HR upon administration of the respective doses.

hydrochloride (2 mg/kg) that significantly blocked the induced-reductions of MAP, SBP, and DBP by the positive control drug, isoproterenol hydrochloride (1.2 µg/kg), by 85.52 ± 7.17% ( $P < 0.001$ ), 76.17 ± 21.09% ( $P < 0.01$ ), and 86.44 ± 9.90% ( $P < 0.001$ ) did not significantly affect AESP-induced reductions of MAP, SBP, DBP, and HR (see Figures 7(a), 7(b), and 7(c)). In another set of experiments, propranolol hydrochloride (2 mg/kg) that significantly blocked the induced-reductions of MAP, SBP, and DBP by the positive control drug, isoproterenol hydrochloride (1.2 µg/kg), by 80.30 ± 14.03% ( $P < 0.01$ ), 73.63 ± 21.17% ( $P < 0.05$ ), and 86.12 ± 10.18% ( $P < 0.01$ ) also caused significant attenuations on met-AESP-induced reductions of MAP, SBP, and DBP by 39.27 ± 18.72% ( $P < 0.01$ ), 33.87 ± 24.94% ( $P < 0.05$ ), and 37.86 ± 16.98% ( $P < 0.01$ ), respectively (see Figures 7(a), 7(b), and 7(c)).

**3.6. Effects of Cholinergic Receptors Blockage on Changes in MAP, SBP, DBP, and HR of Anaesthetized WKY.** Atropine sulphate (2 mg/kg) that significantly blocked the reductions of MAP, SBP, and DBP induced by the positive control drug, acetylcholine chloride (5 µg/kg), by 85.77 ± 6.19% ( $P < 0.001$ ), 96.07 ± 3.85% ( $P < 0.001$ ), and 94.35 ± 4.76% ( $P < 0.001$ ) did not significantly affect AESP-induced reductions of MAP, SBP, DBP and HR (see Figures 8(a), 8(b), 8(c), and 8(d)). In another set of experiments, atropine sulphate (2 mg/kg) that significantly blocked the reductions

of MAP, SBP, and DBP induced by the positive control drug, acetylcholine chloride (5 µg/kg), by 83.71 ± 10.86% ( $P < 0.001$ ), 94.78 ± 6.65% ( $P < 0.001$ ), and 96.02 ± 7.68% ( $P < 0.001$ ) also attenuated met-AESP-induced hypotension by 50.62 ± 29.65% ( $P < 0.01$ ), 41.46 ± 28.47% ( $P < 0.01$ ) and 42.68 ± 28.22% ( $P < 0.01$ ), respectively (see Figures 8(a), 8(b), and 8(c)).

**3.7. Effects of eNOS Enzyme Blockage on Changes in MAP, SBP, DBP, and HR of Anaesthetized WKY.** Blockage of endothelial nitric oxide synthase, eNOS, with 20 mg/kg L-NAME caused significant attenuation in met-AESP-induced reductions of MAP, SBP, and DBP by 75.38 ± 19.32% ( $P < 0.001$ ) 69.63 ± 19.79% ( $P < 0.001$ ) and 75.38 ± 19.32% ( $P < 0.001$ ) but not in AESP-induced reductions of MAP, SBP, DBP, and HR (see Figures 9(a), 9(b), 9(c), and 9(d)).

## 4. Discussion

This study has shown that both intravenous injections of AESP and met-AESP boluses induced significant dose-dependent hypotension while only the highest dose of AESP caused significant bradycardia. The occurrence of bradycardia, found to be accompanying the hypotensive effect of AESP, was common in few other ethnomedicinal plants such as *Tacca integrifolia* (Ker-Gawl). [26], *Musanga cecropioides* [27], and *Sida cordifolia* [25]. Nevertheless, there are some

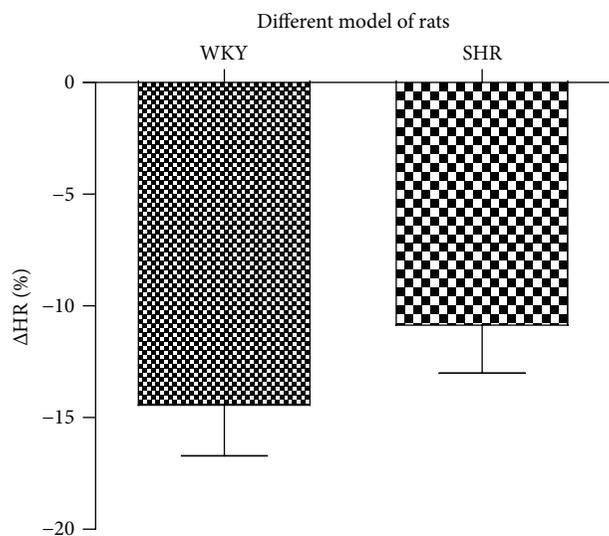


FIGURE 4: Effects of intravenous administrations of 100 mg/kg AESP on the heart rate of WKY and SHR.  $\Delta$ HR: changes of heart rate from baseline (expressed as mean percentage  $\pm$  SD), AESP: aqueous extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats.

other ethnomedicinal plants such as *Andrographis paniculata* [23, 28] and *Bacopa monnieri* [29], elicited hypotensive effects without significant bradycardia as observed during met-AESP-induced hypotension. Besides the appearance of bradycardia for AESP, AESP and met-AESP differed in terms of onset time and sustainability of its action. The onset time for AESP to achieve maximal hypotension was faster than that for met-AESP in both WKY and SHR. AESP-induced hypotension was less sustained than met-AESP-induced hypotension in SHR. This may indicate different composition of active constituent(s) that perhaps acted on different pathways in mediating the hypotensive effects by both extracts. This study also showed that AESP-induced hypotension was more potent in WKY whereby it exhibited a comparable potency in SHR as compared to met-AESP-induced hypotension.

This study has shown that both extracts reduced MAP, SBP, and DBP of both WKY and SHR. However, the recovery time for AESP-induced hypotension and bradycardia was shorter in SHR as compared to in WKY. This can be explained by the preexistence of sympathetic overactivity in SHR as the over activity of sympathetic system in turn might cause a greater reflex pressor response to return the MAP, SBP, DBP, and HR levels to the baseline in SHR. This phenomenon is possible due to an altered number of adrenergic receptors or change in its responsiveness in SHR [30].

Further pharmacological antagonistic studies were then performed to elucidate the postulation that both extracts acted via different mechanism(s) of actions. Since the receptors of ANS are involved in sympathetic and parasympathetic controls of blood pressure and heart rate, their effects were further examined.

In this study, autonomic ganglion blockage was achieved by addition of hexamethonium, a nicotinic receptors blocking agent which blocks ion channels of the autonomic ganglia

resulting in a blockage of the outputs of the sympathetic and parasympathetic pathways [31]. Since autonomic ganglion blockage had attenuated partially the AESP-induced hypotension, it might indicate a partial involvement of ANS in regulating its actions. Further blockages of the two peripheral receptors in ANS (cholinergic and  $\beta$ -adrenergic receptors) with their respective antagonists, propranolol and atropine, did not significantly affect neither AESP-induced hypotension nor its bradycardic effect. However, blockage of  $\alpha$ -adrenergic receptors with phentolamine, a competitive blocker of  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptors, had partially attenuated AESP-induced hypotension and bradycardia. Hence, it is suggested that AESP-induced hypotension and bradycardia in this study were partially acted via ANS, mediated by  $\alpha$ -adrenergic receptors' pathways. It is known that  $\alpha_1$ -adrenergic receptor mediates vasoconstriction. On the other hand,  $\alpha_2$ -receptors play a prominent role in lowering blood pressure by inhibiting the synaptic release of neurotransmitter that mediates the renin production [32]. Alpha 2-adrenergic receptors stimulation also modulates vagally-induced baroreflex bradycardia [33]. Besides, hypotension with accompanying bradycardia is a common effect of  $\alpha_2$ -agonists such as clonidine, guanfacine, and  $\alpha$ -methyldopa [33, 34]. Thus, it is plausible to suggest that AESP-induced hypotension was partially mediated by the  $\alpha_2$ -receptors.

As for met-AESP, blockage of autonomic ganglion significantly attenuated its hypotensive effect which may suggest that its action was primarily mediated *via* ANS. Blockages of the peripheral  $\beta$ -adrenergic receptors with propranolol, a nonselective  $\beta$ -adrenergic receptors' blocker [35], caused a significant attenuation in met-AESP-induced hypotension. Hence, it is suggested that met-AESP partially acted via  $\beta$ -adrenergic receptors of the ANS. Among the  $\beta$ -adrenergic receptors' subtypes,  $\beta_1$  subtype is involved in increasing the blood pressure and heart rate while  $\beta_3$  subtype was involved in lipolysis and not related to the regulation of blood pressure [36]. Thus, it is suggested that  $\beta_2$  subtype receptor which is responsible for smooth vessel relaxation [37] was involved in mediating hypotensive effect of met-AESP. Blockages of the peripheral muscarinic acetylcholine receptors with atropine, a competitive muscarinic acetylcholine receptors' blocker [38], also caused significant attenuations in met-AESP-induced hypotension. Hence, it is also suggested that, besides  $\beta$ -adrenergic receptors, met-AESP-induced hypotension was also partially mediated via muscarinic acetylcholine receptors. Moreover, the degree of attenuation by atropine was much greater than that by propranolol. Hence, it is suggested that met-AESP-induced hypotension was predominant on muscarinic acetylcholine receptor. Muscarinic acetylcholine receptor subtypes include  $M_1$ ,  $M_2$ ,  $M_3$ ,  $M_4$ , and  $M_5$ .  $M_1$  subtype is present in gastric parietal cells whereas  $M_4$  and  $M_5$  subtypes are not being described in blood vessels.  $M_2$  subtype may cause reduction of the cardiac output, blood pressure, and HR as a result of the decrement of firing rate at the sinoatrial node in the heart [39]. Thus, this subtype is unlikely to be involved since met-AESP did not cause any significant reduction of the HR. Therefore, it is suggested that the  $M_3$  subtype was possibly involved since its activation causes endothelium-dependent vasodilatation [40]. This study also

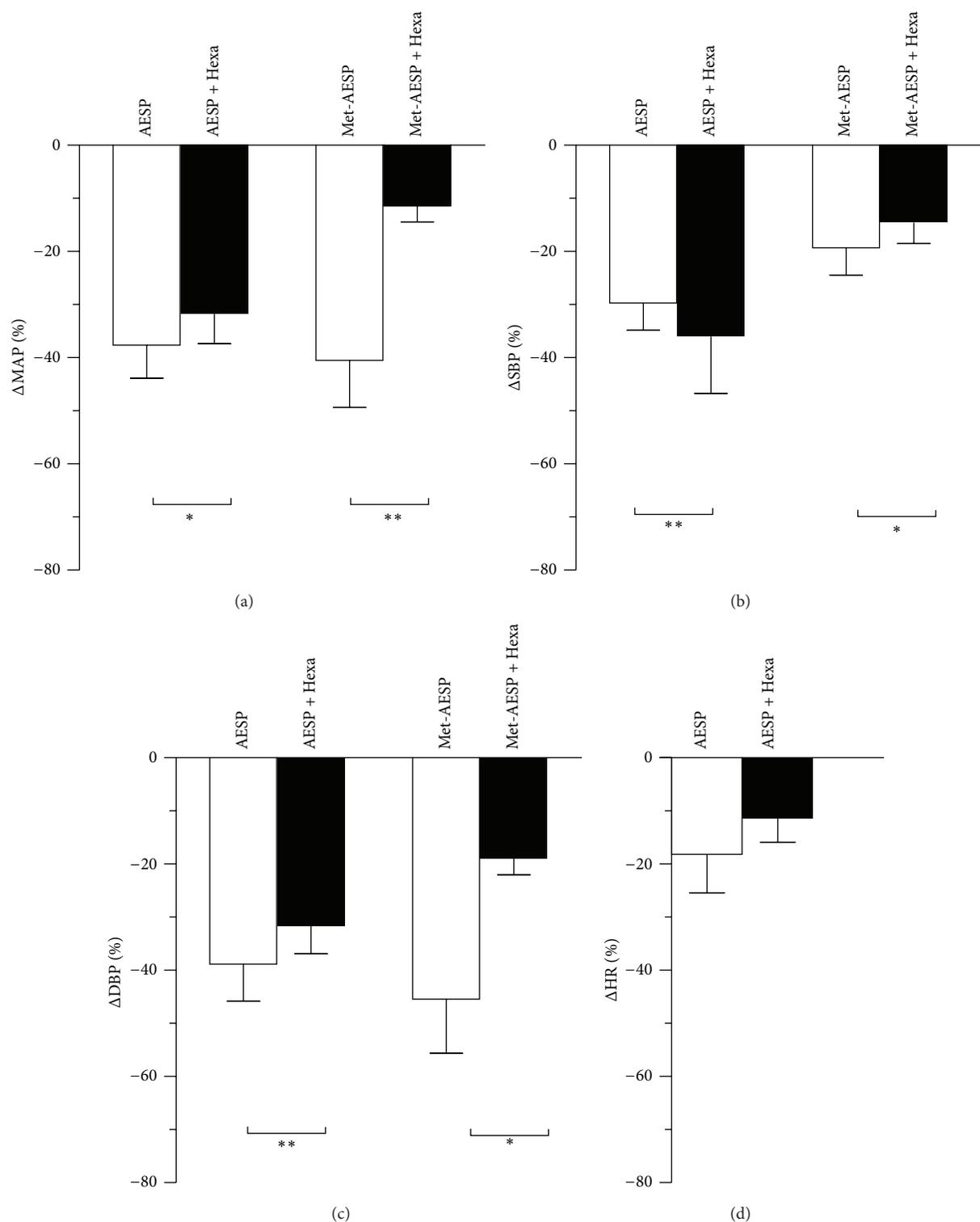


FIGURE 5: Effects of autonomic ganglion receptors' blockages on changes in (a) MAP, (b) SBP, (c) DBP, and (d) heart rate by various treatments in WKY rats ( $n = 5$ ). Hexa: 10 mg/kg hexamethonium bromide,  $\Delta$ MAP/SBP/DBP/HR: changes of MAP/SBP/DBP/HR from baseline (expressed as mean percentage  $\pm$  SD), AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats. \* $P < 0.05$  and \*\* $P < 0.01$ .

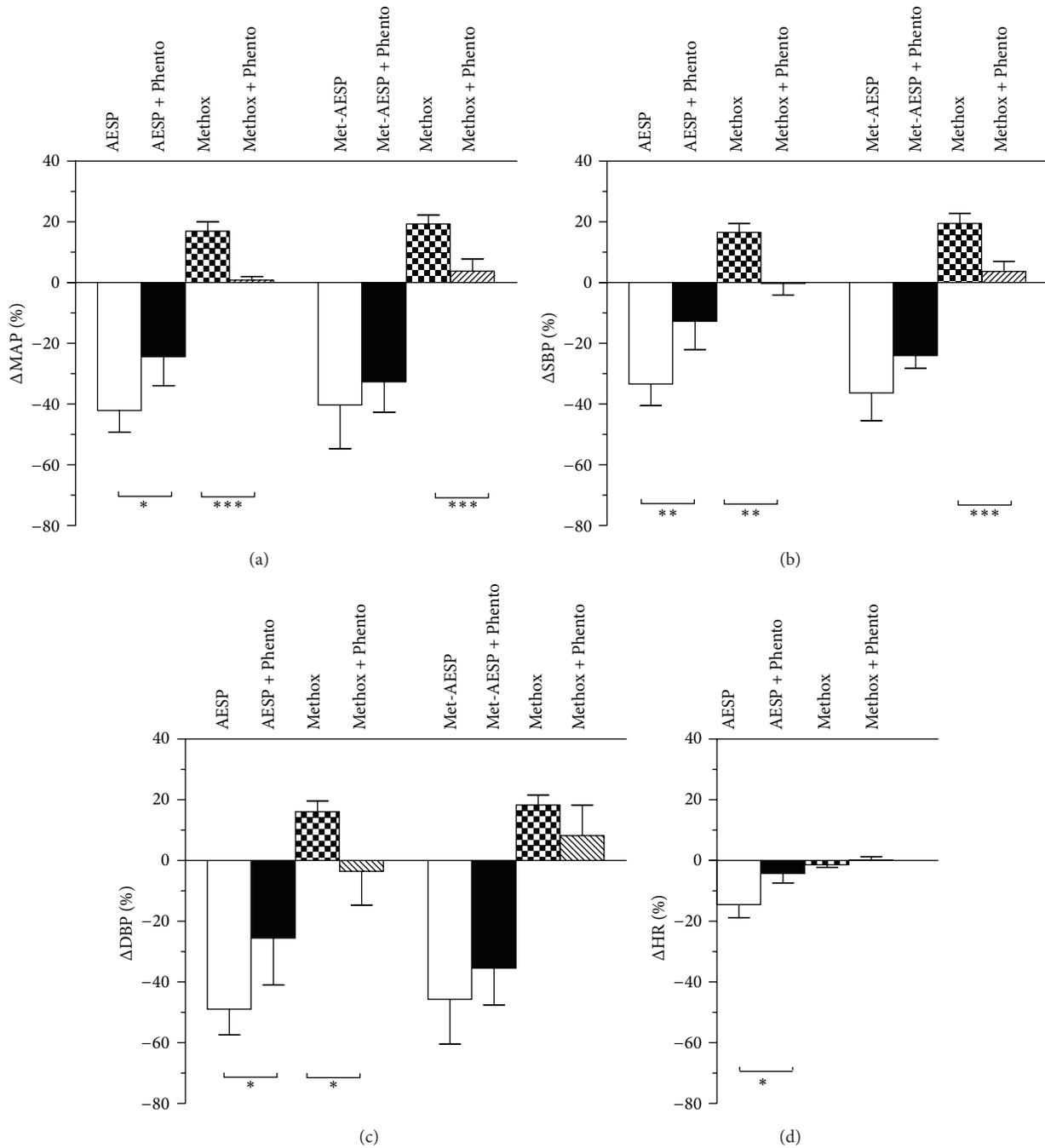


FIGURE 6: Effects of  $\alpha$ -adrenergic receptors' blockade on changes in (a) MAP, (b) SBP, (c) DBP, and (d) HR by various treatments in WKY rats ( $n = 5$ ). Phento: 2 mg/kg phentolamine hydrochloride, Methox: 50  $\mu$ g/kg methoxamine hydrochloride,  $\Delta$ MAP/SBP/DBP: changes of MAP/SBP/DBP/HR from baseline (expressed as mean percentage  $\pm$  SD), AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

showed that the action of met-AESP on ANS was less specific as compared to AESP.

Beta-2-adrenergic and  $M_3$ -muscarinic acetylcholine receptors that are suggested to mediate met-AESP-induced hypotension are associated with endothelium-dependent

vasodilatation or relaxation via increased synthesis of nitric oxide [37, 40, 41]. Therefore, another antagonistic study using L-NAME, a blocker of endothelial nitric oxide synthase (eNOS), was performed. It is found that met-AESP-induced hypotension was attenuated after blockade of eNOS

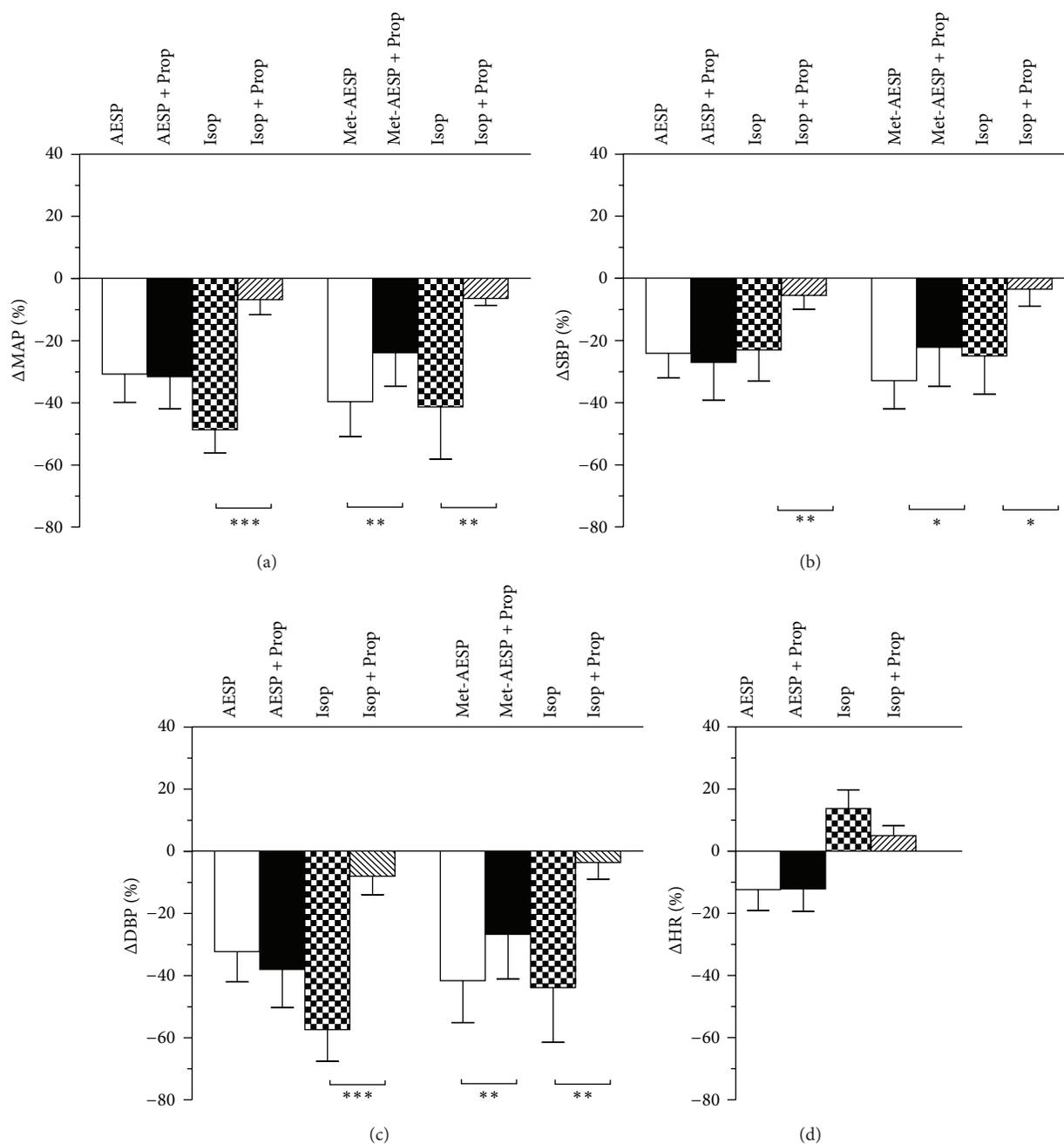


FIGURE 7: Effects of  $\beta$ -adrenergic receptors' blockage on changes in (a) MAP, (b) SBP, (c) DBP, and (d) HR by various treatments in WKY rats ( $n = 5$ ). Prop: 2 mg/kg propranolol hydrochloride, Isop: 1.2  $\mu$ g/kg isoproterenol hydrochloride,  $\Delta$ MAP/SBP/DBP/HR: changes of MAP/SBP/DBP/HR from baseline (expressed as mean percentage  $\pm$  SD), AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

while AESP-induced hypotension and bradycardia were not significantly attenuated after blockage of eNOS. These findings may support the suggestion of the partial involvement of  $\beta_2$ -adrenergic and  $M_3$ -muscarinic acetylcholine receptors in mediating met-AESP-induced hypotension. Besides, these findings may also suggest that AESP-induced hypotension did not involve direct nitric oxide generation.

Taken together, this study may serve as a preliminary verification for the traditional claim of using *S. polyanthum* leaves as a treatment of hypertension. The findings demonstrated that AESP-induced hypotension was more potent than met-AESP in WKY but both were comparably potent in SHR. AESP acted faster in both WKY and SHR. AESP exhibited comparable sustenance as met-AESP in WKY but it was less

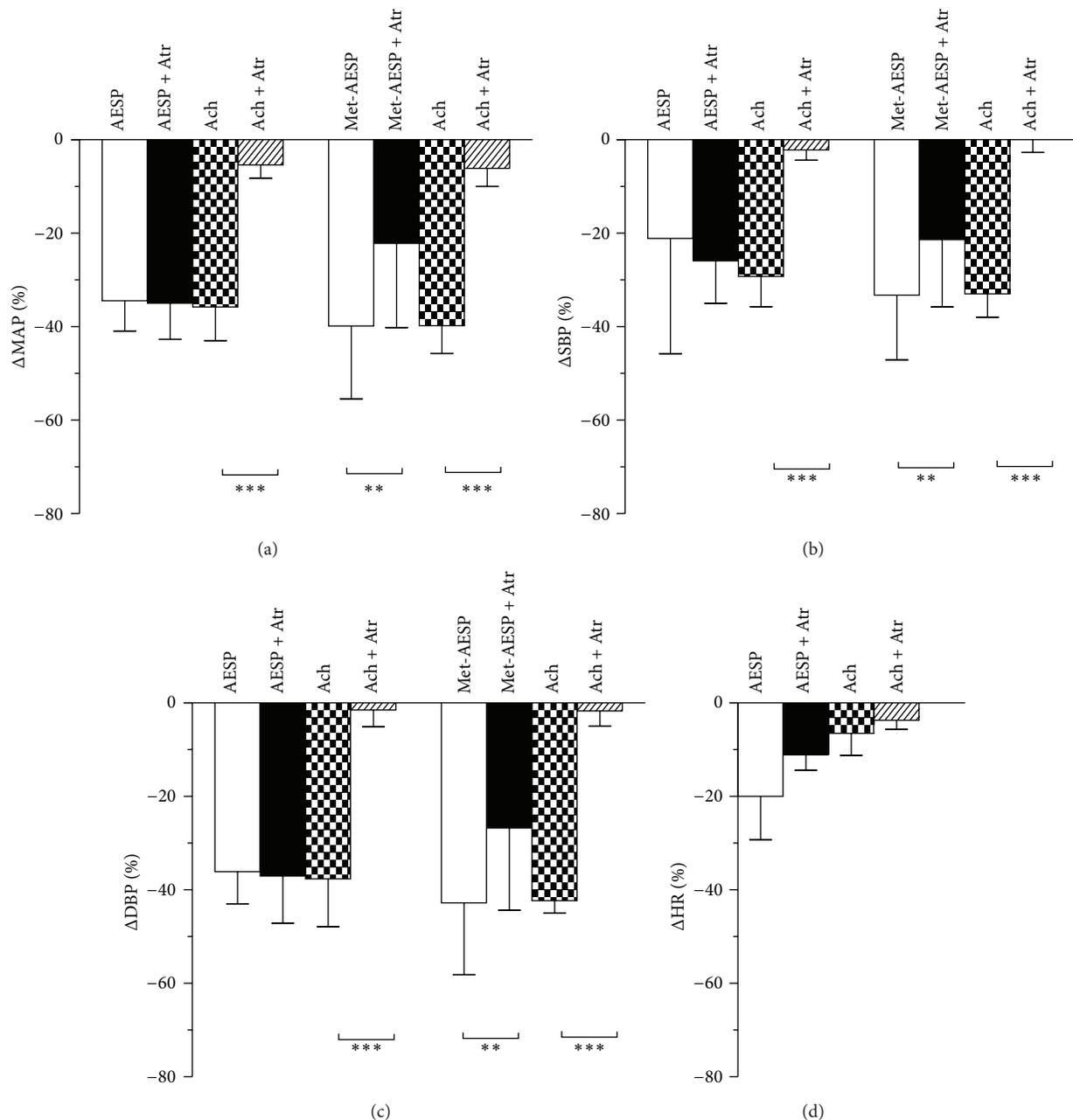


FIGURE 8: Effects of cholinergic receptors blockage on changes in (a) MAP, (b) SBP, (c) DBP, and (d) HR by various treatments in WKY rats ( $n = 5$ ). Atr: 2 mg/kg atropine sulphate, Ach: 5  $\mu$ g/kg acetylcholine chloride.  $\Delta$ MAP/SBP/DBP/HR: changes of MAP/SBP/DBP/HR from baseline (expressed as mean percentage  $\pm$  SD), AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

sustained in SHR. ANS partially mediates AESP actions via  $\alpha$ -receptors and met-AESP via  $\beta$ -adrenergic and muscarinic acetylcholine receptors through NO generation. However, further works using much more subtype-specific antagonists should be done for much more comprehensive verification. Further studies are also warranted to investigate the active compound(s) in AESP and met-AESP responsible for their hypotensive effects.

## Acknowledgments

The study was supported by Incentive Grant (1001/PPSK/8122027), USM-RU-PRGS Grant (1001/PPSK/8144010), and Short Term Grant (304/PPSK/61312059) from University Sains Malaysia. The student was financially sponsored by the International Islamic University Malaysia and the Ministry of Higher Education, Malaysia. This work was dedicated to the

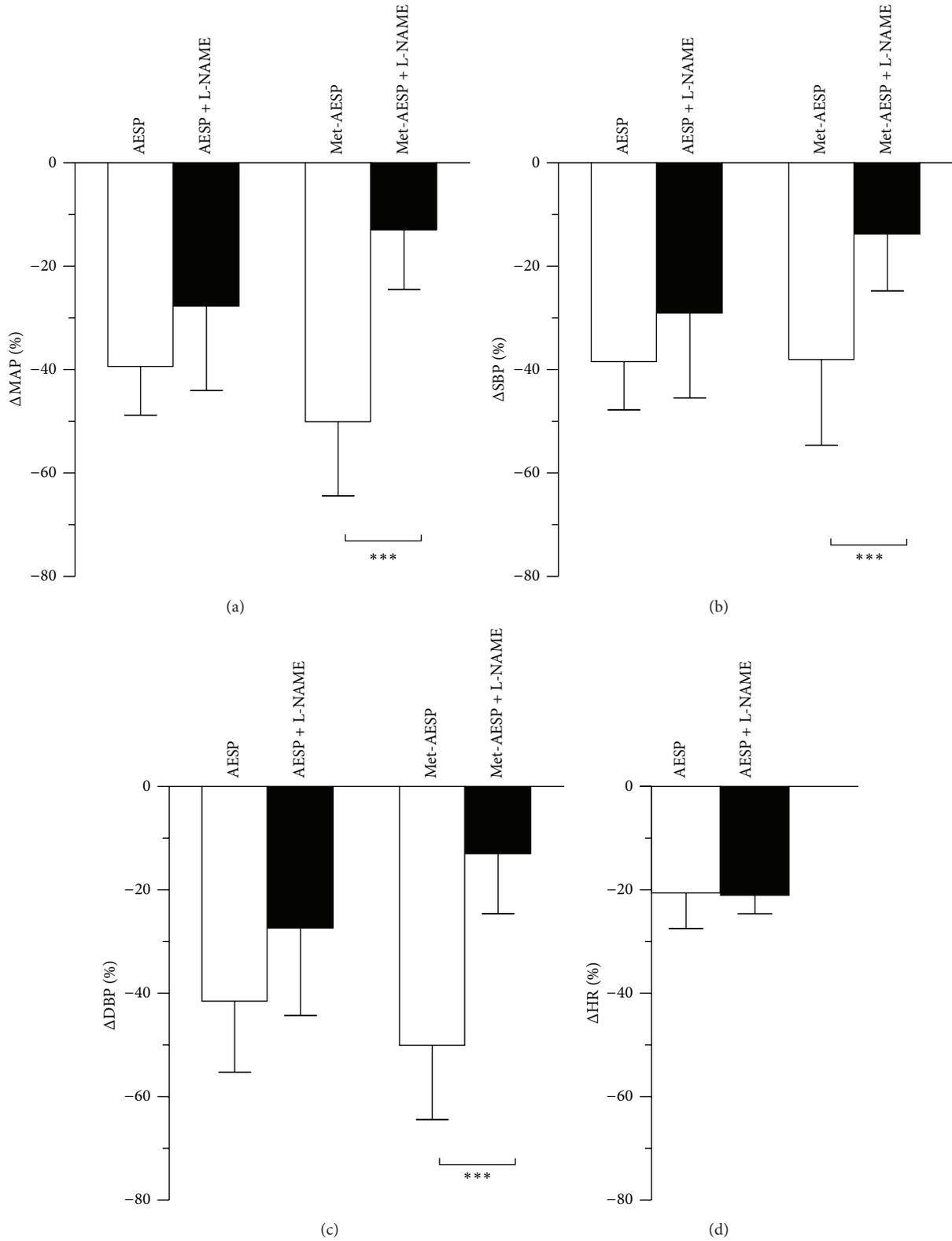


FIGURE 9: Effects of endothelial nitric oxide synthase enzyme blockage on changes in (a) MAP, (b) SBP, (c) DBP, and (d) HR by various treatments in WKY rats (n = 5). L-NAME: N- $\omega$ -nitro-l arginine methyl ester,  $\Delta$ MAP/SBP/DBP/HR: changes of MAP/SBP/DBP/HR from baseline (expressed as mean percentage  $\pm$  SD), AESP: aqueous extract of *S. polyanthum* leaves, met-AESP: residual methanolic extract of *S. polyanthum* leaves, WKY: Wistar-Kyoto rats, SHR: spontaneously hypertensive rats. \*\*\*  $P < 0.001$ .

late Professor Syed Mohsin Syed Sahil Jamalullail for his idea that initiated this project. Special thanks were also extended to Mr. Nik Fakurudin Nik Ali for his technical assistance.

## References

- [1] A. Sumono and A. S. Wulan, "The use of bay leaf (*Eugenia polyantha* Wight) in dentistry," *Dental Journal*, vol. 41, no. 3, pp. 147–150, 2008.
- [2] P. W. Grosvenor, A. Supriono, and D. O. Gray, "Medicinal plants from Riau Province, Sumatra, Indonesia. Part 2: antibacterial and antifungal activity," *Journal of Ethnopharmacology*, vol. 45, no. 2, pp. 97–111, 1995.
- [3] S. Mohamed, S. Saka, S. H. El-Sharkawy, A. M. Ali, and S. Muid, "Antimycotic screening of 58 Malaysian plants against plant pathogens," *Pesticide Science*, vol. 47, no. 3, pp. 259–264, 1996.
- [4] M. M. Mackeen, A. M. Ali, M. A. Abdullah et al., "Antinematodal activity of some Malaysian plant extracts against the pine wood nematode, *Bursaphelenchus xylophilus*," *Pesticide Science*, vol. 51, no. 2, pp. 165–170, 1997.
- [5] A. M. Ali, L. Y. Mooi, K. Yih Yih et al., "Anti-tumor promoting activity of some Malaysian traditional vegetable (ulam) extracts by immunoblotting analysis of Raji cells," *Natural Product Sciences*, vol. 6, no. 3, pp. 147–150, 2000.
- [6] R. A. A. Lelono, S. Tachibana, and K. Itoh, "In vitro antioxidative activities and polyphenol content of *Eugenia polyantha* wight grown in Indonesia," *Pakistan Journal of Biological Sciences*, vol. 12, no. 24, pp. 1564–1570, 2009.
- [7] S. P. Wong, L. P. Leong, and J. H. W. Koh, "Antioxidant activities of aqueous extracts of selected plants," *Food Chemistry*, vol. 99, no. 4, pp. 775–783, 2006.
- [8] I. W. Kusuma, H. Kuspradini, E. T. Arung et al., "Biological activity and phytochemical analysis of three Indonesian medicinal plants, *Murraya koenigii*, *Syzygium polyanthum* and *Zingiber purpurea*," *Journal of Acupuncture and Meridian Studies*, vol. 4, no. 1, pp. 75–79, 2011.
- [9] S. Perumal, R. Mahmud, S. P. Piaru, L. W. Cai, and S. Ramanathan, "Potential antiradical activity and cytotoxicity assessment of *Ziziphus mauritiana* and *Syzygium polyanthum*," *International Journal of Pharmacology*, vol. 8, no. 6, pp. 535–541, 2012.
- [10] S. Dalimartha, "Salam (*Syzygium polyanthum* [Wight.] Walp.)," in *Atlas Tumbuhan Obat Indonesia*, T. Agriwidya, Ed., vol. 2, pp. 161–165, 2000.
- [11] F. Gautier, C. Kamel, S. Calsamiglia, and P. Doane, "Food additives for ruminants based on eugenol and cinnamaldehyde," United States Patent, 2011.
- [12] L. F. L. Interaminense, D. M. Jucá, P. J. C. Magalhães, J. H. Leal-Cardoso, G. P. Duarte, and S. Lahlou, "Pharmacological evidence of calcium-channel blockade by essential oil of *Ocimum gratissimum* and its main constituent, eugenol, in isolated aortic rings from DOCA-salt hypertensive rats," *Fundamental and Clinical Pharmacology*, vol. 21, no. 5, pp. 497–506, 2007.
- [13] C. E. N. Damiani, L. V. Rossoni, and D. V. Vassallo, "Vasorelaxant effects of eugenol on rat thoracic aorta," *Vascular Pharmacology*, vol. 40, no. 1, pp. 59–66, 2003.
- [14] H. Nishijima, R. Uchida, K. Kameyama, N. Kawakami, T. Ohkubo, and K. Kitamura, "Mechanisms mediating the vasorelaxing action of eugenol, a pungent oil, on rabbit arterial tissue," *Japanese Journal of Pharmacology*, vol. 79, no. 3, pp. 327–334, 1999.
- [15] S. Lahlou, L. F. L. Interaminense, P. J. C. Magalhães, J. H. Leal-Cardoso, and G. P. Duarte, "Cardiovascular effects of eugenol, a phenolic compound present in many plant essential oils, in normotensive rats," *Journal of Cardiovascular Pharmacology*, vol. 43, no. 2, pp. 250–257, 2004.
- [16] N. M. Wartini, "Senyawa penyusun ekstrak flavor daun salam (*Eugenia polyantha* Wight) hasil distilasi uap menggunakan pelarut n-heksana dan tanpa n-heksana," *Agrotekno*, vol. 15, no. 2, pp. 72–77, 2009.
- [17] I. A. C. Menezes, C. M. N. Barreto, Â. R. Antonioli, M. R. V. Santos, and D. P. de Sousa, "Hypotensive activity of terpenes found in essential oils," *Journal of Biosciences*, vol. 65, no. 9–10, pp. 562–566, 2010.
- [18] K. E. H. El Tahir, M. F. Al-Ajmi, and A. M. Al-Bekairi, "Some cardiovascular effects of the dethymoquinonated *Nigella sativa* volatile oil and its major components  $\alpha$ -pinene and p-cymene in rats," *Saudi Pharmaceutical Journal*, vol. 11, no. 3, pp. 104–110, 2003.
- [19] C. C. Chiueh and I. J. Kopin, "Hyperresponsivity of spontaneously hypertensive rat to indirect measurement of blood pressure," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 234, no. 6, pp. H690–H695, 1978.
- [20] S. Lahlou, A. F. Figueiredo, P. J. C. Magalhães, J. H. Leal-Cardoso, and P. D. Gloria, "Cardiovascular effects of methyl-eugenol, a natural constituent of many plant essential oils, in normotensive rats," *Life Sciences*, vol. 74, no. 19, pp. 2401–2412, 2004.
- [21] S. Lahlou, J. H. Leal-Cardoso, P. J. C. Magalhães, A. N. Coelho-de-Souza, and G. P. Duarte, "Cardiovascular effects of the essential oil of *Croton nepetaefolius* in rats: role of the autonomic nervous system," *Planta Medica*, vol. 65, no. 6, pp. 553–557, 1999.
- [22] S. Lahlou, A. F. Figueiredo, P. J. C. Magalhães, and J. H. Leal-Cardoso, "Cardiovascular effects of 1,8-cineole, a terpenoid oxide present in many plant essential oils, in normotensive rats," *Canadian Journal of Physiology and Pharmacology*, vol. 80, no. 12, pp. 1125–1131, 2002.
- [23] C. Y. Zhang and B. K. H. Tan, "Mechanisms of cardiovascular activity of *Andrographis paniculata* in the anesthetized rat," *Journal of Ethnopharmacology*, vol. 56, no. 2, pp. 97–101, 1997.
- [24] J.-P. Fluckiger, M. Sonnay, N. Boillat, and J. Atkinson, "Attenuation of the baroreceptor reflex by general anesthetic agents in the normotensive rat," *European Journal of Pharmacology*, vol. 109, no. 1, pp. 105–109, 1985.
- [25] I. A. Medeiros, M. R. V. Santos, N. M. S. Nascimento, and J. C. Duarte, "Cardiovascular effects of *Sida cordifolia* leaves extract in rats," *Fitoterapia*, vol. 77, no. 1, pp. 19–27, 2006.
- [26] N. Kitjaroennirut, C. Jansakul, and P. Sawangchote, "Cardiovascular effects of *Tacca integrifolia* Ker-Gawl extract in rats," *The Songklanakarin Journal of Science and Technology*, vol. 27, no. 2, pp. 281–289, 2005.
- [27] A. A. Adeneye, O. P. Ajagbonna, F. B. O. Mojiminiyi et al., "The hypotensive mechanisms for the aqueous stem bark extract of *Musanga cecropioides* in sprague-dawley rats," *Journal of Ethnopharmacology*, vol. 106, no. 2, pp. 203–207, 2006.
- [28] C. Y. Zhang and B. K. H. Tan, "Hypotensive activity of aqueous extract of *Andrographis paniculata* in rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 23, no. 8, pp. 675–678, 1996.
- [29] N. Kamkaew, C. N. Scholfield, K. Ingkaninan et al., "*Bacopa monnieri* and its constituents is hypotensive in anesthetized rats and vasodilator in various artery types," *Journal of Ethnopharmacology*, vol. 137, no. 1, pp. 790–795, 2011.

- [30] M. C. Michel, O.-R. Brodde, and P. A. Insel, "Peripheral adrenergic receptors in hypertension," *Hypertension*, vol. 16, no. 2, pp. 107–120, 1990.
- [31] M. W. Holladay, M. J. Dart, and J. K. Lynch, "Neuronal nicotinic acetylcholine receptors as targets for drug discovery," *Journal of Medicinal Chemistry*, vol. 40, no. 26, pp. 4169–4194, 1997.
- [32] M. T. Piascik, E. E. Soltis, M. M. Piascik, and L. B. Macmillan, " $\alpha$ -adrenoceptors and vascular regulation: molecular, pharmacologic and clinical correlates," *Pharmacology and Therapeutics*, vol. 72, no. 3, pp. 215–241, 1996.
- [33] P. A. Van Zwieten, M. J. Thoolen, and P. B. Timmermans, "The hypotensive activity and side effects of methyl dopa, clonidine, and guanfacine," *Hypertension*, vol. 6, no. 5, pp. II28–II33, 1984.
- [34] A. De Jonge, P. B. Timmermans, and P. A. Van Zwieten, "Participation of cardiac presynaptic  $\alpha_2$ -adrenoceptors in the bradycardiac effects of clonidine and analogues," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 317, no. 1, pp. 8–12, 1981.
- [35] L. L. Darga, M. J. Hakim, C. P. Lucas, and B. A. Franklin, "Comparison of the effects of guanadrel sulfate and propranolol on blood pressure, functional capacity, serum lipoproteins and glucose in systemic hypertension," *American Journal of Cardiology*, vol. 67, no. 7, pp. 590–596, 1991.
- [36] T. Berg, B. W. Piercey, and J. Jensen, "Role of  $\beta_{1-3}$ -adrenoceptors in blood pressure control at rest and during tyramine-induced norepinephrine release in spontaneously hypertensive rats," *Hypertension*, vol. 55, no. 5, pp. 1224–1230, 2010.
- [37] L. R. Queen, Y. Ji, B. Xu et al., "Mechanisms underlying  $\beta_2$ -adrenoceptor-mediated nitric oxide generation by human umbilical vein endothelial cells," *Journal of Physiology*, vol. 576, no. 2, pp. 585–594, 2006.
- [38] A. Kumari, S. Sreetama, and K. P. Mohanakumar, "Atropine, a muscarinic cholinergic receptor antagonist increases serotonin, but not dopamine levels in discrete brain regions of mice," *Neuroscience Letters*, vol. 423, no. 2, pp. 100–103, 2007.
- [39] J. Gomeza, H. Shannon, E. Kostenis et al., "Pronounced pharmacologic deficits in  $M_2$  muscarinic acetylcholine receptor knockout mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 4, pp. 1692–1697, 1999.
- [40] C. M. Boulanger, K. J. Morrison, and P. M. Vanhoutte, "Mediation by  $M_3$ -muscarinic receptors of both endothelium-dependent contraction and relaxation to acetylcholine in the aorta of the spontaneously hypertensive rat," *British Journal of Pharmacology*, vol. 112, no. 2, pp. 519–524, 1994.
- [41] G. Tobin, D. Giglio, and O. Lundgren, "Muscarinic receptor subtypes in the alimentary tract," *Journal of Physiology and Pharmacology*, vol. 60, no. 1, pp. 3–21, 2009.

## Research Article

# Ginsenoside Rb1 Reduces Isoproterenol-Induced Cardiomyocytes Apoptosis *In Vitro* and *In Vivo*

Xiu-feng Wang,<sup>1</sup> Xin-jun Liu,<sup>2</sup> Qian-mei Zhou,<sup>3</sup> Jia Du,<sup>3</sup>  
Tian-ling Zhang,<sup>3</sup> Yi-yu Lu,<sup>3</sup> and Shi-bing Su<sup>3</sup>

<sup>1</sup> Fujian Academy of Traditional Chinese Medicine, Fuzhou, Fujian 350003, China

<sup>2</sup> Obstetrics & Gynecology Hospital of Fudan University, Shanghai 200090, China

<sup>3</sup> Research Center for Traditional Chinese Medicine Complexity System, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Pudong, Shanghai 201203, China

Correspondence should be addressed to Shi-bing Su; shibingsu07@163.com

Received 10 January 2013; Revised 30 September 2013; Accepted 7 October 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Xiu-feng Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Cardiomyocytes apoptosis can lead to heart failure. Conventional and alternative drugs, such as Chinese herbal remedies, have been developed to target cardiomyoblast cells apoptosis. In this study, we investigated the effects of ginsenoside Rb1 (Rb1), an active compound, which is isolated from Notoginseng and Ginseng on isoproterenol-(ISO-) induced apoptosis in rat cardiomyocytes and its mechanism *in vivo* and *in vitro*. Rb1 reduced the ISO-induced apoptosis in rat cardiomyocytes and H9c2 cells. The effect of Rb1 was significantly suppressed by H89 (inhibitor for PKA), but not by C-1 (inhibitor for PKC). Based on in-cell blot analysis, the ISO-induced PKA and PKC expressions were decreased by Rb1, which was inhibited by H89, but not by C-1. The expressions of caspase-3 and caspase-9 were decreased after treatment with both ISO and Rb1, but with no change for caspase-8. Our results indicated that Rb1 reducing ISO-induced rat cardiomyocytes apoptosis may be involved in PKA and caspase-9 pathways.

## 1. Introduction

Cardiomyocytes apoptosis is a potential mechanism in the heart disease. It has been known that stimulation of the beta-adrenergic agonists causes hypertrophy and apoptosis in cardiomyocytes [1, 2], which leads to further deterioration of cardiac function [3] and so far to an intensification of heart failure [4, 5]. Although adult cardiomyocytes are terminally differentiated and have lost their ability to divide, cardiomyocytes apoptosis may play an important role in heart disease. It can be considered a new approach to reduce or prevent inappropriate cardiac cell death in finding effective drugs as a therapeutic means of slowing down the loss of myocytes.

Recently, it was reported that there are some active compounds in Chinese herbal medicines which could inhibit cardiovascular disease-associated cell apoptosis or protect cardiomyocytes death. For example, silibinin efficiently protected beta-adrenergic agonist-induced rat neonatal cardiomyocytes injury [6, 7]. In H9c2 cardiomyocytes,

reservation decreased apoptosis, ROS production, and intracellular calcium mobilization induced by treatment with As<sub>2</sub>O<sub>3</sub> [8].

Ginsenoside Rb1 (Rb1) (Figure 1(a)) is an active compound, which is isolated from Notoginseng and Ginseng in Chinese herbal medicine. It has been reported to attenuate atherosclerosis in rats by regulating the blood lipid profile and an anti-inflammatory action [9]. Moreover, Rb1 clearly alleviated cardiac dysfunction and remodeling in the cTnT<sup>R141W</sup> transgenic mouse, attenuated cardiac hypertrophy, interstitial fibrosis, ultrastructural degeneration, and intercalated disc remodeling in dilated cardiomyopathy hearts [10], and promoted glucose-stimulated insulin secretion and survival in Min6 cells through PKA which augmented IRS2 expression to enhance insulin/IGF-1 signaling [11]. It is also resistant to anoikis and blocked Erk1/2 phosphorylation in the TKO MEFs [12], inhibited calcineurin signalling pathway in cardiomyocyte hypertrophy induced by prostaglandin

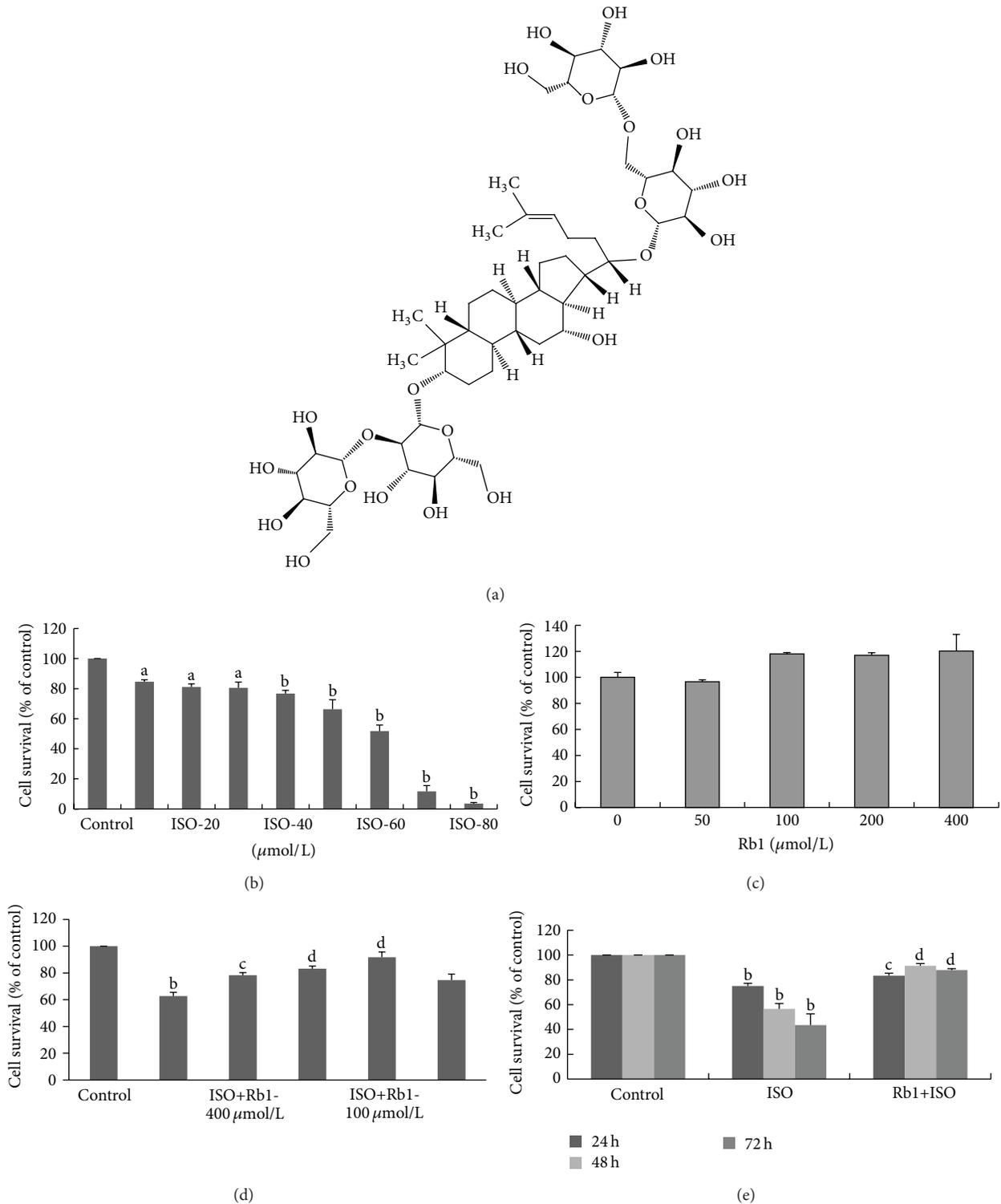


FIGURE 1: The effect of Rb1 on the survival of H9c2 cells. The cells were cultured in either presence or absence of ISO (60 μmol/L). Data was expressed as percent of control and was the mean ± SD of three replicates. (a) The molecular structure of Rb1. (b) Cell survival rates on the different concentrations of ISO treatment. (c) Cell survival rates on the different concentration of Rb1 treatment. (d) The effects of Rb1 on the different concentrations of ISO treatment. (e) Cell survival rates on the Rb1 (100 μmol/L) and/or ISO treatment for 24 h, 48 h, and 72 h. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  versus control, <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  versus ISO.

F2alpha [13], and protected cardiomyocytes against  $\text{CoCl}_2$ -induced apoptosis in neonatal rats by inhibiting mitochondria permeability transition pore opening [14]. However, whether Rb1 reduces cardiomyocytes apoptosis and what are the molecular mechanisms remain poorly understood.

In this study, we hypothesized that Rb1 is a novel agent for reducing isoproterenol-(ISO-) induced apoptosis. We aimed to examine the effects of Rb1 on ISO-induced cardiomyocytes apoptosis *in vivo* and *in vitro* and determined the underlying apoptosis-related signaling mechanisms.

## 2. Materials and Methods

**2.1. Reagents.** Rb1 was obtained from the Standardization Center of Chinese Medicines Centre (Shanghai, China). The purity of Rb1 was measured by HPLC and was determined to be about 99%. Rb1 was dissolved in deionized water to make a stock solution. Caspase-3, caspase-8, caspase-9, GAPDH, and PKA antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). ISO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Hoechst 33258 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**2.2. Animals and Treatment Protocol.** Sprague-Dawley rats, male,  $210 \pm 10$  g, were provided by the Experimental Animal Center, Shanghai University of Traditional Chinese Medicine (Shanghai, China). They were fed in standard cages and maintained on a standard laboratory diet. The rats were treated by ISO as a myocytes apoptosis model [4, 15]. Control treatment group was injected with saline (1 mL/(kg·d), i.p.,  $n = 10$ ). The treatment groups were respectively treated by Rb1 (20 mg/(kg·d), i.p.,  $n = 6$ ) for 7 days, ISO was administered intraperitoneally with one-daily injections (5 mg/(kg·d)) for the last 3 days. After 7 days of the experimental regimen, the hearts were excised under anesthesia using sodium pentobarbital (50 mg/kg, i.p.). Then, left ventricle (LV) tissues were separated up, rinsed in iced sterile saline, placed in 10% buffered formalin, and processed for TUNEL staining.

**2.3. Cell Line and Culture.** H9c2 cells, a cardiomyoblast cell line derived from embryonic rat heart tissue, were obtained from the Shanghai Biological Sciences Institutes (Shanghai, China). The cells were maintained in DMEM (Gibco, Scotland, UK) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 100 U/mL penicillin/streptomycin in a 5%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  in a humidified atmosphere.

**2.4. Cell Viability Assay.** Cell viability was assessed by MTT. Cells were seeded on 96-well plates at a density of  $5 \times 10^3$  cells per well. After 12 h, medium was changed to DMEM plus 5% fetal bovine serum with ISO (60  $\mu\text{mol/L}$ ) or ISO (60  $\mu\text{mol/L}$ ) + Rb1 (100  $\mu\text{mol/L}$ ) with or without H89 or C-1 for 24, 48, and 72 h, respectively. Rb1 was added for 40 min prior to ISO treatment. Then, cells were incubated with MTT (1 mg/mL) for 4 h. The cells viability was assessed at 490 nm absorbance using a 96-well plate reader (Biotek, VT, USA).

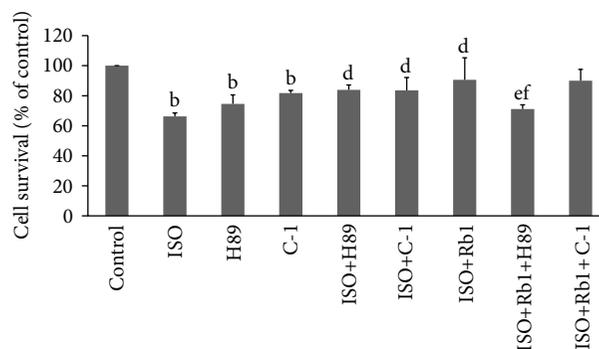


FIGURE 2: Cell survivals by the treatment of PKA and PKC inhibitors. The survival of H9c2 cells was evaluated by MTT assay after the treatments of Rb1 and/or ISO alone or combined with H89 or C-1. The tripleexperiment of results was expressed as mean  $\pm$  SD, <sup>b</sup> $P < 0.01$ , versus control; <sup>d</sup> $P < 0.01$ , versus ISO; <sup>e</sup> $P < 0.01$ , versus Rb1+ISO; <sup>f</sup> $P < 0.01$ , versus Rb1+ISO+C-1.

The viability was calculated as  $\text{viability (\%)} = \frac{(A_{490,\text{sample}} - A_{490,\text{blank}})}{(A_{490,\text{control}} - A_{490,\text{blank}})} \times 100$ .

**2.5. Flow Cytometry Analysis.** H9c2 cells were seeded in 60 mm dishes in DMEM plus 10% FBS. After 12 h, medium was changed to DMEM plus 5% FBS with ISO (60  $\mu\text{mol/L}$ ) or ISO (60  $\mu\text{mol/L}$ ) + Rb1 (100  $\mu\text{mol/L}$ ). Cells were treated without or with H89 (5  $\mu\text{mol/L}$ ) and without or with C-1 (100 nmol/L) for 48 h. Rb1 was added for 40 min prior to ISO treatment. Cells were collected after 48 h. The first stained with FITC-conjugated Annexin V for 30 min, and then stained with propidium iodide (PI) before 1 min and analyzed by FACScan (Beckman Coulter, FL, USA). The stainings were carried out using Annexin V/PI apoptosis kit (Beckman Coulter) according to the manufacture. Detection and quantification of apoptotic cells were obtained by flow cytometry analysis software (Cell Lab Quanta Analysis, Beckman Coulter).

**2.6. Hoechst 33258 Staining.** H9c2 cells ( $5 \times 10^4$ /well) were seeded in 6-well plates with cover slips and left overnight. When the cells anchored to the plates, ISO (60  $\mu\text{mol/L}$ ) and/or Rb1 (100  $\mu\text{mol/L}$ ) were added. Cells were treated without or with H89 (5  $\mu\text{mol/L}$ ) for 24 h. Rb1 was added for 40 min prior to ISO treatment. After incubation for 24 h, the cells were fixed with 1 mL of 4% paraformaldehyde for 20 min. Then, the cells were incubated in 1 mL PBS containing 10  $\mu\text{mol/L}$  Hoechst 33258 at  $37^\circ\text{C}$  for 30 min and observed using fluorescence microscopy (Olympus, Tokyo, Japan) at  $\times 400$  magnification.

**2.7. In Situ Labeling of DNA Fragments.** DNA fragmentation in the myocytes of LV tissues was detected *in situ* by using terminal deoxyribonucleotide transferase-(TdT-) mediated dUTP nick-end labeling (TUNEL) kit (Kai-ji, Nanjing, Jiangsu, China). Briefly, after incubation with proteinase K (20 mg/mL), DNA fragments in the tissues sections were labeled with 2 nmol/L biotin-conjugated dUTP and 0.1 U/mL

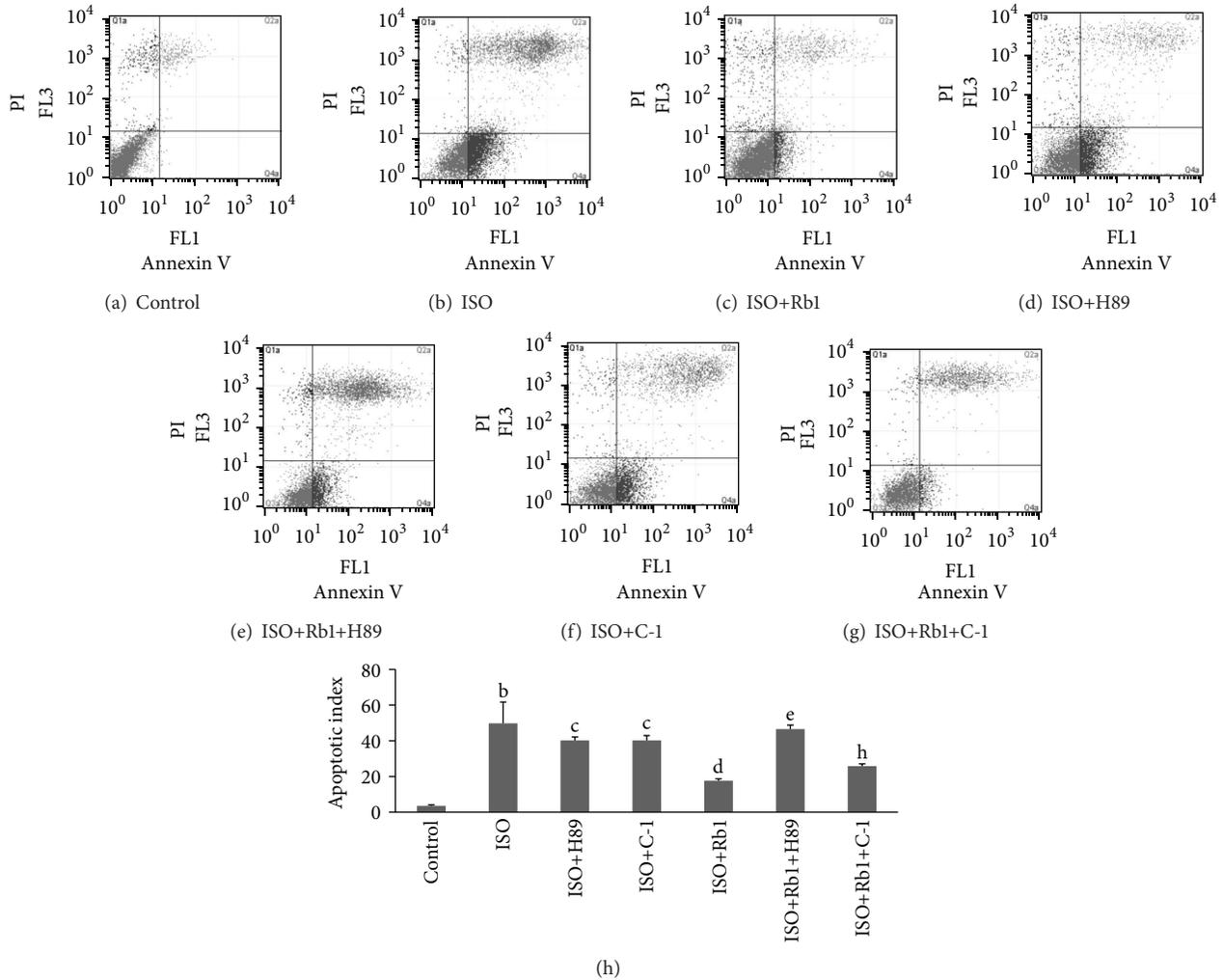


FIGURE 3: Apoptotic rates in H9c2 cells by flow cytometry assays. H9c2 cells were incubated with Annexin V-FITC and PI and analyzed in flow cytometry. (a) Control. (b)–(g) represent each treatment group. X-axis indicated the numbers of Annexin V-FITC stained cells as FL-1. The Y-axis indicated the numbers of PI strained cells as FL-3. The percentages indicated on the graph are the percent of double positive PI and annexin V-stained cells. (h) Statistical graph of annexin V-FITC/PI staining. The tripleexperiment of results was expressed as mean  $\pm$  SD, <sup>b</sup> $P < 0.01$  versus control; <sup>d</sup> $P < 0.01$ , <sup>c</sup> $P < 0.05$  versus ISO; <sup>e</sup> $P < 0.01$  versus Rb1+ISO; <sup>h</sup> $P < 0.01$  versus ISO+Rb1+C-1.

TdT at 37°C for 1 h. Nuclei exhibiting DNA fragmentation were visualized by incubation in 3,3-diamino benzidine (DAB). The sections were observed by light microscopy. The nuclei of apoptotic cells were stained dark brown. At the same magnification ( $\times 400$ ), a minimum of 10 fields with myocytes cut in cross section from each LV tissues were examined to count TUNEL-positive cardiomyocytes.

**2.8. In-Cell Western Assay.** The in-cell protein levels were determined by in-cell western assay as a previous report [16]. The cells ( $1 \times 10^4$ /well) were seeded on 96-well plate and incubated for 72 h. Then cells were incubated with vehicle, ISO (60  $\mu\text{mol/L}$ ), ISO (60  $\mu\text{mol/L}$ ) + Rb1 (100  $\mu\text{mol/L}$ ), without or with H89 (5  $\mu\text{mol/L}$ ) and without or with C-1 (100 nmol/L) for 24 h in DMEM plus 5% FBS. Rb1 was added for 40 min prior to ISO. Then the cells were immediately fixed with 4% formaldehyde for 20 min. After washing with 0.1% Triton,

cells were blocked by 10% nonfat milk for 90 min. The cells were then incubated with diluted primary antibodies PKA and PKC (1:100), caspases 3, 8 and 9 (1:200), respectively. GAPDH was added to each well at the same time as control. After being treated at 4°C overnight, the cells were then incubated with corresponding second IRDye<sup>TM</sup>700DX (red) or IRDye<sup>TM</sup>800DX (green) fluorescence antibody for 2 h. The image was obtained by Odyssey Infrared Imaging System (Licor Biosciences, NE, USA). The protein levels were calculated as the ratio of the intensity of PKA, PKC, caspase-3, caspases-8 and caspases-9 to that of GAPDH. The experiments were carried out in triplicate and repeated three times.

**2.9. Statistical Analysis.** All data were presented as mean  $\pm$  SD and were analyzed using SPSS 11.5 software. Comparisons among groups were made by an unpaired Student's *t*-test. A value of  $P < 0.05$  was considered statistically significant.

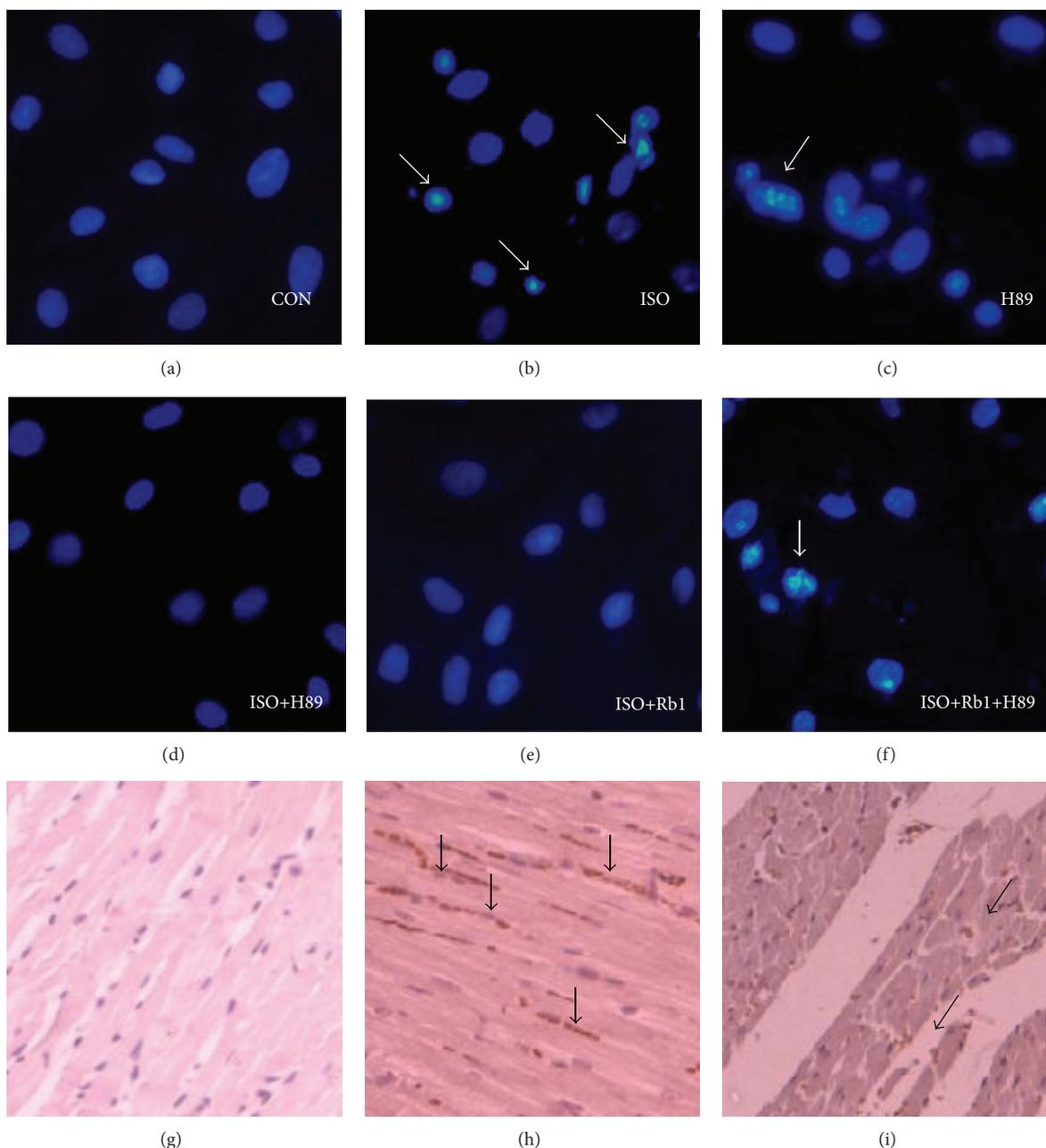


FIGURE 4: Morphologic changes in H9c2 cells and in LV tissues of rats. H9c2 cells were stained with Hoechst 33258 and the sections of LV tissue were stained with TUNEL. They were visualized under fluorescence or light microscope (magnifications:  $\times 400$ ). (a) Control cells; (b) ISO-treated H9c2 cells; (c) H89-treated H9c2 cells; (d) ISO+H89-treated H9c2 cells; (e) ISO+Rb1-treated H9c2 cells; (f) ISO+Rb1+H89-treated H9c2 cells; (g) control LV tissues in rat; (h) ISO-treated LV tissues in rat; (i) ISO+Rb1-treated LV tissues in rat. Arrows indicate apoptotic cells.

### 3. Results

#### 3.1. Rb1 Reduced ISO-Induced Cell Death in H9c2 Cells.

According to previous reports [2, 7] that ISO could induce cell death and that it was carried by  $\beta$ -adrenergic receptor ( $\beta$ -AR) in H9c2 cells, in our study, the effect of Rb1 on the survival of H9c2 cells was evaluated by MTT assay. As expected, it was shown to have an ISO concentration-dependent decrease (Figure 1(b)). Rb1 alone at 50, 100, 200, and 400  $\mu\text{mol/L}$

increased cell growth (Figure 1(c)), and Rb1 increased cell survival under ISO (60  $\mu\text{mol/L}$ , a closed to IC50 concentration) for 48 h. The best effect was 100  $\mu\text{mol/L}$  (Figure 1(d)). It markedly counteracted ISO-induced cell death and restored survival up to 91.78%. Furthermore, the effects of Rb1 on the survival of H9c2 cells were evaluated for 24 h, 48 h, and 72 h, respectively. The survival rates were 83.32% for 24 h, 91.37% for 48 h, and 87.89% for 72 h (Figure 1(e)). These results suggested that Rb1 reduced ISO-induced H9c2 cell death.

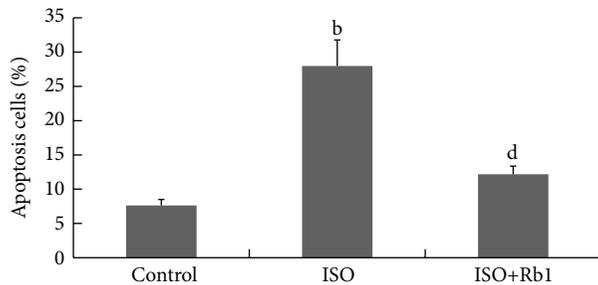


FIGURE 5: The apoptotic index of cardiomyocytes in rat. By TUNEL assay, 10 fields with myocytes cut in cross section from each LV tissue were examined to count TUNEL-positive cardiomyocytes. The apoptotic index was measured by TUNEL-positive cardiomyocytes to the total of cardiomyocytes, and results are expressed as means  $\pm$  SD, <sup>b</sup> $P < 0.01$  versus control; <sup>d</sup> $P < 0.01$  versus ISO.

**3.2. Rb1 Reduced ISO-Induced H9c2 Cell Death via PKA Pathway.** It has been reported that PKA or PKC pathway plays an important role in cardiomyocytes cells survival [7, 17, 18]. In this study, H89, a PKA inhibitor, and C-1, a PKC inhibitor were used to investigate the relationship between Rb1 effect and PKA or PKC pathway. As shown in Figure 2, the ISO-induced H9c2 cell death was significantly decreased not only by H89 but also by C-1, compared to ISO-treated cells ( $P < 0.01$ ). However, there was no significant difference between ISO+H89-treated cells and H89-treated cells ( $P > 0.05$ ), as well as ISO+C-1-treated cells and C-1-treated cells ( $P > 0.05$ ). When Rb1 was present, the ISO-induced H9c2 cell death was significantly decreased, compared to ISO-treated cells ( $P < 0.01$ ). Moreover, the ISO+Rb1-treated H9c2 cell death was significantly increased by H89 ( $P < 0.01$ ), and not by C-1 ( $P > 0.05$ ), compared to ISO+Rb1-treated H9c2 cells. Furthermore, there was significant difference between ISO+Rb1+H89-treated cells and ISO+Rb1+C-1-treated cells ( $P < 0.01$ ). These findings indicated that the Rb1 reduced ISO-induced cell death which may be mainly through the PKA pathway, rather than PKC signaling pathway.

**3.3. Rb1 Reduced ISO-Induced Apoptosis in H9c2 Cells by Flow Cytometry Assay.** To further reveal the effect of Rb1 on the apoptosis event, we next examined apoptosis on Rb1-treated H9c2 cells in response to ISO-treated by evaluating the percentage of PI and annexin-V stained cells. As shown in Figure 3, the percentages of the annexin V/PI-double positive-stained cells in ISO-treated group dramatically increased compared to control group ( $49.8 \pm 11.90\%$  versus  $3.54 \pm 0.60\%$ ,  $P < 0.01$ ) (Figures 3(a) and 3(b)). H89 and C-1 significantly decreased positive-stained cells by ISO-treated compared to only ISO-treated cells ( $40.21 \pm 1.99\%$  versus  $49.8 \pm 11.90\%$ ;  $40.23 \pm 2.69\%$  versus  $49.8 \pm 11.90\%$ ,  $P < 0.01$ ) (Figures 3(d) and 3(f)), Rb1-treated positive-stained cells were remarkably lower than ISO-treated cells ( $17.65 \pm 0.99\%$  versus  $49.8 \pm 11.90\%$ ,  $P < 0.01$ ) (Figure 3(c)). However, the ISO+Rb1-treated positive-stained cells were significantly increased by H89 ( $46.53 \pm 2.25\%$  versus  $17.65 \pm 0.99\%$ ,  $P < 0.01$ ) (Figure 3(e)), and not by C-1 ( $25.80 \pm 1.31\%$

versus  $17.65 \pm 0.99\%$ ,  $P > 0.05$ ) (Figure 3(g)), compared to ISO+Rb1-treated cells. Furthermore, there was significant difference between ISO+Rb1+C-1-treated cells and ISO+C-1-treated cells ( $25.80 \pm 1.31\%$  versus  $40.23 \pm 2.69\%$ ,  $P < 0.01$ ). These results suggested that Rb1 reduced ISO-induced H9c2 cells apoptosis via PKA pathway.

**3.4. Rb1 Reduced H9c2 Cell Apoptosis by the Morphological Observation.** To further verify the effects of Rb1 on ISO-induced apoptosis in H9c2 cells, Hoechst 33258 staining was performed. Without ISO treatment (control group), the nuclei were stained a less bright blue and the color was homogeneous (Figure 4(a)). However, when cells were treated with ISO ( $60 \mu\text{mol/L}$ ) for 24 h, the staining showed the morphological changes in the nuclear chromatin and showed that the blue emission light in apoptotic cells was much brighter than that in the control cells. The condensed chromatin and fragmented nuclei were found in many treated cells, as the classic characteristics of apoptotic cells (Figure 4(b)). In the Rb1 pretreated group, the morphological changes were not observed (Figure 4(e)). When cells were treated with H89 alone (inhibitor for PKA), the condensed chromatin and fragmented nuclei were increased (Figure 4(c)). When H89 was further added, Rb1-pretreated morphological changes were not found (Figure 4(f)). These results suggested that Rb1 reduced apoptotic cells stimulated by ISO, which can be inhibited by H89.

**3.5. Rb1 Reduced TUNEL-Positive Cardiomyocytes in Rat.** To evaluate the cardiomyocytes apoptosis *in vivo*, the sections of LV tissue were detected by TUNEL assay. It revealed only small numbers of TUNEL-positive cells in the control group (Figure 4(g)). The cardiomyocytes in the normal part were of regular shape, and counterstaining was blue. There was many TUNEL positive cardiomyocytes (brown) in ISO-treated group (Figure 4(h)). However, there was only a few TUNEL positive cardiomyocytes in Rb1-treated group (Figure 4(i)). As shown in Figure 5, the apoptotic index (the ratio of apoptotic myocytes to the total of cardiomyocytes) was significantly higher in ISO-treated group than in control group ( $P < 0.01$ ) and was significantly lower in Rb1-treated group than in ISO-treated group ( $P < 0.01$ ). These results suggested that Rb1 reduced ISO-induced cardiomyocytes apoptosis in rats.

**3.6. Rb1 Reduced ISO-Induced the Expressions of PKA Further, PKC, Caspase-3, and Caspase-9 in H9c2 Cells.** In order to clarify the effects of Rb1 on ISO-induced expressions of PKA, PKC, caspase-8, and caspase-9 in H9c2 cells, an in-cell western blot assay was carried out. As shown in Figure 6, ISO increased PKA expression compared to control ( $P < 0.01$ ). H89 and C-1 significantly decreased ISO-induced PKA expression, compared to ISO-treated cells ( $P < 0.01$ ). Rb1 prevented the expressions of PKA and PKC which was increased by ISO, compared to ISO-treated cells ( $P < 0.01$ ). Preincubation with H89 significantly decreased the beneficial effects of Rb1 on PKA expression, compared to ISO+Rb1-treated cells ( $P < 0.01$ ) (Figure 6(a)). But preincubation

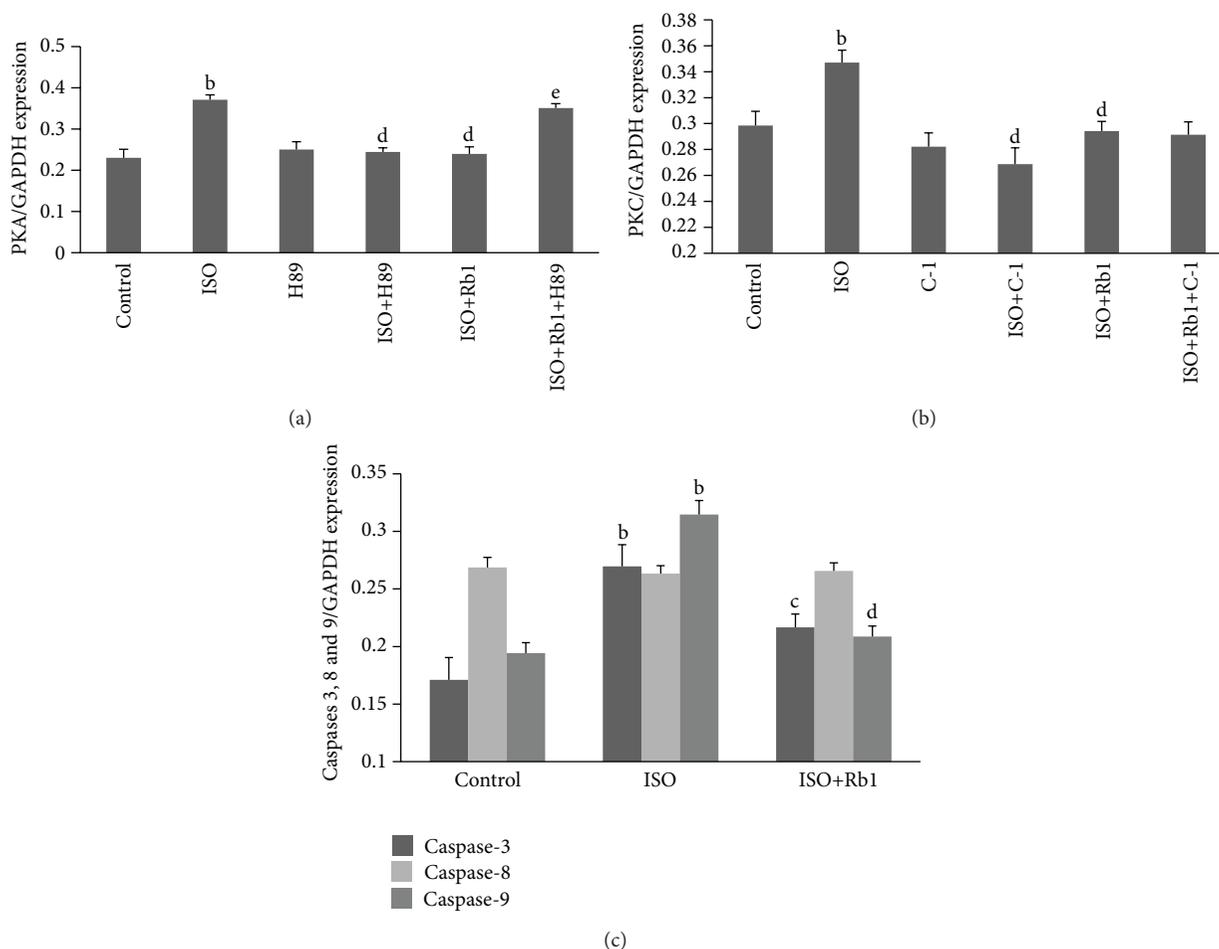


FIGURE 6: The expressions of PKA, PKC, caspase-3, caspase-8, and caspase-9. The protein expressions were assessed by in-cell Western Blot assay and Odyssey Infrared Imaging System after the treatments of Rb1 and/or ISO alone or combined H89 or C-1. The protein expressions were normalized to GAPDH and reported as percent of basal. (a) PKA expression; (b) PKC expression; (c) caspase-3, caspase-8 and caspase-9 expressions. The pictures were from the representative one of three individual experiments. <sup>b</sup> $P < 0.01$  versus control; <sup>d</sup> $P < 0.01$ , <sup>e</sup> $P < 0.05$  versus ISO; <sup>c</sup> $P < 0.05$  versus Rb1+ISO.

with C-1 did not inhibit the beneficial effects of Rb1 on PKC expression (Figure 6(b)). Moreover, the expressions of caspases 3 and 9 increased significantly in ISO-treated cells, compared to control ( $P < 0.01$ ). Rb1 effectively reduced the expressions of caspases 3 and 9 induced by ISO, compared to ISO-treated cells ( $P < 0.01$ ). But no change was seen for caspase-8 (Figure 6(c)). These results suggested that Rb1 reduced ISO-induced expressions of PKA, PKC, caspase-3, and caspase-9, but not caspase-8 in H9c2 cells.

#### 4. Discussion and Conclusions

The inhibition of cardiac apoptosis, which can lead to heart failure [19, 20], holds a promise as an effective therapeutic way for cardiovascular disease. There are many reasons for cardiac apoptosis, such as hypoxia, ischemia, and  $\beta$ -adrenergic receptor ( $\beta$ -AR) stimulation. The  $\beta$ -AR stimulation is a common reason to reduce cardiac cell survival [21, 22]. In fact, the concentration of norepinephrine gradually increased with

the aggravation of heart failure in clinical [23], different  $\beta$ -AR antagonists have been observed in clinical to treat heart failure, such as carvedilol and metoprolol [24].

Natural products are one of the most important fields of drug discovery, such as Notoginseng and Ginseng which still have popular application in traditional Chinese medicine to cardiovascular diseases. Rb1, as a compound of Notoginseng and Ginseng, has been reported to inhibit neonatal rat cardiomyocytes apoptosis [15] and protect against ischaemia/reperfusion injury [25]. However, whether Rb1 reduces cardiomyocyte death through  $\beta$ -AR-stimulated apoptosis is not disclosed.

It has been reported that ISO-induced cell death was carried out by  $\beta$ -AR in cardiomyoblast H9c2 cells [2, 7]. In this study, the effect of Rb1 on the survival of H9c2 cells was evaluated by MTT assay and showed that Rb1 significantly reduced the ISO-induced cell death. Furthermore, the anti-apoptotic effect of Rb1 was verified by TUNEL assay in the left ventricle of rats and by Hoechst 33258 staining and Flow

cytometry assays in H9c2 cells. It was suggested that Rb1 reduced ISO-induced cardiomyocytes apoptosis.

Previous reports has provided evidence that ISO stimulation have been described to induce cardiac cell apoptosis which depended on PKA pathway or PKC pathway [7, 18, 19, 26]. Rb1 protective effect was also known to be involved in cAMP/PKA [27, 28]. Our study demonstrated that Rb1 increased cell survival and reduced cell apoptosis stimulated with ISO, which can be inhibited partly by H89, a PKA inhibitor, but not by C-1, a PKC inhibitor. H89 increased cell death and induced apoptosis in Figures 2 and 4, respectively. However, the cotreatment of ISO+Rb1+H89 inhibited cell growth more significantly than H89 alone. This increased cells death by cotreatment may not be explained by the cytotoxicity of H89 alone. The induced cells inhibition by cotreatment of ISO+Rb1+H89 may be involved in PKA pathway. In addition, in-cell western blot assay showed that Rb1, in presence of ISO, decreased intracellular PKA and PKC expressions in H9c2 cells. Moreover, H89 decreased the beneficial effect of Rb1 on PKA expression, but C-1 did not inhibit this effect of Rb1 on PKC expression. Obviously, these findings indicated that Rb1 inhibited myocyte apoptosis induced by ISO, through PKA signaling, but not PKC signaling pathway.

Cysteine-dependent aspartate-specific proteases (caspase) have been demonstrated to be crucial mediators in apoptotic pathway [29]. There are two well-characterized mammalian caspase activation pathways [30, 31], including the death receptor pathway (extrinsic pathway) and the mitochondria/cytochrome *c*-mediated pathway (intrinsic pathway); in the death receptor pathway, the death signal proteins activated the initiator caspase, caspase-8, which in turn activated downstream effector caspases such as caspase-3. In the mitochondria-mediated pathway, caspase-9 can be activated, which activated the central executioner, caspase-3.

It has been also reported that caspase family proteases played an essential role in ISO-induced apoptosis [32, 33]. Concerning Rb1 infusion with experimental cerebral ischemia/reperfusion, caspase-3 was significantly reduced compared to ischemia rats [33]. In this study, we have shown that caspases 3 and 9 proteins were upregulated along with the occurrence of apoptosis in cardiomyocytes by treatment with ISO. This upregulation was effectively abrogated by the cotreatment with Rb1. However, there was no different expression for caspase-8. It was suggested that Rb1-reduced apoptosis was likely mediated by caspase-9 pathways, rather than caspase-8 pathways.

In conclusion, the present study showed that Rb1 inhibited H9c2 cardiac cells against ISO-induced apoptosis. Rb1 survival effects involved PKA signaling pathway and caspase-9 pathways. In addition to its effects *in vitro*, Rb1 reduced rat heart apoptosis cells number subjected to ISO injury.

## Conflict of Interests

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work and there is no potential

conflict of interests that include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications and registrations, and grants or other funding.

## Acknowledgments

This work was supported by the National Natural Science Funds (no. 81073134) and E-institutes of Shanghai Municipal Education Commission (no. E 03008). Xin-jun Liu is coauthor.

## References

- [1] Y. J. Geng, Y. Ishikawa, D. E. Vatner et al., "Apoptosis of cardiac myocytes in  $G\alpha$  transgenic mice," *Circulation Research*, vol. 84, no. 1, pp. 34–42, 1999.
- [2] P. Krishnamurthy, V. Subramanian, M. Singh, and K. Singh, " $\beta$ 1 integrins modulate  $\beta$ -adrenergic receptor-stimulated cardiac myocyte apoptosis and myocardial remodeling," *Hypertension*, vol. 49, no. 4, pp. 865–872, 2007.
- [3] Y. T. Jin, N. Hasebe, T. Matsusaka et al., "Magnesium attenuates isoproterenol-induced acute cardiac dysfunction and  $\beta$ -adrenergic desensitization," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 3, pp. H1593–H1599, 2007.
- [4] G. C. Fan, Q. Yuan, G. Song et al., "Small heat-shock protein Hsp20 attenuates  $\beta$ -agonist-mediated cardiac remodeling through apoptosis signal-regulating kinase 1," *Circulation Research*, vol. 99, no. 11, pp. 1233–1242, 2006.
- [5] G. Y. Oudit, M. A. Crackower, U. Eriksson et al., "Phosphoinositide 3-Kinase  $\gamma$ -Deficient Mice Are Protected From Isoproterenol-Induced Heart Failure," *Circulation*, vol. 108, no. 17, pp. 2147–2152, 2003.
- [6] B. Zhou, L. J. Wu, S. I. Tashiro, S. Onodera, F. Uchiumi, and T. Ikejima, "Silibinin protects rat cardiac myocyte from isoproterenol-induced DNA damage independent on regulation of cell cycle," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 9, pp. 1900–1905, 2006.
- [7] B. Zhou, L. J. Wu, S. I. Tashiro, S. Onodera, F. Uchiumi, and T. Ikejima, "Activation of extracellular signal-regulated kinase during silibinin-protected, isoproterenol-induced apoptosis in rat cardiac myocytes is tyrosine kinase pathway-mediated and protein kinase C-dependent," *Acta Pharmacologica Sinica*, vol. 28, no. 6, pp. 803–810, 2007.
- [8] X. Y. Zhao, G. Y. Li, Y. Liu et al., "Resveratrol protects against arsenic trioxide-induced cardiotoxicity in vitro and in vivo," *British Journal of Pharmacology*, vol. 154, no. 1, pp. 105–113, 2008.
- [9] Y. G. Zhang, H. G. Zhang, G. Y. Zhang et al., "Panax notoginseng saponins attenuate atherosclerosis in rats by regulating the blood lipid profile and an anti-inflammatory action," *Clinical and Experimental Pharmacology and Physiology*, vol. 35, no. 10, pp. 1238–1244, 2008.
- [10] H. P. Zhao, L. Dan, W. Zhang et al., "Ginsenoside-Rb1 attenuates dilated cardiomyopathy in cTnT<sup>R141W</sup> Transgenic mouse," *Journal of Pharmacological Sciences*, vol. 112, no. 2, pp. 214–222, 2010.
- [11] S. Park, I. S. Ahn, D. Y. Kwon, B. S. Ko, and W. K. Jun, "Ginsenosides Rb1 and Rg1 suppress triglyceride accumulation in 3T3-L1 adipocytes and enhance  $\beta$ -cell insulin secretion and viability in min6 cells via PKA-dependent pathways," *Bioscience*,

- Biotechnology and Biochemistry*, vol. 72, no. 11, pp. 2815–2823, 2008.
- [12] S. El-Naggar, Y. Liu, and D. C. Dean, “Mutation of the Rb1 pathway leads to overexpression of mTor, constitutive phosphorylation of Akt on serine 473, resistance to anoikis, and a block in c-Raf activation,” *Molecular and Cellular Biology*, vol. 29, no. 21, pp. 5710–5717, 2009.
- [13] Q. S. Jiang, X. N. Huang, G. Z. Yang, X. Y. Jiang, and Q. X. Zhou, “Inhibitory effect of ginsenoside Rb1 on calcineurin signal pathway in cardiomyocyte hypertrophy induced by prostaglandin F<sub>2α</sub>,” *Acta Pharmacologica Sinica*, vol. 28, no. 8, pp. 1149–1154, 2007.
- [14] H. L. Kong, Z. Q. Li, Y. J. Zhao et al., “Ginsenoside Rb1 protects cardiomyocytes against CoCl<sub>2</sub>-induced apoptosis in neonatal rats by inhibiting mitochondria permeability transition pore opening,” *Acta Pharmacologica Sinica*, vol. 31, no. 6, pp. 687–695, 2010.
- [15] A. Hu, X. Jiao, E. Gao et al., “Chronic β-adrenergic receptor stimulation induces cardiac apoptosis and aggravates myocardial ischemia/reperfusion injury by provoking inducible nitric-oxide synthase-mediated nitrate stress,” *Journal of Pharmacology and Experimental Therapeutics*, vol. 318, no. 2, pp. 469–475, 2006.
- [16] W. H. Zhou, M. R. Du, L. Dong et al., “Cyclosporin A increases expression of matrix metalloproteinase 9 and 2 and invasiveness in vitro of the first-trimester human trophoblast cells via the mitogen-activated protein kinase pathway,” *Human Reproduction*, vol. 22, no. 10, pp. 2743–2750, 2007.
- [17] B. Ding, J. I. Abe, H. Wei et al., “A positive feedback loop of phosphodiesterase 3 (PDE3) and inducible cAMP early repressor (ICER) leads to cardiomyocyte apoptosis,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 41, pp. 14771–14776, 2005.
- [18] C. Yan, B. Ding, T. Shishido et al., “Activation of extracellular signal-regulated kinase 5 reduces cardiac apoptosis and dysfunction via inhibition of a phosphodiesterase 3A/inducible cAMP early repressor feedback loop,” *Circulation Research*, vol. 100, no. 4, pp. 510–519, 2007.
- [19] V. G. Sharov, H. N. Sabbah, H. Shimoyama, A. V. Goussev, M. Lesch, and S. Goldstein, “Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure,” *American Journal of Pathology*, vol. 148, no. 1, pp. 141–149, 1996.
- [20] J. Narula, N. Haider, R. Virmani et al., “Apoptosis in myocytes in end-stage heart failure,” *New England Journal of Medicine*, vol. 335, no. 16, pp. 1182–1189, 1996.
- [21] R. Granata, L. Trovato, M. P. Gallo et al., “Growth hormone-releasing hormone promotes survival of cardiac myocytes in vitro and protects against ischaemia-reperfusion injury in rat heart,” *Cardiovascular Research*, vol. 83, no. 2, pp. 303–312, 2009.
- [22] M. Zaugg, W. Xu, E. Lucchinetti, S. A. Shafiq, N. Z. Jamali, and M. A. Q. Siddiqui, “β-Adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes,” *Circulation*, vol. 102, no. 3, pp. 344–350, 2000.
- [23] J. N. Cohn, T. B. Levine, and M. T. Olivari, “Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure,” *New England Journal of Medicine*, vol. 311, no. 13, pp. 819–823, 1984.
- [24] J. M. Cruickshank, “Beta-blockers and heart failure,” *Indian heart journal*, vol. 62, no. 2, pp. 101–110, 2010.
- [25] S. Pasupathy and S. Homer-Vanniasinkam, “Ischaemic preconditioning protects against Ischaemia/Reperfusion injury: emerging concepts,” *European Journal of Vascular and Endovascular Surgery*, vol. 29, no. 2, pp. 106–115, 2005.
- [26] H. Tomita, M. Nazmy, K. Kajimoto, G. Yehia, C. A. Molina, and J. Sadoshima, “Inducible cAMP early repressor (ICER) is a negative-feedback regulator of cardiac hypertrophy and an important mediator of cardiac myocyte apoptosis in response to β-adrenergic receptor stimulation,” *Circulation Research*, vol. 93, no. 1, pp. 12–22, 2003.
- [27] W. Liang, S. Ge, L. Yang et al., “Ginsenosides Rb1 and Rg1 promote proliferation and expression of neurotrophic factors in primary Schwann cell cultures,” *Brain Research*, vol. 1357, pp. 19–25, 2010.
- [28] J. F. Xue, Z. J. Liu, J. F. Hu, H. Chen, J. T. Zhang, and N. H. Chen, “Ginsenoside Rb1 promotes neurotransmitter release by modulating phosphorylation of synapsins through a cAMP-dependent protein kinase pathway,” *Brain Research*, vol. 1106, no. 1, pp. 91–98, 2006.
- [29] D. Lu, H. Lian, X. Zhang et al., “LMNA E82K mutation activates FAS and mitochondrial pathways of apoptosis in heart tissue specific transgenic mice,” *PLoS ONE*, vol. 5, no. 12, Article ID e15167, 2010.
- [30] B. F. Lv, C. F. Yu, Y. Y. Chen et al., “Protein tyrosine phosphatase interacting protein 51 (PTPIP51) is a novel mitochondria protein with an N-terminal mitochondrial targeting sequence and induces apoptosis,” *Apoptosis*, vol. 11, no. 9, pp. 1489–1501, 2006.
- [31] K. Saji, Y. Fukumoto, J. Suzuki, S. Fukui, J. Nawata, and H. Shimokawa, “Colchicine, a microtubule depolymerizing agent, inhibits myocardial apoptosis in rats,” *Tohoku Journal of Experimental Medicine*, vol. 213, no. 2, pp. 139–148, 2007.
- [32] W. Q. Tan, J. X. Wang, Z. Q. Lin, Y. R. Li, Y. Lin, and P. F. Li, “Novel cardiac apoptotic pathway the dephosphorylation of apoptosis repressor with caspase recruitment domain by calcineurin,” *Circulation*, vol. 118, no. 22, pp. 2268–2276, 2008.
- [33] S. N. Orlov, N. Thorin-Trescases, N. O. Dulin et al., “Activation of cAMP signaling transiently inhibits apoptosis in vascular smooth muscle cells in a site upstream of caspase-3,” *Cell Death and Differentiation*, vol. 6, no. 7, pp. 661–672, 1999.

## Review Article

# Tai Chi Chuan Exercise for Patients with Cardiovascular Disease

Ching Lan,<sup>1</sup> Ssu-Yuan Chen,<sup>1</sup> May-Kuen Wong,<sup>2</sup> and Jin Shin Lai<sup>1</sup>

<sup>1</sup> Department of Physical Medicine and Rehabilitation, National Taiwan University Hospital and National Taiwan University, College of Medicine, 7 Chung-Shan South Road, Taipei 100, Taiwan

<sup>2</sup> Department of Physical Medicine and Rehabilitation, Chang-Gung Memorial Hospital and Department of Physical Therapy, Post-Graduate Institute of Rehabilitation Science, Chang-Gung University, Taoyuan 333, Taiwan

Correspondence should be addressed to Ching Lan; [clan@ntu.edu.tw](mailto:clan@ntu.edu.tw)

Received 17 December 2012; Revised 19 August 2013; Accepted 23 September 2013

Academic Editor: Ka Kit Hui

Copyright © 2013 Ching Lan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Exercise training is the cornerstone of rehabilitation for patients with cardiovascular disease (CVD). Although high-intensity exercise has significant cardiovascular benefits, light-to-moderate intensity aerobic exercise also offers health benefits. With lower-intensity workouts, patients may be able to exercise for longer periods of time and increase the acceptance of exercise, particularly in unfit and elderly patients. Tai Chi Chuan (Tai Chi) is a traditional Chinese mind-body exercise. The exercise intensity of Tai Chi is light to moderate, depending on its training style, posture, and duration. Previous research has shown that Tai Chi enhances aerobic capacity, muscular strength, balance, and psychological well-being. Additionally, Tai Chi training has significant benefits for common cardiovascular risk factors, such as hypertension, diabetes mellitus, dyslipidemia, poor exercise capacity, endothelial dysfunction, and depression. Tai Chi is safe and effective in patients with acute myocardial infarction (AMI), coronary artery bypass grafting (CABG) surgery, congestive heart failure (HF), and stroke. In conclusion, Tai Chi has significant benefits to patients with cardiovascular disease, and it may be prescribed as an alternative exercise program for selected patients with CVD.

## 1. Introduction

Regular exercise is beneficial to cardiovascular health and longevity. The Centers for Disease Control and Prevention and the American College of Sports Medicine (ACSM) recommend a minimum of 30 minutes of moderate-intensity physical activity on most days of the week [1]. According to the National Institutes of Health-American Association of Retired Persons (NIH-AARP) Diet and Health Study [2], achievement of activity levels approximate the recommendations for moderate activity (at least 30 minutes on most days of the week) may decrease 27% of mortality in men and women. In developing countries, the fast increase of CVD mortality may partly be attributed to the decrease of physical activity. The death rate of CVD in China, for example, nearly increased 50% from 1990 to 2009, and the decrease of exercise participation played an important role in the increase of mortality. A recent investigation in 9 provinces and cities of China showed that the physical activity in 2006 declined by 27.8% in men and by 36.9% in women in comparison with those in 1997 [3].

Nonvigorous exercise training is a central focus of health promotion and is the core component of rehabilitation for patients with CVD. According to a recent meta-analysis evaluating the effect of light-to-moderate physical activity [4], 2.5 hour/week moderate-intensity activity (equivalent to 30 min exercise 5 days a week) compared with no physical activity was associated with a reduction in mortality risk of 19%, while 7 hour/week of moderate activity compared with no activity reduced the mortality risk by 24%.

Tai Chi Chuan (Tai Chi) is a Chinese traditional mind-body exercise. Although the exercise intensity of Tai Chi is low to moderate [5, 6], previous studies have shown that it offers benefits for aerobic capacity [7–9], muscular strength [10, 11], balance, and cardiovascular risk factors. Further, Tai Chi appears to be safe and effective for patients with acute myocardial infarction, coronary artery bypass grafting surgery, congestive heart failure, and stroke. From the standpoint of exercise prescription, Tai Chi is a suitable exercise for patients with CVD because it is easily accessible and of low cost and can be easily implemented in the community setting. The aim of this literature review is to provide an overview of

Tai Chi benefits on cardiovascular health and to introduce the potential application of Tai Chi for patients with CVD.

## 2. Effect of Tai Chi on Cardiovascular Risk Factors

**2.1. Hypertension.** Hypertension is a major risk factor of coronary artery disease, heart failure, stroke, and peripheral vascular disease. About 54% of stroke and 47% of ischaemic heart disease worldwide were attributable to hypertension [12]. Lowering blood pressure (BP) in hypertensive individuals significantly reduces cardiovascular morbidity and mortality. Regular aerobic exercise and lifestyle change are important for preventing and treating high blood pressure. Systemic review of randomized clinical trials indicated that aerobic exercise training leads to reductions in resting BP of 5–7 mm Hg [13] and the reductions appear to be more pronounced in hypertensive subjects [14, 15]. The American College of Sports Medicine recommends the following exercise guidelines for individuals with hypertension: (1) frequency: aerobic exercise on most, preferably all days of the week; resistance exercise 2–3 days per week; (2) intensity: moderate-intensity aerobic (i.e., 40%–60% heart rate reserve or oxygen uptake reserve) and resistance exercise (60–80% of one repetition maximum); (3) time: 30–60 minutes per day of aerobic exercise and resistance training at least one set of 8–12 repetitions for each of the major muscle groups [13].

Tai Chi is a moderate-intensity exercise program fulfilling the ACSM recommendations and thus Tai Chi may be beneficial to individuals with hypertension. In most of the Tai Chi intervention studies, 6- to 12-week training programs have been shown to lower the systolic and diastolic BP at rest or after exercise, and hypertensive patients exhibit the most favorable improvement [16–20]. The decrease of BP during submaximal exercise may lower the rate pressure product, which indicates the decrease of myocardial oxygen consumption. In a recent pooled analysis of 26 studies (11 in English, 15 in Chinese), Yeh and colleagues [21] reported positive effect of Tai Chi on BP control. In individuals with hypertension, Tai Chi training may reduce systolic BP (range: –7 to –32 mm Hg) and diastolic BP (–2.4 to –18 mm Hg). In studies for noncardiovascular populations or healthy patients, the decreases ranged from –4 to –18 mm Hg in systolic BP and from –2.3 to –7.5 mm Hg in diastolic BP.

**2.2. Diabetes Mellitus.** Type 2 diabetes mellitus (DM) is a fast growing risk factor for cardiovascular disease. Exercise is a key to lifetime management of Type 2 diabetes or impaired glucose tolerance [22–24]. The benefits of regular exercise in individuals with Type 2 DM and prediabetes include improved glucose tolerance, increased insulin sensitivity, and decreased Glycated hemoglobin (HbA<sub>1c</sub>). Additionally, exercise can help prevent the development of diabetes in patients with impaired glucose tolerance. In the Da Qing Diabetes Prevention Study for people with impaired glucose tolerance [25], lifestyle intervention groups (diet and exercise) had a 43% lower incidence of DM over the 20-year follow-up period. The Diabetes Prevention Program study in

America [26] also found that participants who lost a modest amount of weight through dietary changes and increased physical activity reduced the incidence of DM by 58%.

Several studies have shown the benefits of Tai Chi for diabetic patients. In a pilot study for 12 patients with DM, Wang [27] reported that an 8 wk Tai Chi program could decrease blood glucose. Additionally, high- and low-affinity insulin receptor numbers and low-affinity insulin receptor binding capacity were increased. In another study, Zhang and Fu [28] randomly assigned 20 female diabetic patients to Tai Chi or control group. The exercise protocol was one-hour Tai Chi per day and 5 days a week. After 14 weeks of training, the Tai Chi group had significantly lower fasting plasma glucose and glycated serum proteins and higher fasting plasma insulin compared to the control group. The results showed that Tai Chi could be used as an exercise intervention to improve diabetic control. For obese patients with diabetes, Chen and colleagues [29] reported that a 12-week of Chen Tai Chi training induced significant improvement in body mass index, triglyceride, and high-density lipoprotein cholesterol. In addition, serum malondialdehyde (oxidative stress indicator) and C-reactive protein (inflammation indicator) decreased significantly.

In diabetic patients complicated with peripheral neuropathy, Ahn and Song [30] recruited 59 diabetic patients with neuropathy and assigned them into a Tai Chi group or a control group. The Tai Chi group participated in an exercise program comprised 1 hour of Tai Chi twice a week for 12 weeks. After training, patients in the Tai Chi group showed improvement in glucose control, balance, neuropathic symptoms, and some dimensions of the quality of life compared to the control group. However, the dropout rate was high (34%) in this study.

A 12-week Tai Chi program for diabetic patients might obtain significant benefits in the quality of life. In a study reported by Liu and colleagues [31], 41 patients with diabetes were randomly assigned to Tai Chi ( $n = 20$ ) or usual care group ( $n = 21$ ). After training, the Tai Chi group revealed significant improvements in the Short Form 36-item Health Survey (SF-36) subscales of physical functioning, role physical, bodily pain, and vitality.

A recent meta-analysis conducted by Yan and colleagues [32] pooled 4 randomized controlled trials (RCT) and 5 nonrandomized controlled trials (NRCT) to assess the effect of Tai Chi in patients with Type 2 DM. The weighted mean differences from RCT were –14.82 mg/dL ( $P = 0.40$ ) for fasting blood glucose, –0.19% ( $P = 0.09$ ) for HbA<sub>1c</sub>, and –0.34 units ( $P = 0.80$ ) for homeostasis model assessment of insulin resistance (HOMA) index (indicator of insulin resistance). The weighted mean differences from NRCT were –11.22 mg/dL ( $P = 0.003$ ) for FBG and –0.41% ( $P < 0.00001$ ) for HbA<sub>1c</sub> and –0.60 units ( $P = 0.16$ ). Because most Tai Chi studies have only a small group of subjects, further large-scale randomized trials are needed to clarify the potential health effect of Tai Chi for diabetic patients.

**2.3. Dyslipidemia.** Regular exercise may ameliorate the trend toward abnormal blood lipid profile. A meta-analysis of 31 randomized controlled trials with exercise training reported

a significant decrease in total cholesterol, low density lipoprotein cholesterol (LDL-C), and triglyceride (TG) and an increase in high-density lipoprotein cholesterol (HDL-C) [33].

Several studies have shown beneficial effects of Tai Chi training on lipid profile. In a study reported by Tsai and colleagues [19], 88 patients with Stage I hypertension were randomly assigned to a Tai Chi or a sedentary control group. After 12 weeks of classical Yang Tai Chi training, total cholesterol, TG, and LDL-C concentration decreased by 15.2, 23.8, and 19.7 mg/dL, respectively, and HDL-C increased 4.7 mg/dL. In healthy elderly individuals, however, Thomas and colleagues [34] reported no significant change in total cholesterol, TG, LDL-C, and HDL-C after 12 months of Tai Chi training. In another study, Zhang and Fu [28] randomly assigned 20 female diabetic patients to Tai Chi or control group. After 14 weeks of training, the Tai Chi group only showed significantly lower TG compared to the control group, whereas there were no significant differences in total cholesterol, HDL-C, and LDL-C. All these studies were not specifically focused on dyslipidemic patients, and difference among results may be attributed to differences in study design, baseline lipid concentrations, training amount and intensity, changes in body composition, or the adjunctive interventions such as diet or lipid-lowering agents.

In a recent study, Lan and colleagues [35] enrolled 70 severe dyslipidemic patients to attend a one-year Yang Tai Chi training program. The inclusion criteria were as follows: (1) patients' initial level of total cholesterol > 300 mg/dL and/or TG > 500 mg/dL prior to medical treatment, (2) patients who had received lipid-lowering medication and diet therapy for at least 6 months, but the lipid profile remained abnormal (total cholesterol > 200 mg/dL or TG > 150 mg/dL). After training, the Tai Chi group showed a significant decrease of 26.3% in TG (From  $224.5 \pm 216.5$  to  $165.9 \pm 147.8$  mg/dL), 7.3% in total cholesterol (from  $228.0 \pm 41.0$  to  $211.4 \pm 46.5$  mg/dL), and 11.9% in LDL-C (from  $134.3 \pm 40.3$  to  $118.3 \pm 41.3$  mg/dL), whereas the HDL-C did not increase significantly. In addition, the Tai Chi group also showed a significant decrease in fasting insulin, HOMA index, and high-sensitivity C-reactive protein. Conversely, the usual care group showed no significant improvement in these cardiovascular risk factors.

**2.4. Low Exercise Capacity.** Low exercise capacity is a strong predictor of cardiac and all-cause mortality. Myers and colleagues [36] reported that the peak exercise capacity measured in metabolic equivalents (MET) was the strongest predictor of the risk of death. Each 1-MET increase in exercise capacity conferred a 12% improvement in survival. Kavanagh and colleagues [37] have reported that the risk of death for coronary patients had inverse relationship with the values of peak oxygen uptake ( $\dot{V}O_{2peak}$ ) during exercise testing.  $\dot{V}O_{2peak}$  values of 15, 15–22, and >22 mL·kg<sup>-1</sup>·min<sup>-1</sup> yielded respective multivariate adjusted hazard ratios of 1.00, 0.62, and 0.39 of cardiac death, respectively.

Regular Tai Chi training for older individuals may improve aerobic capacity. In a cross-sectional study, Lan and colleagues [7] reported that elderly Tai Chi practitioners

displayed 18–19% greater  $\dot{V}O_{2peak}$  than their sedentary counterpart. In a 5-year follow-up study, Lan and colleagues [38] reported that regular Tai Chi training attenuated the age-related decline in  $\dot{V}O_{2peak}$  for nearly 40% compared with the control group. Entering a Tai Chi program can also improve the aerobic capacity for sedentary elderly individuals. After one year of Tai Chi training, elderly participants showed an increase of 16.1% and 21.3% in  $\dot{V}O_{2peak}$  in men and women, respectively [8].

A meta-analysis on 14 studies conducted by Taylor-Piliae [39] reported that Tai Chi was effective in improving aerobic capacity. Large significant effects of Tai Chi on aerobic capacity were found for subjects enrolled in the cross-sectional studies, in both genders, among adults  $\geq 55$  years old and when comparing sedentary subjects with those in Tai Chi exercise groups. In a recent meta-analysis study [40], however, the existing evidence does not indicate that Tai Chi is an effective way of increasing aerobic capacity. This study pooled 5 randomized studies and included 124 Tai Chi participants, and the training only included 5 to 15 Tai Chi movements. Most of the training protocols were 12-week “Tai Chi calisthenics,” and the exercise intensity appeared significantly lower than classical Tai Chi. Classical Tai Chi consists of 108 movements and it takes long time to learn and practice. Lan and colleagues [5] reported that the average HR during classical Yang Tai Chi practice was 58% of the heart rate reserve (HRR) and the oxygen uptake was 55% of the peak oxygen uptake. Tai Chi participants usually need 12 weeks of intensive training (with an exercise frequency 5–7 times per week) to familiarize all movements. During the familiarization phase, the exercise intensity and amount of training are inconsistent. If the goal of Tai Chi training is to increase aerobic capacity, a classical Tai Chi program for at least 6 months may be more appropriate than short-term Tai Chi-like calisthenics.

**2.5. Endothelial Dysfunction.** Nitric oxide (NO) is an endothelium-dependent vasodilator and plays an important role in the vasodilatory response during exercise. Lack of exercise is associated with endothelial dysfunction and atherosclerosis due to low shear stress status. Low shear stress to the vessel wall predisposes to endothelial proliferative status and may lead to the pathogenesis of atherosclerosis.

Regular practice of Tai Chi may enhance endothelium-dependent dilation in skin vasculature of older individuals. Wang and colleagues [41] have reported that older Tai Chi practitioners displayed a higher skin blood flow and level of plasma NO metabolite than sedentary subjects at rest and after maximal exercise. In addition, Tai Chi subjects had higher arterial blood flow and acetylcholine-induced cutaneous perfusion than the sedentary controls.

Tai Chi training also has benefits to microcirculation. Wang and colleagues [42] measured skin blood flow and vascular conductance in elderly men before and after a maximal exercise by using impedance plethysmography. Additionally, different doses of 1% acetylcholine and 1% sodium nitroprusside were iontophoretically applied to the skin of subjects' lower legs, and cutaneous microvascular perfusion responses were determined by laser Doppler measurements.

In comparison with older individuals with sedentary life, older Tai Chi participants had higher lower leg arterial blood flow (LABF), LABF in response to reactive hyperemia, and lower leg venous capacity, tone, and blood flow. Additionally, the older Tai Chi group displayed similar arterial and venous hemodynamic variables to the younger sedentary group. The older Tai Chi group showed a higher ACh-induced cutaneous perfusion and a higher ratio of ACh- to SNP-induced cutaneous perfusion than those of the older sedentary group. The results showed that regular practice of Tai Chi is associated with enhanced endothelium-dependent dilation in the skin vasculature of older individuals. Moreover, Tai Chi training may delay the age-related decline of venous compliance and hyperemic arterial response.

**2.6. Depression.** Depression and depressive symptoms are prevalent in patients with coronary heart disease. Depression in patients with AMI showed 4–6-fold increase in risk of death than in patients with no depression [43, 44]. Major depression following AMI runs a chronic course if untreated, but the prevalence significantly decreased following exercise training [45]. Depressed coronary patients who completed rehabilitation had lower mortality compared with those who did not complete training (8% versus 30%).

Jimenez and colleagues [46] reviewed 35 Tai Chi intervention articles and reported that Tai Chi training could improve psychological function. In those studies, 9 out of 11 studies confirmed significant improvements in mood and depressive symptoms; 7 out of 8 studies showed reduction in anger and tension; 6 out of 10 studies displayed improvements in anxiety reduction.

In a recent study, Yeung and colleagues [47] randomized 39 patients with major depressive disorder into a 12 wk Tai Chi intervention or a wait-listed control group. The results showed trends toward improvement in the Tai Chi intervention group, compared with the control group, in positive treatment-response rate (24% versus 0%) and remission rate (19% versus 0%).

Taylor-Piliae and colleagues [48] have applied a 60-minute Tai Chi program (3 times per week for 12 weeks) to 39 subjects with cardiovascular risk factors. Improvement in mood state, reduction in anxiety, anger-tension, and perceived stress were found after training. Tai Chi training also benefits psychological function for patients with heart failure. In a recent study, Yeh and colleagues [49] enrolled 100 outpatients with systolic heart failure (New York Heart Association, NYHA classes I–III) and randomly assigned them to a 12-week Tai Chi exercise group or time-matched education group. After training, patients in the Tai Chi group showed greater improvements in quality of life, exercise self-efficacy, and mood than the controls.

### 3. Application of Tai Chi in Cardiovascular Disease

Exercise is a major component of rehabilitation for patients with cardiovascular disease. The benefits of cardiac rehabilitation (CR) exercise training include exercise tolerance

enhancement, amelioration of CVD risk factors, improvement of psychological well-being, and reduction of mortality [50]. Cardiac rehabilitation usually begins during hospitalization (phase I), followed by supervised outpatient program lasting 3–6 months (phase II), and continues in a lifetime maintenance stage in minimally supervised or unsupervised setting (phase III). Tai Chi can be prescribed as an alternative exercise training program for patients who need cardiac rehabilitation. In a recent cross-sectional study, Taylor-Piliae and colleagues [51] evaluated 51 patients who participated in a CR program. Twenty-three patients attended a group-based Wu Tai Chi class plus CR, while 28 subjects attended conventional CR only. Subjects attending Tai Chi plus CR showed better balance, perceived physical health, and Tai Chi self-efficacy compared to those attending CR only. The results suggest that Tai Chi can be easily implemented in the community setting or in CR facility and may offer additional exercise options for cardiac patients.

**3.1. Coronary Artery Disease.** Acute myocardial infarction is the most common cause of mortality in patients with CVD but exercise significantly reduces the mortality rate of patients with AMI. In a recent Cochrane review [52] involving 47 studies randomizing 10,794 patients with AMI to exercise-based cardiac rehabilitation or usual care, patients receiving exercise training reduced the risk for total mortality by 13%, the risk for cardiovascular mortality by 26%, and the risk for hospital admissions by 31%. Patients recovering from AMI are recommended to receive cardiac rehabilitation services; however, many patients feel inconvenient to attend CR courses. Tai Chi is easily accessible and can be practiced individually or in group settings. Channer and colleagues [16] randomly assigned 126 patients with AMI to a Tai Chi, an aerobic exercise, or a nonexercise support group. The Tai Chi group and the aerobic exercise group participated in an 8 wk training program, attended twice weekly for three weeks and then weekly for a further five weeks. The results of this study showed that Tai Chi was effective for reducing systolic and diastolic BP and was safe for patients after AMI.

Lan and colleagues [9] applied Tai Chi in the treatment of patients after CABG and found improvement of aerobic capacity. Patients with CABG participated in a 12-month classical Yang Tai Chi program 3 times weekly as a Phase III CR program. After training, the Tai Chi group showed a significant improvement of oxygen uptake at the peak exercise and the ventilatory threshold. At the peak exercise, the Tai Chi group showed 10.3% increase in  $\dot{V}O_2$  (from  $26.2 \pm 4.4$  to  $28.9 \pm 5.0$  mL·kg<sup>-1</sup>·min<sup>-1</sup>) and 11.8% increase in peak work rate, while the control group did not show any improvement. At the ventilatory threshold, the Tai Chi group increased 17.6% in  $\dot{V}O_2$  while the control group did not display significant change. It should be noted that even a small increase in  $\dot{V}O_2$  at the ventilatory threshold improves the functional level in activities of daily living.

**3.2. Congestive Heart Failure.** Congestive heart failure (HF) is characterized by the inability of the heart to deliver sufficient oxygenated blood to tissue. Exercise training improves

TABLE 1: Effect of Tai Chi in patients with heart failure.

Author	Design	Patients	Intervention	Outcomes
Yeh et al. (2004) [54]	RCT (randomized controlled trial)	30 patients with HF 64 ± 13 y/o	Tai Chi group ( <i>n</i> = 15): 12-week Tai Chi exercise, twice a week for 16 weeks Usual care group ( <i>n</i> = 15): pharmacologic therapy, diet, and exercise counseling	Tai Chi group showed improved quality of life scores, increased 6-min walking distance, and decreased serum B-type natriuretic peptide levels compared with patients in the control group
Barrow et al. (2007) [53]	RCT	52 patients with HF 68.9 y/o (NYHA classes II-III)	Tai Chi group ( <i>n</i> = 32): 16-week Tai Chi exercise, 1-hour twice weekly Medical care group ( <i>n</i> = 33): no exercise	Tai Chi group had an improvement in symptom scores of heart failure and depression scores compared with those patients in the control group
Yeh et al. (2008) [55]	RCT	18 patients with HF 59 ± 14 y/o (NYHA classes I-III, mean EF: 24 ± 8%)	Tai Chi group ( <i>n</i> = 8): 12-week Tai Chi exercise, 1-hour twice weekly Usual care group ( <i>n</i> = 10): pharmacologic therapy and dietary and exercise counseling	ECG-based sleep spectrogram showed that Tai Chi group had a significant increase in high-frequency coupling and significant reduction in low-frequency coupling, which indicated improved sleep stability and better disease-specific quality of life
Yeh et al. (2011) [49]	RCT	100 patients with systolic HF 67 ± 11 y/o (NYHA classes I-III, mean EF: 29 ± 8%)	Tai Chi group ( <i>n</i> = 50): 12-week Tai Chi exercise program Control group ( <i>n</i> = 50): time-matched education	No significant changes in 6-minute walking distance and peak oxygen uptake Tai chi group had greater improvements in quality of life, exercise self efficacy, mood, and total mood disturbance
Caminiti et al. (2011) [56]	RCT	60 HF patients 73.8 ± 6 y/o, M/F 51/9,	Combined training group (CT, <i>n</i> = 30): Tai Chi + endurance training Endurance training group (ET, <i>n</i> = 30): endurance training only All patients performed 4 sessions of exercise per week for 12 weeks	6-minute walking increased in both groups Systolic BP and BNP decreased in the CT group compared to ET CT group had a greater improvement in physical perception and peak torque of knee extensor compared to ET group
Yeh et al. (2013) [57]	RCT	16 HF patients with preserved ejection fraction 66 ± 12 y/o,	Tai Chi group ( <i>n</i> = 8): 12-week Tai Chi exercise program Aerobic exercise group ( <i>n</i> = 8)	Change in peak oxygen uptake was similar between groups 6-minute walk distance increased more with Tai Chi Depression scores improved more with Tai Chi Both groups had improved Minnesota Living With Heart Failure scores and self-efficacy
Redwine et al. (2012) [60]	Non-RCT	28 HF patients (NYHA class II) 67.0 ± 11.9 y/o	Tai Chi group ( <i>n</i> = 16): 12-week Yang Tai Chi short form 60 minutes twice per week Usual care control group ( <i>n</i> = 12)	Tai Chi group reduced total depression scores and somatic/affective symptoms of depression compared to usual care patients

functional capacity and symptoms in patients with HF, and the increase in exercise tolerance may be attributed to increased skeletal muscle oxidative enzymes and mitochondrial density. Previous studies have shown that low-intensity Tai Chi training has benefits to patients with HF (Table 1). In a study by Barrow and associates [53], 52 patients with HF (NYHA classes II-III) were randomly assigned to either a Tai Chi group or a standard medical care group. The Tai Chi group practiced Tai Chi twice a week for 16 weeks. After training, the Tai Chi group did not show significant increase in exercise tolerance but had improvement in symptom scores

of heart failure and depression scores compared with the control group.

Yeh and colleagues [54, 55] reported that a 12-week Tai Chi training in patients with HF revealed improvement in quality of life, sleep stability, 6-minute walking distance, and decreased serum B-type natriuretic peptide (BNP). BNP is produced by ventricular cardiomyocytes and correlates with left ventricular dysfunction. In a recent study, Yeh and colleagues [49] randomly assigned 100 patients with systolic HF to a Tai Chi group or a control group. Tai Chi participants practiced 5 basic simplified Yang Tai Chi

TABLE 2: Effect of Tai Chi in patients with stroke.

Author	Design	Patients	Intervention	Outcomes
Hart et al. (2004) [62]	RCT (randomized controlled trial)	18 men average of 27 months after the onset of the stroke	Tai Chi group ( $n = 9$ ): Tai Chi for 1 h twice weekly Control group ( $n = 9$ ): group exercises focusing on improvement of balance	Tai Chi group improved in general functioning and social functioning but did not exhibit changes in balance or speed of walking Control group improved in balance, speed of walking, climbing stairs, and the Up and Go Test but showed no changes in general and social functioning
Au-Yeung et al. (2009) [63]	RCT	136 men 6 months after stroke	Tai Chi group ( $n = 56$ ): 12 weeks of short-form Tai Chi (Sun style). 1 hour of group practice weekly; 3 hours of self-practice per week Control group ( $n = 52$ ), breathing and stretching exercises; active mobilization of muscles and joints of the limbs and trunk	Tai Chi group showed greater COG excursion amplitude in leaning forward, backward, and toward the affected and nonaffected sides as well as faster reaction time in moving the COG toward the nonaffected side The Tai Chi group demonstrated better reliance on vestibular integration for balance control
Wang et al. (2010) [64]	RCT	34 elderly patients after stroke	Tai Chi group ( $n = 17$ ): group sessions once a week for 12 weeks Rehabilitation group ( $n = 17$ ): conventional rehabilitation program	No significant effects of interaction between group and time in the time courses of P300 amplitudes and latencies Significant time-by-group interactions for Sleep Quality, general health total score, anxiety/insomnia score, and depression score
Taylor-Piliae and Coull (2012) [65]	RCT	28 subjects aged $69 \pm 11$ years 3 months after stroke	Tai Chi group ( $n = 13$ ): Yang style 24-posture short-form Tai Chi exercise 150 min/week for 12 weeks Usual care group ( $n = 12$ ): weekly phone calls along with written materials for participating in community-based physical activity	No falls or other adverse events The changes in balance, endurance, and quality of life scores were in favor of the Tai Chi intervention The changes in overall physical functioning, strength, and gait speed were greater for usual care subjects

movements twice weekly, while the control group participated in a HF education program. After 12 weeks of training, the Tai Chi group showed greater improvements in quality of life, exercise self-efficacy, and mood. For patients with HF, low-intensity exercise such as simplified Tai Chi may increase the acceptance. Interval training protocol by using selected Tai Chi movements is suitable for HF patients with very low endurance.

Caminiti and colleagues enrolled 60 HF patients and randomly assigned them into a combined training group (CT) performing Tai Chi plus endurance training and an endurance training group (ET) [56]. After 12 weeks of training, 6-minute walking distance increased in both groups with significant between-groups differences. Systolic BP and BNP decreased in the CT group compared to the ET group. The Tai Chi group had a greater significant improvement in physical perception and peak torque of knee extensor compared to the ET group.

In patients with heart failure with preserved ejection fraction (HFPEF), Yeh and colleagues [57] randomly assigned 16 subjects to 12-week Tai Chi or aerobic exercise. Change in  $\dot{V}O_{2peak}$  was the same between groups, but 6-minute walking distance increased more after Tai Chi training, which implied

improvement in exercise endurance at submaximal workload. Both groups had improved Minnesota Living With Heart Failure scores and self-efficacy, but the Tai Chi group showed a decrease in depression scores in contrast to an increase in the aerobic exercise group. Overall, the Tai Chi group displayed similar improvement as the aerobic exercise group despite a lower training workload.

In a recent meta-analysis study, Pan and colleagues [58] pooled data from four randomized controlled trials ( $n = 242$ ). The results found that Tai Chi significantly improved quality of life but is not associated with significant reduction in BNP, systolic/diastolic blood pressure, improved 6-minute walking distance, or peak oxygen uptake. Further larger randomized controlled trials are needed to prove the beneficial effects of Tai Chi to HF patients.

Depressive disorders are prevalent in patients with heart failure. Patients with depression are associated with increased mortality, clinical events, and hospitalization [59]. Depression-related somatic symptoms such as fatigue and sleep disturbances may lead to physical inactivity and create a spiraling decline in physical and cardiac function. In a recent study, Redwine and colleagues [60] assigned patients with HF to a 12-week Tai Chi training group ( $n = 16$ ) or a usual care

group ( $n = 12$ ). After training, the Tai Chi group showed a reduction in somatic symptoms of depression but not in cognitive symptoms of depression.

**3.3. Stroke.** Stroke results in a significant decrease in quality of life, which is determined not only by the neurological deficits but also by impairment of cognitive function. In a recent meta-analysis study, Stoller and colleagues [61] reported that stroke patients benefited from exercise by improving peak oxygen uptake and walking distance. Stroke patients usually have impaired balance and motor function, thus Tai Chi exercise may have potential benefits in rehabilitation. Studies of Tai Chi in the treatment of stroke are summarized in Table 2.

Hart and colleagues [62] enrolled 18 community-dwelling stroke patients and assigned them to a Tai Chi group or a control group. The study group received Tai Chi one hour twice weekly for 12 weeks, while the control group received conventional physical therapy. After training, the Tai Chi group showed improvement in social and general functioning whereas the control group showed improvement in balance and speed of walking. The results implied that physical therapy should be served as a main treatment program for stroke patients but Tai Chi can be used as an alternative exercise program.

Au-Yeung and colleagues [63] randomly assigned 136 stroke patients to a Tai Chi group or a control group practicing general exercises. The Tai Chi group practiced 12 short-form Tai Chi for 12 weeks. After training, the Tai Chi group showed greater excursion in the center of gravity (COG) amplitude in leaning forward, backward, and toward the affected and nonaffected sides as well as faster reaction time in moving the COG toward the nonaffected side. The result indicated that 12 weeks of Tai Chi training improved the standing balance for stroke patients.

Wang and colleagues [64] randomly assigned 34 stroke patients to Tai Chi exercise or conventional rehabilitation in group sessions once a week for 12 weeks. After training, significant time-by-group interactions were found for sleep quality, general health score, anxiety/insomnia score, and depression score. The results implied that Tai Chi exercise is beneficial to cognitive function in stroke patients.

In a recent study, Taylor-Piliae and Coull [65] recruited 28 stroke patients to participate in a community-based Yang Tai Chi training program. Patients practiced Tai Chi exercise  $\geq 150$  minutes/week. The results showed good satisfaction, and the adherence rates were high ( $\geq 92\%$ ). There were no falls or other adverse events. Tai Chi appears to be safe and can be considered as a community-based exercise program for stroke patients.

#### 4. Conclusion

Tai Chi exercise may promote cardiovascular health and can be considered as an alternative exercise program for patients with CVD. Previous studies prove that Tai Chi is safe and effective for patients with acute myocardial infarction, coronary artery bypass grafting surgery, congestive heart

failure, and stroke. In addition, Tai Chi has benefits to cardiovascular risk factors, such as hypertension, diabetes, dyslipidemia, poor exercise capacity, endothelial dysfunction, and depression. However, the study design and training protocols among Tai Chi studies vary significantly, and hence the results are difficult to compare. In future research, large-scale randomized controlled trials using standardized training protocols should be performed in accordance with the guidelines of exercise prescription for patients with cardiovascular disease.

#### References

- [1] R. R. Pate, M. Pratt, S. N. Blair et al., "Physical activity and public health: a recommendation from the Centers for Disease Control and Prevention and the American College of Sports Medicine," *Journal of the American Medical Association*, vol. 273, no. 5, pp. 402–407, 1995.
- [2] M. F. Leitzmann, Y. Park, A. Blair et al., "Physical activity recommendations and decreased risk of mortality," *Archives of Internal Medicine*, vol. 167, no. 22, pp. 2453–2460, 2007.
- [3] S. S. Hu, L. Z. Kong, R. L. Gao et al., "Outline of the report on cardiovascular disease in China, 2010," *Biomedical and Environmental Sciences*, vol. 25, no. 3, pp. 251–256.
- [4] J. Woodcock, O. H. Franco, N. Orsini, and I. Roberts, "Non-vigorous physical activity and all-cause mortality: systematic review and meta-analysis of cohort studies," *International Journal of Epidemiology*, vol. 40, no. 1, pp. 121–138, 2011.
- [5] C. Lan, S.-Y. Chen, J.-S. Lai, and M.-K. Wong, "Heart rate responses and oxygen consumption during Tai Chi Chuan practice," *American Journal of Chinese Medicine*, vol. 29, no. 3–4, pp. 403–410, 2001.
- [6] C. Lan, S.-Y. Chen, and J.-S. Lai, "Relative exercise intensity of Tai Chi Chuan is similar in different ages and gender," *American Journal of Chinese Medicine*, vol. 32, no. 1, pp. 151–160, 2004.
- [7] C. Lan, J.-S. Lai, M.-K. Wong, and M.-L. Yu, "Cardiorespiratory function, flexibility, and body composition among geriatric Tai Chi Chuan practitioners," *Archives of Physical Medicine and Rehabilitation*, vol. 77, no. 6, pp. 612–616, 1996.
- [8] C. Lan, J.-S. Lai, S.-Y. Chen, and M.-K. Wong, "12-month Tai Chi training in the elderly: its effect on health fitness," *Medicine and Science in Sports and Exercise*, vol. 30, no. 3, pp. 345–351, 1998.
- [9] C. Lan, S.-Y. Chen, J.-S. Lai, and M.-K. Wong, "The effect of Tai Chi on cardiorespiratory function in patients with coronary artery bypass surgery," *Medicine and Science in Sports and Exercise*, vol. 31, no. 5, pp. 634–638, 1999.
- [10] C. Lan, J.-S. Lai, S.-Y. Chen, and M.-K. Wong, "Tai Chi Chuan to improve muscular strength and endurance in elderly individuals: a pilot study," *Archives of Physical Medicine and Rehabilitation*, vol. 81, no. 5, pp. 604–607, 2000.
- [11] G. Wu, F. Zhao, X. Zhou, and L. Wei, "Improvement of isokinetic knee extensor strength and reduction of postural sway in the elderly from long-term Tai Chi exercise," *Archives of Physical Medicine and Rehabilitation*, vol. 83, no. 10, pp. 1364–1369, 2002.
- [12] C. M. Lawes, S. V. Hoorn, and A. Rodgers, "Global burden of blood-pressure-related disease, 2001," *The Lancet*, vol. 371, no. 9623, pp. 1513–1518, 2008.
- [13] L. S. Pescatello, B. A. Franklin, R. Fagard, W. B. Farquhar, G. A. Kelley, and C. A. Ray, "American College of Sports Medicine

- position stand. Exercise and hypertension," *Medicine and Science in Sports and Exercise*, vol. 36, no. 3, pp. 533–553, 2004.
- [14] S. P. Whelton, A. Chin, X. Xin, and J. He, "Effect of aerobic exercise on blood pressure: a meta-analysis of randomized, controlled trials," *Annals of Internal Medicine*, vol. 136, no. 7, pp. 493–503, 2002.
- [15] R. H. Fagard, "Exercise characteristics and the blood pressure response to dynamic physical training," *Medicine and Science in Sports and Exercise*, vol. 33, no. 6, supplement, pp. S484–S492, 2001.
- [16] K. S. Channer, D. Barrow, R. Barrow, M. Osborne, and G. Ives, "Changes in haemodynamic parameters following Tai Chi Chuan and aerobic exercise in patients recovering from acute myocardial infarction," *Postgraduate Medical Journal*, vol. 72, no. 848, pp. 349–351, 1996.
- [17] R. E. Taylor-Piliae, W. L. Haskell, and E. Sivarajan Froelicher, "Hemodynamic responses to a community-based Tai Chi exercise intervention in ethnic Chinese adults with cardiovascular disease risk factors," *European Journal of Cardiovascular Nursing*, vol. 5, no. 2, pp. 165–174, 2006.
- [18] E. W. Thornton, K. S. Sykes, and W. K. Tang, "Health benefits of Tai Chi exercise: improved balance and blood pressure in middle-aged women," *Health Promotion International*, vol. 19, no. 1, pp. 33–38, 2004.
- [19] J.-C. Tsai, W.-H. Wang, P. Chan et al., "The beneficial effects of Tai Chi Chuan on blood pressure and lipid profile and anxiety status in a randomized controlled trial," *Journal of Alternative and Complementary Medicine*, vol. 9, no. 5, pp. 747–754, 2003.
- [20] D. R. Young, L. J. Appel, S. Jee, and E. R. Miller III, "The effects of aerobic exercise and T'ai Chi on blood pressure in older people: results of a randomized trial," *Journal of the American Geriatrics Society*, vol. 47, no. 3, pp. 277–284, 1999.
- [21] G. Y. Yeh, C. Wang, P. M. Wayne, and R. S. Phillips, "The effect of Tai Chi exercise on blood pressure: a systematic review," *Preventive Cardiology*, vol. 11, no. 2, pp. 82–89, 2008.
- [22] J. Tuomilehto, J. Lindström, J. G. Eriksson et al., "Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance," *The New England Journal of Medicine*, vol. 344, no. 18, pp. 1343–1350, 2001.
- [23] W. C. Knowler, E. Barrett-Connor, S. E. Fowler et al., "Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin," *The New England Journal of Medicine*, vol. 346, no. 6, pp. 393–403, 2002.
- [24] J. Lindström, P. Ilanne-Parikka, M. Peltonen et al., "Sustained reduction in the incidence of type 2 diabetes by lifestyle intervention: follow-up of the Finnish Diabetes Prevention Study," *The Lancet*, vol. 368, no. 9548, pp. 1673–1679, 2006.
- [25] G. Li, P. Zhang, J. Wang et al., "The long-term effect of lifestyle interventions to prevent diabetes in the China Da Qing Diabetes Prevention Study: a 20-year follow-up study," *The Lancet*, vol. 371, no. 9626, pp. 1783–1789, 2008.
- [26] W. C. Knowler, E. Barrett-Connor, S. E. Fowler et al., "Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin," *The New England Journal of Medicine*, vol. 346, no. 6, pp. 393–403, 2002.
- [27] J. H. Wang, "Effects of Tai Chi exercise on patients with type 2 diabetes," *Medicine and Sport Science*, vol. 52, pp. 230–238, 2008.
- [28] Y. Zhang and F. H. Fu, "Effects of 14-week Tai Ji quan exercise on metabolic control in women with type 2 diabetes," *American Journal of Chinese Medicine*, vol. 36, no. 4, pp. 647–654, 2008.
- [29] S.-C. Chen, K.-C. Ueng, S.-H. Lee, K.-T. Sun, and M.-C. Lee, "Effect of T'ai Chi exercise on biochemical profiles and oxidative stress indicators in obese patients with type 2 diabetes," *Journal of Alternative and Complementary Medicine*, vol. 16, no. 11, pp. 1153–1159, 2010.
- [30] S. Ahn and R. Song, "Effects of tai chi exercise on glucose control, neuropathy scores, balance, and quality of life in patients with type 2 diabetes and neuropathy," *Journal of Alternative and Complementary Medicine*, vol. 18, no. 12, pp. 1172–1178, 2012.
- [31] X. Liu, Y. D. Miller, N. W. Burton, J. H. Chang, and W. J. Brown, "The effect of Tai Chi on health-related quality of life in people with elevated blood glucose or diabetes: a randomized controlled trial," *Quality of Life Research*, vol. 22, no. 7, pp. 1783–1786, 2012.
- [32] J. H. Yan, W. J. Gu, and L. Pan, "Lack of evidence on Tai Chi-related effects in patients with type 2 diabetes mellitus: a meta-analysis," *Experimental and Clinical Endocrinology and Diabetes*, vol. 121, no. 5, pp. 266–271, 2013.
- [33] J. A. Halbert, C. A. Silagy, P. Finucane, R. T. Withers, and P. A. Hamdorf, "Exercise training and blood lipids in hyperlipidemic and normolipidemic adults: a meta-analysis of randomized, controlled trials," *European Journal of Clinical Nutrition*, vol. 53, no. 7, pp. 514–522, 1999.
- [34] G. N. Thomas, A. W. L. Hong, B. Tomlinson et al., "Effects of Tai Chi and resistance training on cardiovascular risk factors in elderly Chinese subjects: a 12-month longitudinal, randomized, controlled intervention study," *Clinical Endocrinology*, vol. 63, no. 6, pp. 663–669, 2005.
- [35] C. Lan, T.-C. Su, S.-Y. Chen, and J.-S. Lai, "Effect of T'ai Chi Chuan training on cardiovascular risk factors in dyslipidemic patients," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 7, pp. 813–819, 2008.
- [36] J. Myers, M. Prakash, V. Froelicher, D. Do, S. Partington, and J. Edwin Atwood, "Exercise capacity and mortality among men referred for exercise testing," *The New England Journal of Medicine*, vol. 346, no. 11, pp. 793–801, 2002.
- [37] T. Kavanagh, D. J. Mertens, L. F. Hamm et al., "Prediction of long-term prognosis in 12 169 men referred for cardiac rehabilitation," *Circulation*, vol. 106, no. 6, pp. 666–671, 2002.
- [38] C. Lan, S.-Y. Chen, and J.-S. Lai, "Changes of aerobic capacity, fat ratio and flexibility in older TCC practitioners: a five-year follow-up," *American Journal of Chinese Medicine*, vol. 36, no. 6, pp. 1041–1050, 2008.
- [39] R. Taylor-Piliae, "The effectiveness of Tai Chi exercise in improving aerobic capacity: an updated meta-analysis," *Medicine and Sport Science*, vol. 52, pp. 40–53, 2008.
- [40] M. S. Lee, E.-N. Lee, and E. Ernst, "Is tai chi beneficial for improving aerobic capacity? A systematic review," *British Journal of Sports Medicine*, vol. 43, no. 8, pp. 569–573, 2009.
- [41] J.-S. Wang, C. Lan, and M.-K. Wong, "Tai Chi Chuan training to enhance microcirculatory function in healthy elderly men," *Archives of Physical Medicine and Rehabilitation*, vol. 82, no. 9, pp. 1176–1180, 2001.
- [42] J.-S. Wang, C. Lan, S.-Y. Chen, and M.-K. Wong, "Tai Chi Chuan training is associated with enhanced endothelium-dependent dilation in skin vasculature of healthy older men," *Journal of the American Geriatrics Society*, vol. 50, no. 6, pp. 1024–1030, 2002.
- [43] N. Frasure-Smith, F. Lesperance, and M. Talajic, "Depression following myocardial infarction: impact on 6-month survival," *Journal of the American Medical Association*, vol. 270, no. 15, pp. 1819–1825, 1993.

- [44] N. Frasure-Smith, F. Lesperance, and M. Talajic, "Depression and 18-month prognosis after myocardial infarction," *Circulation*, vol. 91, no. 4, pp. 999–1005, 1995.
- [45] R. V. Milani and C. J. Lavie, "Impact of cardiac rehabilitation on depression and its associated mortality," *American Journal of Medicine*, vol. 120, no. 9, pp. 799–806, 2007.
- [46] P. J. Jimenez, A. Melendez, and U. Albers, "Psychological effects of Tai Chi Chuan," *Archives of Gerontology and Geriatrics*, vol. 55, no. 2, pp. 460–467, 2012.
- [47] A. Yeung, V. Lepoutre, P. Wayne, G. Yeh et al., "Tai chi treatment for depression in Chinese Americans: a pilot study," *American Journal of Physical Medicine and Rehabilitation*, vol. 91, no. 10, pp. 863–870, 2012.
- [48] R. E. Taylor-Piliae, W. L. Haskell, C. M. Waters, and E. S. Froelicher, "Change in perceived psychosocial status following a 12-week Tai Chi exercise programme," *Journal of Advanced Nursing*, vol. 54, no. 3, pp. 313–329, 2006.
- [49] G. Y. Yeh, E. P. McCarthy, P. M. Wayne et al., "Tai chi exercise in patients with chronic heart failure: a randomized clinical trial," *Archives of Internal Medicine*, vol. 171, no. 8, pp. 750–757, 2011.
- [50] Agency for Health Care Policy and Research Cardiac Rehabilitation, "Clinical Practice Guidelines," AHCPR Publication 96-0672, U.S. Department of Health and Human Services, Public Health Services, Agency for Health Care Policy and Research, Rockville, Md, USA, 1995.
- [51] R. E. Taylor-Piliae, E. Silva, and S. P. Sheremeta, "Tai Chi as an adjunct physical activity for adults aged 45 years and older enrolled in phase III cardiac rehabilitation," *European Journal of Cardiovascular Nursing*, vol. 11, no. 1, pp. 34–43, 2010.
- [52] B. S. Heran, J. M. Chen, S. Ebrahim et al., "Exercise-based cardiac rehabilitation for coronary heart disease," *Cochrane Database of Systematic Reviews*, no. 7, Article ID CD001800, 2011.
- [53] D. E. Barrow, A. Bedford, G. Ives, L. O'Toole, and K. S. Channer, "An evaluation of the effects of Tai Chi Chuan and Chi Kung training in patients with symptomatic heart failure: a randomised controlled pilot study," *Postgraduate Medical Journal*, vol. 83, no. 985, pp. 717–721, 2007.
- [54] G. Y. Yeh, M. J. Wood, B. H. Lorell et al., "Effects of Tai Chi mind-body movement therapy on functional status and exercise capacity in patients with chronic heart failure: a randomized controlled trial," *American Journal of Medicine*, vol. 117, no. 8, pp. 541–548, 2004.
- [55] G. Y. Yeh, J. E. Mietus, C.-K. Peng et al., "Enhancement of sleep stability with Tai Chi exercise in chronic heart failure: preliminary findings using an ECG-based spectrogram method," *Sleep Medicine*, vol. 9, no. 5, pp. 527–536, 2008.
- [56] G. Caminiti, M. Volterrani, G. Marazzi et al., "Tai chi enhances the effects of endurance training in the rehabilitation of elderly patients with chronic heart failure," *Rehabilitation Research and Practice*, vol. 2011, Article ID 761958, 2011.
- [57] G. Y. Yeh, M. J. Wood, P. M. Wayne et al., "Tai Chi in patients with heart failure with preserved ejection fraction," *Congestive Heart Failure*, vol. 19, no. 2, pp. 77–84, 2013.
- [58] L. Pan, J. Yan, Y. Guo, and J. Yan, "Effects of Tai Chi training on exercise capacity and quality of life in patients with chronic heart failure: a meta-analysis," *European Journal of Heart Failure*, vol. 15, no. 3, pp. 316–323, 2013.
- [59] T. Rutledge, V. A. Reis, S. E. Linke, B. H. Greenberg, and P. J. Mills, "Depression in Heart Failure. A meta-analytic review of prevalence, intervention effects, and associations with clinical outcomes," *Journal of the American College of Cardiology*, vol. 48, no. 8, pp. 1527–1537, 2006.
- [60] L. S. Redwine, M. Tsuang, A. Rusiewicz et al., "A pilot study exploring the effects of a 12-week tai chi intervention on somatic symptoms of depression in patients with heart failure," *Journal of Alternative and Complementary Medicine*, vol. 18, no. 8, pp. 744–748, 2012.
- [61] O. Stoller, E. D. de Bruin, R. H. Knols, and K. J. Hunt, "Effects of cardiovascular exercise early after stroke: systematic review and meta-analysis," *BMC Neurology*, vol. 12, article 45, 2012.
- [62] J. Hart, H. Kanner, R. Gilboa-Mayo, O. Haroeh-Peer, N. Rozenthal-Sorokin, and R. Eldar, "Tai Chi Chuan practice in community-dwelling persons after stroke," *International Journal of Rehabilitation Research*, vol. 27, no. 4, pp. 303–304, 2004.
- [63] S. S. Y. Au-Yeung, C. W. Y. Hui-Chan, and J. C. S. Tang, "Short-form tai chi improves standing balance of people with chronic stroke," *Neurorehabilitation and Neural Repair*, vol. 23, no. 5, pp. 515–522, 2009.
- [64] W. Wang, M. Sawada, Y. Noriyama et al., "Tai Chi exercise versus rehabilitation for the elderly with cerebral vascular disorder: a single-blinded randomized controlled trial," *Psychogeriatrics*, vol. 10, no. 3, pp. 160–166, 2010.
- [65] R. E. Taylor-Piliae and B. M. Coull, "Community-based Yang-style Tai Chi is safe and feasible in chronic stroke: a pilot study," *Clinical Rehabilitation*, vol. 26, no. 2, pp. 121–131, 2012.

## Research Article

# The Protective Role of Resveratrol against Arsenic Trioxide-Induced Cardiotoxicity

Wei-qian Zhang,<sup>1</sup> Chang-ming Guo,<sup>2</sup> Ruifeng Gao,<sup>2</sup> Ming Ge,<sup>1</sup>  
Yanzhu Zhu,<sup>3</sup> and Zhigang Zhang<sup>1</sup>

<sup>1</sup> College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, China

<sup>2</sup> College of Animal Science and Veterinary, Medicine, Jilin University, Changchun 130062, China

<sup>3</sup> Institute of Special Economic Animal and Plant Science, Chinese Academy of Agricultural Sciences, Jilin 132109, China

Correspondence should be addressed to Zhigang Zhang; zzgneau@yahoo.com.cn

Received 2 February 2013; Revised 30 August 2013; Accepted 17 September 2013

Academic Editor: Ka Kit Hui

Copyright © 2013 Wei-qian Zhang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Arsenic trioxide ( $\text{As}_2\text{O}_3$ ) shows substantial anticancer activity in patients with acute promyelocytic leukemia (APL). Unfortunately, limiting the application of this effective agent to APL patients is severe cardiotoxicity. Resveratrol, the natural food-derived polyphenolic compound, is well known for its antioxidant properties and protects the cardiovascular system. But the potential role of resveratrol against  $\text{As}_2\text{O}_3$  in heart via nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) is unclear. The present study evaluated the effects of pretreatment with resveratrol and  $\text{As}_2\text{O}_3$  on oxidative stress and cardiac dysfunction in rat. In the present study, resveratrol decreased  $\text{As}_2\text{O}_3$ -induced reactive oxygen species generation, oxidative DNA damage, and pathological alterations. In addition, cardiac dysfunction parameters, intracellular calcium and arsenic accumulation, glutathione redox ratio, and cAMP deficiency levels were observed in  $\text{As}_2\text{O}_3$ -treated rats; these changes were attenuated by resveratrol. Furthermore, resveratrol significantly prohibited the downregulation of both Nrf2 and HO-1 gene expressions that were downregulated by  $\text{As}_2\text{O}_3$ , whereas resveratrol did not alter  $\text{As}_2\text{O}_3$ -induced nitric oxide formation. Thus, the protective role of resveratrol against  $\text{As}_2\text{O}_3$ -induced cardiotoxicity is implemented by the maintenance of redox homeostasis (Nrf2-HO-1 pathway) and facilitating arsenic efflux. Our findings suggest coadministration with resveratrol, and  $\text{As}_2\text{O}_3$  might provide a novel therapeutic strategy for APL.

## 1. Introduction

High concentration of dietary exposure to arsenic and arsenic compounds is considered to increase the risk of human carcinogenesis [1]. However, arsenic has attracted worldwide interest because it shows substantial anticancer activity in individuals with acute promyelocytic leukemia (APL). Unfortunately, the use of these drugs is associated with cardiotoxicity (including a prolonged QT interval and prolonged action potential), *torsades de pointes*, and sudden cardiac death [2–5]. This may involve multiple mechanisms, including the generation of reactive oxygen species (ROS) in cardiomyocytes, oxidative DNA damage, and arsenic accumulation, [6–8]. However, proper drug that has the protective ability of the heart to protect against arsenic toxicity in the clinical practice is insufficient.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a plant-derived polyphenolic compound belonging to a class of stilbenes, found abundantly in certain grapes, roots, berries, and peanuts. Resveratrol has been shown to exert various cardiovascular protective effects in myocardial ischemic-reperfusion injury and atherosclerosis [9, 10], metabolic diseases [11], and in aged mice [12–14]. It was also reported that resveratrol could protect against cardiotoxicity in  $\text{As}_2\text{O}_3$ -exposed mouse by the increase in the activities of antioxidant enzymes in the heart and antiapoptotic activity in H9c2 cardiomyocytes [15]. However, whether resveratrol can attenuate the  $\text{As}_2\text{O}_3$ -induced cardiotoxicity mediated by improving cardiac function through redox signaling mechanisms and the decrease in arsenic accumulation is yet to be determined. The present study was undertaken to explore this problem.

## 2. Materials and Methods

**2.1. Animals and Chemicals.** All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Six-week-old male Wistar rats from the Experimental Animal Centre of Harbin Medical University (Harbin, China) were housed in the Animal Quarters of the Northeast Agricultural University at 22°C on a 12-hour light-dark cycle. They were allowed free access to standard rodent chow and tap water. Thirty-two rats were randomly assigned to four groups: control, As<sub>2</sub>O<sub>3</sub>-treated, As<sub>2</sub>O<sub>3</sub> + resveratrol, and resveratrol-treated. All treatments were given via the caudal vein on alternate days for 4 days (i.e., days: 1, 3, 5, and 7) with measurements being made on the 8th day. As<sub>2</sub>O<sub>3</sub> (Harbin Yida Pharmaceutical Co. Ltd., Harbin, China) was administered 3 mg/kg; resveratrol (Sigma-Aldrich, St. Louis, MO, USA) was administered 8 mg/kg; in the As<sub>2</sub>O<sub>3</sub> + resveratrol group, rats were given resveratrol 1 h prior to As<sub>2</sub>O<sub>3</sub> administration. Dose selection is based on the literature [14]. An equal amount of 0.9% normal saline was administered as vehicles to control rats. On the 8th day, rats were given ether anesthesia and sacrificed.

**2.2. Biochemical Analysis.** Blood was collected from puncturing the retro-orbital venous sinus and immediately centrifuged at 8,000 ×g for 10 min at 4°C to separate serum. Serum lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase MB (CK-MB), and aspartate aminotransferase (AST) were measured using a commercial kit from Jiancheng Bio-engineering Institute (Nanjing, China), following the manufacturer's instructions.

**2.3. Measurement of ROS, 8-Hydroxy-2-deoxyguanosine (8-OHdG) and the Ratio Reduced Glutathione (GSH) to Oxidized Glutathione (GSSG).** Cardiac tissues were homogenized in phosphate-buffered saline (pH 7.4) using an Ultrathurax T25 Homogenisator and centrifuged at 10,000 ×g for 10 min at 4°C. ROS production of cardiac tissue was determined by 2', 7'-dichlorofluorescein diacetate (DCF-DA, Invitrogen) assay, in which highly fluorescent DCF can be converted by cellular peroxides, as previously reported by Maxwell et al. [16]. The DNA of each sample was extracted using a DNeasy tissue kit (QIAGEN, Valencia, CA, USA), and 8-OHdG was measured using an oxidative DNA damage enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolaboratories, San Diego, CA, USA), following the manufacturer's instructions. Supernatant glutathione was determined by the method as described in [17], and the ratio of GSH to GSSG was calculated.

**2.4. Histological Analysis.** Cardiac tissues were quickly removed. For light microscopic (BX-FM; Olympus, Tokyo, Japan) observation, cardiac tissues were fixed by immersion in 10% formaldehyde solution for 24 h at 37°C; then paraffin sections (4 μm) were cut and stained with hematoxylin and eosin.

**2.5. Determination of Arsenic Accumulation in the Heart.** The arsenic contents in cardiac tissues of all rats were analyzed following the method in the literature [18] with an atomic

fluorescence spectrometry system (AFS930; Beijing Jitian Instrument Co. Ltd., Beijing, China).

**2.6. Measurements of Cytosolic Free Calcium Ion Ca<sup>2+</sup> Level.** Cardiac cells were digested with 0.25% trypsinase. The cell suspension was washed twice with Tyrode's Solution (in mM: NaCl, 137; KCl, 5.4; MgCl<sub>2</sub>, 1; glucose, 10; HEPES, 10; CaCl<sub>2</sub>, 2; pH 7.4) and loaded with 2 μM Fura-2/AM for 30 min at 37°C in culture medium. The cells were washed three times then incubated for an additional 30 min at 37°C to complete probe de-esterification and resuspended in loading buffer at a density of 10<sup>6</sup> cells/mL. Fluorescence was monitored with a 970 CRT spectrofluorophotometer at 488 nm for excitation and 530 nm for emission. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 5 mM CaCl<sub>2</sub>) and 10 mM EGTA sequentially. Ca<sup>2+</sup> levels were calculated as previously described [19].

**2.7. cAMP and Nitric Oxide (NO) Concentration Assay.** The supernatant of homogenized cardiac tissue was assayed for cAMP and NO concentration using the enzyme immunoassay kit and the method of chemical colorimetry, respectively, following the manufacturer's instructions (Jiancheng Bio-engineering Institute (Nanjing, China)).

**2.8. Determination of Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) and Heme Oxygenase-1 (HO-1) mRNA Level by Quantitative Real-Time PCR Assay.** Total RNA was extracted from the cardiac tissue samples using the RNeasy 200 Kit (Fastagen, China). The concentration of total RNA in the extract was quantified spectrophotometrically. RNA integrity was evaluated by the proportion of the ribosomal bands (28S:18S) after electrophoresis on 1% agarose gel in the presence of ethidium bromide. cDNA was synthesized using 5 μL of total RNA using the reverse transcriptase M-M LV (Promega), as described by the manufacturer's system [20]. Quantitative real-time PCR was carried out using a SYBR Green PCR kit (Biotek, Peking, China), and PCR amplification was conducted on an ABI PRISM 7500 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primer sequences for the genes are as follows: Nrf-2 Forward: 5'-ACT CAT CGA TCC CCT CAC TG-3', Reverse: 5'-CTA ATG GCA GCA GAG GAA GG-3'; HO-1 Forward: 5'-AAG AGG CTA AGA CCG CCT TC-3', Reverse: 5'-GCA TAA ATT CCC ACT GCC AC-3'; GAPDH Forward: 5'-GCA TGG CCT TCC GTG TTC C-3', Reverse: 5'-CTC ATT CTT TGG GAC GTG GTG GG-3'. The expression of mRNA level in each sample was normalized against its GAPDH mRNA level.

**2.9. Statistical Analysis.** Statistical analysis was performed using SPSS ver19.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (Duncan's multiple comparison) was used for the determination of differences in measurements between groups. *P* < 0.05 was considered significant.

TABLE 1: The effect of resveratrol on As<sub>2</sub>O<sub>3</sub>-induced biochemical makers.

	Control	As <sub>2</sub> O <sub>3</sub>	As <sub>2</sub> O <sub>3</sub> + resveratrol	Resveratrol
AST (U/L)	156.23 ± 56.78	282.69 ± 60.43 <sup>a**</sup>	225.78 ± 58.36 <sup>b*</sup>	155.34 ± 53.41
LDH (U/L)	1050.25 ± 370.36	2700.45 ± 380.13 <sup>a**</sup>	1680.34 ± 470.68 <sup>b*</sup>	1051.37 ± 360.42
CK (U/L)	336.98 ± 48.04	1463.31 ± 452.16 <sup>a**</sup>	597.28 ± 90.54 <sup>b**</sup>	335.62 ± 50.26
CK-MB (U/L)	205.83 ± 32.32	812.94 ± 129.85 <sup>a**</sup>	407.59 ± 67.40 <sup>b**</sup>	204.30 ± 30.02

Values are expressed as mean ± S.E. for eight rats in each group.

\*  $P < 0.05$ , \*\*  $P < 0.01$ ; <sup>a</sup>comparison of control with As<sub>2</sub>O<sub>3</sub>; <sup>b</sup>comparison of As<sub>2</sub>O<sub>3</sub> with As<sub>2</sub>O<sub>3</sub> + resveratrol.

### 3. Results

**3.1. The Contents of LDH, AST, CK, and CK-MB in Serum.** As shown in Table 1, LDH, AST, CK, and CK-MB release from cardiac cells, in the rat treated with As<sub>2</sub>O<sub>3</sub>, were markedly increased compared with those in the control group ( $P < 0.05$ ). Pretreatment of resveratrol resulted in a significant decrease in plasma LDH, AST, CK, and CK-MB release, 20.21%, 37.78%, 59.19%, and 49.88%, respectively, compared with that in the As<sub>2</sub>O<sub>3</sub>-treated group, whereas resveratrol alone did not show significant effect on LDH, AST, CK, and CK-MB activity.

**3.2. Effects of Resveratrol on As<sub>2</sub>O<sub>3</sub>-Induced ROS, 8-OHd, and GSH/GSSG.** After exposure to arsenic for 4 days, the remarkable increase in ROS and 8-OHdG generation was observed in rats' hearts, compared with that in the control group (Figures 1(a) and 1(b)). However, pretreatment with resveratrol partly abolished these changes. In addition, treatment with resveratrol exposure significantly reversed the decrease in As<sub>2</sub>O<sub>3</sub>-induced the ration GSH/GSSG ( $P < 0.01$ ) (Figure 1(c)).

**3.3. Effect of Resveratrol on As<sub>2</sub>O<sub>3</sub>-Induced Cardiomyopathy.** Histopathological assessments of different cardiac tissues of rats are shown in Figure 2. Compared with those in the control group, myofibrillar loss and cardiomyocyte necrosis were observed in the hearts of the As<sub>2</sub>O<sub>3</sub>-treated rats (Figure 2(b)). Structural abnormalities in the hearts of As<sub>2</sub>O<sub>3</sub>-treated rats were partly prevented by pretreatment with resveratrol and showed slight myocardial hemorrhage. Resveratrol-treated rats had normal myocardial morphology (data not shown).

**3.4. The Contents of Total Arsenic in the Heart.** Figure 3 has shown the contents of total arsenic in the heart. Our results showed that total arsenic content in the heart appeared to be obviously increased compared with As<sub>2</sub>O<sub>3</sub>-treated rats. Pretreatment with resveratrol significantly attenuated arsenic accumulation in the heart compared with that seen in the As<sub>2</sub>O<sub>3</sub>-treated group ( $P < 0.05$ ) (Figure 3).

**3.5. Effect of Resveratrol on As<sub>2</sub>O<sub>3</sub>-Induced Intracellular Calcium Accumulation.** The effects of resveratrol on As<sub>2</sub>O<sub>3</sub>-induced intracellular calcium accumulation in the heart are shown in Figure 4(b). Our data indicated Ca<sup>2+</sup> content was markedly greater in As<sub>2</sub>O<sub>3</sub>-treated group than that in the control group ( $P < 0.01$ ), and resveratrol significantly

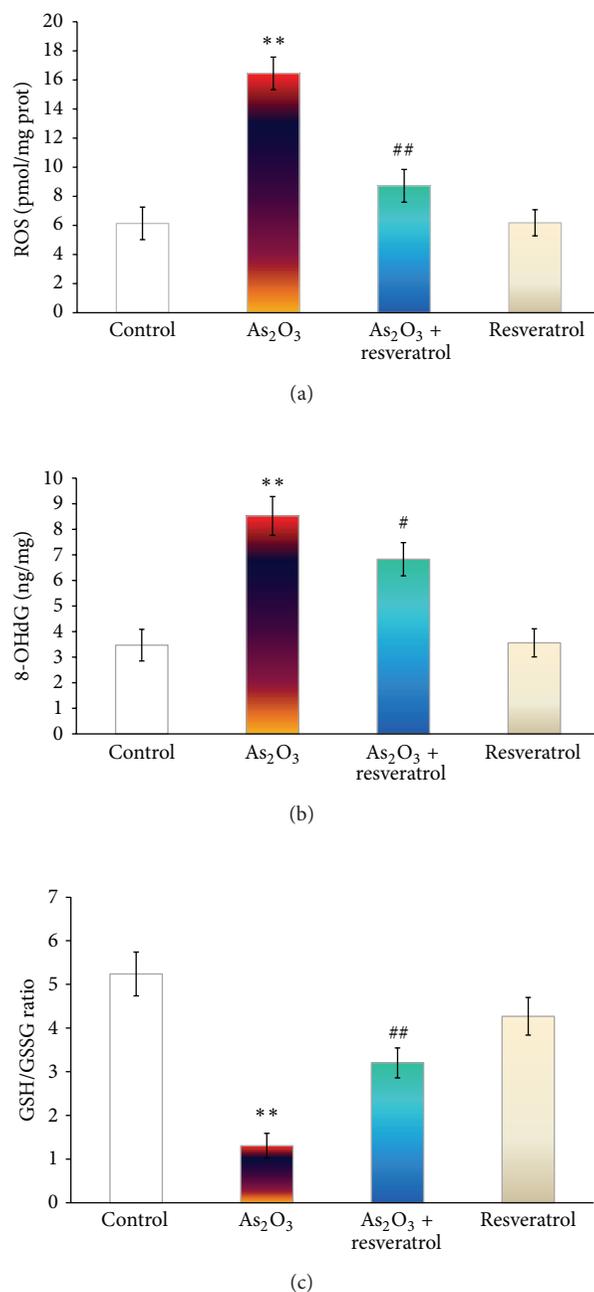


FIGURE 1: The effect of resveratrol and As<sub>2</sub>O<sub>3</sub> on ROS (a), 8-OHdG (b), and GSH/GSSG (c) ratio in the heart tissue from control, As<sub>2</sub>O<sub>3</sub>-treated, resveratrol + As<sub>2</sub>O<sub>3</sub>, and resveratrol-treated groups. Values are mean ± S.E. mean;  $n = 8$ . \*  $P < 0.05$  or \*\*  $P < 0.01$  versus control group, #  $P < 0.05$  or ##  $P < 0.01$  versus As<sub>2</sub>O<sub>3</sub>-treated group.

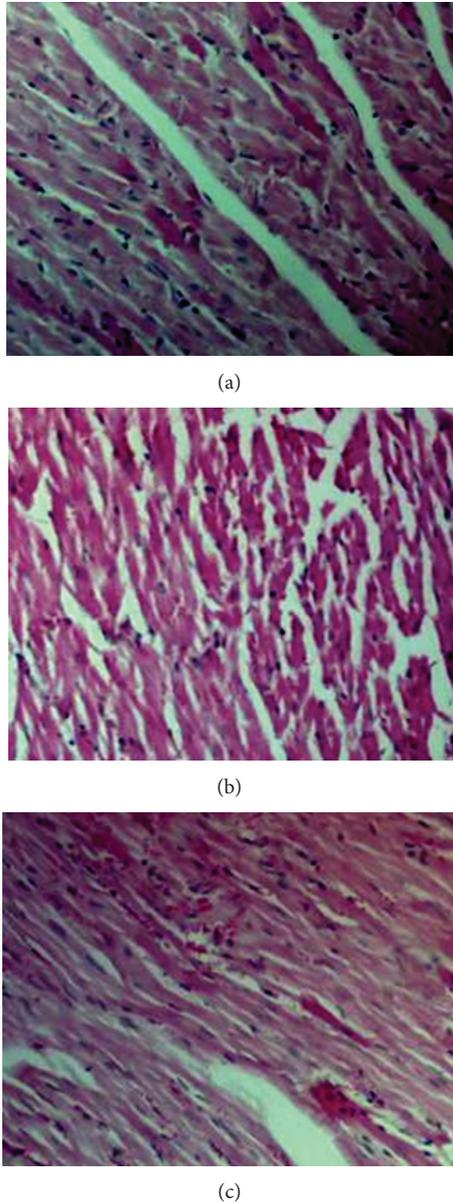


FIGURE 2: The effect of resveratrol and  $As_2O_3$  on cardiac histology. Paraffin sections of heart tissues from control (a),  $As_2O_3$ -treated (b), and resveratrol +  $As_2O_3$  (c) were stained with hematoxylin and eosin ( $\times 100$  magnification).

inhibited this  $As_2O_3$ -induced that  $Ca^{2+}$  accumulation ( $P < 0.05$ ).

**3.6. Effects of Resveratrol on  $As_2O_3$ -Induced cAMP and NO in the Heart.** To determine the effect of resveratrol on  $As_2O_3$ -induced cAMP, concentrations in rats treated with  $As_2O_3$  and pretreatment with resveratrol were measured. As expected, treatment of rats with  $As_2O_3$  (3 mg/kg i.v.) significantly decreased cAMP concentrations in the heart compared with saline-treated control group, and pretreatment of resveratrol could partly abolish this decrease in cAMP concentration (Figure 4(a)). In contrast, NO concentration in the heart has

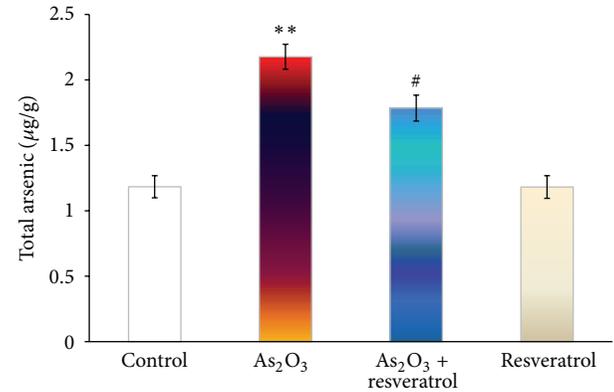


FIGURE 3: The effect of resveratrol and  $As_2O_3$  on total arsenic in the heart. The total arsenic of heart tissues from control,  $As_2O_3$ -treated, resveratrol +  $As_2O_3$ , and resveratrol-treated groups was quantified by high-performance liquid chromatography-hydride generation-atomic fluorescence spectrometry. Values are mean  $\pm$  S.E. mean;  $n = 8$ . \* $P < 0.05$  or \*\* $P < 0.01$  versus control group, # $P < 0.05$  or ## $P < 0.01$  versus  $As_2O_3$ -treated group.

no statistical change during exposure to  $As_2O_3$  and with or without the administration of resveratrol (Figure 4(b)).

**3.7. Effects of Resveratrol on the mRNA Level of HO-1 and Nrf2.** To further explore the possible mechanism of  $As_2O_3$ -induced cardiotoxicity, HO-1 and Nrf2 mRNA with antioxidant and protective properties were selected to determine the effect of resveratrol on cytotoxicity in  $As_2O_3$ -treated rats. After exposure to  $As_2O_3$  on alternate 4 days, both Nrf2 and HO-1 gene expressions in the heart were significantly downregulated, compared with the control group (Figure 5). Treatment with resveratrol significantly prohibited the downregulated Nrf2 and HO-1 gene expressions that were downregulated by  $As_2O_3$ , compared with  $As_2O_3$ -treated rats ( $P < 0.01$ ).

## 4. Discussion

In this study, we investigated cardiac function associated with Nrf2-HO-1 pathway and arsenic accumulation for the protection of resveratrol against  $As_2O_3$ -induced cardiac injury in Wistar rats *in vivo*.

Aposhian and Aposhian [21] described that exposure to inorganic arsenic induces cellular oxidative stress through ROS generation. Several studies have indicated that cardiovascular diseases, such as endothelial dysfunction, ischaemia-reperfusion injury, and atherosclerosis, are linked to the release of intracellular ROS [7, 22]. In our studies, LDH, AST, CK, and CK-MB release, which are the most important markers of myocardial injury, disorder and necrosis in response to  $As_2O_3$  treatment, were increased, especially CK-MB, which is a more sensitive marker of myocardial injury than total CK activity. In addition, there were various oxidative damages in Wistar rat's heart indicated with the increase of ROS, 8-OHdG formation, and percentage of GSSG/GSH, which resulted in the severe histological alterations, including myofibrillar loss, cardiomyocyte necrosis,

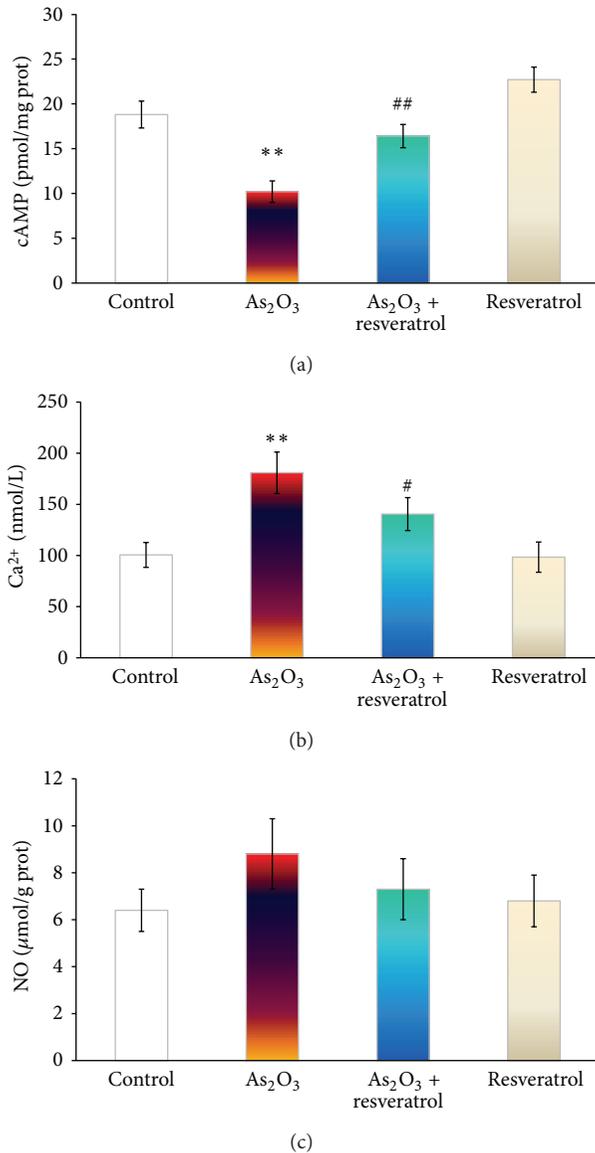


FIGURE 4: The effect of resveratrol on As<sub>2</sub>O<sub>3</sub>-induced cAMP (a), Ca<sup>2+</sup> (b), and NO (c) in the heart from control, As<sub>2</sub>O<sub>3</sub>-treated, resveratrol + As<sub>2</sub>O<sub>3</sub>, and resveratrol-treated groups. Values are mean  $\pm$  S.E. mean;  $n = 8$ . \* $P < 0.05$  or \*\* $P < 0.01$  versus control group, # $P < 0.05$  or ## $P < 0.01$  versus As<sub>2</sub>O<sub>3</sub>-treated group.

and myocardial hemorrhage. Consistent with Ermak and Davies's research on ROS-induced Ca<sup>2+</sup> dyshomeostasis in the heart [23], our data showed that intracellular calcium accumulation after exposure to As<sub>2</sub>O<sub>3</sub> is at least partially due to ROS formation induced by As<sub>2</sub>O<sub>3</sub>.

Under normal circumstances, cells can defend against ROS damage by means of endogenous oxidants, such as glutathione, vitamin C, and vitamin E, as well as with the involvement of various peroxidases in the cellular antioxidant systems. Glutathione redox state correlates with the biological status of the cell [24]. On the other hand, Nrf2 has been demonstrated to be a critical transcription factor that binds

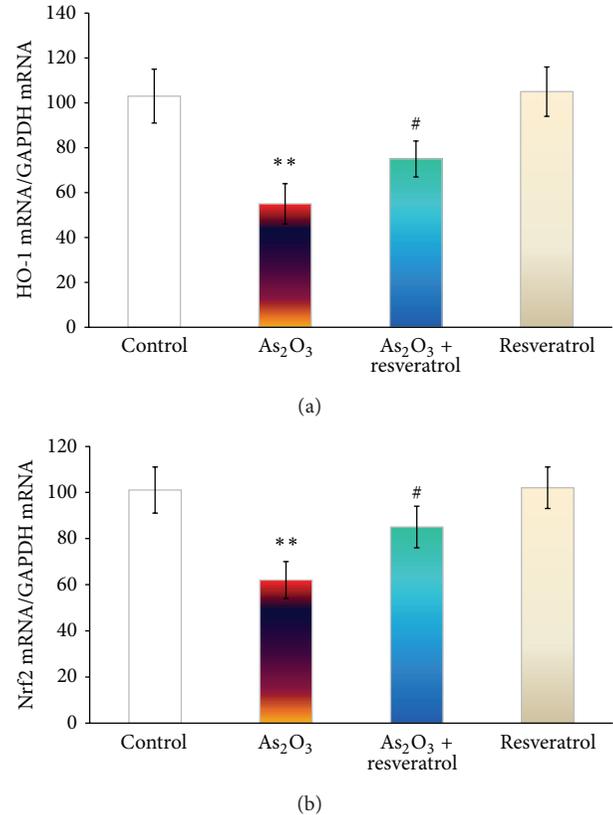


FIGURE 5: The effect of resveratrol on As<sub>2</sub>O<sub>3</sub>-induced HO-1 and Nrf2 on the mRNA level in the heart from the control, As<sub>2</sub>O<sub>3</sub>-treated, resveratrol + As<sub>2</sub>O<sub>3</sub>, and resveratrol-treated groups. (a) Real-time quantitative PCR analyses of gene expression levels of HO-1 in cardiac myocytes. (b) Real-time quantitative PCR analyses of gene expression levels of Nrf2 in cardiac myocytes. Values are mean  $\pm$  S.E. mean;  $n = 8$ . \* $P < 0.05$  or \*\* $P < 0.01$  versus control group, # $P < 0.05$  or ## $P < 0.01$  versus As<sub>2</sub>O<sub>3</sub>-treated group.

to the antioxidant response element in the promoter region of a number of genes, encoding for phase I and phase II antioxidative enzymes and cytoprotective proteins, such as NAD(P)H:quinone acceptor oxidoreductase 1, glutathione S-transferases, the glutamyl cysteine ligase catalytic subunit, and multidrug resistance-associated protein [25]. Hence, Nrf2 pathway is presumably the most important pathway in cells to deal with oxidative stress generated from exposure to exogenous and endogenous chemicals [20]. HO-1 is an enzyme with antioxidant and protective properties during cellular stress [20]. After exposure to As<sub>2</sub>O<sub>3</sub> (3 mg/kg every alternative day for 4 days), the antioxidant defense system in rats cannot maintain the depletion. Consequently, we observed significantly a decrease in GSH/GSSG ratio and mRNA expression of Nrf2 and HO-1 downregulation (Figure 1).

There is a considerable interest in the role of constituent in dietary supplement in the prevention and treatment of cardiovascular disease [23]. Furthermore, natural compounds also modulate ROS accounting for the reduction of cell injury in pathological conditions in heart diseases [26].

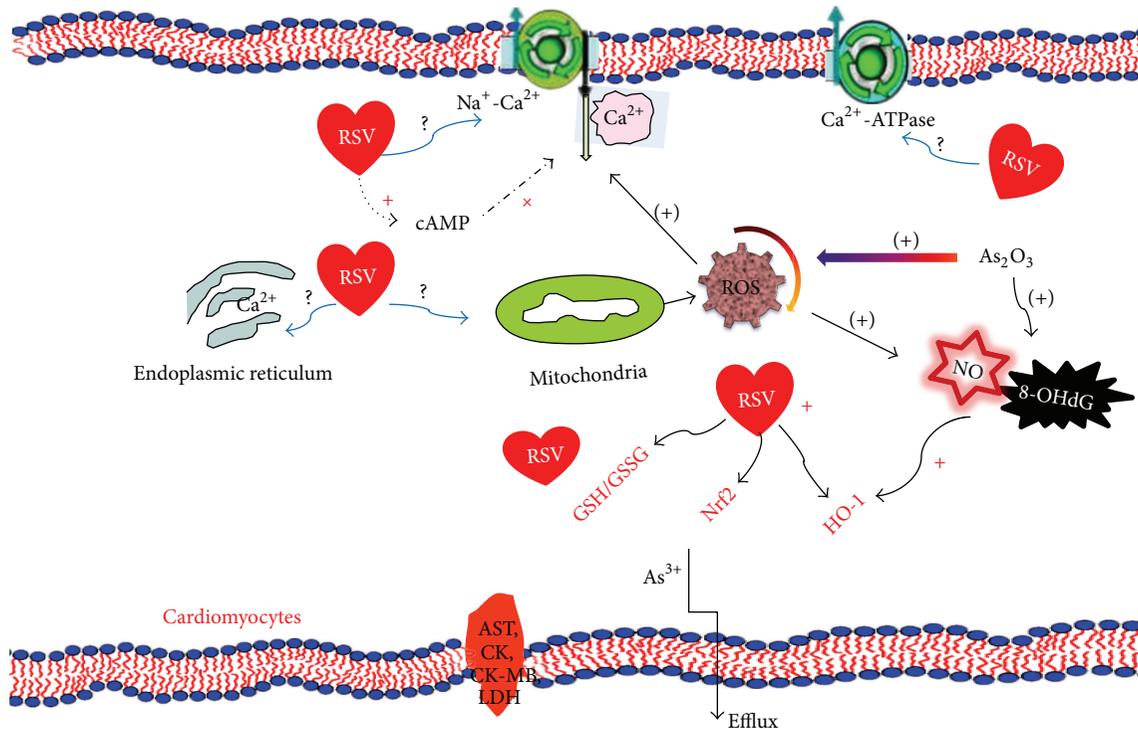


FIGURE 6: Summary indicating involvement of oxidative stress responses and the possible mechanism associated with Nrf2-HO-1 in  $\text{As}_2\text{O}_3$ -induced injury.  $\text{As}_2\text{O}_3$  induces the increase of ROS production from mitochondria in rat cardiac myocytes. ROS triggers  $\text{Ca}^{2+}$  accumulation, 8-OHdG formation, and GSH deficiency in cardiocytes. RSV scavenges ROS, reduces DNA damage (indicated with 8-OHdG), and preserves GSH and  $\text{Ca}^{2+}$  homeostasis. Additionally, Nrf2-HO-1, a key signaling pathway involved in cellular oxidative responses, is inhibited by RSV.  $\text{As}_2\text{O}_3$ -induced downregulation. Taken together, RSV protects the integrity of cardiac myocytes after exposure to  $\text{As}_2\text{O}_3$ , thereby decreasing AST, CK, CK-MB, and LDH release, as well as facilitating arsenic efflux. Future studies are required to clarify the mechanism for protecting RSV against  $\text{As}_2\text{O}_3$ -induced cardiotoxicity in endoplasmic reticulum and mitochondria. ROS: reactive oxygen species; RSV: resveratrol; + or (-) stands for positive improvement or negative improvement.

Unfortunately, several approaches which were able to reduce tissue damage in animal or cell culture models are either not applicable to humans or failed to be beneficial in clinical trials [27, 28].

Resveratrol, is an antioxidant found in grapes, red wine, and some other botanical sources with a wide range of biological and pharmacological properties, for example, anti-inflammatory, cardioprotection activity, and anticancer properties [29, 30]. Haskó and Pacher [31] had demonstrated that resveratrol regulated endothelial Nrf2 activation. Therefore, resveratrol administration before  $\text{As}_2\text{O}_3$  treatment diminished  $\text{As}_2\text{O}_3$ -induced ROS and 8-OHdG generation mediated by the partly maintenance of GSH/GSSH ratio and mRNA expression of Nrf2 and HO-1 (Figure 5).

Resveratrol has been shown to regulate cAMP through the competitive inhibition of cAMP-degrading phosphodiesterases, though it has not been clearly demonstrated in myocardial systems [13]. The intracellular cAMP-dependent modulate L-type  $\text{Ca}^{2+}$  channel has been widely recognized. In cardiac tissue, elevation of  $\text{Ca}^{2+}$  has been linked to various functional abnormalities, such as ventricular arrhythmia and contractile dysfunction. Also the increase of  $\text{Ca}^{2+}$  has been suggested to be one of the key signals leading to

apoptosis [11, 32]. In our studies, pretreatment with resveratrol attenuated  $\text{As}_2\text{O}_3$ -induced calcium overload and cAMP deficiency, suggesting that this might be attributed to the maintenance  $\text{Ca}^{2+}$  homeostasis by multiple possible ways (Figure 4). Notably, resveratrol administrated had no effect on  $\text{As}_2\text{O}_3$ -induced NO overload because of NO dual role in cardiac cells. Taken together, pretreatment with resveratrol ameliorated  $\text{As}_2\text{O}_3$ -induced myocardial damage in the heart (Figure 2). We cannot rule out that this result could be due to the improvements in cardiocyte function by heightening their aerobic capacity and autophagy to maintain tissue metabolic homeostasis in the presence of resveratrol [33, 34].

Sumi et al. [35] reported that cardiomyocytes have a weak ability to excrete arsenic into the extracellular space. This sensitivity was attributed to the modest activation of Nrf2, leading to a decrease in the metabolism and excretion of arsenic. It is plausible that resveratrol can facilitate arsenic efflux to reduce the burden of arsenic in the heart mediated by the suppression from  $\text{As}_2\text{O}_3$ -induced Nrf2 downregulation (Figure 6).

In conclusion, the protective role of resveratrol against  $\text{As}_2\text{O}_3$ -induced cardiotoxicity is found by the maintenance of redox homeostasis via Nrf2-HO-1 pathway and facilitation of arsenic efflux. Resveratrol has been shown to have

antiproliferative effects in various leukemic cell lines [36, 37]. To the best of our knowledge, our findings suggest that coadministration with resveratrol and  $As_2O_3$  may be a novel therapeutic strategy for APL. Further investigation is warranted to elucidate another potential signal mechanism by which resveratrol protects  $As_2O_3$ -induced cardiac injury and another animal model.

## Acknowledgments

This study was supported by the National Science Foundation Committee of China (31101868), Heilongjiang Province Foundation for Young Scholars (QC2010057), Heilongjiang Province Foundation for Postdoctor (LBH-Z10256), China Postdoctoral Science Foundation (20100481040), Special Foundation of China Postdoctoral Science Foundation (2012T50302), and Northeast Agricultural University Doctoral Foundation (2010RCB41) and Program for New Century Excellent Talents in Heilongjiang Provincial University (1253-NCET-007).

## References

- [1] Y.S. Chang, K.H. Lu, H.J. Lee et al., "Synergistic apoptosis-inducing antileukemic effects of arsenic trioxide and mucuna macrocarpa stem extract in human leukemic cells via a reactive oxygen species-dependent mechanism," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 921430, 14 pages, 2012.
- [2] K. Ohnishi, H. Yoshida, K. Shigeno et al., "Prolongation of the QT interval and ventricular tachycardia in patients treated with arsenic trioxide for acute promyelocytic leukemia," *Annals of Internal Medicine*, vol. 133, no. 11, pp. 881–885, 2000.
- [3] B. S. L. Soignet, S. R. Frankel, D. Douer, M. S. Tallman, H. Kantarjian, and E. Calleja, "United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia," *Journal of Clinical Oncology*, vol. 19, no. 18, pp. 3852–3860, 2001.
- [4] P. Westervelt, R. A. Brown, D. R. Adkins et al., "Sudden death among patients with acute promyelocytic leukemia treated with arsenic trioxide," *Blood*, vol. 98, no. 2, pp. 266–271, 2001.
- [5] E. Ficker, Y. A. Kuryshv, A. T. Dennis et al., "Mechanisms of arsenic-induced prolongation of cardiac repolarization," *Molecular Pharmacology*, vol. 66, no. 1, pp. 33–44, 2004.
- [6] S. Hirano, X. Cui, S. Li et al., "Difference in uptake and toxicity of trivalent and pentavalent inorganic arsenic in rat heart microvessel endothelial cells," *Archives of Toxicology*, vol. 77, no. 6, pp. 305–312, 2003.
- [7] J.T. Hwang, D. Y. Kwon, O. J. Park, and M. S. Kim, "Resveratrol protects ROS-induced cell death by activating AMPK in H9c2 cardiac muscle cells," *Genes and Nutrition*, vol. 2, no. 4, pp. 323–326, 2008.
- [8] P. Manna, M. Sinha, and P. C. Sil, "Arsenic-induced oxidative myocardial injury: protective role of arjunolic acid," *Archives of Toxicology*, vol. 82, no. 3, pp. 137–149, 2008.
- [9] J. Dudka, R. Gieroba, A. Korga et al., "Different effects of resveratrol on dose-related Doxorubicin-induced heart and liver toxicity," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 606183, 10 pages, 2012.
- [10] S. Das and D. K. Das, "Resveratrol: a therapeutic promise for cardiovascular diseases," *Recent Patents on Cardiovascular Drug Discovery*, vol. 2, no. 2, pp. 133–138, 2007.
- [11] M. Sulaiman, M. J. Matta, N. R. Sunderesan, M. P. Gupta, M. Periasamy, and M. Gupta, "Resveratrol, an activator of SIRT1, upregulates sarcoplasmic calcium ATPase and improves cardiac function in diabetic cardiomyopathy," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 298, no. 3, pp. H833–H843, 2010.
- [12] J. A. Baur, K. J. Pearson, N. L. Price et al., "Resveratrol improves health and survival of mice on a high-calorie diet," *Nature*, vol. 444, no. 7117, pp. 337–342, 2006.
- [13] S.J. Park, F. Ahmad, A. Philp et al., "Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases," *Cell*, vol. 148, no. 3, pp. 421–433, 2012.
- [14] R. I. Tennen, E. Michishita Kioi, and K. F. Chua, "Finding a target for resveratrol," *Cell*, vol. 148, no. 3, pp. 387–389, 2012.
- [15] X.Y. Zhao, G.Y. Li, Y. Liu et al., "Resveratrol protects against arsenic trioxide-induced cardiotoxicity in vitro and in vivo," *British Journal of Pharmacology*, vol. 154, no. 1, pp. 105–113, 2008.
- [16] D. P. Maxwell, Y. Wang, and L. McIntosh, "The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 14, pp. 8271–8276, 1999.
- [17] J. Lakritz, C. G. Plopper, and A. R. Buckpitt, "Validated high-performance liquid chromatography-electrochemical method for determination of glutathione and glutathione disulfide in small tissue samples," *Analytical Biochemistry*, vol. 247, no. 1, pp. 63–68, 1997.
- [18] I. Csanaky and Z. Gregus, "Effect of selenite on the disposition of arsenate and arsenite in rats," *Toxicology*, vol. 186, no. 1-2, pp. 33–50, 2003.
- [19] S. Kimura, "New generation of  $Ca^{2+}$  indicators with greatly improved fluorescence properties," *Tanpakushitsu Kakusan Koso*, vol. 52, no. 13, pp. 1758–1759, 2007.
- [20] X. Shi and B. Zhou, "The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos," *Toxicological Sciences*, vol. 115, no. 2, pp. 391–400, 2010.
- [21] H. V. Aposhian and M. M. Aposhian, "Arsenic toxicology: five questions," *Chemical Research in Toxicology*, vol. 19, no. 1, pp. 1–15, 2006.
- [22] G. Y. Oudit, M. G. Trivieri, N. Khaper et al., "Taurine supplementation reduces oxidative stress and improves cardiovascular function in an iron-overload murine model," *Circulation*, vol. 109, no. 15, pp. 1877–1885, 2004.
- [23] Y. Yang, S. W. Chan, M. Hu, R. Walden, and B. Tomlinson, "Effects of some common food constituents on cardiovascular disease," *ISRN Cardiology*, vol. 2011, Article ID 397136, 16 pages, 2011.
- [24] E. Bassenge, O. Sommer, M. Schwemmer, and R. Bünger, "Antioxidant pyruvate inhibits cardiac formation of reactive oxygen species through changes in redox state," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 279, no. 5, pp. H2431–H2438, 2000.
- [25] D.X. Hou, Y. Korenori, S. Tanigawa et al., "Dynamics of Nrf2 and Keap1 in ARE-mediated NQO1 expression by wasabi 6-(methylsulfinyl)hexyl isothiocyanate," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 22, pp. 11975–11982, 2011.
- [26] J. A. Baur and D. A. Sinclair, "Therapeutic potential of resveratrol: the in vivo evidence," *Nature Reviews Drug Discovery*, vol. 5, no. 6, pp. 493–506, 2006.
- [27] I. El Hamamsy, L.M. Stevens, M. Carrier et al., "Effect of intravenous N-acetylcysteine on outcomes after coronary

- artery bypass surgery: a randomized, double-blind, placebo-controlled clinical trial," *Journal of Thoracic and Cardiovascular Surgery*, vol. 133, no. 1, pp. 7–12, 2007.
- [28] S. Kinlay, D. Behrendt, J. C. Fang et al., "Long-term effect of combined vitamins E and C on coronary and peripheral endothelial function," *Journal of the American College of Cardiology*, vol. 43, no. 4, pp. 629–634, 2004.
- [29] M. Joseph, H. Tze chen, and Z. R. Wang, "Cardioprotection by resveratrol: a review of effects/targets in cultured cells and animal tissues," *American Journal of Cardiovascular Disease*, vol. 1, pp. 38–47, 2011.
- [30] W. Zhang, J. Xue, M. Ge, M. Yu, L. Liu, and Z. Zhang, "Resveratrol attenuates hepatotoxicity of rats exposed to arsenic trioxide," *Food and Chemical Toxicology*, vol. 51, pp. 87–92, 2013.
- [31] G. Haskó and P. Pacher, "Endothelial Nrf2 activation: a new target for resveratrol?" *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 299, no. 1, pp. H10–H12, 2010.
- [32] R. M. Shaw and Y. Rudy, "Ionic mechanisms of propagation in cardiac tissue: roles of the sodium and L-type calcium currents during reduced excitability and decreased gap junction coupling," *Circulation Research*, vol. 81, no. 5, pp. 727–741, 1997.
- [33] M. Lagouge, C. Argmann, Z. Gerhart-Hines et al., "Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 $\alpha$ ," *Cell*, vol. 127, no. 6, pp. 1109–1122, 2006.
- [34] S. Orrenius, V. O. Kaminsky, and B. Zhivotovsky, "Autophagy in toxicology: cause or consequence?" *The Annual Review of Pharmacology and Toxicology*, vol. 53, pp. 275–97, 2013.
- [35] D. Sumi, T. Sasaki, H. Miyataka, and S. Himeno, "Rat H9c2 cardiac myocytes are sensitive to arsenite due to a modest activation of transcription factor Nrf2," *Archives of Toxicology*, vol. 85, no. 12, pp. 1509–1516, 2011.
- [36] M. Athar, J. H. Back, X. Tang et al., "Resveratrol: a review of preclinical studies for human cancer prevention," *Toxicology and Applied Pharmacology*, vol. 224, no. 3, pp. 274–283, 2007.
- [37] J. M. Matés, J. A. Segura, F. J. Alonso, and J. Márquez, "Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis," *Archives of Toxicology*, vol. 82, no. 5, pp. 273–299, 2008.

## Research Article

# Effect of KIOM-79 on Diabetes-Induced Myocardial Fibrosis in Zucker Diabetic Fatty Rats

**Junghyun Kim, Eunjin Sohn, Chan-Sik Kim, Yun Mi Lee, Kyuhyung Jo, and Jin Sook Kim**

*Korean Medicine Based Herbal Drug Development Group, Herbal Medicine Research Division, Korea Institute of Oriental Medicine, 1672 Yuseongdae-ro, Yuseong-gu, Daejeon 305-811, Republic of Korea*

Correspondence should be addressed to Jin Sook Kim; [jskim@kiom.re.kr](mailto:jskim@kiom.re.kr)

Received 15 February 2013; Revised 25 September 2013; Accepted 29 September 2013

Academic Editor: Keji Chen

Copyright © 2013 Junghyun Kim et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

KIOM-79, a herbal mixture of parched *Puerariae radix*, gingered *Magnoliae cortex*, *Glycyrrhizae radix*, and *Euphorbiae radix*, has a strong inhibitory effect on advanced glycation end products (AGEs) formation. We investigated the beneficial effects of KIOM-79 on cardiac fibrosis in Zucker diabetic fatty (ZDF) rats. KIOM-79 (50 or 500 mg/kg/day) was orally administered for 13 weeks. AGEs formation and collagen expression in the myocardium were assessed by immunohistochemistry. The expression levels of the receptor for AGEs (RAGE), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), collagen IV, fibronectin, urotensin II, and urotensin II receptor were examined in the myocardial tissue of ZDF rats. KIOM-79 treatment at 500 mg/kg inhibited the accumulation of AGEs, reduced RAGE mRNA and protein expression, and reduced the upregulation of cardiac fibrogenic factors, such as fibronectin and collagen IV, in heart of ZDF rats. Additionally, KIOM-79 ameliorated urotensin II/receptor gene expression in the cardiac tissue of ZDF rats. Our findings indicate that KIOM-79 diminishes cardiac fibrosis in ZDF rats by preventing AGEs accumulation and RAGE overexpression and by modulating the cardiac urotensin II/receptor pathway, which decreases the amount of profibrotic factors, such as TGF- $\beta$ 1, fibronectin, and collagen in cardiac tissue.

## 1. Introduction

Diabetic cardiomyopathy is characterized by myocardial fibrosis, which leads to decreased elasticity and impaired contractile function of the heart. The link between hyperglycemia and the development of diabetic cardiomyopathy involves the accumulation of advanced glycation end products (AGEs) in cardiac tissue. Within the cells, AGEs and their precursors modify molecules and produce irreversible cross-linkages between extracellular matrix proteins [1, 2], which compromises tissue compliance and produces myocardial stiffness [3, 4]. AGEs-mediated modification of matrix proteins disrupts matrix-matrix and matrix-cell interactions, which contributes to their profibrotic properties. AGEs play an important role in cell signaling through their interaction with specific receptors, namely, the receptor for advanced glycation end products (RAGE), which activates adhesion molecules, proinflammatory cytokines, and growth factors, and contributes to the pathogenesis of diabetic complications [5].

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is regarded as the main profibrotic factor and upregulation of TGF- $\beta$ 1 protein expression was observed in the myocardium of rodents with diabetic cardiomyopathy [6]. Profibrotic factors have been pathogenetically linked to the excessive accumulation of collagenous matrix in a wide range of organ and disease states, including the diabetic heart [7].

Recently, the potent vasoconstrictor peptide urotensin II has emerged as a contributor to the pathology of cardiovascular disease. It has been suggested that urotensin II also plays a role in cardiac fibrosis, and it has been identified within the heart [8], where there is an abundant expression of urotensin II receptor [9]. Recent studies reported that the upregulated expression of urotensin II and its receptor were accompanied with profibrotic factor TGF- $\beta$ 1 expression and extracellular matrix accumulation in the kidney and myocardium of diabetic animal models [10–12].

KIOM-79 is a new herbal prescription and composed of four medicinal herbs: parched *Puerariae Radix*, gingered *Magnolia Cortex*, *Glycyrrhiza Radix*, and *Euphorbia Radix*,

TABLE 1: Body weight, blood glucose, and heart weight at the end of the experiment.

Group	Body weight (g)	Blood glucose (mg/dL)	Heart weight (mg/100 g)
ZL	338.5 ± 40.5	92.93 ± 10.76	352.4 ± 11.4
ZDF	433.2 ± 69.4*	489.8 ± 038.0**	339.9 ± 14.1
KIOM-79-50	414.6 ± 45.0	391.70 ± 113.5	348.8 ± 20.9
KIOM-79-500	422.9 ± 49.1	390.80 ± 79.55	339.9 ± 15.3

All data were expressed as the mean ± S.E.M. \* $P < 0.05$  compared to ZL rats; \*\* $P < 0.01$  compared to ZL rats.

and each herb has been used in traditional Korean medicine and other countries for a variety of medical purposes, including diabetes [13, 14]. Pueraria radix has potential medicinal benefits in diabetes and cardiovascular disease [15]. Honokiol, a major bioactive compound of *Magnolia officinalis*, has anti-inflammatory and antifibrotic effects [16]. Glycyrrhizin, a major bioactive compound of Glycyrrhizae radix, inhibited hepatic fibrogenesis [17]. Euphorbiae radix has antihyperglycemic [18] and anti-AGEs effects [19]. Our previous studies reported that KIOM-79 has a pharmacological effects on diabetic condition [20–23]. In addition, KIOM-79 prevented S100b-induced TGF- $\beta$ 1, fibronectin, and NF- $\kappa$ B expression in mesangial cells cultured under diabetic conditions and the progression of diabetic nephropathy in type 2 diabetic rats [24, 25].

Thus, the aim of the current study is to examine the pharmacological effects of KIOM-79 on diabetic cardiac fibrosis in Zucker diabetic fatty (ZDF) rats, which is a genetic animal model of type 2 diabetes.

## 2. Materials and Methods

**2.1. KIOM-79 Preparation.** The cortex of *Magnolia officinalis* Rehd. et Wils. (Magnoliaceae), radix of *Pueraria lobata* Ohwi (Leguminosae), radix of *Glycyrrhiza uralensis* Fisch (Leguminosae), and radix of *Euphorbia pekinensis* Ruprecht (Euphorbiaceae) were collected from the Gamsuk province in China in 2003 after identification by botanist Professor J. H. Kim (Department of Life Science, Kyungwon University, Korea). All voucher specimens were stored at the herbarium of the Korea Institute of Oriental Medicine (Nos. 1240, 2, 7, and 207, resp.). Magnoliae cortex (100 g) was simmered with 3 g of Zingiberis rhizoma for 60 min. Puerariae radix (100 g) was stir-roasted at 75°C for 45 min; when its surface became yellow with brown spots, it was removed and cooled. Equal amounts of gingered Magnoliae cortex, parched Puerariae radix, Glycyrrhizae radix, and Euphorbiae Radix were mixed, pulverized, extracted in 80% EtOH for 1 week at room temperature, concentrated with a rotary evaporator, and lyophilized. The entire procedure was repeated for four times. The quality of KIOM-79 was controlled by HPLC [19].

**2.2. Animal Treatment.** Male Zucker diabetic fatty (*fa/fa*, ZDF) and Zucker lean (*fa/+* or *+/+*, ZL) rats were obtained at 6 weeks of age from Charles River Laboratory (Wilmington, MA, USA). Rats were allowed free access to water and food. KIOM-79 was dissolved in water and orally administered to the rats for 13 weeks. Animals were divided into four

groups: Zucker lean rats (ZL,  $n = 7$ ); Zucker diabetic fatty rats (ZDF,  $n = 7$ ); Zucker diabetic fatty rats treated with KIOM-79-50 (50 mg/kg body weight, ZDF + KIOM-79,  $n = 8$ ); Zucker diabetic fatty rats treated with KIOM-79-500 (500 mg/kg body weight, ZDF + KIOM-79,  $n = 8$ ). The dosage of freeze-dried powder was calculated based on the human equivalent dosage of raw herbs. At the end of experimental period, the body weight and heart weight of the rats were measured. The ZDF rats were anesthetized with diethyl ether, and blood and tissue samples were taken for glucose measurement and histological examination. All experimental protocols involving the use of animals were conducted in accordance with National Institutes of Health (NIH) guidelines and approved by the Committee on Animal Care of our institute.

**2.3. Western Blot Analysis.** Cardiac tissue (0.1–0.2 g) from the rats was lysed with a homogenizer at 3000 rpm in a solution containing 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mM potassium phosphate buffer at pH 7.6. Equal amounts of protein (50  $\mu$ g) were analyzed with immunoblotting techniques with the indicated antibodies. The antibodies used were as follows: TGF- $\beta$ 1, RAGE, collagen IV (1:1000, Santa Cruz Biotechnology, CA, USA), and  $\beta$ -actin (1:3000, Sigma, USA). A horseradish peroxidase-conjugated secondary antibody was used and detected with an enhanced chemiluminescence detection system (iNTRON Biotechnology, Korea). Protein expression levels were assessed by analyzing the signal from the PVDF membranes using an image analyzer (Las-3000, Fuji photo, Tokyo, Japan).

**2.4. RNA Isolation and RT-PCR.** Total RNA was isolated using TRIzol reagent (MCRC, Cincinnati, OH, USA) according to the manufacturer's instructions. cDNA was synthesized with 3  $\mu$ g of RNA using RT-premix (Bioneer, Korea). RNA was reverse transcribed using a Takara PCR Thermal Cycler (Japan). Primer sequences are summarized in Table 1. RT-PCR products were separated by electrophoresis with 1.2% agarose gels containing ethidium bromide (EtBr), and DNA band intensities were quantified using densitometry (Las-3000, Fuji photo, Tokyo, Japan).

**2.5. Immunohistochemistry.** To determine collagen deposition in the cardiac tissue, paraffin-embedded sections were deparaffinized, sectioned, and stained using Masson's

TABLE 2: Primer sequences for RT-PCR.

Gene	Sequence (5'-3')
RAGE	5'-ACT ACC GAG TCC GAG TCT ACC A-3' 5'-GCT CTG ACC GAA GCG TGA-3'
TGF- $\beta$ 1	5'-CGA GGT GAC CTG GGC ACC ATC CAT GAC-3' 5'-CTG CTC CAC CTT GGG CTT GCG ACC CAC-3'
Fibronectin	5'-CAG GCT CAG CAA ATC GTG CA-3' 5'-CCC CAC GAC CTA GGA AGT C-3'
Urotensin II	5'-TGC CTG CTC TTC GTA GGA CT-3' 5'-AGA GCC TTC CTC AAG CTT-3'
Urotensin II receptor	5'-CTG TGA CTG AGC TGC CTG GTG AC-3' 5'-GGT GGC TAT GAT GAA GGG AAT GC-3'
$\beta$ -actin	5'-TCA TTG ACC TCA ACT ACA-3' 5'-CAA AGT TGT CAT GGA TGA CC-3'

trichrome. For AGEs immunohistochemistry, the deparaffinized sections were hydrated and treated with 1% H<sub>2</sub>O<sub>2</sub> in methanol. Sections were incubated with anti-AGEs antibody (1:100, Transgenic Inc. Kobe, Japan) for 2 h at room temperature using a standard manual immunoperoxidase procedure with streptavidin-peroxidase (LSAB 2 kit, Dako, CA, USA). The stained sections were observed using a light microscopy (Olympus BX51, Japan) equipped with an Olympus DP 71 camera. The intensity of the staining tissue sections was analyzed using Image J software (NIH).

**2.6. Statistical Analysis.** Data are expressed as the mean  $\pm$  S.E.M and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or by an unpaired Student's *t*-test using GraphPad Prism 4.0 software (Graph pad, San Diego, CA, USA). Differences with a value of  $P < 0.05$  were considered to be statistically significant.

### 3. Results

**3.1. Glucose Level, Body Weight, and Organ Weight of Experimental Rats.** Table 2 shows the effect of KIOM-79 treatment on the general biochemical parameters of blood glucose levels, body weight, and heart weight. Untreated ZDF rats exhibited markedly increased blood glucose levels compared to control ZL rats ( $P < 0.05$ ) and did not exhibit differences in glucose levels compared to ZDF rats treated with KIOM-79. Body weight gain in untreated ZDF rats was significantly higher than that of control ZL rats. KIOM-79 treatment did not change the body weight of ZDF rats when compared to untreated ZDF rats. There were no significant changes in heart weight in the rats in all groups.

**3.2. Effect on AGEs Accumulation and RAGE Expression in Heart Tissue.** Immunohistochemistry was used to determine whether KIOM-79 inhibits AGEs accumulation in the heart. Untreated ZDF rats had significantly higher levels of AGEs accumulation in the heart when compared to control ZL rats ( $P < 0.05$ ). However, KIOM-79 treatment suppressed AGEs accumulation markedly in ZDF rats compared to untreated ZDF rats ( $P < 0.05$ ) (Figures 1(a) and 1(b)). The protein expression of AGEs in cardiac tissue is shown in Figures

1(c) and 1(d). The multiple and intensive bands for advanced glycation adducts were detected in diabetic cardiac tissues from ZDF rats. The elevated level of AGEs was significantly decreased by the administration of 500 mg/kg body weight per day of KIOM-79 ( $P < 0.05$ ). In addition, RAGE expression in untreated ZDF rats was significantly increased when compared to its expression in control ZL rats. However, KIOM-79 treatment at the higher concentration significantly reversed the enhanced RAGE expression in ZDF rats ( $P < 0.05$ ) (Figure 2).

**3.3. Effect on Accumulation of Collagen in Heart.** The degree of cardiac fibrosis was assessed using Masson's trichrome staining, and representative images are shown in Figure 3(a). Positive stained fibrotic area (blue color) was evaluated under a microscope. Collagen was detected in the interstitium of the heart of untreated ZDF rats at a greater level than in control ZL rats, and the collagen expression was lower in the heart of ZDF rats treated with KIOM-79 at the higher concentration.

**3.4. Effect on Expression of Fibrogenic Factor in Heart.** Expression of a cardiac fibrosis factor, TGF- $\beta$ 1, was assessed by western blot and RT-PCR. The expression of TGF- $\beta$ 1 was found to be significantly higher in ZDF rats compared to control ZL rats ( $P < 0.05$ ). High concentration KIOM-79 treatment in ZDF rats significantly inhibited the increase in TGF- $\beta$ 1 protein and gene expressions in ZDF rats (Figures 4(a) and 4(b)). In addition, fibronectin and collagen are major components of the extracellular matrix that play an important role in abnormal cardiac muscle function. Therefore, we examined their gene and protein expression in heart. Fibronectin gene and collagen IV protein expression were significantly increased in the heart of ZDF rats compared to control ZL rats ( $P < 0.05$ ). Treatment with KIOM-79 at the higher concentration reduced the expressions of fibronectin gene and collagen IV protein when compared to control ZL rats ( $P < 0.05$ ) (Figures 4(c) and 4(d)).

**3.5. Effect on Expression of Urotensin II Gene in Heart.** To investigate the molecular mechanism underlying the anti-fibrogenic effect of KIOM-79 in ZDF rats, we examined the cardiac expression of urotensin II and its receptor. Cardiac

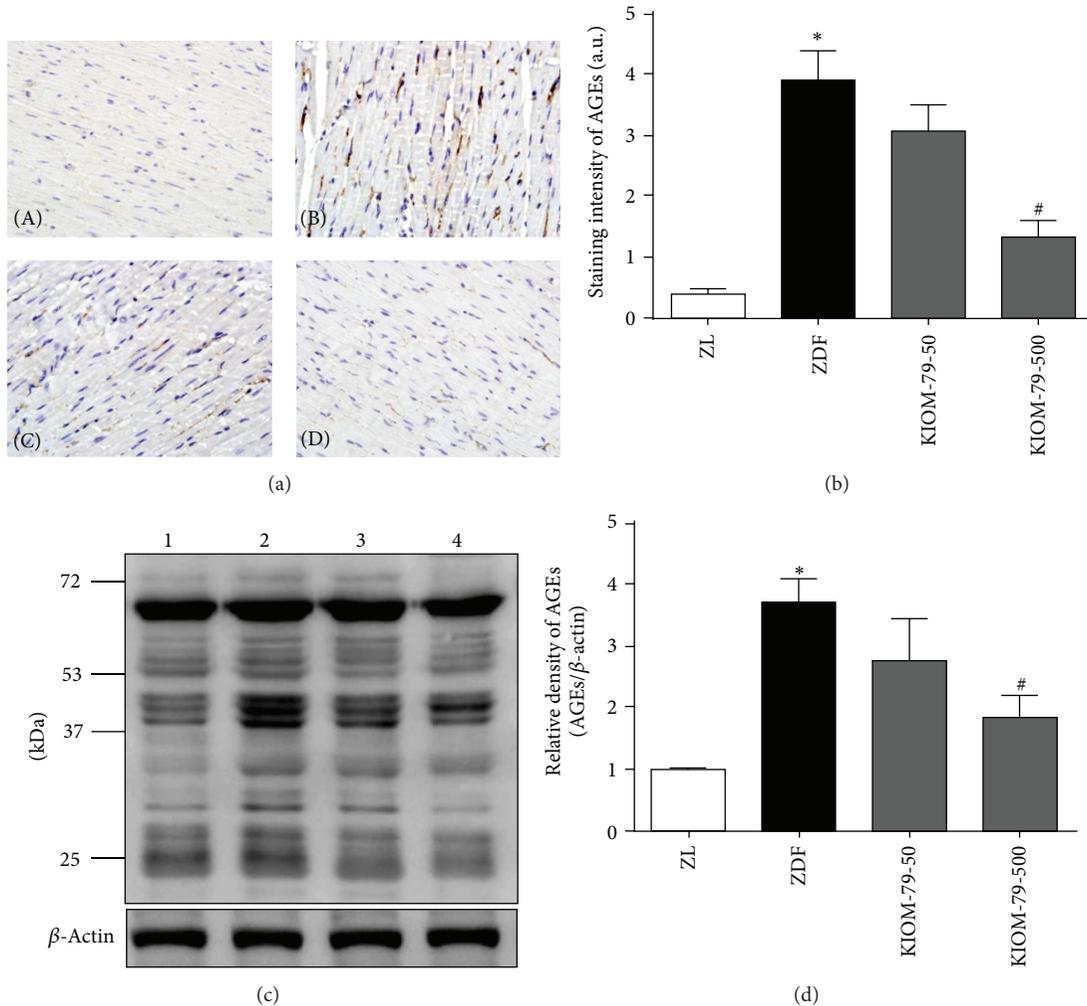


FIGURE 1: Effect of KIOM-79 treatment on AGEs accumulation in the heart. (a) Representative immunohistochemistry images for AGEs (a) staining in the heart of ZL rats (A), untreated ZDF rats (B), ZDF rats treated with KIOM-79 (50 mg/kg) (C), and ZDF rats treated with KIOM-79 (500 mg/kg) (D). Original magnification:  $\times 400$ . (b) Quantitative analysis of AGEs stain was calculated. ((c) and (d)) Western blot analysis of AGEs in cardiac tissue. The nonenzymatic reaction of the amino groups of cellular proteins with reducing sugars forms a variety of AGEs. Thus, the multiple bands for advanced glycation adducts were detected. All data were expressed as the mean  $\pm$  S.E.M. \* $P < 0.05$  compared to ZL rats; # $P < 0.05$  compared to untreated ZDF rats.

gene expression of urotensin II in ZDF rats was markedly increased compared to control ZL rats ( $P < 0.05$ ). The treatment of KIOM-79 dose-dependently reduced the expression of urotensin II. In addition, the elevated urotensin II receptor level in untreated ZDF rats was also reduced with treatment of KIOM-79 in a dose-dependent manner (Figure 5).

#### 4. Discussion

The present study demonstrates that KIOM-79 treatment decreased AGEs accumulation and RAGE expression in the heart without hypoglycemia. Although the levels of fibrogenic factors were unchanged with low concentrations of KIOM-79, mRNA and protein levels of fibronectin, collagen IV, and TGF- $\beta 1$  were significantly reduced in cardiac tissues of ZDF rats treated with high concentrations of KIOM-79.

Furthermore, KIOM-79 treatment reduced urotensin II and urotensin II receptor mRNA expression. These results suggest that KIOM-79 treatment may modulate AGEs accumulation and the urotensin pathway in cardiac tissues, which may play a role in reducing cardiac fibrosis in ZDF rats.

KIOM-79 was developed based on the known ability of each herb to treat diabetes in traditional Korean medicine, and each herb also has anti-inflammatory, anti-AGEs, and antifibrotic effects [15–19]. In a 90-day repeated oral dose toxicity study in rats, the no observed adverse effect level (NOAEL) of KIOM-79 was at least 2000 mg/kg/day in both males and females (data not shown). Although the administration of KIOM-79 at 50 mg/kg body weight dose not significantly alter the level of AGEs formation and RAGE expression, our study showed that KIOM-79 dose-dependently inhibits AGEs accumulation and RAGE expression in cardiac tissues of ZDF rats. This finding agrees with the observation

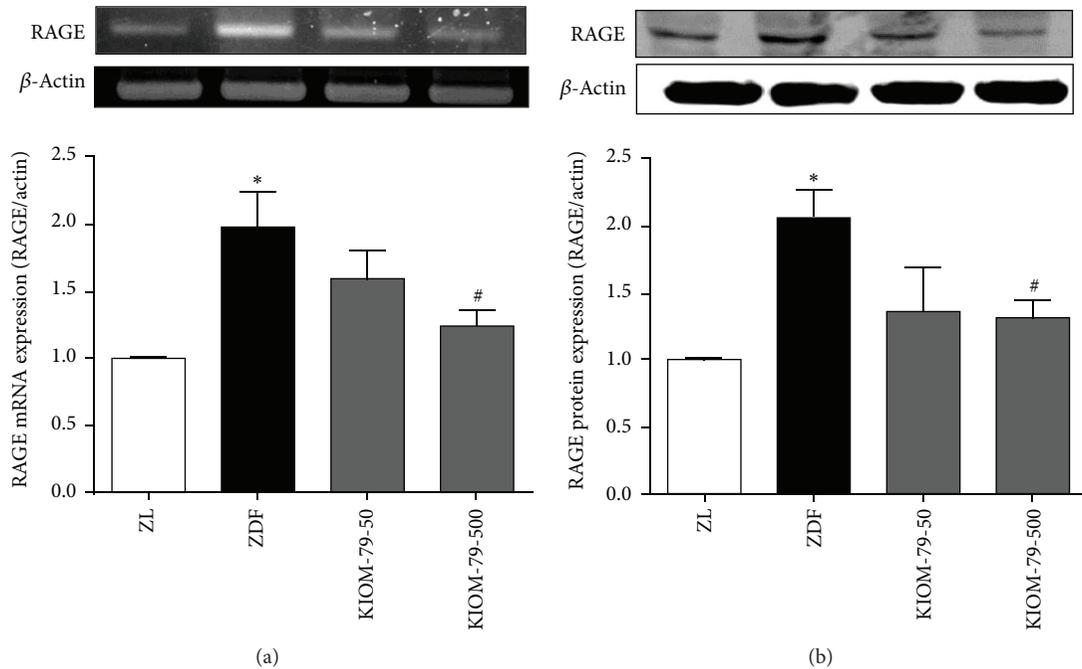


FIGURE 2: Effect of KIOM-79 treatment on RAGE expression in the heart. RAGE mRNA expression (a) and RAGE protein expression (b) in Zucker lean rat (ZL), Zucker diabetic rat (ZDF), ZDF rat treated with KIOM-79 at 50 mg/kg (KIOM-79-50), and ZDF rat treated with KIOM-79 at 500 mg/kg (KIOM-79-500). All data were expressed as the mean ± S.E.M. \**P* < 0.05 compared to ZL rats; #*P* < 0.05 compared to untreated ZDF rats.

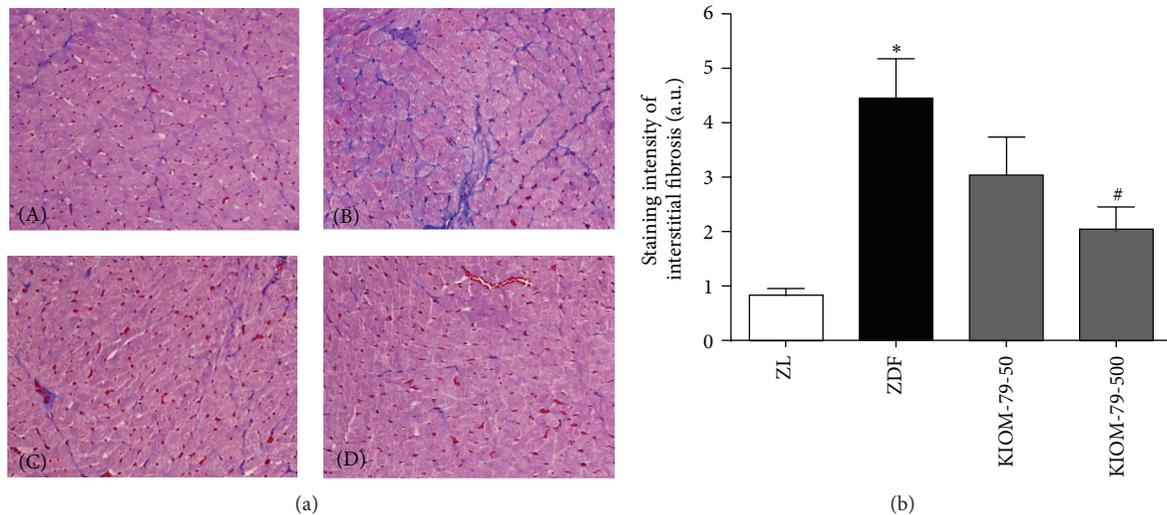


FIGURE 3: Effect of KIOM-79 on cardiac interstitial fibrosis. (a) Representative images for Masson's trichrome staining in the heart of ZL rats (A), untreated ZDF rats (B), ZDF rats treated with KIOM-79 (50 mg/kg) (C) and ZDF rats treated with KIOM-79 (500 mg/kg) (D). Positive staining is visible in blue. Original magnification: ×200. (b) Quantitative analysis. The positive stained area was calculated. All data were expressed as the mean ± S.E.M. \**P* < 0.05 compared to ZL rats; #*P* < 0.05 compared to untreated ZDF rats.

of our previous reports that showed that KIOM-79 has a strong inhibitory effect on AGEs formation and demonstrated beneficial effects of KIOM-79 on AGEs formation in the kidney [26] and retina [22, 23] of diabetic rats. Consistent with these studies, the present study shows that KIOM-79 inhibits AGEs accumulation in the heart of ZDF rats.

Prior studies have shown strong evidence for the positive role of AGEs in the process of the fibrogenesis [27–29]. In

addition, some of the pathogenic effects of AGEs appear to be mediated by its interaction with AGE receptors [30]. TGF-β1 is a primary agent in fibrosis and the induction of TGF-β1 expression by AGEs results in collagen and fibronectin accumulation in the tissue, which is linked to the progression of diabetic complications including those observed in the heart [3, 6, 31]. In this study, treatment with KIOM-79 in ZDF rats suppressed the overexpression of cardiac TGF-β1,

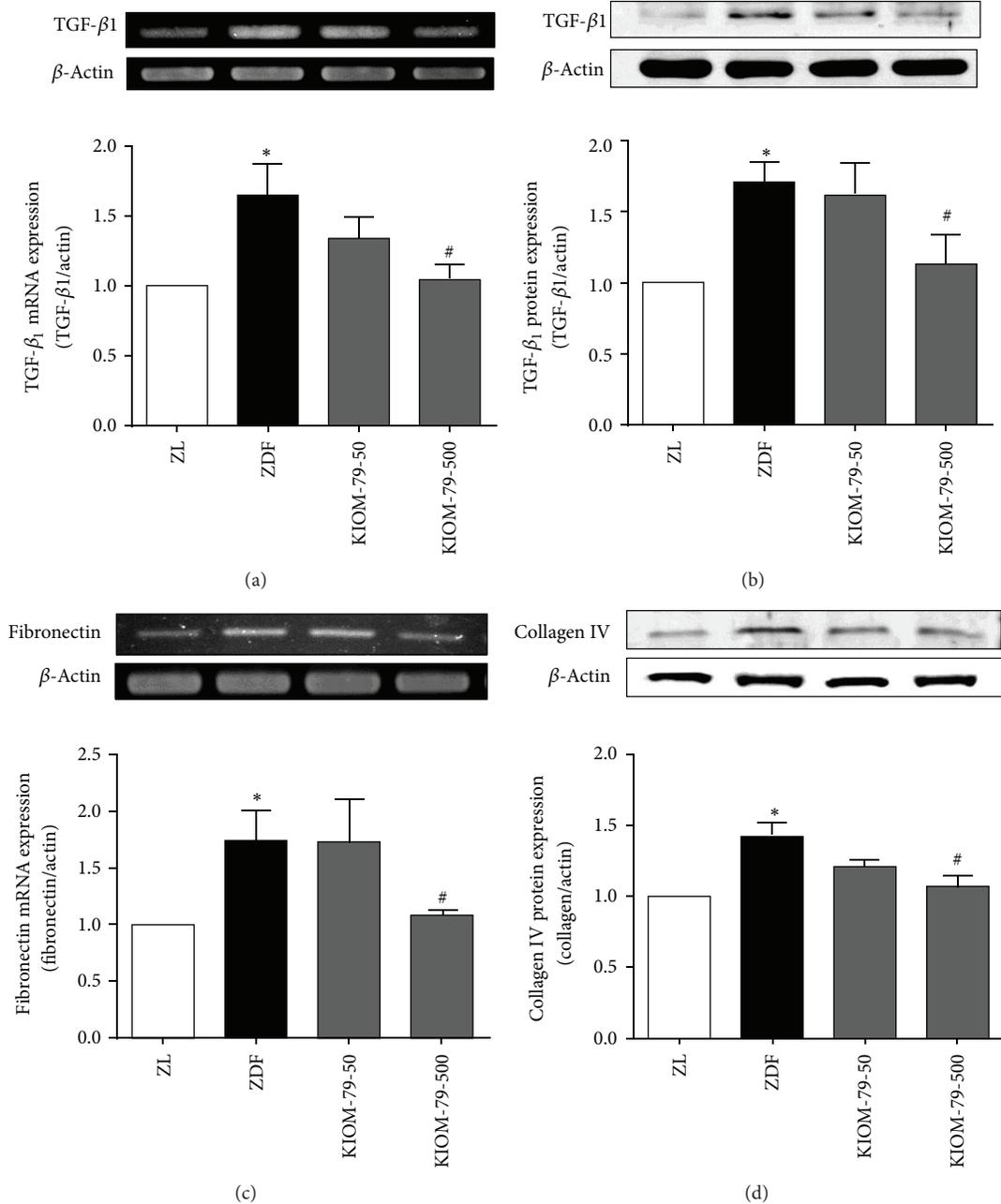


FIGURE 4: Effect of KIOM-79 treatment on the expression of TGF- $\beta_1$ , fibronectin, and collagen IV in the heart. (a) TGF- $\beta_1$  mRNA expression, (b) TGF- $\beta_1$  protein expression, (c) fibronectin mRNA expression, and (d) collagen IV protein expression. The results were normalized to  $\beta$ -actin. All data were expressed as the mean  $\pm$  S.E.M. \*  $P < 0.05$  compared to ZL rats; #  $P < 0.05$  compared to untreated ZDF rats.

fibronectin, and collagen IV. Additionally, accumulation of collagen in the interstitium of the heart was ameliorated by KIOM-79 treatment. Our recently published study demonstrated that KIOM-79 inhibits expression of AGEs-induced TGF- $\beta_1$  and fibronectin in a cultured mesangial cell line [25]. The results presented in this study confirm that KIOM-79 prevents the hyperglycemia-induced accumulation of AGEs and the expression of fibrogenic factors, such as TGF- $\beta_1$ , fibronectin, and collagen IV, in the cardiac tissue of ZDF rats.

Recently, a number of studies have suggested that the urotensin/urotensin receptor system may play an important role in the pathogenesis of diabetic cardiomyopathy. Expression of urotensin II and its receptor is localized to cardiomyocytes, endothelial cells, smooth muscle cells, and cardiac fibroblast of the diabetic hearts [12, 32]. Experimental and clinical studies have revealed an increased expression of urotensin II in animals with experimentally induced myocardial infarction and diabetes and in patients with diabetes [33, 34], as well as a promotion of cell proliferation and

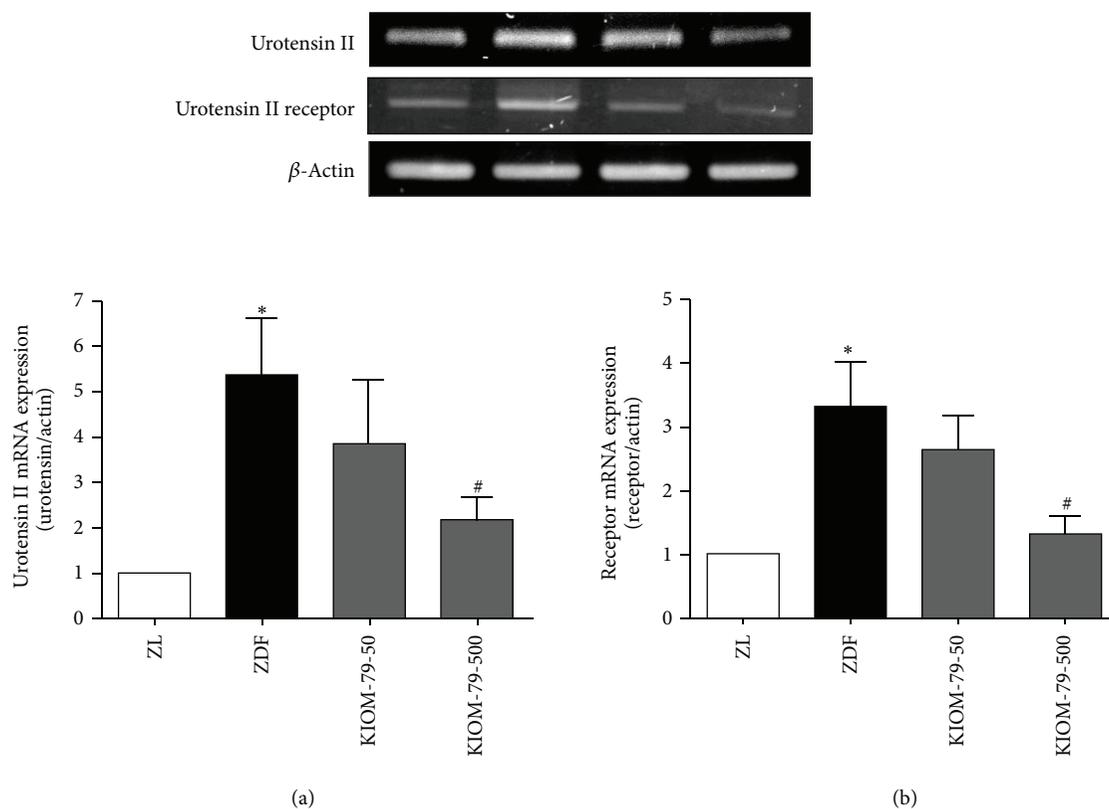


FIGURE 5: Effect of KIOM-79 treatment on the expressions of urotensin II and urotensin II receptor gene in the heart. (a) Urotensin II mRNA expression and (b) urotensin II receptor mRNA expression. The results were normalized to  $\beta$ -actin. All data were expressed as the mean  $\pm$  S.E.M. \*  $P < 0.05$  compared to ZL rats; #  $P < 0.05$  compared to untreated ZDF rats.

stimulated extra cellular matrix in diabetic animal models [8, 10, 35]. Consistent with these studies, the present study shows, for the first time, that urotensin II and its receptor gene expression are increased in the cardiac tissue of ZDF rats. Interestingly, KIOM-79 treatment dose-dependently inhibits the upregulation of urotensin II and its receptor gene expression in ZDF rats. These data suggest that KIOM-79 may regulate the cardiac urotensin pathway and may be partially involved in the regulation of profibrotic factor proteins.

Taken together, our finding demonstrate that KIOM-79 diminishes cardiac fibrosis in ZDF rats by preventing accumulation of AGEs and RAGE overexpression and by modulating the cardiac urotensin II/receptor pathway, which produces a decrease in profibrotic factors such as TGF- $\beta$ 1, fibronectin, and collagen in cardiac tissue. These data suggest that KIOM-79 may be a promising anti-fibrogenic agent in the diabetic heart and may delay diabetes-related cardiac complication caused by fibrosis.

## Acknowledgment

This research was supported by a Grant [K12010] from the Korea Institute of Oriental Medicine (KIOM).

## References

- [1] R. Candido, J. M. Forbes, M. C. Thomas et al., "A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes," *Circulation Research*, vol. 92, no. 7, pp. 785–792, 2003.
- [2] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.
- [3] M. Aragno, R. Mastrocola, G. Alloatti et al., "Oxidative stress triggers cardiac fibrosis in the heart of diabetic rats," *Endocrinology*, vol. 149, no. 1, pp. 380–388, 2008.
- [4] D. Aronson, "Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes," *Journal of Hypertension*, vol. 21, no. 1, pp. 3–12, 2003.
- [5] R. M. Mason and N. A. Wahab, "Extracellular matrix metabolism in diabetic nephropathy," *Journal of the American Society of Nephrology*, vol. 14, no. 5, pp. 1358–1373, 2003.
- [6] D. Westermann, S. Rutschow, S. Jäger et al., "Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of angiotensin type 1 receptor antagonism," *Diabetes*, vol. 56, no. 3, pp. 641–646, 2007.
- [7] K. J. Way, K. Isshiki, K. Suzuma et al., "Expression of connective tissue growth factor is increased in injured myocardium associated with protein kinase C  $\beta$ 2 activation and diabetes," *Diabetes*, vol. 51, no. 9, pp. 2709–2718, 2002.

- [8] A. Tzanidis, R. D. Hannan, W. G. Thomas et al., "Direct actions of Urotensin II on the heart: implications for cardiac fibrosis and hypertrophy," *Circulation Research*, vol. 93, no. 3, pp. 246–253, 2003.
- [9] R. S. Ames, H. M. Sarau, J. K. Chambers et al., "Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14," *Nature*, vol. 401, no. 6750, pp. 282–286, 1999.
- [10] L. Tian, C. Li, J. Qi et al., "Diabetes-induced upregulation of urotensin II and its receptor plays an important role in TGF- $\beta$ 1-mediated renal fibrosis and dysfunction," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 295, no. 5, pp. E1234–E1242, 2008.
- [11] R. G. Langham, D. J. Kelly, R. M. Gow et al., "Increased expression of urotensin II and urotensin II receptor in human diabetic nephropathy," *American Journal of Kidney Diseases*, vol. 44, no. 5, pp. 826–831, 2004.
- [12] H.-Y. Dai, X.-G. Guo, Z.-M. Ge et al., "Elevated expression of urotensin II and its receptor in diabetic cardiomyopathy," *Journal of Diabetes and its Complications*, vol. 22, no. 2, pp. 137–143, 2008.
- [13] W. L. Li, H. C. Zheng, J. Bukuru, and N. De Kimpe, "Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus," *Journal of Ethnopharmacology*, vol. 92, no. 1, pp. 1–21, 2004.
- [14] J. Hur, *Donguibogam Paralled Version*, Committee of Dongui Bogam Translation, Bupin publishing Co., Seoul, Korea, 1999.
- [15] K. H. Wong, G. Q. Li, K. M. Li, V. Razmovski-Naumovski, and K. Chan, "Kudzu root: traditional uses and potential medicinal benefits in diabetes and cardiovascular diseases," *Journal of Ethnopharmacology*, vol. 134, no. 3, pp. 584–607, 2011.
- [16] C.-K. Chiang, M.-L. Sheu, Y.-W. Lin et al., "Honokiol ameliorates renal fibrosis by inhibiting extracellular matrix and pro-inflammatory factors in vivo and in vitro," *British Journal of Pharmacology*, vol. 163, no. 3, pp. 586–597, 2011.
- [17] C. T. Tu, J. Li, F. P. Wang, L. Li, J. Y. Wang, and W. Jiang, "Glycyrrhizin regulates CD4+ T cell response during liver fibrogenesis via JNK, ERK and PI3K/AKT pathway," *International Immunopharmacology*, vol. 14, no. 4, pp. 410–421, 2012.
- [18] F. J. Alarcon-Aguilara, R. Roman-Ramos, S. Perez-Gutierrez, A. Aguilar-Contreras, C. C. Contreras-Weber, and J. L. Flores-Saenz, "Study of the anti-hyperglycemic effect of plants used as antidiabetics," *Journal of Ethnopharmacology*, vol. 61, no. 2, pp. 101–110, 1998.
- [19] J. Kim, C. S. Kim, E. Sohn, Y. M. Lee, K. Jo, and J. S. Kim, "KIOM-79 protects AGE-induced retinal pericyte apoptosis via inhibition of NF-kappaB activation in vitro and in vivo," *PLoS One*, vol. 7, no. 8, article e43591, 2012.
- [20] K. Ah Kang, J. S. Kim, R. Zhang et al., "Induction of heme oxygenase-1 by plant extract KIOM-79 via Akt pathway and NF-E2 related factor 2 in pancreatic  $\beta$ -cells," *Journal of Toxicology and Environmental Health A*, vol. 71, no. 20, pp. 1392–1399, 2008.
- [21] K. A. Kang, K. H. Lee, S. Y. Kim, H. S. Kim, J. S. Kim, and J. W. Hyun, "Cytoprotective effects of KIOM-79 on streptozotocin induced cell damage by inhibiting ERK and AP-1," *Biological and Pharmaceutical Bulletin*, vol. 30, no. 5, pp. 852–858, 2007.
- [22] Y. S. Kim, D. H. Jung, N. H. Kim et al., "KIOM-79 inhibits high glucose or AGEs-induced VEGF expression in human retinal pigment epithelial cells," *Journal of Ethnopharmacology*, vol. 112, no. 1, pp. 166–172, 2007.
- [23] E. J. Sohn, Y. S. Kim, C.-S. Kim, Y. M. Lee, and J. S. Kim, "KIOM-79 prevents apoptotic cell death and AGEs accumulation in retinas of diabetic db/db mice," *Journal of Ethnopharmacology*, vol. 121, no. 1, pp. 171–174, 2009.
- [24] D. H. Jung, Y. S. Kim, and J. S. Kim, "KIOM-79 prevents S100b-induced TGF- $\beta$ 1 and fibronectin expression in mouse mesangial cells," *Journal of Ethnopharmacology*, vol. 125, no. 3, pp. 374–379, 2009.
- [25] J. S. Kim, Y. S. Kim, J. Kim et al., "KIOM-79, an inhibitor of AGEs-protein cross-linking, prevents progression of nephropathy in Zucker diabetic fatty rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 761859, 10 pages, 2011.
- [26] C.-S. Kim, E. J. Sohn, Y. S. Kim et al., "Effects of KIOM-79 on hyperglycemia and diabetic nephropathy in type 2 diabetic Goto-Kakizaki rats," *Journal of Ethnopharmacology*, vol. 111, no. 2, pp. 240–247, 2007.
- [27] J. S. Huang, J. Y. Guh, H. C. Chen, W. C. Hung, Y. H. Lai, and L. Y. Chuang, "Role of receptor for advanced glycation end-product (RAGE) and the JAK/STAT-signaling pathway in AGE-induced collagen production in NRK-49F cells," *Journal of Cellular Biochemistry*, vol. 81, no. 1, pp. 102–113, 2001.
- [28] C.-I. Lee, J.-Y. Guh, H.-C. Chen et al., "Leptin and connective tissue growth factor in advanced glycation end-product-induced effects in NRK-49F cells," *Journal of Cellular Biochemistry*, vol. 93, no. 5, pp. 940–950, 2004.
- [29] G. Zhou, C. Li, and L. Cai, "Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor  $\beta$ -independent pathway," *American Journal of Pathology*, vol. 165, no. 6, pp. 2033–2043, 2004.
- [30] S. F. Yan, R. Ramasamy, Y. Naka, and A. M. Schmidt, "Glycation, inflammation, and RAGE: a scaffold for the macrovascular complications of diabetes and beyond," *Circulation Research*, vol. 93, no. 12, pp. 1159–1169, 2003.
- [31] J. M. Forbes, V. Thallas, M. C. Thomas et al., "The breakdown of preexisting advanced glycation end products is associated with reduced renal fibrosis in experimental diabetes," *The FASEB Journal*, vol. 17, no. 12, pp. 1762–1764, 2003.
- [32] S. A. Douglas, L. Tayara, E. H. Ohlstein, N. Halawa, and A. Giaid, "Congestive heart failure and expression of myocardial urotensin II," *The Lancet*, vol. 359, no. 9322, pp. 1990–1997, 2002.
- [33] K. Totsune, K. Takahashi, Z. Arihara et al., "Elevated plasma levels of immunoreactive urotensin II and its increased urinary excretion in patients with Type 2 diabetes mellitus: association with progress of diabetic nephropathy," *Peptides*, vol. 25, no. 10, pp. 1809–1814, 2004.
- [34] P. N. Sidharta, F. D. Wagner, H. Bohnemeier et al., "Pharmacodynamics and pharmacokinetics of the urotensin II receptor antagonist palosuran in macroalbuminuric, diabetic patients," *Clinical Pharmacology and Therapeutics*, vol. 80, no. 3, pp. 246–256, 2006.
- [35] Y.-G. Zhang, Y.-G. Li, B.-G. Liu et al., "Urotensin II accelerates cardiac fibrosis and hypertrophy of rats induced by isoproterenol," *Acta Pharmacologica Sinica*, vol. 28, no. 1, pp. 36–43, 2007.

## Review Article

# Ten Years' Research on a Cardiovascular Tonic: A Comprehensive Approach—From Quality Control and Mechanisms of Action to Clinical Trial

**Ping-Chung Leung,<sup>1,2</sup> Chi-Man Koon,<sup>1,2</sup> Clara Bik-San Lau,<sup>1,2</sup>  
Ping Chook,<sup>1</sup> William King-Fai Cheng,<sup>1</sup> Kwok-Pui Fung,<sup>1,2,3</sup>  
Timothy Chi-Yui Kwok,<sup>4</sup> and Kam-Sang Woo<sup>5,6</sup>**

<sup>1</sup> Institute of Chinese Medicine, The Chinese University of Hong Kong, Hong Kong

<sup>2</sup> State Key Laboratory of Phytochemistry and Plant Resources in West China, The Chinese University of Hong Kong, Hong Kong

<sup>3</sup> School of Medical Sciences, The Chinese University of Hong Kong, Hong Kong

<sup>4</sup> Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong

<sup>5</sup> School of Life Sciences, The Chinese University of Hong Kong, Hong Kong

<sup>6</sup> School of Life Sciences, Biochemistry Programme, The Chinese University of Hong Kong, Hong Kong

Correspondence should be addressed to Ping-Chung Leung; pingcleung@cuhk.edu.hk

Received 19 March 2013; Revised 21 August 2013; Accepted 19 September 2013

Academic Editor: Ka Kit Hui

Copyright © 2013 Ping-Chung Leung et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

*Objective.* Mortality arising from cardiovascular pathologies remains one of the highest. Maintenance of cardiovascular health therefore remains a universal concern. Interventional therapies and medications have made impressive advances, but preventive measures would be of the same importance. *Method.* Ten years' search for a simple herbal formula has resulted in a two-herb combination, consisting of *Salviae Miltiorrhizae Radix et Rhizoma* and *Puerariae Lobatae Radix*. The formula has been studied extensively on cardiovascular biological platforms and then put on three clinical trials. *Results.* In the laboratory, the formula was found to have the biological effects of anti-inflammation, anti-oxidation, anti-foam cell formation on vascular endothelium, and vasodilation. Clinical trials using ultrasonic carotid intima thickness as a surrogate marker showed very significant benefits. No significant adverse effects were encountered. *Conclusion.* It is therefore recommended that the herbal formula could be used as an adjuvant therapy in cardiac patients under treatment or as a preventive agent among the susceptible.

## 1. Introduction

The aging population commonly suffers from deteriorating cardiovascular health. Indeed, mortalities related to cardiac failure and cerebral vascular accidents have remained the major causes of death. Although remedial measures are available to maintain cardiovascular well-being, from therapeutic measures to highly sophisticated revascularisation skills, adverse drug effects and recurrences of obstructions are still inevitable [1]. The search for agents that protect the cardiovascular system on a broad base, without being too specific, is a logical attempt [2].

Some herbs in the Chinese medicine have been widely used for the promotion of “circulatory strength,” which in modern terms should mean cardiovascular health. A wide variety of proprietor herbal preparations are available in market and people in the Chinese communities have been using them either in combination with pharmaceuticals like aspirin and statins or as prophylactic agents for blood cholesterol control and/or vascular integrity [3, 4]. We intend to choose, among the many popular herbs traditionally used for cardiovascular problems, the least number to form a simple combined formula to be used as an effective cardiovascular protective tonic. The mechanisms of action need to be

explored properly before the formula would be put on an evidence-based clinical trial.

**1.1. The Two-Herb Formula—Danshen and Gegen (D&G).** Among the many medicinal herbs *Salviae Miltiorrhizae Radix et Rhizoma* (Danshen) stands out as the most frequently used one. Its clinical values and vascular protective effects have such strong historical background that users take it for granted for its efficacy claims [5]. Given a full respect to the philosophy of clinical treatment in the Chinese medicine, we need to identify one more herb to form a combined formula and to gain enhanced effects or synergies.

A deceased respectable herbal expert and clinician, Shi Jin-mo (1882–1968), was well known for his expertise on selecting twin combinations of herbs in the formation of simple, synergistic formulae. Shi advocated the use of *Salviae Miltiorrhizae Radix et Rhizoma* (Danshen) together with *Puerariae Lobatae Radix* (Gegen) for the promotion of a good circulation [6]. Many proprietary manufactures have since made more complicated formulae, based on Shi's recommendation of Danshen and Gegen (D&G).

Danshen and Gegen together, therefore, constitute a simple herbal formula (D&G) favourable for further study on biological platforms to prove its efficacy.

**1.2. Quality Control and Chemical Fingerprint.** Danshen was purchased from Sichuan province and Gegen from Guangdong province of China. Both places are noted for the best quality supply of the respectable herb. Large batches, estimated to be sufficient for both the laboratory and later clinical trials, were acquired to ensure uniformity.

The raw herbs were morphologically authenticated by a botanist and chemically using the thin layer chromatography in accordance with the Chinese Pharmacopoeia 2005. Small quantities of the two raw herbs were deposited as voucher specimens in the sample bank of the Institute of Chinese Medicine, The Chinese University of Hong Kong, with voucher specimen numbers of 2008-3166a and 2008-3167a for Danshen and Gegen, respectively.

They were then washed, cut into small pieces, and weighed in the ratio of 7 : 3. The herbs were soaked with 10-fold of water (v/w) for 1.5 hr, followed by extraction at 100°C for 1 hr. Two subsequent extractions were carried out with 10-fold of water (v/w) for another 1 hr and 0.5 hr. The extracts were combined and concentrated under reduced pressure to give dry D&G powdered extract.

Accurately weighed 0.5 g sample was sonicated with 20 mL methanol for 30 min at 40°C. The solution was filtered and evaporated to dryness. The residue was re-dissolved in 5 mL methanol and filtrated through a 0.45 µm syringe filter. The final extract was further diluted 5 times for LC-DAD-MS analysis.

Chemical analysis was done and recorded using LC-DAD-MS instrumentation with set conditions. An Agilent 1100 Series LC/MSD Trap VL (Agilent Technologies, USA) coupled with a photodiode array detector was used. The mass spectra were acquired using ion trap instrument with an ESI source. The ESI source was operated at a sheath gas

(N<sub>2</sub>) flow of 30 psi, auxiliary gas (N<sub>2</sub>) flow of 10.0 L/min, an ion spray voltage of 3.5 kV, and a capillary temperature of 340°C. For chromatographic separation, the column consisted of a Thermo ODS hypersil reserved-phase column (5 µm, 250 mm × 4.6 mm) and a Thermo ODS hypersil guard column (5 µm, 10 mm × 4.6 mm). The sample injection volume was 10 µL. The detection wavelength was set at 254 and 280 nm, the flow rate was 0.8 mL/min and the column temperature was maintained at 20°C. The mobile phase consisted of 0.8% acetic acid (A) and acetonitrile (B) and operated at gradient separation. The initial condition was A-B (98 : 2, v/v) and remains unchanged for 10 min. Over the next 50 min, the percentage of mobile-phase B increased linearly to 30%. Then the percentage of mobile-phase B increased linearly to 50% on the next 20 min [7–9].

The chemical fingerprint of D&G was thence established and registered.

## 2. Methods

**2.1. Biological Studies.** If D&G was cardiovascular protective, one could expect it to be anti-inflammatory, anti-oxidative, and might be anti-coagulant. These biological activities were serially verified on *in vitro* cell line models. The tests include the following.

### 2.1.1. Anti-Inflammatory and Anti-Oxidative Tests

- (i) Inhibition of LPS—induced nitric oxide production [10].
- (ii) Inhibition of iNOS, COX<sub>2</sub>, and NFκB protein expression using Western blot [11].
- (iii) Inhibition of inflammatory cytokines using ELISA [12].
- (iv) Inhibition of NFκB translocation using electrophoretic motility shift assay (EMSA) [11].
- (v) Inhibition of iNOS and COX<sub>2</sub> inflammatory cytokines gene expressions using real-time PCR [12].
- (vi) Inhibition of foam cell formation using macrophages (RAW 264.7) acetylated low-density lipoprotein uptake [13].

### 2.1.2. Vascular Protection Tests

- (i) Effect of D&G on blood pressure, using spontaneous hypertensive rats (SHRs) [12].
- (ii) Effect of D&G on vasodilation using *ex vivo* aortic ring of rats [5].
- (iii) Effect of D&G on balloon injury-induced neointimal media thickness [14, 15].
- (iv) Effect of D&G on cerebral blood flow using the middle cerebral artery occlusion rat model to evaluate neurological deficit, brain infarct, and anti-oxidative effects on brain tissues [16–19].
- (v) Effect of D&G on myocardium [20–22].

### 2.1.3. Tests on Cardiac Effects (Zebrafish Embryo)

- (i) D&G effects on heart rate [20].
- (ii) D&G effects on acetylcholine and on  $\beta$  adrenergic activities [17].
- (iii) D&G effects on cardiac toxicity.

### 2.1.4. Functional Genomic Studies

- (i) Using rat cardiac myoblast cell line H9c2 exposed to different doses of D&G to check cell proliferation and cell cycles, and using cDNA microarray analysis to identify the 5 categories of genes, namely, cardiovascular, apoptosis, cell proliferation, cytokine and inflammation, and anti-oxidants.
- (ii) Variations were induced through hypoxia treatment and pretreatment with D&G.
- (iii) Tissue specific gene expression pattern, protein expression profiles, and signaling pathways involved were also studied [23, 24].

**2.1.5. Herb-Drug Interaction: Whether D&G Interfere with Systemic Anti-Coagulant (Warfarin).** It is important to understand whether D&G might enhance or lower the anti-coagulant effects of standard, maintenance therapies that many patients are receiving.

- (i) D&G was given together with warfarin to rats, to check tail bleeding time, prothrombin time, and platelet agglutination.
- (ii) Basic pharmacodynamics studies also included interaction with aspirin and diclofenac sodium.
- (iii) Basic bioavailability of D&G using their marker compounds was also studied [25, 26].

**2.2. Clinical Trials.** The three clinical trials were designed as randomized, double-blind, placebo-controlled clinical studies.

**Trial 1.** The clinical trial was aimed to evaluate the efficacy and safety of *Salvia miltiorrhiza* (Danshen) and *Pueraria lobata* (Gegen) in secondary prevention. One hundred (100) eligible coronary patients were randomized to take 6 capsules of the D&G preparation (3 g) or 6 capsules of placebo capsules daily, in a double-blind and parallel fashion for 24 weeks. Brachial flow-mediated dilation (FMD) and carotid intima-media thickness (IMT) were measured using ultrasound technology.

**Trial 2.** Atherosclerosis commonly occurs in patients with hypertension. We hypothesized that Danshen and Gegen (D&G) have beneficial effects on the atherogenesis of high-risk hypertensive subjects. 90 patients with essential hypertension (SBP 160/90 mmHg before treatment) were studied. All subjects were randomized to receive either oral D&G capsules 1g/day, D&G capsules 2g/day, or placebos, in a double-blind parallel fashion for 12 months. Brachial flow-mediated dilation (endothelium-dependent dilation, FMD)

and carotid intima-media thickness (IMT) were measured using ultrasound technology.

**Trial 3.** This clinical study was designed to demonstrate the safety and effectiveness of D&G in the prevention of atherosclerosis in postmenopausal women with early hypercholesterolemia. 165 postmenopausal women were randomized to take the D&G preparation (2 capsules) or placebo capsules (2 capsules) daily, in a double-blind and parallel fashion for 12 months. Carotid intima-media thickness (IMT) was measured using ultrasound technology. The lipid profile was also tested.

## 3. Results

### 3.1. Biological Studies

**3.1.1. Anti-Inflammatory Effects.** The direct anti-inflammatory effects and the indirect effects through anti-oxidative mechanisms, foam cell inhibition, and so forth were all positively demonstrated [13].

**3.1.2. Vascular Protection.** The anti-hypertensive effects of D&G on spontaneous hypertensive rats (SHRs) were clearly shown. The endothelium-independent vasodilatation effects and the nitric oxide related mechanisms were shown in *ex vivo* isolated rat aorta rings model [5, 12, 16–19].

The balloon injury model demonstrated the inhibitory effects of D&G on the deposition of atheromatous plugs [14, 15].

**3.1.3. Cardiac Effects.** D&G reduced acetylcholinesterase activity in zebrafish embryonic hearts. D&G induced bradycardia in zebrafish embryos through the regulation of muscarinic and  $\beta$  adrenergic pathways [17, 20].

**3.1.4. Functional Genomic Studies.** To understand the molecular mechanism of the cardio protection effect of D&G, the functional specific cDNA microarray was used to study the expression profile of genes related to cardiac disease biomarker, apoptosis, cell cycle and proliferation, cytokine and inflammation, and anti-oxidants. A homemade rat cDNA microarray containing 100 genes was fabricated to study gene expression profiles of H9c2 cells upon 50  $\mu$ g/mL of D&G treatment for 24 hr. After data analysis, it was found that 14 and 11 relevant genes were either upregulated or downregulated by D&G treatment, respectively [23, 24].

Our study demonstrated that D&G could promote the expression of apolipoprotein D (*ApoD*), lecithin cholesterol acyltransferase (*Lcat*), and intercellular adhesion molecule 1 (*Icam1*), which are well-known cardiac biomarkers. D&G could also upregulate the expression of inducible nitric oxide synthase (*iNos*) and downregulate the selection expression.

The results suggested that the D&G might exert its protective effects on myocardial cells by regulating NO and the selection expression.

The study demonstrated that both IRS-1 and AKT were activated in the D&G-treated myocardial cells. It is well

known that AKT could promote cell survival and oppose apoptosis by a variety of routes. The study suggested that several routes might be involved in the cardiac protection effect of D&G. For example, the induction of the phosphorylation of I kappa B leads to the activation of the transcription factor nuclear factor kappa B (NF- $\kappa$ B) to suppress apoptosis. The promotion of the expression of nitric oxide synthase, which can catabolize L-arginine to NO, triggers many physiological actions in the cardiovascular system.

The study showed that D&G could negatively regulate the expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) at both gene and protein expression levels. Tissue specific genes, protein expression patterns, and signaling pathways, involved in SHR aorta and heart, treated with D&G were found.

Furthermore, to examine the treatment group- and tissue-specific gene expression profiling induced by D&G, the differentially expressional genes from different groups of tissues were studied, and the results resembled observations described as above.

**3.1.5. Pharmacokinetic Study.** With regard to the pharmacokinetic study of the identified markers after oral administration of D&G, the whole pharmacokinetic profiles of the important chemicals like danshensu, puerarin, and daidzein could be obtained, whereas salvianolic acid B, protocatechuic aldehyde, and daidzin could not be detected possibly because of extremely low quantity. Moreover, the assay for the simultaneous determination of R-warfarin and S-warfarin and their metabolites in rat plasma had been developed. The results showed that coadministration of D&G with warfarin/ aspirin would result in significant pharmacokinetic and pharmacodynamic (prothrombin time and bleeding time were increased) interactions in the rat. More in-depth studies would be required in future before the wide clinical uses of D&G.

### 3.2. Clinical Trials

**Trial 1.** The baseline characteristics were similar between the 2 groups. After 6 months' treatment, there were no significant changes in blood pressures, blood hematological and biochemical profiles, folate, and homocysteine levels in both groups when compared with the baseline but a mild decrease in low density lipoprotein cholesterol in both groups ( $(2.6 \pm 0.7)$  mmol/L versus  $(2.7 \pm 0.9)$  mmol/L,  $P < 0.05$ ;  $(2.5 \pm 0.7)$  mmol/L versus  $(2.8 \pm 0.8)$  mmol/L,  $P < 0.05$ ). The brachial FMD was improved after treatment in the D&G group ( $5.9\% \pm 1.0\%$  versus  $5.3\% \pm 1.2\%$ ,  $P < 0.001$ ), and it was less improved in control group ( $5.5\% \pm 1.0\%$  versus  $5.3\% \pm 1.1\%$ ,  $P < 0.05$ ). Improvement in carotid IMT was observed in the D&G group only, and it has significance ( $(0.96 \pm 0.32)$  mm versus  $(0.98 \pm 0.30)$  mm,  $P < 0.05$ ). After the open-label D&G treatment for 6 more months, further improvement in both brachial FMD and carotid IMT was observed in the D&G group, and they had significance ( $5.91\% \pm 0.95\%$  versus  $5.35\% \pm 1.21\%$ ,  $P < 0.05$ ;  $(0.964 \pm 0.316)$  mm versus  $(0.979 \pm 0.303)$  mm,  $P < 0.05$ ). Eight adverse events were reported: 2 in the D&G group; 6 in

the control group, among which, 2 patients required treatment termination.

**The Conclusion Made.** Danshen and Gegen adjunctive treatment for patients with coronary arterial diseases was well tolerated and effective in improving vascular function and structure [27, 28].

**Trial 2.** To evaluate the potential of D&G for primary atherosclerosis prevention in high-risk hypertensive patients, 90 patients (74.4% male) with hypertension associated with left ventricular hypertrophy (63.3%), diabetes mellitus (62.5%), and renal insufficiency (30%) were randomized to receive D&G herbal capsules (2 gm/day) or (1 gm/day) or identical placebo capsules in double-blind and parallel fashion for 12 months on top of their anti-hypertensive treatments. Flow-mediated dilation (endothelium-dependent dilation, FMD) and nitroglycerin-induced dilation (endothelium-independent dilation, NTG) of brachial artery and carotid intima-media thickness (surrogate atherosclerosis marker, IMT) were measured by high resolution B-mode ultrasound.

Results showed that their mean age was  $55 \pm 8$  years. After 12 months and compared with the baseline, there were no significant changes in blood pressure, heart rate, blood cholesterol (TC), haematological, glucose (HBA1c), and creatinine profiles in both placebo and D&G groups. FMD and IMT but not NTG improved significantly after D&G ( $P < 0.001$ ) and not after placebo treatment. No significant difference in FMD and IMT changes in the 2 D&G groups with different dosages was seen. The studied herbal drugs were well tolerated in both groups, with no significant adverse events reported.

**The Conclusion Made.** Danshen and Gegen adjunctive treatment was well tolerated and significantly improved the atherogenic process in high-risk hypertensive patients. There was potential in the primary prevention of atherosclerosis [29–32].

**Trials 3.** A population based sample of 165 postmenopausal women aged 47 to 65 was included in the trial. Only women who experienced menopause for more than 12 months were recruited.

Results showed that the baseline characteristics were comparable between the 2 groups. After 12 months, there were no significant changes in blood pressures and general biochemical profiles in both groups. However, there was a remarkable decrease in serum low density lipoprotein (LDL) cholesterol ( $-6.92\%$ ) and total cholesterol (TC) ( $-5.85\%$ ) from the baseline in the D&G group, when compared with placebo group ( $-3.21\%$  and  $-3.42\%$ ). The carotid intima-media thickness (IMT) decreased 1.52% from the baseline in the D&G group ( $P < 0.004$ ), and the decrease was only 1.13% for the placebo treatment group ( $P = 0.009$ ) after a 12-month treatment. Twelve adverse events were reported: 6 in the placebo group and 6 in the D&G group; no one of them was significant.

*The Conclusions Made.* Postmenopausal women with early hypercholesterolemia tolerated D&G well. The D&G improved the carotid intima and lowered LDL and total cholesterol. D&G therefore may be recommended for the prevention of atherosclerosis in postmenopausal women with hypercholesterolemia [33].

#### 4. Discussion

The comprehensive approach to the creation of an evidence based simple herbal formula for cardiovascular health has taken ten years to reach the present state of maturity. In the laboratory, through a variety of *in vitro* platforms, we have demonstrated the multiple biological activities of D&G, namely, anti-inflammation, anti-oxidation, and anti-form cell formation on the vascular endothelium. The different mechanistic channels leading to these favourable cardiovascular protective events have also been demonstrated in the extensive cytokine studies. Through a variety of animal studies, D&G has been demonstrated to provide control on hypertension, atherosclerosis, and vasodilatation. D&G appears to be both cardiac protective and vascular protective.

The traditional Chinese medicine is characterized by its complexity and holism in both diagnostic and therapeutic approaches. DNA microarray technology could be a powerful tool to study the functional genomics of the traditional Chinese medicine. It might be useful in the identification and characterization of the active components of the complex mixtures to provide significant information for understanding the efficacy of the herbs from the genomic point of view in a systematic way and to hunt for candidate disease genes or characterization of tissue-specific genes. In this study, the genomic and proteomic signatures of D&G treated samples either *in vitro* or *in vivo* were investigated using cDNA microarray and iTRAQ labeled LC/MS/MS techniques, respectively, which provide better understanding of the mechanism of action of Danshen-Gegen. Our future challenge is to integrate the information to give a more complete picture of the interaction between the herbal formula and the living organisms.

We look forward to the more sophisticated microarray studies which might lead to more definitive identification of sub-fractionations within the D&G extract to give more targeted preparations.

With regard to the bioavailability study of the D&G oral administration, the whole pharmacokinetic profiles of the important chemicals like danshensu, puerarin, and daidzein could be obtained, whereas salvianolic acid B, protocatechuic aldehyde, and daidzin could not be detected or were found under the limit of quantification. Moreover, the assay for the simultaneous determination of R-warfarin and S-warfarin and their metabolites in the rat plasma was developed. The results showed that the coadministration of D&G with warfarin/ aspirin would result in significant pharmacokinetic and pharmacodynamic changes which would require more studies.

We have conducted three randomized controlled clinical trials using the same surrogate markers on different target populations. Firstly, we chose the coronary type II patients

who were at high risks. D&G served them well. Next, we chose the less risky patients (those with hypertension and/or diabetes mellitus). D&G also gave good results. Lastly, we recruited postmenopausal women with borderline increase of cholesterol. D&G helped maintain the low cholesterol level. At this stage, we are sure that D&G did not give rise to serious adverse effects. It is a safe preparation and deserves further in-depth studies.

#### 5. Conclusion

This safe preparation has been developed from very popular edible medicinal herbs. The formula has been advocated by a respectable Chinese medicine physician. The current preparation with a modified ratio has shown multiple mechanisms of biological activities which are beneficial to the cardiovascular system. Now that we have reliable means to maintain the quality of the two herbs through careful assessment of their chemical and biological profiles, we could confidently recommend that D&G is a safe and an effective choice for cardiovascular protection. Its further development could follow the direction of a proprietary medicine for a proper hospital and specialist use or as a specific health supplement, targeting towards cardiovascular health.

#### Acknowledgments

The work reported in this paper was partially supported by an Area of Excellence Grant from the University Grants Committee of the Hong Kong Special Administration Region, China (Project no. AoE/B-10/01), and it is related to the joint partnership between the Institute of Chinese Medicine, The Chinese University of Hong Kong, and the Kunming Institute of Botanical Research, a state key Laboratory joint venture of China.

#### References

- [1] J. B. Muhlestein, "Post-hospitalization management of high-risk coronary patients," *American Journal of Cardiology A*, vol. 85, no. 5, pp. 13B–20B, 2000.
- [2] H. Dalal, P. H. Evans, and J. L. Campbell, "Recent developments in secondary prevention and cardiac rehabilitation after acute myocardial infarction," *British Medical Journal*, vol. 328, no. 7441, pp. 693–697, 2004.
- [3] X. Y. Ji, B. K. Tan, and Y. Z. Zhu, "Salvia miltiorrhiza and ischemic diseases," *Acta Pharmacologica Sinica*, vol. 21, no. 12, pp. 1089–1094, 2000.
- [4] G. Zheng, X. Zhang, J. Zheng, W. Gong, X. Zheng, and A. Chen, "Hypocholesterolemic effect of total isoflavones from Pueraria lobata in ovariectomized rats," *Zhong Yao Cai*, vol. 25, no. 4, pp. 273–275, 2002.
- [5] D. P. Sieveking, K. Woo, K. P. Fung, P. Lundman, S. Nakhla, and D. S. Celermajer, "Chinese herbs Danshen and Gegen modulate key early atherogenic events *in vitro*," *International Journal of Cardiology*, vol. 105, no. 1, pp. 40–45, 2005.
- [6] J. S. Lv, "Study of Shi Jin Mo's pair drugs," *Shanxi Journal of Traditional Chinese Medicine*, vol. 24, no. 3, pp. 31–34, 2008.

- [7] Z. D. Zeng, Y. Z. Liang, T. Zhang, F. T. Chau, and Y. L. Wang, "Orthogonal projection (OP) technique applied to pattern recognition of fingerprints of the herbal medicine *houlttuynia cordata* Thunb. and its final injection products," *Analytical and Bioanalytical Chemistry*, vol. 385, no. 2, pp. 392–400, 2006.
- [8] D. K. W. Mok and F. Chau, "Chemical information of Chinese medicines: a challenge to chemist," *Chemometrics and Intelligent Laboratory Systems*, vol. 82, no. 1-2, pp. 210–217, 2006.
- [9] Q. Chang, L. Sun, R. Zhao, M. S. S. Chow, and Z. Zuo, "Simultaneous determination of ten active components in traditional Chinese medicinal products containing both gegen (*Pueraria lobata*) and danshen (*Salvia miltiorrhiza*) by high-performance liquid chromatography," *Phytochemical Analysis*, vol. 19, no. 4, pp. 368–375, 2008.
- [10] P. Y. Chiu, H. Y. Leung, P. K. Leong et al., "Danshen-Gegen decoction protects against hypoxia/reoxygenation-induced apoptosis by inhibiting mitochondrial permeability transition via the redox-sensitive ERK/Nrf2 and PKC $\epsilon$ /mKATP pathways in H9c2 cardiomyocytes," *Phytomedicine*, vol. 19, no. 2, pp. 99–110, 2012.
- [11] H. M. Lam, W. S. Yam, L. K. Leung et al., "Antioxidative and vasodilative effects of Danshen and Gegen," *Journal of Molecular and Cellular Cardiology*, vol. 38, no. 5, p. 840, 2005.
- [12] C. F. Ng, C. M. Koon, D. W. S. Cheung et al., "The anti-hypertensive effect of Danshen (*Salvia miltiorrhiza*) and Gegen (*Pueraria lobata*) formula in rats and its underlying mechanisms of vasorelaxation," *Journal of Ethnopharmacology*, vol. 137, no. 3, pp. 1366–1372, 2011.
- [13] Y. L. Chan, K. S. Woo, P. C. Leung, and K. P. Fung, "Traditional Chinese medicine Danshen and Gegen combination formula improves Atherogenic pathophysiology: an in-vitro and ex-vivo study," *Journal of the Hong Kong College of Cardiology*, vol. 14, no. 2, p. 68, 2006.
- [14] C. M. Koon, K. S. Woo, P. C. Leung, and K. P. Fung, "Salviae Miltiorrhizae Radix and *Puerariae Lobatae* Radix herbal formula mediates anti-atherosclerosis by modulating key atherogenic events both in vascular smooth muscle cells and endothelial cells," *Journal of Ethnopharmacology*, vol. 138, no. 1, pp. 175–183, 2011.
- [15] P. Y. Chiu, S. M. Wong, H. Y. Leung et al., "Acute treatment with Danshen-Gegen decoction protects the myocardium against ischemia/reperfusion injury via the redox-sensitive PKC $\epsilon$ /mKATP pathway in rats," *Phytomedicine*, vol. 18, no. 11, pp. 916–925, 2011.
- [16] P. Y. Chiu, S. M. Wong, H. Y. Leung et al., "Long-term treatment with danshen-gegen decoction protects the myocardium against Ischemia/reperfusion injury via the redox-sensitive protein kinase C- $\epsilon$ /mKATP pathway in rats," *Rejuvenation Research*, vol. 14, no. 2, pp. 173–184, 2011.
- [17] S. Y. Deng, F. F. Y. Lam, E. S. K. Ng et al., "Mechanisms of the dilator action of a Danshen and Gegen formula on rat basilar artery," *Basic and Clinical Pharmacology and Toxicology*, vol. 107, supplement 1, pp. 254–255, 2010.
- [18] Y. Deng, E. S. K. Ng, J. H. K. Yeung et al., "Mechanisms of the cerebral vasodilator actions of isoflavonoids of Gegen on rat isolated basilar artery," *Journal of Ethnopharmacology*, vol. 139, no. 1, pp. 294–304, 2012.
- [19] F. F. Lam, S. Y. Deng, E. S. Ng et al., "Mechanisms of the relaxant effect of a Danshen and Gegen formulation on rat isolated cerebral basilar artery," *Journal of Ethnopharmacology*, vol. 132, no. 1, pp. 186–192, 2010.
- [20] W. J. Xia, M. Yang, T. F. Fok et al., "Partial neuroprotective effect of pretreatment with tanshinone IIA on neonatal hypoxia-ischemia brain damage," *Pediatric Research*, vol. 58, no. 4, pp. 784–790, 2005.
- [21] C. C. Fong, F. Wei, Y. Chen et al., "Danshen-Gegen decoction exerts proliferative effect on rat cardiac myoblasts H9c2 via MAPK and insulin pathways," *Journal of Ethnopharmacology*, vol. 138, no. 1, pp. 60–66, 2011.
- [22] S. M. Wong, P. Y. Chiu, H. Y. Leung et al., "Myocardial post-conditioning with Danshen-Gegen decoction protects against isoproterenol-induced myocardial injury via a PKC $\epsilon$ /mKATP-mediated pathway in rats," *Chinese Medicine*, vol. 6, p. 7, 2011.
- [23] Q. Zhang and M. M. Yang, "DNA microarray technology and traditional Chinese medicines," *Progress in Nutrition*, vol. 12, no. 1, pp. 6–12, 2010.
- [24] L. Zhou, Z. Zuo, and M. S. S. Chow, "Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use," *Journal of Clinical Pharmacology*, vol. 45, no. 12, pp. 1345–1359, 2005.
- [25] L. Zhou, M. S. Chow, and Z. Zuo, "Effect of sodium caprate on the oral absorptions of danshensu and salvianolic acid B," *International Journal of Pharmaceutics*, vol. 379, no. 1-2, pp. 109–118, 2009.
- [26] L. Zhou, M. S. Chow, and Z. Zuo, "Improved quality control method for Danshen products-Consideration of both hydrophilic and lipophilic active components," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 41, no. 3, pp. 744–750, 2006.
- [27] P. Chook, W. Y. Tam, L. T. Chan et al., "Efficacy and safety of Danshen and Gegen as adjunctive secondary prevention therapy in coronary artery disease," *South China Journal of Cardiovascular Diseases*, vol. 17, no. 1, pp. 48–52, 2011.
- [28] P. Chook, W. Y. Tam, Y. K. Poon et al., "Danshen and Gegen as cardiovascular tonic in coronary patients: a novel strategy for secondary atherosclerosis prevention," *South China Journal of Cardiovascular Diseases*, vol. 15, pp. 56–64, 2009.
- [29] W. Y. Tam, P. Chook, Y. K. Poo et al., "Danshen and Gegen as cardiovascular tonic in coronary patients: a novel strategy for secondary atherosclerosis prevention," *Journal of the Hong Kong College of Cardiology*, vol. 12, p. 32, 2004.
- [30] W. Y. Tam, P. Chook, M. Qiao et al., "The efficacy and tolerability of adjunctive alternative herbal medicine (*Salvia miltiorrhiza* and *Pueraria lobata*) on vascular function and structure in coronary patients," *Journal of Alternative and Complementary Medicine*, vol. 15, no. 4, pp. 415–421, 2009.
- [31] T. W. C. Yip, P. Chook, S. K. Kwong et al., "Adjunctive Danshen and Gegen therapy improves atherogenic process: a final report of double-blind placebo control trial in high risk hypertension," *Journal of the Hong Kong College of Cardiology*, vol. 17, p. 12, 2009.
- [32] K. S. Woo, T. W. C. Yip, P. Chook et al., "Cardiovascular protective effects of adjunctive alternative medicine (*Salvia miltiorrhiza* and *Pueraria lobata*) in high-risk hypertension," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 132912, 8 pages, 2013.
- [33] T. C. Y. Kwok, P. C. Leung, C. Lam, S. Ho, C. K. Wong, and P. Chook, "A randomized placebo controlled trial of an Innovative Herbal Formula in the prevention of atherosclerosis in postmenopausal women with Borderline hypercholesterolemia," *Complementary Therapies in Medicine*. In press.

## Research Article

# Effects of Wenxin Keli on the Action Potential and L-Type Calcium Current in Rats with Transverse Aortic Constriction-Induced Heart Failure

Yu Chen,<sup>1,2</sup> Yang Li,<sup>3</sup> Lili Guo,<sup>2</sup> Wen Chen,<sup>2</sup> Mingjing Zhao,<sup>4</sup> Yonghong Gao,<sup>4</sup>  
Aiming Wu,<sup>4</sup> Lixia Lou,<sup>4</sup> Jie Wang,<sup>2</sup> Xiaoqiu Liu,<sup>1</sup> and Yanwei Xing<sup>2</sup>

<sup>1</sup>Shenyang Pharmaceutical University, Shenyang, Liaoning 110016, China

<sup>2</sup>Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100053, China

<sup>3</sup>Institute of Geriatric Cardiology of Chinese PLA General Hospital, Beijing 100853, China

<sup>4</sup>The Key Laboratory of Chinese Internal Medicine of the Ministry of Education, Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100700, China

Correspondence should be addressed to Xiaoqiu Liu; liuxiaoqiu3388@tom.com and Yanwei Xing; xingyanwei12345@163.com

Received 21 March 2013; Revised 8 September 2013; Accepted 10 September 2013

Academic Editor: Hao Xu

Copyright © 2013 Yu Chen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Objective.** We investigated the effects of WXKL on the action potential (AP) and the L-type calcium current ( $I_{Ca-L}$ ) in normal and hypertrophied myocytes. **Methods.** Forty male rats were randomly divided into two groups: the control group and the transverse aortic constriction- (TAC-) induced heart failure group. Cardiac hypertrophy was induced by TAC surgery, whereas the control group underwent a sham operation. Eight weeks after surgery, single cardiac ventricular myocytes were isolated from the hearts of the rats. The APs and  $I_{Ca-L}$  were recorded using the whole-cell patch clamp technique. **Results.** The action potential duration (APD) of the TAC group was prolonged compared with the control group and was markedly shortened by WXKL treatment in a dose-dependent manner. The current densities of the  $I_{Ca-L}$  in the TAC group treated with 5 g/L WXKL were significantly decreased compared with the TAC group. We also determined the effect of WXKL on the gating mechanism of the  $I_{Ca-L}$  in the TAC group. We found that WXKL decreased the  $I_{Ca-L}$  by accelerating the inactivation of the channels and delaying the recovery time from inactivation. **Conclusions.** The results suggest that WXKL affects the AP and blocked the  $I_{Ca-L}$ , which ultimately resulted in the treatment of arrhythmias.

## 1. Introduction

Cardiovascular diseases (CVD) are the most common threat to human health worldwide. From 1999 to 2009, the relative rates of death attributable to CVD decreased by 32.7%. However, CVD still accounted for 32.3% of deaths in the United States in 2009. Thus, the burden of this disease remains high. Epidemiological data show that an estimated 5.1 million Americans greater than 20 years of age exhibited heart failure from 2007 to 2010. Projections show that the prevalence of heart failure by 2030 will increase by 25% from the 2013 estimates [1]. In many cardiovascular diseases, cardiac hypertrophy is a common pathological process, and the disorder of the heart rhythm that is induced by

cardiac hypertrophy is the most common cause of sudden cardiovascular death. A previous study indicates that 36% of the 690 athletes died as a result of cardiac hypertrophy [2]. This high incidence of cardiac hypertrophy has caused widespread concern, and these data highlight the importance of finding suitable drugs for treating cardiac hypertrophy-induced arrhythmias.

The currently electrophysiological research on cardiac hypertrophy mainly focuses on changes in the action potentials and the related ionic mechanisms. In recent years, the role of Ca signalling in cardiac myocytes was studied with respect to electrophysiology, such as the effect of Ca signalling on arrhythmias and action potentials [3].  $Ca^{2+}$  enters the cell via the L-type  $Ca^{2+}$  current, and the sarcoplasmic reticulum

(SR) releases  $\text{Ca}^{2+}$  via the ryanodine receptor (RyR) to increase the intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) and thus activate cardiac contraction. However, the overloading of the SR with  $\text{Ca}^{2+}$  can induce arrhythmias [4]. Blocking  $\text{Ca}^{2+}$  channel and reducing the  $\text{Ca}^{2+}$  overload will thus benefit the treatment of arrhythmias and heart failure. The most important finding in animal models of left ventricular hypertrophy is the significant prolongation of the action potential duration (APD) at low but not at high frequencies. Recent model results suggest that even subtle changes in AP morphology that may result from remodeling of membrane transporter expression in disease may have major impact on the temporal waveform of  $\text{Ca}^{2+}$  transients, thus influencing tissue level electromechanical function [5]. The prolongation of the APD would increase the incidence of triggered activity and the early after-depolarization (EAD), which would result in increasing the incidence of arrhythmias [6, 7]. Using ion channel antagonist drugs to treat arrhythmias in patients with structural heart disease does not reduce their mortality [8, 9]. In addition, most antiarrhythmic drugs have shown potential lethal proarrhythmic effects that result in the aggravation of arrhythmias and potentially induce ventricular arrhythmias. Thus, there is an urgent need to develop effective and safer antiarrhythmic agents. In particular, traditional Chinese medicine has been well recognized for its antiarrhythmic potential.

Wenxin Keli (WXKL) is a Chinese herb extract developed by Guang'anmen Hospital at the Chinese Academy of Chinese Medical Sciences and is the first antiarrhythmic Chinese medicine to be approved by the state. It is composed of five main components: *Nardostachys chinensis* Batal extract, *Codonopsis*, *Notoginseng*, amber, and *Rhizoma Polygonati*. Since its clinical application, this drug had been proven to be of benefit in the treatment of various diseases, such as cardiac arrhythmias, cardiac inflammation, and chronic heart failure [10]. Previous studies have shown that WXKL is a safe and effective treatment, which does not result in significant adverse reactions for the premature ventricular contractions caused by viral myocarditis, as evaluated by improvements in the clinical symptoms, signs, and ECG [11]. Some studies have investigated the effects of irbesartan, amiodarone, and WXKL, applied either alone or in combination, on sinus rhythm maintenance in patients with atrial fibrillation after conversion. It was shown that a combined therapy of Chinese and Western medicines exhibits a synergistic antiarrhythmia effect that improves the conversion rate of atrial fibrillation, shortens the conversion time, and avoids adverse reaction [12, 13]. A fascinating electrophysiologic study of the effects of WXKL suggests that this agent can depress the sodium channel-dependent parameters in canine isolated coronary-perfused preparations and effectively manages and prevents the induction of atrial fibrillation [14]. In addition, the action of notoginseng, which is one of the components of WXKL, was demonstrated to have antiarrhythmic properties, as demonstrated in rats with ischemia arrhythmias [15]. As a result, notoginseng further enhances the antiarrhythmic properties of WXKL. Moreover, it has been reported that WXKL exhibits beneficial effects

on isoproterenol- (ISO-) induced heart failure in rats. In addition, WXKL can greatly improve ISO-induced cardiac dysfunction and protect against aconitine-induced arrhythmia in rats [16]. A recent study suggested that long-term treatment with WXKL may have seen it attenuate ischemia-induced ventricular arrhythmias in rats and inhibit  $I_{\text{Ca-L}}$  and  $I_{\text{to}}$  in a concentration-dependent manner, which may contribute to the observed attenuation [17].

Although a number of electrophysiological studies have analysed the use of WXKL for the treatment of cardiac hypertrophy, the effects of WXKL on the APs and  $I_{\text{Ca-L}}$  of normal and hypertrophied myocytes have not been reported. Thus, the goal of this study was to evaluate the effect of WXKL on the APs and  $I_{\text{Ca-L}}$  of normal and hypertrophic ventricular myocytes using whole-cell patch clamp recording techniques and to explore the mechanism through which WXKL benefits the treatment of cardiac hypertrophy, which would provide better insights into the effects of antiarrhythmic drugs.

## 2. Materials and Methods

**2.1. Animals.** Forty male Sprague-Dawley rats (body weight = 140–160 g), which were purchased from Vital River Experimental Animal Centre (license number SYXK (E) 200420007, Beijing, China) were randomly divided into two groups: the TAC group ( $n = 25$ ) and the control group ( $n = 15$ ). The TAC rats underwent transverse aortic constriction (TAC) surgery, and the control group underwent an identical procedure but without the application of the ligation. All of the following experiments conformed to the Guiding Principles for the Care and Use of Laboratory Animals issued by the National Committee of Science and Technology of China.

**2.2. Drugs and Solutions.** WXKL was provided by Shandong Buchang Pharmaceuticals Co., Ltd., China. The drug was dissolved with  $\text{Ca}^{2+}$ -free Tyrode solution at concentrations of 0.5 g/L, 1 g/L, 5 g/L, 10 g/L, and 20 g/L prior to the experiment. The  $\text{Ca}^{2+}$ -free Tyrode solution contained (in mmol/L) 137 NaCl, 5.4 KCl, 1.0  $\text{MgCl}_2$ , 0.33  $\text{NaH}_2\text{PO}_4$ , 10 hydroxyethyl piperazine ethanesulfonic acid (HEPES), and 10 glucose (pH 7.35, adjusted with NaOH). The Krebs buffer (KB) solution for cell storage contained (in mmol/L) 40 KCl, 20  $\text{KH}_2\text{PO}_4$ , 3.0  $\text{MgCl}_2$ , 70 KOH, 50 L-glutamic acid, 10 HEPES, 20 taurine, 10 glucose, and 0.5 EGTA (pH 7.35, adjusted with KOH). For the AP recordings, the internal pipette solution contained (in mmol/L) 120 K aspartate, 20 KCl, 1.0  $\text{MgCl}_2$ , 4.0  $\text{Na}_2\text{ATP}$ , 10 glucose, and 10 HEPES, and the bath solution contained (in mmol/L) 140 NaCl, 1.0  $\text{CaCl}_2$ , 1.0  $\text{MgCl}_2$ , 4 KCl, 10 HEPES, and 5 glucose. For the  $I_{\text{Ca-L}}$  recordings, the internal pipette solution contained (in mmol/L) 120 CsCl, 1.0  $\text{CaCl}_2$ , 5.0  $\text{MgCl}_2$ , 5.0  $\text{Na}_2\text{ATP}$ , 11 EGTA, 10 HEPES, and 11 glucose (pH 7.3, adjusted with CsOH), and the bath solution was the Tyrode solution supplemented with 1.8 mmol/L  $\text{CaCl}_2$ .

**2.3. Creation of the TAC Model.** The transverse aortic constriction (TAC) surgery was performed in male Sprague-Dawley rats as described previously [18, 19]. Briefly, the rats were anesthetised with 3% chloral hydrate (300 mg/kg,

intraperitoneally). The thorax was opened, and a 4-0 silk suture was passed under the aorta between the origin of the right innominate and the left common carotid arteries. A 6 G needle was placed on the ascending aorta, and the suture was snugly tied around the needle and the aorta. The probe was then quickly removed. The skin was closed, and the rats were maintained in a heating pad until they recovered from the anesthesia. The sham-operated animals underwent an identical procedure but without the application of the ligation. After surgery, both groups were fed tap water and normal fodder in different cages for 8 weeks. To characterise the model, echocardiographic measurements were obtained 8 weeks after surgery using a Vivid 7 Dimension cardiovascular ultrasound system (GE Healthcare, Fairfield, Connecticut, United States) as described previously [20]. The detection indicator was the left ventricular posterior wall thickness (LPWD), and the parameters measured are shown in Figure 1(e).

**2.4. Cardiac Ventricular Myocytes Isolation.** Single cardiac ventricular myocytes were isolated from the hearts of the rats as previously described [21] with slight modifications. Briefly, 5 minutes after the rats were heparinised (100 U/mL 1 mL/100 g i.p.), the animals were anaesthetised with 3% chloral hydrate (0.5 mL/100 g i.p). The heart was rapidly excised and mounted on the Langendorff apparatus and perfused via the aorta with oxygenated  $\text{Ca}^{2+}$ -free Tyrode solution for 5 minutes and then with  $\text{Ca}^{2+}$ -free Tyrode solution containing collagenase II (0.6 mg/mL, Worthington, USA), trypsin (0.24 mg/mL, Amresco, USA), and proteinase E (0.08 mg/mL, Amresco, USA) for 15–20 minutes at 37°C. Subsequently, the ventricular tissue was excised, cut into small pieces in a dish containing KB solution, and blown gently to obtain single ventricular myocytes. The cells were maintained at 4°C in KB solution until use. All of the solutions were continuously gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  at 37°C. The single ventricular myocyte selected for electrophysiological measurements is rod-shaped, quiescent,  $\text{Ca}^{2+}$ -tolerant, and had clear cross-striations and a smooth and glossy surface.

**2.5. Histological Examination.** The rat heart samples were cut into transverse sections and routinely stained with haematoxylin and eosin (H&E) as described previously [22]. The stained sections were examined under a light microscope (OLYMPUS BX51, Japan) and photographed at 400x magnification for morphological analysis.

**2.6. Electrophysiological Recording.** The whole-cell patch clamp technique was used to record the APs and  $I_{\text{Ca-L}}$  using an Axopatch 700B amplifier (Axon Instruments, USA) with the pCLAMP 9.2 software (Axon Instruments, USA). Borosilicate glass patch pipettes (resistance = 3–5 M $\Omega$ ) were pulled using a vertical pipette puller (Narishige pp-830, Japan). The cells were maintained in external solution for 5 to 10 minutes after perfusion and the data were recorded after entering the cell for 5 minutes to stabilise the current. All of the recordings were performed at room temperature

(22°C) within 25 minutes to avoid current rundown. The APs were elicited in the current-clamp mode at a rate of 1.0 Hz using 30 trains of suprathreshold current pulses. The membrane capacitance was calculated using the manual whole-cell capacitance controls on the Axopatch amplifier. The  $I_{\text{Ca-L}}$  was recorded in the voltage-clamp mode and elicited through step depolarisation from –40 to +50 mV in 10-mV increments for 250 ms.

**2.7. Statistical Analysis.** Off-line leak correction was performed on all of the amplitude data. The pCLAMP 9.2 software (Axon Instruments, USA) and the Origin 6.1 software (Microcal Software, USA) were used for the data acquisition and analysis. The data are presented as the mean  $\pm$  SE, where  $n$  represents the number of cells analysed. The statistical comparisons between different groups were performed with ANOVA and Student's  $t$ -test. Differences with a value of  $P$  less than 0.05 were considered statistically significant.

### 3. Results

**3.1. Echocardiographic and Histological Characteristics.** Eight weeks after the TAC surgery, the cardiac structure and function were measured through echocardiographic and histological examinations. Compared with the control group, the heart was slightly enlarged (Figure 1(a)), and the wall of ventricle was thickened (Figure 1(c)) in the TAC group. A significant difference was found in the left ventricular apical biopsy between the control group and the TAC group (Figure 1(b), HE staining 400x magnification). The single ventricular myocytes in the TAC group were larger than those in the control group (Figure 1(d)). We evaluated the cardiac systolic and diastolic functions by measuring the left ventricular posterior wall thickness (LPWD) of the control group and the TAC group (Figure 1(e)). Compared with the control group, the LPWD of the TAC group was significantly increased ( $0.22 \pm 0.02$  cm versus  $0.31 \pm 0.03$  cm,  $n = 10$ ,  $P < 0.01$ , Figure 1(e3)).

**3.2. Effects of WXKL on the APs in the Control Group and the TAC Group.** The APs were recorded by applying a 900-pA current pulse with duration of 3 ms at 1 Hz in the current-clamp mode. The APD was significantly prolonged in the TAC group compared with the control group (Figure 2(a)). The APDs obtained with 20%, 50%, and 90% repolarisation ( $\text{APD}_{20}$ ,  $\text{APD}_{50}$ , and  $\text{APD}_{90}$  in ms) in the control group and the TAC group were the following:  $48.5 \pm 3.5$  ms versus  $81.9 \pm 4.3$  ms ( $n = 6$ ,  $P < 0.01$ ),  $98.7 \pm 8.8$  ms versus  $137.2 \pm 13.4$  ms ( $n = 6$ ,  $P < 0.01$ ), and  $142.0 \pm 9.3$  ms versus  $163.7 \pm 2.9$  ms ( $n = 6$ ,  $P < 0.05$ ), respectively (Figure 2(b)).

After treatment with different dose of WXKL, the APD in the TAC group exhibited significant changes (Figure 2(c)). The  $\text{APD}_{20}$ ,  $\text{APD}_{50}$ , and  $\text{APD}_{90}$  in the TAC group were markedly shortened by WXKL in a dose-dependent manner. After perfusion with 1 g/L WXKL, the  $\text{APD}_{20}$ ,  $\text{APD}_{50}$ , and  $\text{APD}_{90}$  were shortened from  $81.9 \pm 4.3$  ms to  $67.4 \pm 6.2$  ms ( $n = 6$ ,  $P < 0.05$ ),  $137.2 \pm 13.4$  ms to  $122.8 \pm 11.6$  ms ( $n = 6$ ,  $P < 0.05$ ), and  $163.7 \pm 2.9$  ms to  $144.4 \pm 3.2$  ms

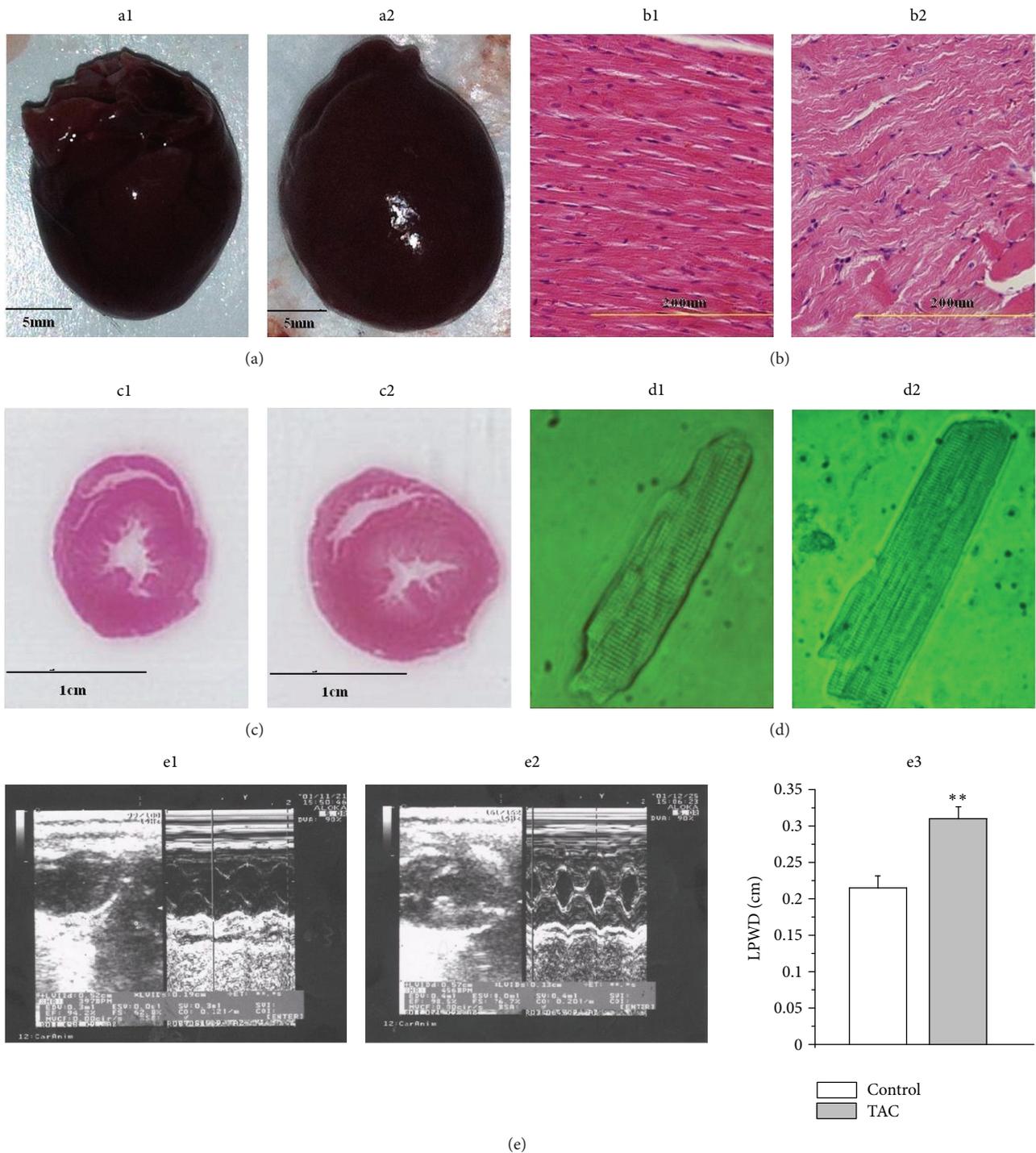


FIGURE 1: (a) Preparation of the hearts from the control group (a1) and the TAC group (a2). (b) Left ventricular apical biopsy of the control group ((b1), HE staining 400x magnification) and the TAC group ((b2), HE staining 400x magnification). (c) Pathological section of the largest cross-section of the control group (c1) and the TAC group (c2). (d) Single ventricular myocytes from the control group (d1) and the TAC group (d2). (e) Typical echocardiography images from the control group (e1) and the TAC group (e2). Eight weeks after the TAC surgery, the cardiac structure and function were measured through echocardiography. We evaluated the cardiac systolic and diastolic functions by measuring the left ventricular posterior wall thickness (LPWD) of the control group and the TAC group (e3). \*\* $P < 0.01$  versus the control group.

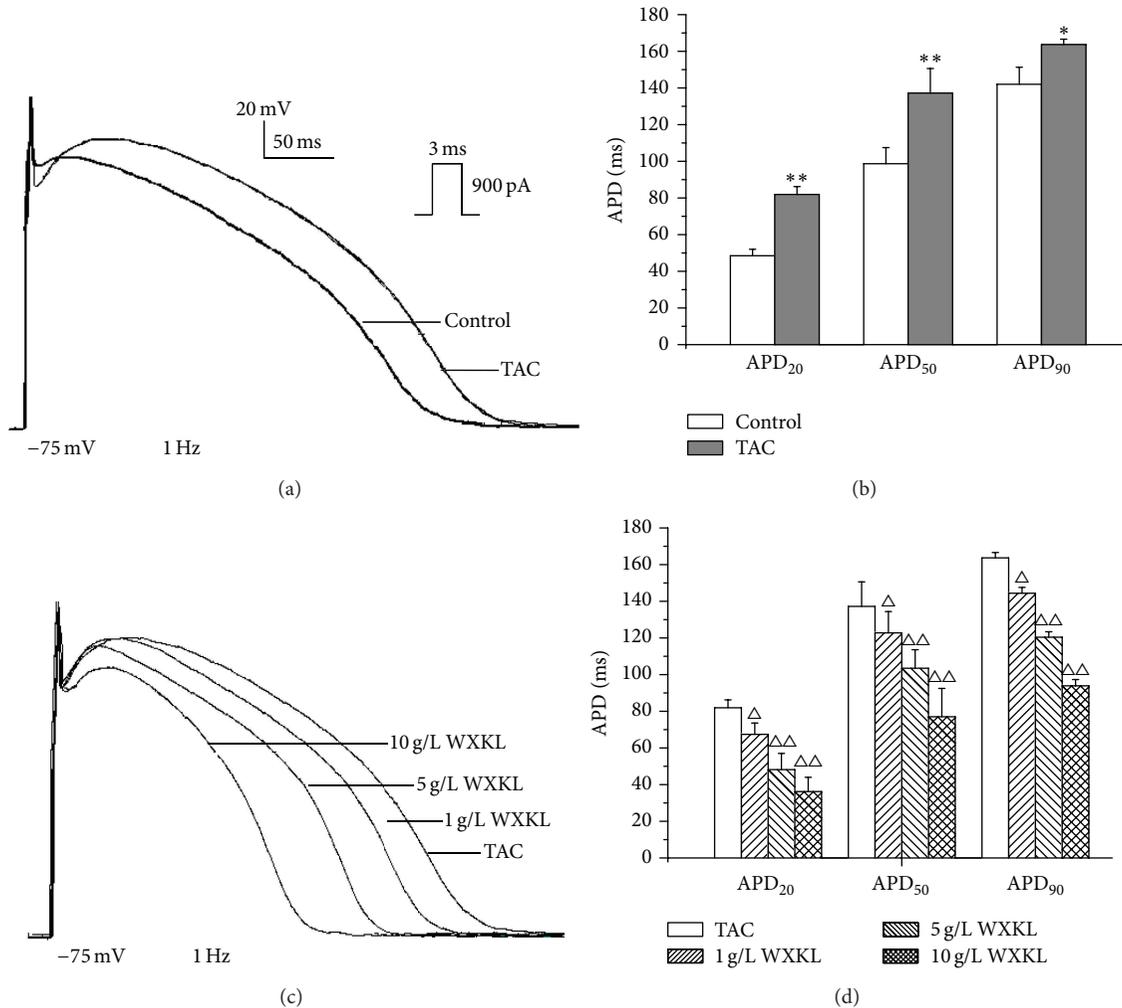


FIGURE 2: Representative AP traces recorded from the control and the TAC group and effects of different concentrations of WXXL on the APs in the TAC group. (a) APs of the control group and the TAC group. (b) APD<sub>20</sub>, APD<sub>50</sub>, and APD<sub>90</sub> of the control group and the TAC group. (c) Effects of 1, 5, and 10 g/L WXXL on the APs in the TAC group. (d) APD<sub>20</sub>, APD<sub>50</sub>, and APD<sub>90</sub> of the TAC group after treatment with 1, 5, and 10 g/L WXXL. \**P* < 0.05 and \*\**P* < 0.01 versus the control group. Δ*P* < 0.05 and ΔΔ*P* < 0.01 versus the TAC group.

(*n* = 6, *P* < 0.05), respectively. When treated with 5 g/L WXXL, the APD<sub>20</sub>, APD<sub>50</sub>, and APD<sub>90</sub> were shortened from 81.9 ± 4.3 ms to 48.2 ± 8.8 ms (*n* = 6, *P* < 0.01), 137.2 ± 13.4 ms to 103.5 ± 10.1 ms (*n* = 6, *P* < 0.01), and 163.7 ± 2.9 ms to 120.4 ± 3.0 ms (*n* = 6, *P* < 0.01), respectively. When treated with 10 g/L WXXL, the APD<sub>20</sub>, APD<sub>50</sub>, and APD<sub>90</sub> were shortened from 81.9 ± 4.3 ms to 36.2 ± 7.8 ms (*n* = 6, *P* < 0.01), 137.2 ± 13.4 ms to 77.0 ± 15.5 ms (*n* = 6, *P* < 0.01), and 163.7 ± 2.9 ms to 93.9 ± 3.4 ms (*n* = 6, *P* < 0.01), respectively (Figure 2(d)). To understand the mechanisms that were responsible for the observed changes in the APD in the TAC group, we examined the *I*<sub>Ca-L</sub> in the control group and the TAC group.

**3.3. Effects of WXXL on the *I*<sub>Ca-L</sub> in the Control Group.** To avoid the influence of current rundown, all of the recordings were obtained within 25 minutes. The current traces were obtained during a depolarising pulse from the holding potential of -40 mV to 0 mV over 250 ms. After WXXL (0.5, 1, 5, 10,

and 20 g/L) treatment, the *I*<sub>Ca-L</sub> in control group significantly decreased by 27.45 ± 2.51%, 40.57 ± 1.77%, 48.15 ± 1.95%, 58.22 ± 2.96%, and 71.68 ± 2.63%, respectively (*n* = 10, *P* < 0.01, Figure 3(a)). Thus, we concluded that WXXL significantly decreased the *I*<sub>Ca-L</sub> of the control group in a concentration-dependent manner. The IC<sub>50</sub> of WXXL was found to be 6.23 g/L (Figure 3(b)). The time-dependent curve showed that the effects of WXXL on the *I*<sub>Ca-L</sub> remained stable after 5 minutes (Figure 3(c)).

**3.4. Effects of WXXL on the *I*<sub>Ca-L</sub> in the TAC Group.** The current-voltage (*I*-*V*) curves were obtained by applying voltage steps in 10-mV increments (-40 mV to +50 mV) for 250 ms from a holding potential of -40 mV. The representative *I*<sub>Ca-L</sub> traces show that the amplitude of the *I*<sub>Ca-L</sub> was higher in the TAC group compared with the control group (Figures 4(a) and 4(b)). After treatment with 5 g/L WXXL, the *I*<sub>Ca-L</sub> was significantly reduced in the TAC group (Figure 4(c)). The *I*-*V* curves also show that the current

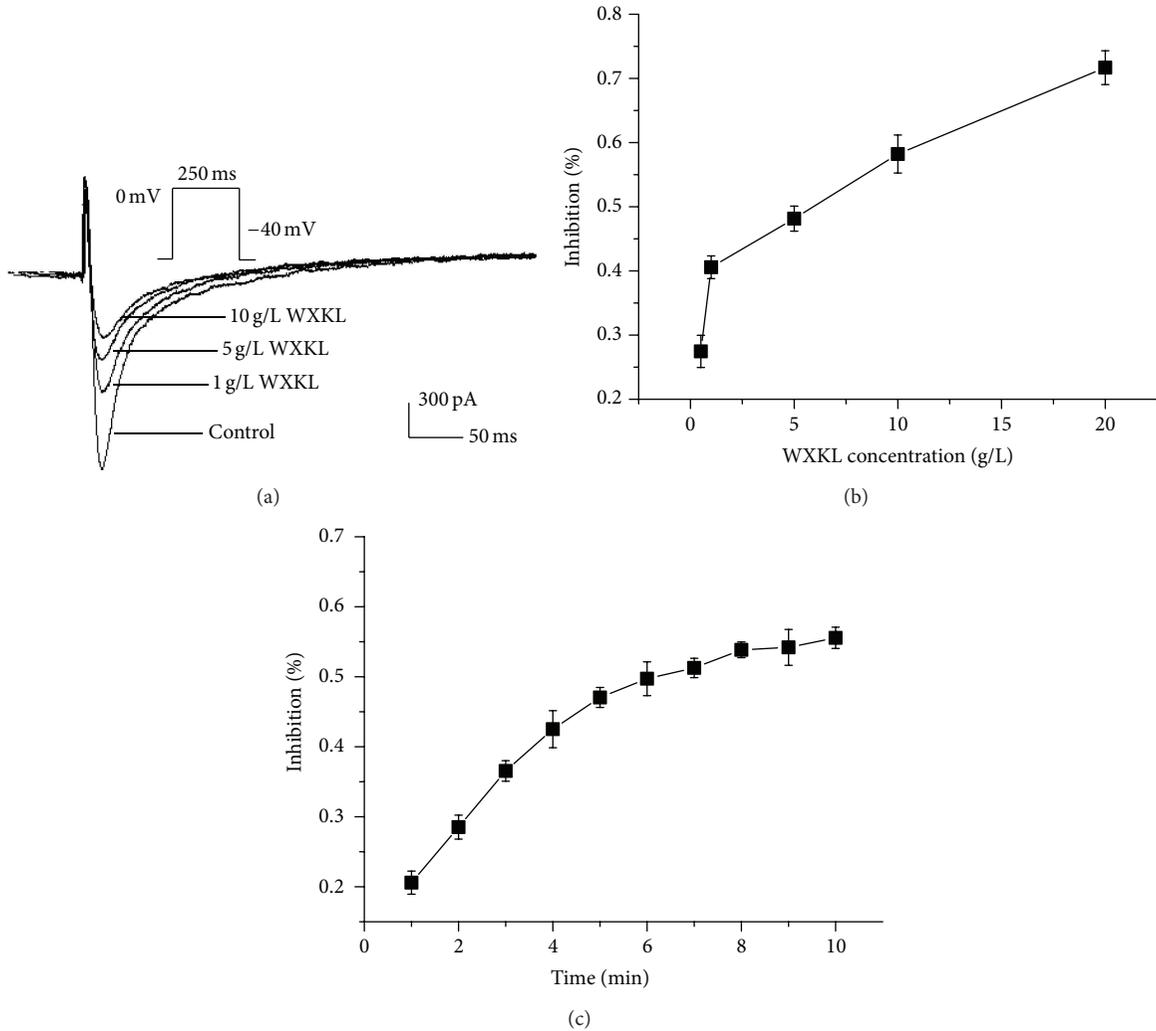


FIGURE 3: Effects of WXXL on the  $I_{Ca-L}$  in the control group. (a) Effects of 1, 5, and 10 g/L WXXL on the  $I_{Ca-L}$  in the control group. After treatment with WXXL, the current amplitudes of the control group were significantly reduced. (b) Concentration-dependent effects of WXXL on the  $I_{Ca-L}$  in the control group ( $IC_{50} = 6.23$  g/L). (c) Time-dependent effects of 5 g/L WXXL on the  $I_{Ca-L}$  in the control group. \* $P < 0.05$  and \*\* $P < 0.01$  versus the control group.

densities in the TAC group were significantly increased by a range of  $-10$  mV to  $+10$  mV compared with the control group ( $n = 10$ ,  $P < 0.01$ ). The current densities of the TAC group after treatment with 5 g/L WXXL were significantly decreased by a range of  $-10$  mV to  $+40$  mV compared with untreated TAC group ( $n = 10$ ,  $P < 0.01$ , Figure 4(d)). The mean current densities at 0 mV were  $-8.56 \pm 0.20$  pA/pF in the control group and  $-9.52 \pm 0.40$  pA/pF in the TAC group ( $n = 10$ ,  $P < 0.05$ , Figure 4(e)). In contrast, the mean current density at 0 mV in the TAC group after treatment with 5 g/L WXXL was decreased to  $-5.86 \pm 0.69$  pA/pF ( $n = 10$ ,  $P < 0.01$ , Figure 4(e)).

**3.5. Effects of WXXL on the Steady-State Activation and Inactivation Kinetics of  $I_{Ca-L}$  in the TAC Group.** The steady-state activation curves of  $I_{Ca-L}$  were determined using pulses from  $-40$  mV to  $+50$  mV at 10-mV increments for 250 ms. The steady-state inactivation curves of  $I_{Ca-L}$  were determined

using pulses from  $-60$  mV to  $+30$  mV at 10-mV increments for 1,000 ms. The steady-state activation curves was described assuming that a Boltzmann function:

$$\left( \frac{G}{G_{\max}} = \frac{1}{1 + \exp((V_{1/2, \text{ack}} - V_{\text{rev}})/k)} \right). \quad (1)$$

The steady-state inactivation curves was described assuming that a Boltzmann function:

$$\left( \frac{I}{I_{\max}} = \frac{1}{1 + \exp((V_{\text{rev}} - V_{1/2, \text{inact}})/k)} \right). \quad (2)$$

The steady-state activation curve in each group exhibited no significant difference (Figure 5(a)). The half-activation potentials ( $V_{1/2, \text{act}}$ , at which 50% of the channels are activated) in the control and the TAC groups were  $12.01 \pm 0.91$  mV and  $13.55 \pm 1.55$  mV, respectively ( $n = 10$ ,  $P > 0.05$ ). Treatment

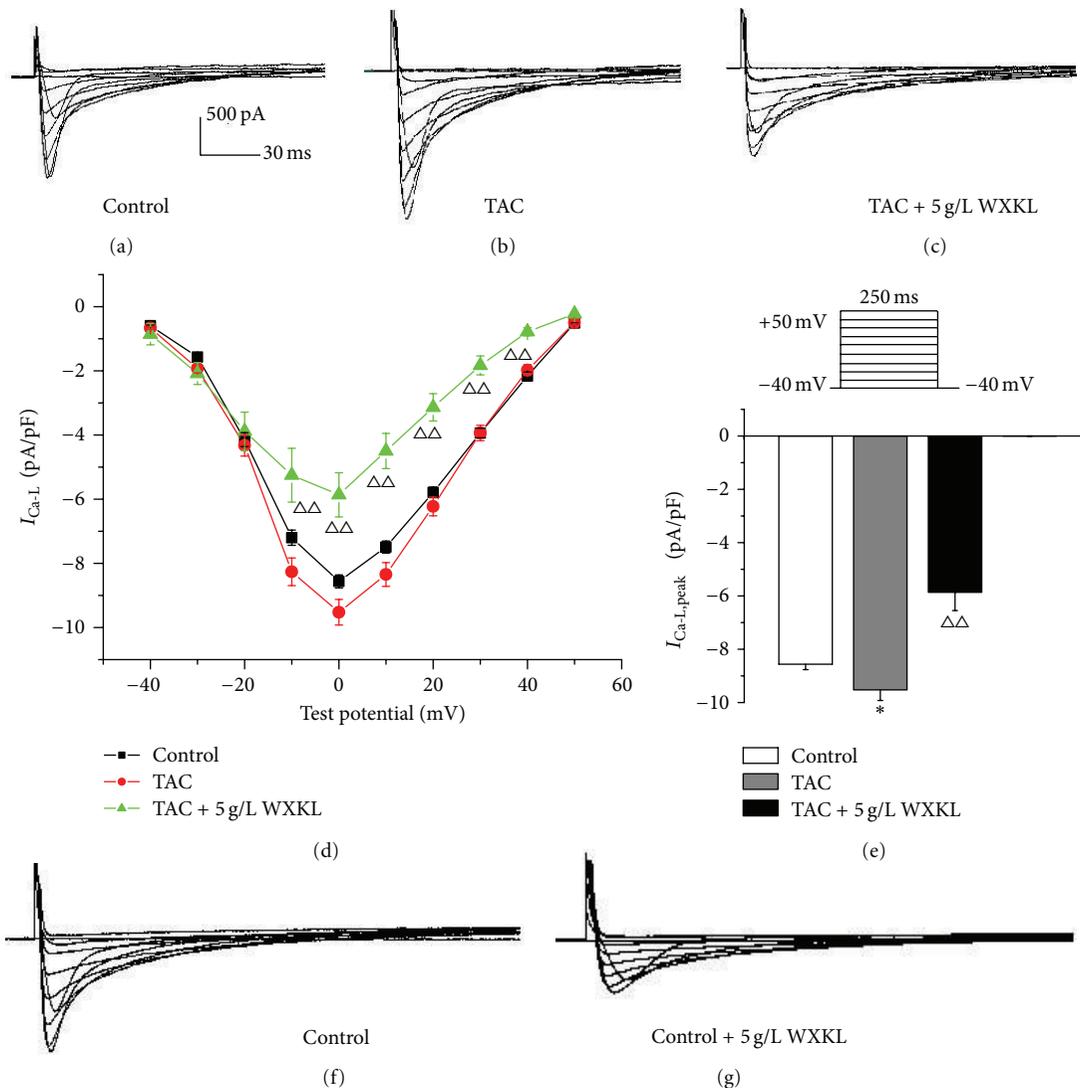


FIGURE 4: Effects of WXKL on the  $I_{Ca-L}$  in the TAC group. (a) Representative  $I_{Ca-L}$  traces recorded from the control group. (b) Representative  $I_{Ca-L}$  traces recorded from the TAC group. (c) Representative  $I_{Ca-L}$  traces recorded from the TAC group in the presence of 5 g/L WXKL. (d) The peak current density-voltage relationship showed that the current densities in the TAC group were significantly increased by a range of  $-10$  mV to  $+10$  mV, and the current densities in the TAC group after treatment with 5 g/L WXKL were significantly reduced by a range of  $-10$  mV to  $+40$  mV. (e) The peak current densities in each group exhibited significant differences. ((f) and (g)) Representative  $I_{Ca-L}$  traces recorded from the control group and after treatment with 5 g/L WXKL. \* $P < 0.05$  and \*\* $P < 0.01$  versus the control group.  $\Delta P < 0.05$  and  $\Delta\Delta P < 0.01$  versus the TAC group.

with 5 g/L WXKL shifted the  $V_{1/2,act}$  from  $13.55 \pm 1.55$  mV to  $12.50 \pm 1.57$  mV in the TAC group ( $n = 10$ ,  $P > 0.05$ , Figure 5(b)). The slope factor ( $k_{act}$ ) activation values in the control group, the TAC group, and the TAC group after treatment with 5 g/L WXKL were  $18.22 \pm 1.04$  mV,  $19.89 \pm 1.79$  mV, and  $20.39 \pm 1.96$  mV, respectively. These values were not significantly different ( $n = 10$ ,  $P > 0.05$ , Figure 5(c)).

However, compared with the control group, the steady-state inactivation curve in the TAC group shifted to a more positive potential. In the presence of 5 g/L WXKL, the steady-state inactivation curve of the TAC group was shifted to a more negative potential (Figure 5(d)). The half-inactivation potentials ( $V_{1/2,inact}$ , at which 50% of the channels are inactivated) in the control and the TAC groups were shifted from

$-11.60 \pm 1.15$  mV and  $-3.83 \pm 0.66$  mV, respectively ( $n = 10$ ,  $P < 0.01$ , Figure 5(e)). The half-inactivation potential in the TAC group after treatment with 5 g/L WXKL was  $-16.89 \pm 2.24$  mV ( $n = 15$ ,  $P < 0.01$ , Figure 5(e)). The slope factor ( $k_{inact}$ ) inactivation values in the control group, the TAC group, and the TAC group after treatment with 5 g/L WXKL were  $9.89 \pm 0.98$  mV,  $11.81 \pm 0.38$  mV, and  $13.55 \pm 1.11$  mV, respectively ( $n = 15$ ,  $P < 0.01$ , Figure 5(f)). These results revealed that WXKL reduced the current by accelerating inactivation of the channels.

**3.6. Effects of WXKL on the Recovery of  $I_{Ca-L}$  from Inactivation in the TAC Group.** The time course of recovery from inactivation of  $I_{Ca-L}$  was evaluated using a paired-pulse protocol:

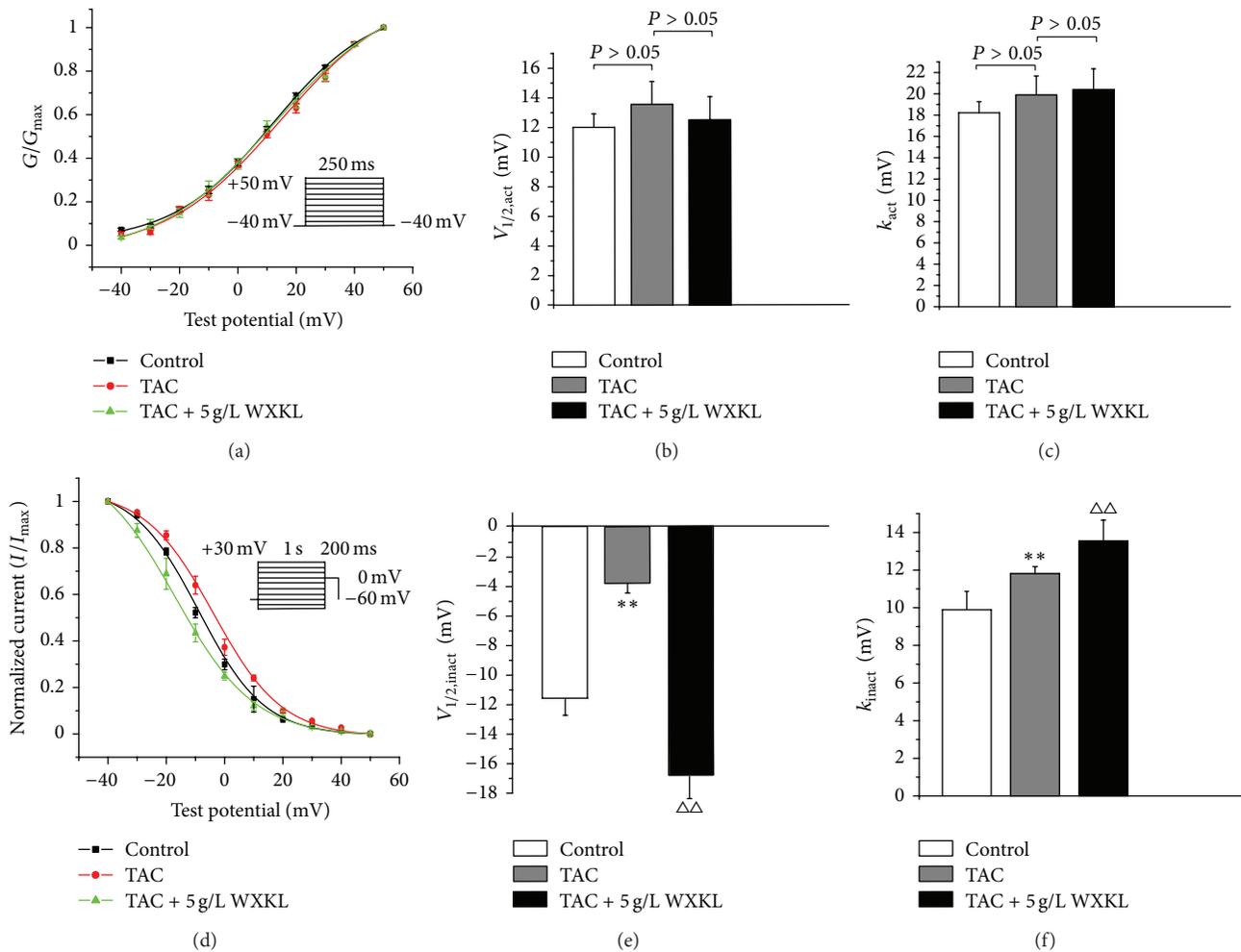


FIGURE 5: Effects of WXKL on the steady-state activation and inactivation kinetics of the  $I_{Ca-L}$  in the TAC group. (a) Steady-state activated curve of the control group, the TAC group and the TAC group treated with 5 g/L WXKL. The steady-state activation curve of each group did not exhibit significant differences. (b) The  $V_{1/2,act}$  of each group did not exhibit significant differences. (c) The  $k_{act}$  of each group did not exhibit significant differences. (d) The steady-state inactivation curve of the TAC group was shifted to a more negative potential, whereas the steady-state inactivation curve of the TAC group treated with 5 g/L WXKL was shifted to a more active potential. (e) The  $V_{1/2,inact}$  in each group exhibited significant differences. (f) The  $k_{inact}$  of each group exhibited significant differences. \* $P < 0.05$  and \*\* $P < 0.01$  versus the control group.  $\Delta P < 0.05$  and  $\Delta\Delta P < 0.01$  versus the TAC group.

a conditioning pulse was first applied from a holding potential of  $-80$  mV to  $0$  mV, and a test potential of  $0$  mV was then applied for 250 ms after various interval durations of 0.05, 0.1, 0.5, 1, 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, and 5120 ms. The recovery curve from inactivation was fitted by a single exponential function. The treatment with 5 g/L WXKL shifted the recovery curve from the inactivation of  $I_{Ca-L}$  in the TAC group to the right (Figures 6(a) and 6(b)). The results showed that WXKL delayed the recovery time from inactivation.

#### 4. Discussion

We can draw the following conclusions from the present study: (1) in the TAC group, WXKL treatment can significantly decrease the prolongation of the APD in a dose-dependent manner, and the  $APD_{20}$ ,  $APD_{50}$ , and  $APD_{90}$  were all significantly shortened. (2) The amplitude of the  $I_{Ca-L}$  in

the TAC group was increased compared with the control group, and WXKL treatment significantly reduced the  $I_{Ca-L}$  in the TAC group. (3) WXKL decreased the  $I_{Ca-L}$  by accelerating the inactivation process of the channels and delaying the recovery time from inactivation but had no significant effect on the activation process. These major findings suggest that  $I_{Ca-L}$  may be the target of the antiarrhythmic effect of WXKL.

Cardiac hypertrophy is a common pathological change that increases the incidence and mortality of many cardiovascular diseases. These changes are frequently induced by electrical remodelling and arrhythmogenesis. Most of the drugs that have been used can potentially induce ventricular arrhythmias. Thus, it is necessary to identify more effective and safer drugs for the treatment of arrhythmias induced by cardiac hypertrophy.

The functioning of the heart depends on the normal action potential, and the normal action potential depends

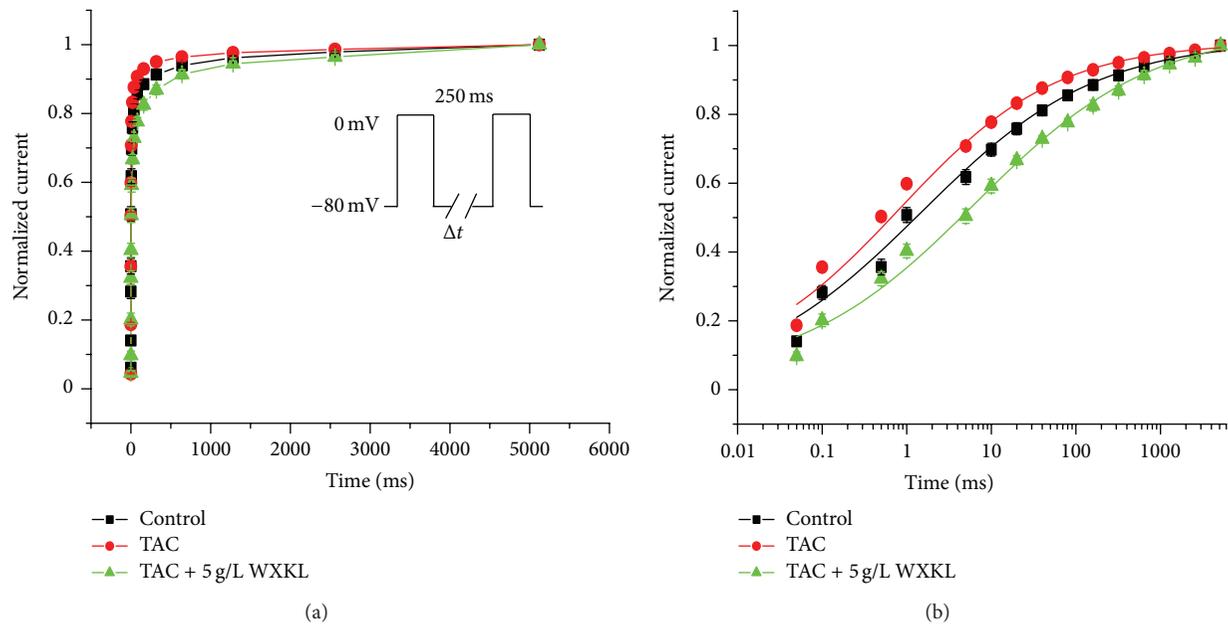


FIGURE 6: Effects of WXKL on the recovery of the  $I_{Ca-L}$  from inactivation in the TAC group. The time course of the recovery from inactivation was fitted with a single exponential function. The recovery from inactivation of the  $I_{Ca-L}$  was changed after exposure to 5 g/L WXKL.

on the normal functioning of ion channels. The abnormality that is most consistently found in animal models of cardiac hypertrophy is the prolongation of the APD [23–25].  $Ca^{2+}$  plays a key role in the excitation-contraction coupling and the activation of  $Ca^{2+}$ -dependent signalling pathways. The APD prolongation may increase the  $Ca^{2+}$  entry via  $I_{Ca-L}$  during the long plateau phase, which would cause an accumulation of  $Ca^{2+}$  in the sarcoplasmic reticulum (SR) and spontaneous SR  $Ca^{2+}$  release [26]. Increases in the intracellular  $Ca^{2+}$  concentration, which can be caused by multiple mechanisms, such as L-type  $Ca^{2+}$  channels coupled with  $Ca^{2+}$ -induced  $Ca^{2+}$  release from the ryanodine receptors, and T-type  $Ca^{2+}$  channels could lead to cardiac hypertrophy [27]. However, the L-type  $Ca^{2+}$  channel is involved in the predominant mechanism responsible for the influx of  $Ca^{2+}$  in cardiac cells [28]. This channel also plays an important role in the generation of AP under physiological and pathophysiological conditions. The blockade of the L-type  $Ca^{2+}$  channels results in antiarrhythmic actions [29]. Thus, blocking the  $Ca^{2+}$  channels, reducing the  $Ca^{2+}$  overload; and weakening the myocardial contractility will benefit the treatment of cardiac hypertrophy and heart failure.

WXKL is the first antiarrhythmic Chinese medicine to be approved by the state. A large number of clinical trials have confirmed that WXKL can improve the left ventricular diastolic function and reduce the degree of left ventricular hypertrophy with high blood pressure, which ultimately leads to a reduction in the incidence of arrhythmias [30]. Most of the previous studies that analysed the antiarrhythmic properties of WXKL were based on normal myocytes or on drug-induced cardiac hypertrophy. However, it was unknown whether the drugs would exhibit the same effect on pathological myocytes, and the experimental studies on this subject

were rare. During these years, an increasing number of studies have been performed using animal models of hypertrophy. A large number of studies have been performed in rats using different interventions to induce hypertrophy [31, 32]. Thus, this paper investigated the antiarrhythmic effects of WXKL on a TAC model using an electrophysiological technology.

Previous experiments have shown that WXKL can reverse cardiac hypertrophy induced by ISO. WXKL significantly reduced HW/BW, LVW/BW and the expression of  $\beta$ -catenin and e-myc. Thus, the use of WXKL for the treatment of patients with hypertension and arrhythmia may be a reasonable and effective choice [33]. Our previous study has shown that WXKL inhibits heart failure and cardiac arrhythmias via a mechanism that may involve the regulation of the CaMKII signal transduction pathway similar to amiodarone. WXKL treatment can increase the calcium transient amplitude in isolated cardiac myocytes from rats with myocardial infarction and reduce the incidence of cardiac arrhythmias in rat myocardial infarction model [34]. In our model, we found that the APD of the TAC group was significantly prolonged compared with the control group, which was in accordance with the results of previous studies [25, 35]. The  $APD_{20}$ ,  $APD_{50}$ , and  $APD_{90}$  were all longer than those of the control group. After WXKL treatment, the  $APD_{20}$ ,  $APD_{50}$ , and  $APD_{90}$  of the TAC group were significantly shortened. WXKL abbreviated the prolongation of the APD in a dose-dependent manner. The change in the APD indicated that the ion channel currents of the TAC group were also changed. Considering the important role of  $Ca^{2+}$  in cardiac hypertrophy, we investigated the effect of WXKL on the L-type  $Ca^{2+}$  channel.

A previous study showed the effect of WXKL on the  $I_{Ca-L}$  and  $I_{to}$  in normal rat ventricular myocytes. WXKL decreased the  $I_{Ca-L}$ , shifted the steady-state activation curve to the

right, and prolonged the recovery time from inactivation [17]. However, the results from studies of changes of the  $I_{Ca-L}$  in cardiac hypertrophy models are inconsistent. These disparities are due in part to the differences in the models used and the variations in the experimental conditions. Our results are consistent with the studies that showed a significant increase in the  $Ca^{2+}$  current [24]. In our study, we found that both the current amplitude and the current density of the  $I_{Ca-L}$  in the TAC group were higher than those in the control group. The acute application of WXKL inhibited the  $I_{Ca-L}$  in a concentration-dependent manner in the control group. The  $IC_{50}$  was found to be 6.23 g/L. WXKL significantly decreased the peak current of the  $I_{Ca-L}$  in the TAC group. It appears that the effect of WXKL can significantly relieve the increase in the  $I_{Ca-L}$  in the TAC group. The experiments revealed that WXKL was able to block the  $I_{Ca-L}$ , which may account for the shortening of the APD and contribute to some of its antiarrhythmic effects. WXKL significantly reduced the  $APD_{20}$ ,  $APD_{50}$ , and  $APD_{90}$  of the TAC group. This result is well explained by the reduction of the  $I_{Ca-L}$ . We demonstrated that WXKL has the potential to attenuate the development of cardiac hypertrophy by affecting the signalling mechanisms of cardiac myocytes. In our study, a WXKL concentration of 5 g/L was used to explore the mechanism through which this agent treats cardiac hypertrophy. This concentration is close to the  $IC_{50}$ . However, the results show that the WXKL treatment decreased the  $I_{Ca-L}$  in the TAC group and in the control group to a level that was lower than the normal level. We should therefore use a lower dose of WXKL and analyse the resulting effects on the  $I_{Ca-L}$  of the TAC group. In addition, it is unclear whether an excessive dose of WXKL can exert a significant antiarrhythmic effect with fewer side effects.

Furthermore, we determined the effect of WXKL on the gating mechanism of  $I_{Ca-L}$  in the TAC group. The steady-state activation curves in each group were not significantly different. Compared with the control group, the steady-state inactivation curve of the TAC group was shifted to a more positive potential. In the presence of 5 g/L WXKL, the steady-state activation curve was shifted to a more negative potential. This result suggests that the voltage-dependent steady-state inactivation of the L-type  $Ca^{2+}$  channels was accelerated. Moreover, 5 g/L WXKL shifted the recovery curve from inactivation of the  $I_{Ca-L}$  in the TAC group to the right. These data suggested that WXKL decreased the  $I_{Ca-L}$  through facilitation of the steady-state inactivation and retardation of the recovery from inactivation. Interestingly, we found that the effects of WXKL on the steady-state activation and inactivation procedures of the L-type  $Ca^{2+}$  channels in the TAC group were not in accordance with its effects on normal rats [17]. An explanation for this discrepancy is that pathological cells may have undergone electrical remodelling, which would change the effect that WXKL would have on these cells. The effects of WXKL on the recovery curve from inactivation of  $I_{Ca-L}$  were consistent and showed that the mechanism underlying the beneficial effects of WXKL may involve the regulation of the  $Ca^{2+}$  channel and a reduction in the  $Ca^{2+}$  influx. WXKL likely affects the L-type  $Ca^{2+}$  channels, alters the cellular  $Ca^{2+}$  regulation, and improves the heart function.

WXKL includes five ingredients: *Nardostachys chinensis* Batal extract, codonopsis, notoginseng, amber, and *Rhizoma Polygonati*. A study had investigated the effects of *Nardostachys chinensis* Batal extract (NcBe) on the activation kinetics of normal rat cardiac sodium channels and transient outward potassium channels. NcBe significantly blocks the  $I_{Na}$  and  $I_{to}$  of normal rat ventricular myocytes [36]. Studies could be conducted to discover more effective drugs. Furthermore, the present study focused on the effect of WXKL in the  $I_{Ca-L}$  of TAC rats. To investigate the mechanism through which WXKL exerts antiarrhythmic effect, it is necessary to study the related signal transduction pathways in further study. In addition to the shortening of the APD after WXKL perfusion and the excessive reduction of the  $I_{Ca-L}$ , other ion currents in hypertrophied myocytes may be modulated by this herbal extract. Therefore, it will be valuable to obtain further insight into the effect of WXKL on the regulation of other ion channels and the related signal transduction pathway.

In summary, the present study evaluated the electrophysiologic effects and antiarrhythmic potential of WXKL in hypertrophied ventricular myocytes. The results demonstrated that WXKL treats cardiac hypertrophy and cardiac arrhythmias via a mechanism that may involve the regulation of the L-type  $Ca^{2+}$  channels. WXKL treatment significantly shortened the prolongation of the APD and reduced the  $I_{Ca-L}$ . Further studies should explore the deeper mechanism through which WXKL treats cardiac hypertrophy to ultimately offer new avenues for the prevention and treatment of this and other related diseases.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Author's Contribution

Yu Chen, Yang Li, Lili Guo, and Wen Chen contributed equally to this paper.

## Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant no. 81001514 and no. 81373835), the Beijing Nova Program (Grant no. 20111110), and the Fundamental Research Funds for the Central Public Welfare Research Institutes, (Grant no. ZZ070802).

## References

- [1] V. L. Roger, A. S. Go, D. M. Lloyd-Jones et al., "Executive summary: heart disease and stroke statistics-2012 update: a report from the American heart association," *Circulation*, vol. 125, no. 1, pp. 188–197, 2012.
- [2] B. J. Maron, J. J. Doerer, T. S. Haas, D. M. Tierney, and F. O. Mueller, "Sudden deaths in young competitive athletes analysis of 1866 deaths in the united states, 1980–2006," *Circulation*, vol. 119, no. 8, pp. 1085–1092, 2009.

- [3] D. M. Bers, "Calcium cycling and signaling in cardiac myocytes," *Annual Review of Physiology*, vol. 70, pp. 23–49, 2008.
- [4] D. A. Eisner, T. Kashimura, L. A. Venetucci, and A. W. Trafford, "From the ryanodine receptor to cardiac arrhythmias," *Circulation Journal*, vol. 73, no. 9, pp. 1561–1567, 2009.
- [5] L. D. Gauthier, J. L. Greenstein, and R. L. Winslow, "Toward an integrative computational model of the Guinea pig cardiac myocyte," *Frontiers in Physiology*, vol. 3, no. 244, pp. 1–19, 2012.
- [6] E. M. Cherry, F. H. Fenton, and R. F. Jr. Gilmour, "Mechanisms of ventricular arrhythmias: a dynamical systems-based perspective," *American Journal of Physiology*, vol. 302, no. 12, pp. H2451–H2463, 2012.
- [7] R. Rouet, M. E. Worou, P. E. Puddu et al., "Nifedipine blocks ondansetron electrophysiological effects in rabbit purkinje fibers and decreases early afterdepolarization incidence," *Current Clinical Pharmacology*, vol. 7, no. 1, pp. 41–48, 2012.
- [8] E. F. Aziz, F. Javed, B. Pratap, and E. Herzog, "Strategies for the prevention and treatment of sudden cardiac death," *Journal of Open Access Emergency Medicine*, vol. 2010, no. 2, pp. 99–114, 2010.
- [9] E. J. Velazquez, J. B. Williams, E. Yow et al., "Long-term survival of patients with ischemic cardiomyopathy treated by coronary artery bypass grafting versus medical therapy," *Annals of Thoracic Surgery*, vol. 93, no. 2, pp. 523–530, 2012.
- [10] J. Kalifa and U. M. R. Avula, "The Chinese herb extract Wenxin Keli: atrial selectivity from the Far East," *Heart Rhythm*, vol. 9, no. 1, pp. 132–133, 2012.
- [11] H.-Y. Sun, "Clinical study of Wenxikeli treatment of viral myocarditis," *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, vol. 23, no. 2, pp. 144–145, 2009.
- [12] P.-Y. Xie and S.-H. Shen, "Effect of combination of Chinese and Western medicines on sinus rhythm maintenance in patients with auricular fibrillation after conversion," *Zhongguo Zhong Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi*, vol. 26, no. 7, pp. 644–646, 2006.
- [13] M. Wang, Y.-B. Yu, and S.-E. Huang, "Clinical observation on effect and safety of combined use of wenxin granule and amiodarone for conversion of auricular fibrillation," *Zhongguo Zhong Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi*, vol. 26, no. 5, pp. 445–448, 2006.
- [14] A. Burashnikov, A. Petroski, D. Hu, H. Barajas-Martinez, and C. Antzelevitch, "Atrial-selective inhibition of sodium-channel current by Wenxin Keli is effective in suppressing atrial fibrillation," *Heart Rhythm*, vol. 9, no. 1, pp. 125–131, 2012.
- [15] J. M. Zhou, Z. G. Ye, X. M. Cui, Z. Jing A Zhao, and L. Zhu, "Pharmacodynamic research of notoginsenoside R1, R2 and ginsenoside Rb1," *Chinese Traditional Patent Medicine*, vol. 32, no. 9, pp. 1494–1497, 2010.
- [16] F. Zhou, S.-J. Hu, and Y. Mu, "Protection effect of Wenxin Keli on isoproterenol induced heart failure in rats," *Zhongguo Zhongyao Zazhi*, vol. 32, no. 16, pp. 1676–1679, 2007.
- [17] X. Wang, X. Wang, Y. Gu, T. Wang, and C. Huang, "Wenxin Keli attenuates ischemia-induced ventricular arrhythmias in rats: involvement of L-type calcium and transient outward potassium currents," *Molecular Medicine Reports*, vol. 7, no. 2, pp. 519–524, 2013.
- [18] Y.-M. Lu, J. Huang, N. Shioda et al., "Camkii $\delta$  mediates aberrant NCX1 expression and the imbalance of NCX1/SERCA in transverse aortic constriction-Induced failing heart," *PLoS ONE*, vol. 6, no. 9, Article ID e24724, 2011.
- [19] Y.-M. Lu, N. Shioda, F. Han et al., "Imbalance between CaM kinase II and calcineurin activities impairs caffeine-induced calcium release in hypertrophic cardiomyocytes," *Biochemical Pharmacology*, vol. 74, no. 12, pp. 1727–1737, 2007.
- [20] J. Wang, N. Xu, X. Feng et al., "Targeted disruption of Smad4 in cardiomyocytes results in cardiac hypertrophy and heart failure," *Circulation Research*, vol. 97, no. 8, pp. 821–828, 2005.
- [21] J. Tytgat, "How to isolate cardiac myocytes," *Cardiovascular Research*, vol. 28, no. 2, pp. 280–283, 1994.
- [22] F. Han, Y.-M. Lu, H. Hasegawa et al., "Inhibition of dystrophin breakdown and endothelial nitric-oxide synthase uncoupling accounts for cytoprotection by 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate (DY-9760e) in left ventricular hypertrophied mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 332, no. 2, pp. 421–428, 2010.
- [23] M. E. Díaz, H. K. Graham, and A. W. Trafford, "Enhanced sarcolemmal Ca<sup>2+</sup> efflux reduces sarcoplasmic reticulum Ca<sup>2+</sup> content and systolic Ca<sup>2+</sup> in cardiac hypertrophy," *Cardiovascular Research*, vol. 62, no. 3, pp. 538–547, 2004.
- [24] W. U. Foltz, M. Wagner, E. Rudakova, and T. Volk, "N-acetylcysteine prevents electrical remodeling and attenuates cellular hypertrophy in epicardial myocytes of rats with ascending aortic stenosis," *Basic Research in Cardiology*, vol. 107, no. 5, p. 290, 2012.
- [25] C. Shi, X. Wang, F. Dong et al., "Temporal alterations and cellular mechanisms of transmural repolarization during progression of mouse cardiac hypertrophy and failure," *Acta Physiologica*, vol. 208, no. 1, pp. 95–110, 2013.
- [26] P. Milberg, M. Fink, C. Pott et al., "Blockade of ICa suppresses early afterdepolarizations and reduces transmural dispersion of repolarization in a whole heart model of chronic heart failure," *British Journal of Pharmacology*, vol. 166, no. 2, pp. 557–568, 2012.
- [27] J. D. Molkenkin, "Dichotomy of Ca<sup>2+</sup> in the heart: Contraction versus intracellular signaling," *Journal of Clinical Investigation*, vol. 116, no. 3, pp. 623–626, 2006.
- [28] G. M. Faber, J. Silva, L. Livshitz, and Y. Rudy, "Kinetic properties of the cardiac L-type Ca<sup>2+</sup> channel and its role in myocyte electrophysiology: a theoretical investigation," *Biophysical Journal*, vol. 92, no. 5, pp. 1522–1543, 2007.
- [29] D. J. Triggle, "Calcium channel antagonists: clinical uses-past, present and future," *Biochemical Pharmacology*, vol. 74, no. 1, pp. 1–9, 2007.
- [30] N. Su, T. Xu, Z. Zhou, and Y. Tang, "Efficacy and safety of wenxin granules in the treatment of congestive heart failure: a systematic review," *China Pharmacy*, vol. 21, no. 7, pp. 637–640, 2010.
- [31] G. Hasenfuss, "Animal models of human cardiovascular disease, heart failure and hypertrophy," *Cardiovascular Research*, vol. 39, no. 1, pp. 60–76, 1998.
- [32] Z. Abassi, I. Goltsman, T. Karram, J. Winaver, and A. Hoffman, "Aortocaval fistula in rat: a unique model of volume-overload congestive heart failure and cardiac hypertrophy," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 729497, 13 pages, 2011.
- [33] G. Q. Wang, W. Jin, M. Y. Qi et al., "The mechanism of the effects of Wenxin Keli on cardiac hypertrophy in rats," *Chinese Journal of Integrative Medicine on Cardio-/Cerebrovascular Disease*, vol. 9, no. 4, pp. 462–463, 2011.
- [34] Y. W. Xing, Y. H. Gao, J. X. Chen et al., "Wenxin-keli regulates the calcium/calmodulin-dependent Protein kinase II Signal

transduction pathway and inhibits cardiac arrhythmia in rats with myocardial infarction,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 464508, 15 pages, 2013.

- [35] C. X. Shi, Y. H. Wang, F. Dong, Y. J. Zhang, and Y. F. Xu, “Transmural L-type calcium current in a pressure-overloaded mouse model with heart failure,” *Sheng Li Xue Bao*, vol. 59, no. 1, pp. 19–26, 2007.
- [36] Y. W. Liu, J. H. Guo, P. Zhang, J. W. Li, and C. Li, “The effects of *Nardostachys chinensis* batal extract on the sodium current and transient outward potassium current of rat ventricular myocytes,” *Chinese Journal of Cardiac Pacing and Electrophysiology*, vol. 23, no. 6, pp. 533–535, 2009.

## Research Article

# Astragalus Polysaccharide Suppresses the Expression of Adhesion Molecules through the Regulation of the p38 MAPK Signaling Pathway in Human Cardiac Microvascular Endothelial Cells after Ischemia-Reperfusion Injury

Zhu Hai-Yan,<sup>1,2</sup> Gao Yong-Hong,<sup>2</sup> Wang Zhi-Yao,<sup>3</sup> Xu Bing,<sup>4</sup> Wu Ai-Ming,<sup>2</sup> Xing Yan-Wei,<sup>5</sup> Liu Bei,<sup>2</sup> Lou Li-Xia,<sup>1</sup> and Chen Li-Xin<sup>6</sup>

<sup>1</sup> Institute for Cardiovascular Disease, Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100700, China

<sup>2</sup> Key Laboratory of Chinese Internal Medicine of Ministry of Education and Beijing, Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100700, China

<sup>3</sup> Eye Hospital, China Academy of Chinese Medical Sciences, Beijing 100040, China

<sup>4</sup> Beijing Tibetan and Ethnic Medicine Hospital, 100053, China

<sup>5</sup> Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100053, China

<sup>6</sup> World Federation of Chinese Medicine Societies, Beijing 100029, China

Correspondence should be addressed to Chen Li-Xin; [wfcms@hotmail.com](mailto:wfcms@hotmail.com)

Received 1 April 2013; Revised 4 September 2013; Accepted 17 September 2013

Academic Editor: Hao Xu

Copyright © 2013 Zhu Hai-Yan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Astragalus polysaccharide is a major component of radix astragali, a vital qi-reinforcing herb medicine with favorable immune-regulating effects. In a previous animal experiment, we demonstrated that astragalus polysaccharide effectively alleviates ischemia-reperfusion injury (IRI) of cardiac muscle through the regulation of the inflammatory reactions. However, the relationship between this herb and the cohesion molecules on the cell surface remains controversial. In this study, human cardiac microvascular endothelial cells (HCMECs) were used to validate the protective effects of astragalus under an IRI scheme simulated through hypoxia/reoxygenation in vitro. The results indicated that astragalus polysaccharide inhibited the cohesion between HCMECs and polymorphonuclear leukocyte (PMN) during IRI through the downregulation of p38 MAPK signaling and the reduction of cohesive molecule expression in HCMECs.

## 1. Introduction

Myocardial ischemia-reperfusion represents a milestone in the management of acute myocardial infarction; however, the management of ischemia-reperfusion injury (IRI) presents a medical conundrum. In IRI, the coronary artery is typically revascularised after reperfusion, although in more severe hemorrhagic infarctions, as observed in cardiac muscles supplied through the artery, the circulation of the distal ischemic area is blocked, resulting in serious microvascular damage.

Until recently, the mechanisms underlying cardiac IRI have not been fully understood. It has been suggested that

IRI results from a specific inflammatory response [1], where leukocyte participation is a pivotal step leading to injury and the microvascular endothelium is a key target. The molecular basis of IRI involves the interaction between vascular endothelial cells (VECs) and adhesion molecules on the surface of leukocytes and the regulation of adhesion molecule expression through associated factors [2, 3].

A recent study showed that the regulation of the signal transduction network is vital to regulate adhesion molecule expression [4]. The transcription of adhesion molecule requires the involvement of the mitogen-activated protein kinase (MAPK) signalling pathway and NF- $\kappa$ B transcription factors [5, 6]. Notably, there is a close association between

p38 MAPK and the injury in VECs [7]. Hypoxia, oxygen radicals, and other stresses stimulate VECs and activate the p38 MAPK-mediated secretion of adhesion molecules. The suppression of the associated signalling pathways significantly alleviates the prognosis of IRI [8].

There are some similarities between Chinese medicine and modern medicine concerning the comprehension of ischemic heart diseases [9]. Specifically, traditional Chinese medicine considers ischemias an obstruction of the heart meridian, contributing to poor blood flow, and the heart meridian is the counterpart of cardiac microvessel. Traditional Chinese medicine considers blood stasis and collateral obstruction as consequences of ischemia, whereas yang and qi deficiencies are the causes of this disease. Consequently, qi-tonifying and blood-activating herbs are commonly used to treat this medical condition. Astragalus is an important qi-tonifying herbal medicine whose unique effect has been fully demonstrated in clinical practice [10]. Astragalus polysaccharide is one of the main effective ingredients of this herb. In a previous study, we showed that astragalus polysaccharide exhibits superior anticardiac IRI action in animals. Specifically, this compound restored cardiac mechanisms, such as coronary blood flow, LPO content, superoxide dismutase activity, and so forth, to favourable levels after reperfusion [11, 12]. However, the influence of astragalus polysaccharide on the expression of adhesion molecules in VECs remains unknown. While some studies have indicated that this compound upregulates the expression of adhesion molecules [13], other studies suggest that astragalus polysaccharide downregulates adhesion molecule expression [14].

To avoid confusion through heterogeneity in cells, organs, and tissues, primary human cardiac microvascular endothelial cells were used as target cells, and *in vitro* hypoxia-reoxygenation was adopted to simulate ischemia-reperfusion under these conditions. The effects of astragalus polysaccharide on the cohesion of HCMECs and PMN during IRI, the expression of major adhesion molecules, and the regulation of signalling pathways were observed.

## 2. Materials and Methods

**2.1. Materials.** Human cardiac microvascular endothelial cells (HCMECs), endothelial cell media, and cell digestion enzymes were purchased from the ScienCell Corporation, USA. Poly-L-lysine and SB203580 (p38 MAPK inhibitor) were obtained from Sigma, USA. Rose Bengal sodium salt was purchased from the Beijing Chemical Reagents Company. Astragalus polysaccharide (batch no.: Z20040086) was obtained from the Tianjin Cinorch Pharmaceutical Co., Ltd., China. Polymorphonuclear leukocyte (PMN) separation medium was obtained from the Institute of Hematology and Blood Diseases Hospital Chinese Academy of Medical Sciences. The SV Total RNA Isolation System and Reverse Transcription System were purchased from the Promega Corporation, USA. Power SYBR Green kits were purchased from ABI. A mouse monoclonal antibody against E-selectin, (product no.: SC-137054), goat polyclonal antibody against P-selectin (product no.: SC-6941), rabbit polyclonal antibody against p38 $\alpha/\beta$

(product no.: SC-7149), and rabbit polyclonal antibody against phosphorylated-p38 (product no.: SC-101759) were purchased from the Santa Cruz Corporation, USA. A rabbit polyclonal antibody against ATF-2 (product no.: AB11031) was purchased from the Abcam Company. HRP-labeled goat anti-rabbit IgG (product no.: ZDR-5306) was obtained from the ZSGB-Bio Corporation, and Protein-marker was obtained from the MBI Company. The developer and fixative solutions were purchased from Appligen Company (Beijing).

### 2.2. Endothelial Cell Culture and the Establishment of the Ischemia-Reperfusion Injury Model

**2.2.1. Endothelial Cell Culture.** A frozen aliquot of cells was obtained from liquid nitrogen and immediately thawed at 37°C. The cells were injected into a polylysine (20 mg/L)-coated culture bottle and cultivated in an incubator with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. The cells were passaged every 2 to 3 days, and the endothelial cells were used in the following experiments at passage 4 or 5.

**2.2.2. Establishment of the Ischemia-Reperfusion Injury Model.** Endothelial cells at 80% confluency were digested into single cell suspension, and the cell density was adjusted to  $1 \times 10^5$ /mL. The cells were subsequently seeded onto polylysine-coated 96-well plates. A volume of 100  $\mu$ L cell suspension was added to each well, and the media was replaced the next day. The IRI model was established at one day later. Ischemia simulation: the cells were washed twice with PBS, changed into sugar-free Earle's solution, and subsequently cultivated in an incubator with 1% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. Reperfusion simulation: the sugar-free Earle's solution was replaced with complete medium, and the cells were further cultivated in a CO<sub>2</sub> incubator.

#### 2.2.3. Evaluation of the IRI Model

(1) *Evaluation of the Endothelial Cell Viability.* The MTT method was used to assess the cell viability. Four hours before the end of the ischemic reperfusion, 20  $\mu$ L of MTT solution was added to each well. Subsequently, the supernatant was discarded prior to the addition of 150  $\mu$ L DMSO, and the cells were gently oscillated for 10 minutes. The cell viability was detected using an enzyme-labeling instrument at 492 nm. The hypoxia duration was 1, 2, and 4 hours, while reoxygenation lasted 6 and 24 hours.

(2) *Rate of Adhesion between HCMECs and PMN.* Preparation of PMN: elbow vein blood was collected from healthy adults into tubes containing heparin anticoagulant and mixed well with an equal volume PBS. The samples were placed onto the liquid surface of PMN separation medium. The mixture was centrifuged at 2000 r/min for 20 minutes. The third layer of PMN was collected and washed 3 times with PBS. The ECM was used to resuspend the PMN, and the cell density was adjusted to  $1 \times 10^9$ /mL. Subsequently, 0.05 mL of the cell suspension was mixed with an equal volume of trypan blue

(10 g/L) for staining, showing 95% PMN viability. Moreover, Giemsa staining showed that the purity of the PMN cell suspension was more than 97%. The cell suspension was stored at 4°C for subsequent use within 2 hours.

HCMECs and PMN adhesion experiment: endothelial cells were treated using the hypoxia/reoxygenation method described above, and the ECM was removed from the 96-well, followed by the addition of 200  $\mu$ L of PMN cell suspension ( $1 \times 10^9$ /L). Subsequently, the cells were incubated in a CO<sub>2</sub> incubator for 1 h. The unadhered PMN were discarded through aspiration, and the remaining cells were washed twice with PBS. A total of 100  $\mu$ L of Rose Bengal sodium salt (2.5 g/L) was added to each well, and the reaction was incubated at room temperature for 10 minutes. The dyeing solution was subsequently discarded, and the cells were washed twice with PBS. The cells were mixed with an equal proportion of PBS (0.1 g/L) and 0.95% ethanol for decolourisation. Subsequently, the cells were stored at room temperature for 1 h, and adhesion was detected using an enzyme-labeling instrument at 550 nm. The hypoxia duration was 2 h, and reoxygenation lasted 6 and 24 h.

### 2.3. Effects of Astragalus Polysaccharide on the Adhesion of HCMECs and PMN during IRI

**2.3.1. The Experiment Comprised Six Groups.** Control group (cells were cultured normally without stimulation); hypoxia/reoxygenation group (2-hour oxygen and glucose deprivation, followed by 24-hour reoxygenation and resupply of glucose); treatment with low, moderate, and high doses of Astragalus Polysaccharide (25, 50, and 100 mg/L of astragalus polysaccharide, respectively, were added during hypoxia/reoxygenation), SB203580 group (50  $\mu$ mol/L SB203580 was added for 30 minutes prior to hypoxia and subsequently supplanted with sugar-free Earl's solution using the same stimulating method as performed in the hypoxia/reoxygenation group). Six replicates were performed in separate wells for each group.

**2.3.2. Rate of Adhesion between HCMECs and PMN.** After 2 hours of hypoxia treatment, followed by up to 4 hours of reoxygenation, the rate of adhesion between HCMECs and PMN was determined using the methods described above.

**2.4. Influence of Astragalus Polysaccharide on Adhesion Molecules (P-Selectin and E-Selectin) and Gene Transcription through the p38 MAPK Signalling Pathway (p38, ATF-2) in HCMECs under IRI.** The groups were assigned in the same manner as described above. The mRNA was extracted, reverse transcribed, and quantified using real-time PCR. The following PCR primers were used:

E-selectin (238 bp): Forward 5'-GCA CAT CTC AGG GAC AAT GGA-3', Reverse 5'-TTG GAC TCA GTG GGA GCT TCA-3';

P-selectin (306 bp): Forward 5'-TTC AGG ACA ATG GAC AGC AGT-3', Reverse 5'-GTC CCA CCC ATT ATC AGA CCT-3';

p38 (260 bp): Forward 5'-GCC GAA GAT GAA CTT TGC GA-3', Reverse 5'-GTG GTG GCA CAA AGC TGA TG-3';

ATF-2 (237 bp): Forward 5'-ATG GTC AGC TGC AGA GTG AAG-3', Reverse 5'-CTG CCT TGG AGG TTG AAC TGA-3';

$\beta$ -actin (302 bp): Forward 5'-TCC TCC CTG GAG AAG AGC TA-3', Reverse 5'-TCA GGA GGA GCA ATG ATC TTG-3'.

The reaction conditions included predenaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, renaturation at 55°C for 30 s, and elongation at 72°C for 30 s. The Stratagene MX3000P System was used to analyse the data and compare the P-selectin and E-selectin mRNA ratios among the different groups.

**2.5. Influence of Astragalus Polysaccharide on Adhesion Molecules (P-Selectin and E-Selectin) and Protein Expression through the p38 MAPK Signalling Pathway (p38/Phosphate-p38, ATF-2) in HCMECs under IRI.** The cells were lysed in cell lysis buffer (15 mM Tris-HCl, pH 7.5, 0.2% TritonX-100, and 150 mM NaCl), and the cellular protein extract was collected. The proteins were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes, which were subsequently incubated with primary antibody (1/1000) at 4°C. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit) (1:2000) for 2 h at room temperature, followed by ECL visualisation. A gel imaging system (Syngene Co.) was used to capture the images, which were subsequently analysed using Image J software (NIH image, Bethesda, MD).

**2.6. Statistical Analyses.** The data were expressed as the means  $\pm$  SEM. The statistical evaluation was performed using SPSS10.0 software. The statistical comparisons were performed using a one-way analysis of variance (ANOVA), and Dunn's method was used to discriminate the differences between different groups.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Establishment of the Ischemia-Reperfusion Model In Vitro

**3.1.1. Endothelial Cell Culture.** HEMECs were grown into a confluent single layer; the cell body was plump and transparent, with a rhombic or polygonal shape and a small round nucleus (Figure 1).

**3.1.2. Viability of Endothelial Cell after Ischemia-Reperfusion.** The cell viability remained unchanged after 1 h hypoxia, followed by 6 h reperfusion. When the reperfusion duration was prolonged to 24 h, the cell activity increased dramatically compared with the 1 h hypoxia group ( $P = 0.000$ ). The comparison between the 2 h hypoxia group and the 2 h hypoxia, followed by 6 and 24 h reperfusion groups, revealed that the viability of latter group was markedly lower ( $P = 0.041, 0.035$ ). However, after 4 h of hypoxia, followed by 6 and

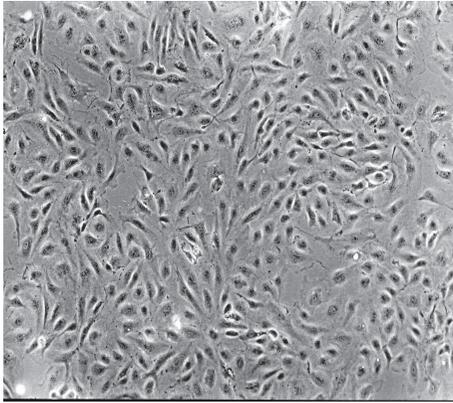


FIGURE 1: HCMECs cultivated for 3 days (100x).

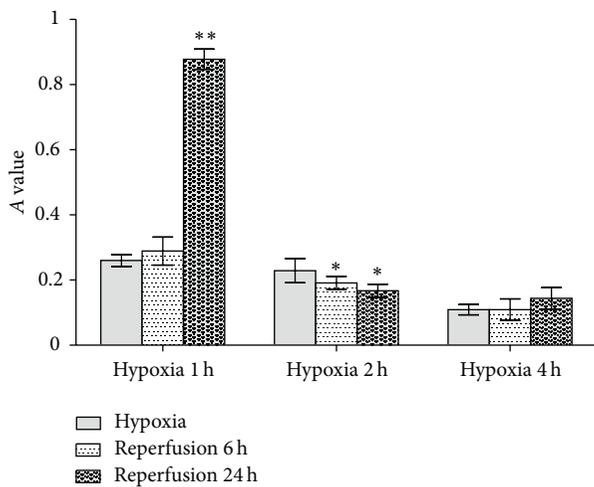


FIGURE 2: Viability of endothelial cells after ischemia-reperfusion. In contrast to 1 h oxygen deprivation alone, 1 h followed by 6 h reperfusion will not cause further injury to HCMEC; however, 24 h reperfusion would lead to apparent IRI to HCMEC. Compared with 2 h hypoxia alone, 2 h hypoxia followed by 6 h reperfusion or 12 h reperfusion both can result in IRI. In comparison with 4 h oxygen deprivation alone, 4 h hypoxia followed by 4 h reperfusion, 6 h reperfusion, or 24 h reperfusion all bring about similar influence on cell proliferation. (\*  $P < 0.05$ , \*\*  $P < 0.01$  versus the corresponding hypoxia-treated groups.)

24 h of reperfusion, the cell activity was similar to that of cells subjected to 4 h hypoxia treatment alone (Figure 2).

### 3.1.3. Rate of Adhesion between Endothelial Cells and PMN.

The adhesion rates after 1 h hypoxia, followed by 6 h reperfusion and 2 h hypoxia, followed by 6 h reperfusion, were significantly different from the corresponding groups were subjected to hypoxia alone ( $P = 0.030, 0.010$ ). Both groups of cells subjected to 4 h hypoxia, and 4 h hypoxia, followed by 4 h reperfusion exhibited low adhesion rates and there was no difference between them ( $P = 0.803$ ) (Figure 3).

### 3.2. Effects of Astragalus Polysaccharide on Adhesion between PMN and HCMECs Subjected to IRI. The adhesion of the

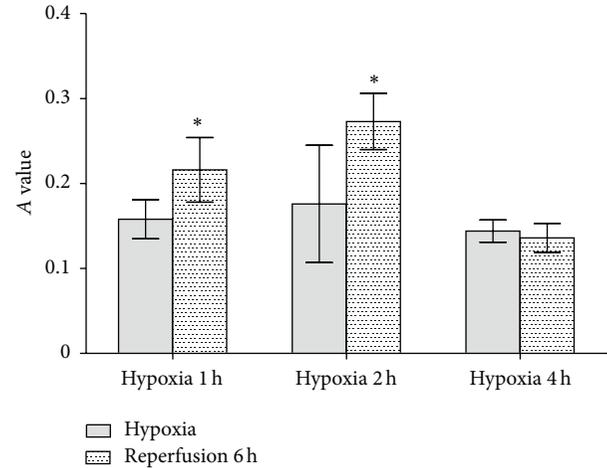


FIGURE 3: Rate of adhesion between endothelial cells and PMN. In contrast to 1 h oxygen deprivation alone, 1 h hypoxia followed by 6 h reperfusion increases the number of neutrophil granulocytes that adhered to HCMEC. Same result happens to comparison between 2 h hypoxia group and 2 h hypoxia followed by 6 h reperfusion group. Which means more neutrophil granulocyte adhere to HCMEC in latter group than in former group. However, things become different in 4 h hypoxia group and 4 h oxygen deprivation followed by 6 h reperfusion group. That is to say cohesion between neutrophil granulocytes and HCMEC in two groups is of no significant difference. (\*  $P < 0.05$  versus the corresponding hypoxia-treated groups.)

cells in the reperfusion groups was obviously higher than that observed in the control group ( $P = 0.001$ ). Treatment with astragalus polysaccharide decreased HCMECs-PMN adhesion. Specifically, the number of attached cells after treatment with high-dose astragalus polysaccharide and SB203580 was markedly different from the number of cells observed in the model group ( $P = 0.011, 0.000$ ) (Figure 4).

**3.3. Influence of Astragalus Polysaccharide on the Gene Transcription of Adhesion Molecules (P-Selectin and E-Selectin) and p38 Signalling Pathway Factors in HCMECs during IRI.** The results demonstrated that the gene transcription of P-selectin, E-selectin, p38, and ATF-2 in the model group was higher than in the control group ( $P = 0.000$ ). In the low- and high-dose astragalus polysaccharide treatment groups and the SB203580 group, the transcription of P-selectin and E-selectin was significantly inhibited compared with the model group ( $P = 0.000$ ). In addition, high-dose astragalus polysaccharide and SB203580 treatments suppressed the transcription of p38 and ATF-2 during IRI (Figure 5).

**3.4. Influence of Astragalus Polysaccharide on the Protein Expression of Adhesion Molecules (P-Selectin and E-Selectin) and p38 Signalling Pathway Factors (p38, p-p38, and ATF-2) in HCMECs during IRI.** Compared with the control group, the expression of the adhesion molecules (P-selectin and E-selectin) and p38 signalling pathway factors (p38, p-p38 and ATF-2) was significantly elevated ( $P < 0.01, P = 0.000$ ,

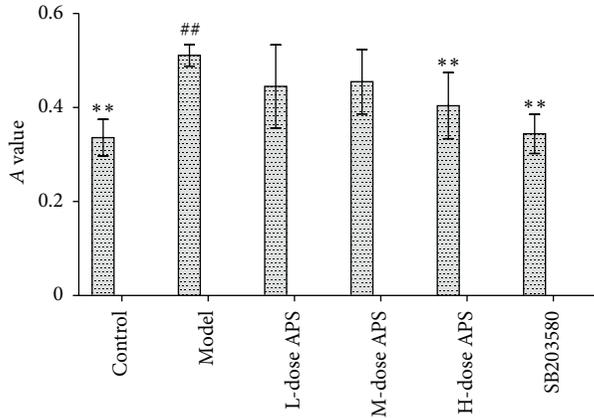


FIGURE 4: Effects of astragalus polysaccharide on the adhesion between PMN and HCMECs subjected to IRI. The number of attached cells after treatment with high-dose astragalus polysaccharide and SB203580 was markedly different from the number of cells observed in the model group. (\*\* $P < 0.01$  versus the control group. \*\* $P < 0.01$  versus the model group.)

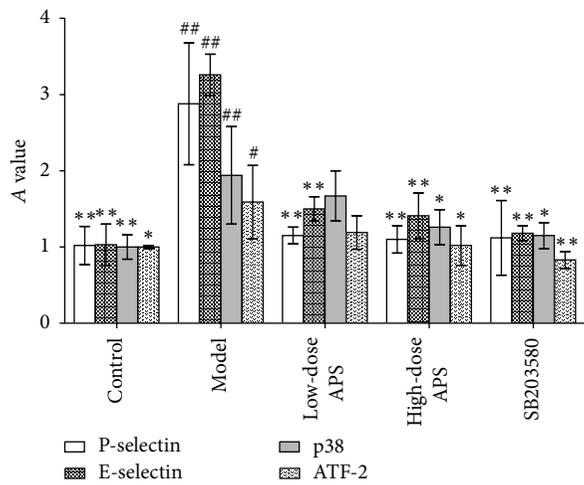


FIGURE 5: Gene transcription of adhesion molecules and p38 signalling pathway-related proteins in HCMECs during IRI. ( $^{\#}P < 0.05$ ,  $^{\#\#}P < 0.01$  versus the control group.  $^*P < 0.05$ ,  $^{**}P < 0.01$  versus the model group.)

0.000, 0.006, 0.000, and 0.000). High-dose treatments with astragalus polysaccharide, SB203580, and adhesion molecule antibodies suppressed the effects on the expression of the 5 proteins described above in HCMECs during IRI ( $P < 0.01$ . P-selectin = 0.133, 0.044, 0.007, 0.001. E-selectin = 0.031, 0.017, 0.001, 0.000. p38 = 0.041, 0.018, 0.007. p-p38 = 0.090, 0.004, 0.000. ATF-2 = 0.190, 0.003, 0.000) (Figures 6, 7, 8, and 9).

#### 4. Discussion

IRI is a type of inflammation reaction mediated through PMN. It has been suggested that this mechanism involves activated leukocytes that adhere to the vascular endothelium and obstruct capillaries, thereby decreasing the blood flow

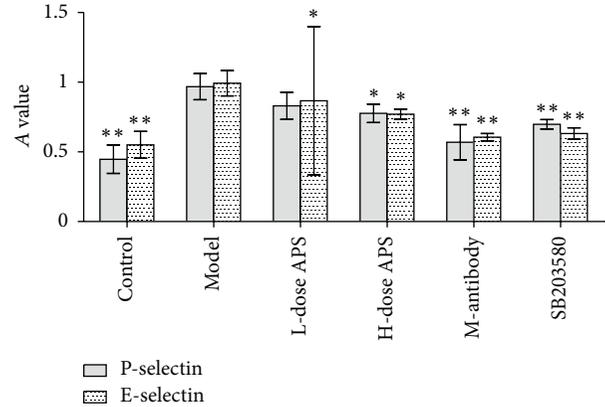


FIGURE 6: Protein expression of adhesion molecules. High-dose astragalus polysaccharide and SB203580 treatments decreased P-selectin and E-selectin expression ( $^*P < 0.05$ ,  $^{**}P < 0.01$  versus the model group.)

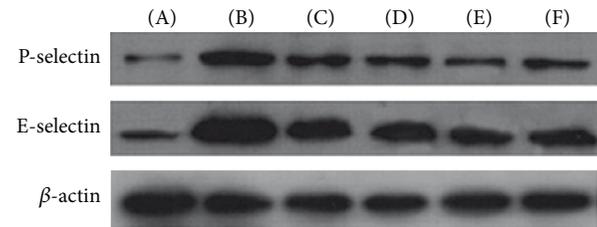


FIGURE 7: Protein expression of adhesion molecules. (A) Normal control group, (B) model group, (C) low-dose AP group, (D) high-dose AP group, (E) cohesion molecule antibody group, and (F) SB203580 group.

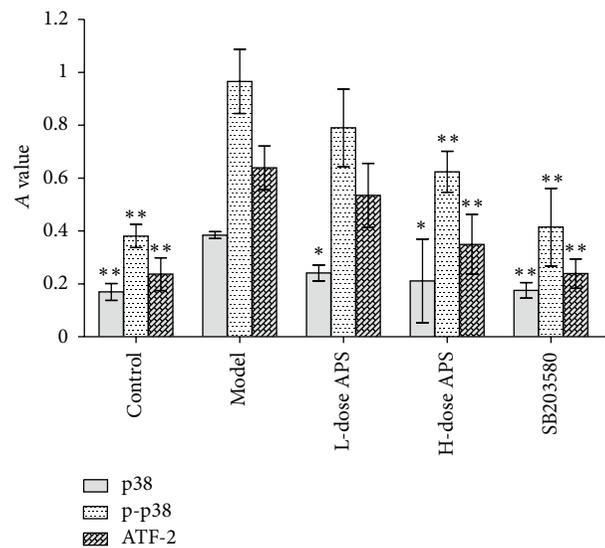


FIGURE 8: Protein expression of the factors involved in p38 MAPK signalling pathways. High-dose astragalus polysaccharide and SB203580 treatments decreased p38, p-p38 and ATF-2 expression ( $^*P < 0.05$ ,  $^{**}P < 0.01$  versus the model group.)

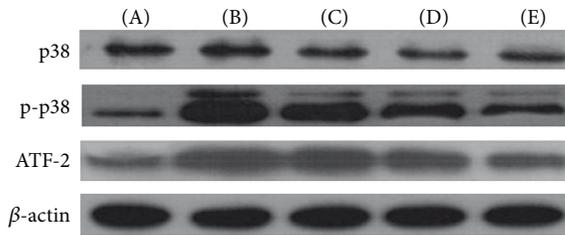


FIGURE 9: Protein expression of the factors involved in p38 MAPK signalling pathways. (A) Normal control group, (B) model group, (C) low-dose AP group, (D) high-dose AP group, and (E) SB203580 group.

and compromising the integrity and function of the capillaries [15, 16]. Apart from the direct damage to endothelial cells, PMN also migrate to ischemic cardiac muscles and release large amount of active substances, leading to elevated vascular permeability, tissue edema, tissue necrosis and cardiac myocyte apoptosis [17, 18]. It is clear that the inhibition of adhesion between PMN and endothelial cells alleviates IRI. In the present study, we showed that astragalus polysaccharide possesses anti-IRI and anti-PMN adhesion effects using an IRI model *in vitro*, consistent with those of the results obtained in our previous animal experiments. Thus, this compound, derived from the herb blood supply, relieves cardiac muscle damage in rats subjected to IRI.

When PMN adhere to the endothelium of coronary arteries after IRI, the molecules mediating adhesion might be the initial factors facilitating cardiac muscle IRI. Under normal conditions, PMN and HCMECs seldom adhere, and occasional adherence is rapidly dissociated [19]. In contrast, after IRI, endothelial cells and adhesion molecules from the PMN surface vary in number and structure, contributing to the substantial attachment of the PMN to the endothelium surface. Unfortunately, the mechanism underlying this phenomena is not yet fully understood. Recent studies have indicated that hypoxia/reoxygenation stimulates endothelial cells to release active substances, such as oxygen radicals, which induce expression and formation changes in adhesion molecules from PMN and activate adhesion molecules on the surface of PMN, thereby potentiating cell adhesion.

In the present study, we demonstrated that astragalus polysaccharide possesses restraining effects on the transcription and expression of two adhesion molecules, P-selectin and E-selectin. P-selectin is expressed in the Weibel-Palade body of endothelial cells and is secreted from the cell interior to the serous membrane on the cell surface minutes after reperfusion occurs, to perform its PMN-recruiting function [20]. Thereafter, P-selectin is degraded into soluble fragments [21]. Hillis and coworkers suggested that elevated soluble P-selectin in blood might serve as an indicator to predict early-stage myocardial ischemia in chest pain patients [22]. In contrast, E-selectin is not expressed under normal conditions but rather is secreted during endothelial cell stimulation. However, the synthesis of E-selectin is slow and is typically initiated at 4 to 6 hours after hypoxia [23]. Thus, E-selectin is not an important factor during the early hours after

IRI; instead, this factor plays a vital role in unconsolidated PMN adhesion during the later stages of IRI [24]. Astragalus polysaccharide reduces P-selectin and E-selectin expression during early- and late-stage IRI, respectively. Thus, astragalus polysaccharide affects the entire course of PMN-endotheliocyte adhesion, and the differences in stimulating methods and cells utilised in this experiment might reflect the inconsistencies between our results and those of others [13]. HCMECs were used in this study, as these cells represent the first battle line in IRI; that is, these cells are the first affected sites in reperfusion. Therefore, changes in HCMECs exert huge influences on the damage or restoration of cardiac muscle [25, 26]. In addition, recent studies have shown that endothelial cells of different organic and histological origins obviously vary in morphology, gene, and function. Consequently, the phenomenon that large vessels and microvessels are derived from endothelial cells in animal deserves further reflection [27–29]. Thus, to avoid the problems described above, we used HCMECs in this study to yield more realistic results.

Based on the fact that the p38 signalling pathway is one of the major channels regulating inflammation reactions, we proposed that astragalus polysaccharide likely regulates this pathway. In the present study, astragalus polysaccharide, isolated from *Scutellariae baicalensis* Georgi, is effective in suppressing p38 phosphorylation and inhibiting the expression of ATF-2, a downstream p38 effector molecule; consequently, the modulatory activity of astragalus polysaccharide affects the transcription and protein synthesis of cohesion molecules.

In a previous animal study, we showed that astragalus polysaccharide exerts a positive effect on the management of myocardial ischemia. Based on the results obtained in the present study, the adhesion molecules in HCMECs are the central targets for the anti-IRI effects of astragalus polysaccharide.

## 5. Conclusions

The results of this study suggest that treatment with astragalus polysaccharide, within a certain dose range, suppresses the adhesion between HCMECs and PMN, and the underlying mechanism of this suppression is associated with the downregulation of the expression and phosphorylation of p38 MAPK, thereby contributing to the subsequent expression of P-selectin and E-selectin. These results are consistent with the findings obtained in previous animal experiments demonstrating that this compound possesses anti-IRI effects. The qi-reinforcing and yang-elevating effects of astragalus polysaccharide treatment for IRI in traditional Chinese medicine can therefore be interpreted as the regulation of the expression of major cohesion molecules in HCMECs and the revascularisation of microcirculation.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contributions

Zhu Hai-Yan, Gao Yong-Hong, Wang Zhi-Yao, Xu Bingand Wu Ai-Ming equally contributed to this work.

## Acknowledgments

This work was supported by funding from the National Natural Science Foundation (30772805).

## References

- [1] A. Frank, M. Bonney, S. Bonney, L. Weitzel, M. Koeppen, and T. Eckle, "Myocardial ischemia reperfusion injury: from basic science to clinical bedside," *Semin Cardiothorac Vasc Anesth*, vol. 16, no. 3, pp. 123–132, 2012.
- [2] S. P. Jones, S. D. Trocha, M. B. Strange et al., "Leukocyte and endothelial cell adhesion molecules in a chronic murine model of myocardial reperfusion injury," *American Journal of Physiology*, vol. 279, no. 5, pp. H2196–H2201, 2000.
- [3] S. Fukushima, S. R. Coppen, A. Varela-Carver et al., "A novel strategy for myocardial protection by combined antibody therapy inhibiting both P-selectin and intercellular adhesion molecule-1 via retrograde intracoronary route," *Circulation*, vol. 114, no. 1, pp. I251–I256, 2006.
- [4] S. K. Quadri, "Cross talk between focal adhesion kinase and cadherins: Role in regulating endothelial barrier function," *Microvascular Research*, vol. 83, no. 1, pp. 3–11, 2012.
- [5] S.-F. Luo, R.-Y. Fang, H.-L. Hsieh et al., "Involvement of MAPKs and NF- $\kappa$ B in tumor necrosis factor  $\alpha$ -induced vascular cell adhesion molecule 1 expression in human rheumatoid arthritis synovial fibroblasts," *Arthritis and Rheumatism*, vol. 62, no. 1, pp. 105–116, 2010.
- [6] Y.-L. Chang, C.-L. Chen, C.-L. Kuo, B.-C. Chen, and J.-S. You, "Glycyrrhetic acid inhibits ICAM-1 expression via blocking JNK and NF- $\kappa$ B pathways in TNF- $\alpha$ -activated endothelial cells," *Acta Pharmacologica Sinica*, vol. 31, no. 5, pp. 546–553, 2010.
- [7] T. Matsumoto, I. Turesson, M. Book, P. Gerwins, and L. Claesson-Welsh, "p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis," *Journal of Cell Biology*, vol. 156, no. 1, pp. 149–160, 2002.
- [8] K. Temming, M. Lacombe, P. Van Der Hoeven et al., "Delivery of the p38 MAPkinase inhibitor SB202190 to angiogenic endothelial cells: development of novel RGD-equipped and PEGylated drug-albumin conjugates using platinum(II)-based drug linker technology," *Bioconjugate Chemistry*, vol. 17, no. 5, pp. 1246–1255, 2006.
- [9] N. Tian, "A study on microcirculation of blood stasis syndrome," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 21, no. 4, pp. 248–251, 2001.
- [10] X. Xu and L. Tang, "Research Progress on cardiovascular effects of Astragalus," *Chinese Journal of New Drugs*, vol. 12, no. 11, 2003.
- [11] Z. Zhang, L. X. Chen, and L. M. Qin, "Effects of Astragalus saponin on hemodynamics and oxygen free radicals of rat cardiac muscle during ischemia reperfusion injury," *Chinese Journal of Information on Traditional Chinese Medicine*, vol. 7, no. 3, 2000.
- [12] Z. Zhang, L. X. Chen, and C. S. Song, "The protective effects of Astragalus polysaccharide on cardiac muscle of rats during ischemia reperfusion injury," *Chinese Journal of Information on Traditional Chinese Medicine*, vol. 14, no. 2, 2007.
- [13] Y. Hao, Q.-Y. Qiu, and J. Wu, "Effect of Astragalus polysaccharides in promoting neutrophil-vascular endothelial cell adhesion and expression of related adhesive molecules," *Zhongguo Zhong Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi*, vol. 24, no. 5, pp. 427–430, 2004.
- [14] P. Li, X. J. He, and Y. Zhang, "Effects of astragalus polysaccharin on fibroblast proliferation and adhesion between HUVECs and white cells," *Chinese Journal of Pathophysiology*, vol. 20, no. 9, 2004.
- [15] H. Morrison, D. McKee, and L. Ritter, "Systemic neutrophil activation in a mouse model of ischemic stroke and reperfusion," *Biological Research for Nursing*, vol. 13, no. 2, pp. 154–163, 2011.
- [16] A. Sievert, "Leukocyte depletion as a mechanism for reducing neutrophil-mediated ischemic-reperfusion injury during transplantation," *Journal of Extra-Corporeal Technology*, vol. 35, no. 1, pp. 48–52, 2003.
- [17] Y. Zhao, A. K. Sharma, D. J. Lapar et al., "Depletion of tissue plasminogen activator attenuates lung ischemia-reperfusion injury via inhibition of neutrophil extravasation," *American Journal of Physiology*, vol. 300, no. 5, pp. L718–L729, 2011.
- [18] J. E. Jordan, Z.-Q. Zhao, and J. Vinten-Johansen, "The role of neutrophils in myocardial ischemia-reperfusion injury," *Cardiovascular Research*, vol. 43, no. 4, pp. 860–878, 1999.
- [19] M. H. Wu, "Endothelial focal adhesions and barrier function," *Journal of Physiology*, vol. 569, no. 2, pp. 359–366, 2005.
- [20] V. S. Dole, W. Bergmeier, H. A. Mitchell, S. C. Eichenberger, and D. D. Wagner, "Activated platelets induce Weibel-Palade-body secretion and leukocyte rolling in vivo: role of P-selectin," *Blood*, vol. 106, no. 7, pp. 2334–2339, 2005.
- [21] A. S. Weyrich, M. Buerke, K. H. Albertine, and A. M. Lefer, "Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of the ischemic feline myocardium," *Journal of Leukocyte Biology*, vol. 57, no. 1, pp. 45–55, 1995.
- [22] G. S. Hillis, C. Terregino, P. Taggart et al., "Elevated soluble P-selectin levels are associated with an increased risk of early adverse events in patients with presumed myocardial ischemia," *American heart journal*, vol. 143, no. 2, pp. 235–241, 2002.
- [23] V. Alamanda, S. Singh, N. J. Lawrence, and S. P. Chellappan, "Nicotine-mediated induction of E-selectin in aortic endothelial cells requires Src kinase and E2F1 transcriptional activity," *Biochemical and Biophysical Research Communications*, vol. 418, no. 1, pp. 56–61, 2012.
- [24] F. Joucher, G.-M. Mazmanian, and M. German-Fattal, "E-selectin early overexpression induced by allogeneic activation in isolated mouse lung," *Transplantation*, vol. 78, no. 9, pp. 1283–1289, 2004.
- [25] K. Laude, V. Richard, and C. Thuillez, "Coronary endothelial cells: a target of ischemia reperfusion and its treatment?" *Archives des Maladies du Coeur et des Vaisseaux*, vol. 97, no. 3, pp. 250–254, 2004.
- [26] P. C. H. Hsieh, M. E. Davis, L. K. Lisowski, and R. T. Lee, "Endothelial-cardiomyocyte interactions in cardiac development and repair," *Annual Review of Physiology*, vol. 68, pp. 51–66, 2006.
- [27] I. Lang, C. Hoffmann, H. Olip et al., "Differential mitogenic responses of human macrovascular and microvascular endothelial cells to cytokines underline their phenotypic heterogeneity," *Cell Proliferation*, vol. 34, no. 3, pp. 143–155, 2001.

- [28] M. Gräfe, W. Auch-Schwelk, H. Hertel et al., "Human cardiac microvascular and macrovascular endothelial cells respond differently to oxidatively modified LDL," *Atherosclerosis*, vol. 137, no. 1, pp. 87–95, 1998.
- [29] S. Murakami, T. Morioka, Y. Nakagawa, Y. Suzuki, M. Arakawa, and T. Oite, "Expression of adhesion molecules by cultured human glomerular endothelial cells in response to cytokines: comparison to human umbilical vein and dermal microvascular endothelial cells," *Microvascular Research*, vol. 62, no. 3, pp. 383–391, 2001.

## Research Article

# Ethanol Extract of *Lepidium apetalum* Seed Elicits Contractile Response and Attenuates Atrial Natriuretic Peptide Secretion in Beating Rabbit Atria

Seung Ju Kim,<sup>1,2</sup> Hye Yoom Kim,<sup>1,2</sup> Yun Jung Lee,<sup>1,2</sup> Hao Zhen Cui,<sup>1,3</sup>  
Ji Yeon Jang,<sup>1</sup> Dae Gill Kang,<sup>1,2</sup> and Ho Sub Lee<sup>1,2</sup>

<sup>1</sup> School of Oriental Medicine & College of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea

<sup>2</sup> Hanbang Body-Fluid Research Center, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea

<sup>3</sup> The College of Chinese Traditional Medicine, Yanbian University, Yanji, Jilin 133002, China

Correspondence should be addressed to Dae Gill Kang; dgkang@wku.ac.kr and Ho Sub Lee; host@wku.ac.kr

Received 15 March 2013; Accepted 9 September 2013

Academic Editor: Hao Xu

Copyright © 2013 Seung Ju Kim et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The seeds of *Lepidium apetalum* Willdenow (called “Tinglizi” in China and “Jungryukza” in Korea) have been used to discharge phlegm and improve dropsy in Oriental medicine. The present study investigated the effects of ethanol extract of the seeds of *Lepidium apetalum* (ELA) on atrial dynamics and atrial natriuretic peptide (ANP) secretion in beating rabbit atria. ELA increased atrial stroke volume, pulse pressure, and cAMP efflux, concomitantly attenuating ANP secretion in a dose-dependent manner. ELA-induced increases in atrial stroke volume, pulse pressure, and cAMP levels and decrease in ANP secretion were not inhibited by pretreatment with staurosporine, a nonspecific protein kinase inhibitor, or diltiazem and verapamil, the L-type Ca<sup>2+</sup> channel blockers, respectively. Helveticoside, a well-known digitalis-like cardiac glycosidic constituent of ELA, also increased atrial dynamics, including stroke volume and pulse pressure, without changing cAMP efflux and ANP secretion, and the effects of helveticoside were not inhibited by pretreatment with staurosporine, diltiazem, and verapamil. These results suggest that the ELA-induced positive inotropic activity in beating rabbit atria might, at least partly, be due to the digitalis-like activity of helveticoside rather than an increase in cAMP efflux.

## 1. Introduction

Cardiac glycosides are a diverse family of naturally derived compounds that bind to and inhibit Na<sup>+</sup>/K<sup>+</sup>-ATPase. Members of this family have been used for many years for the treatment of heart failure and atrial arrhythmia, and the mechanism of their positive inotropic effect is well characterized. There are many different well-described clinical trials of drugs for the treatment of chronic heart failure, including cardiac glycosides, sympathomimetics, phosphodiesterase (PDE) III inhibitors, diuretics, and angiotensin-converting enzyme inhibitors [1]. Stimulation of  $\beta$ -adrenergic receptor with a sympathomimetic agent induces positive inotropic

effects, which are dependent on protein kinases (PKs) and L-type Ca<sup>2+</sup> channels [2, 3]. The increase in cyclic adenosine monophosphate (cAMP) levels induced by PDE III inhibitors also accentuates cardiac contractility via activation of protein kinases and L-type Ca<sup>2+</sup> channels [4]. Treatment of heart failure patients with cardiac glycosides like digitalis, which augment pump function by increasing the contractility of cardiac myocytes, is known to improve hemodynamics and exercise tolerance [5, 6]. In the regulation of cardiac contractility, Ca<sup>2+</sup> plays a pivotal role and has been implicated in the functional mechanism of various agents involved in the modulation of cardiac action [7, 8]. In addition, several signal transduction factors like cAMP, inositol triphosphate (IP<sub>3</sub>),

diacylglycerol (DAG), PK, and adenylyl cyclase (AC) influence the generation of cardiac contractile forces and regulate intracellular  $\text{Ca}^{2+}$  concentrations [9].

Atrial natriuretic peptide (ANP) is synthesized and stored in atrial cardiomyocytes and secreted into the bloodstream by atrial stimulation [10]. The secretion of ANP from cardiomyocytes under mechanical stimulation has been known to regulate body fluid levels through relaxation of vascular smooth muscle and inhibition of water and renal electrolyte reabsorption [11].

The seeds of *Lepidium apetalum* Willdenow (Cruciferae, called "Tinglizi" in China and "Jungryukza" in Korea) have been used to discharge phlegm and improve dropsy in Oriental medicine. From the seeds of *Lepidium apetalum*, compounds such as helveticoside, linoleic acid, and olein have been isolated [12]. Recently, it was reported that an extract of the seeds of *Lepidium apetalum* inhibits skin pigmentation mediated by IL-6-driven signaling. However, to the best of our knowledge, the inotropic effect of *Lepidium apetalum* in perfused beating atria has not been defined. Therefore, we performed this study to elucidate the mechanism of ELA-induced positive inotropic activity in perfused beating rabbit atria.

## 2. Materials and Methods

**2.1. Plant Materials and Extraction.** The seeds of *Lepidium apetalum* Willdenow were commercially available and purchased from the herbal market in Iksan, Jeonbuk Province, and authenticated by professor Tae-Oh Kwon, College of Life Sciences and Natural Resources, Wonkwang University. A herbarium voucher specimen (HBI-048) was deposited in the herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, South Korea. The dried seeds of *Lepidium apetalum* (600 g) were subjected to extraction procedures with 1 L of 95% ethanol thrice, with each extraction being performed for 24 h. The ethanol extract was filtered through a Whatman No. 3 filter paper, concentrated using a rotary evaporator (ELA, 1.5 g), and then used in experiments.

**2.2. Preparation of Perfused Beating Rabbit Atria and Determination of Atrial Stroke Volume and Pulse Pressure.** New Zealand white male rabbits weighing 2 kg were used as the source of rabbit atria. Each rabbit was anesthetized by injecting ketamine-HCl, and its chest was opened. An isolated perfused atrial preparation was prepared by a slightly modified version of Cho's method [13], allowing atrial pacing and measurements of changes in atrial volume during contraction (stroke volume) and cAMP efflux. Briefly, the hearts were rapidly removed and placed in oxygenated warm saline. The left atrium was then dissected. A calibrated transparent atrial cannula containing 2 small catheters was inserted into the left atrium through the atrioventricular orifice. The cannula was secured by ligatures around the atrioventricular sulcus. The outer tip of one of the 2 catheters located in the atrium was used for perfusion, and the other catheter was used to record pressure changes in the atrium. The cannulated atrium was then transferred to an organ chamber containing

3 mL of buffer at 34°C. The pericardial space of the organ chamber was opened to air so as not to restrict atrial dynamics. The atrium was immediately perfused with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer solution by means of a peristaltic pump (1 mL/min). The buffer was prepared using the following constituents: 118 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 25 mM  $\text{NaHCO}_3$ , 10.0 mM glucose, and 10.0 mM HEPES (adjusted to pH 7.4 with 1 M NaOH) and 0.1% bovin serum albumin (BSA). Soon after setup of the perfused atrium, transmural electrical field stimulation at 1.3 Hz (duration, 0.3–0.5 ms; voltage, twice the threshold intensity, 20–30 V; distention, 6.1 cm  $\text{H}_2\text{O}$ ) was started with a luminal electrode. The organ chamber was fixed so as to allow axial rotation to change the height of the atrial cannula and intra-atrial pressure. The perfusate was prewarmed to 34°C by passage through silicone tubing in a mixed gas chamber. The buffer in the organ chamber was oxygenated.

**2.3. Measurement of ANP Levels in Perfusates.** The levels of immunoreactive ANP in the perfusate were measured by radioimmunoassay, as previously described [13]. The radioimmunoassay was performed in tris (hydroxymethyl) aminomethane (Tris)-acetate buffer (0.1 mM EDTA, 0.005% soybean trypsin inhibitor, 0.02% sodium azide, 0.0004% phenylmethylsulfonyl fluoride, and 1% BSA, at pH 7.4). The sample volume used for radioimmunoassay was 50  $\mu\text{L}$ , and the total assay volume was 300  $\mu\text{L}$ . Standard or perfusate samples were incubated with 100  $\mu\text{L}$  of anti-ANP antibody and 100  $\mu\text{L}$  of  $^{125}\text{I}$ -labeled ANP for 24 h at 4°C. The separation of free tracer from antibody-bound tracer was achieved by adding 1.0 mL of dextran-charcoal suspension (charcoal, 6.0 g; Dextran T-70, 0.625 g; phenylmercuric acetate, 34 mg; and neomycin, 2 g in 1 L of Tris-acetate buffer, 0.1 M, pH 7.4). Radioimmunoassay for ANP was performed on the day of the experiments, and all samples in an experiment were analyzed in a single assay. The secreted amount of ANP was expressed as nanograms of ANP per minute per gram of atrial wet weight.

**2.4. Preparation of Samples for cAMP Assay.** To prepare the perfusates for cAMP assay, 100  $\mu\text{L}$  of the perfusate was treated with trichloroacetic acid (100  $\mu\text{L}$ ) to a final concentration of 6% for 15 min at room temperature and centrifuged at 4°C. The supernatant (100  $\mu\text{L}$ ) was transferred to a polypropylene tube, extracted 3 times with water-saturated ether (300  $\mu\text{L}$ ), and dried using a speedVac concentrator (Savant, Farmingdale, NY, USA). The dried samples were resuspended in 50 mM sodium acetate buffer (pH 4.85).

**2.5. Measurement of cAMP Levels in Perfusates.** Production of cAMP was measured in an equilibrated radioimmunoassay, as described previously [14]. Briefly, standards or samples were made up to a final volume of 100  $\mu\text{L}$  in 50 mM sodium acetate buffer (pH 4.8) containing theophylline (8 mM). Then, 100  $\mu\text{L}$  of diluted cAMP antiserum and iodinated 2'-O-monosuccinyl-adenosine 3',5'-cyclic monophosphate tyrosyl methyl ester ( $^{125}\text{I}$ -ScAMP-TME, 10,000 counts/min [cpm])

per 100  $\mu\text{L}$ ) were added, and the mixture was incubated for 24 h at 4°C. For the acetylation reaction, 5  $\mu\text{L}$  of a mixture of acetic anhydride and triethylamine (1 : 2 dilution) was added to the assay tube before adding antiserum and tracer as well. The bound form was separated from the free form by charcoal suspension.  $^{125}\text{I}$ -ScAMP-TME was prepared as described previously [15]. Briefly, 2  $\mu\text{g}$  of ScAMP-TME was introduced into a vial containing 100 mM phosphate buffer (pH 7.4), and 1 mCi of  $^{125}\text{I}$ -Na was added. Chloramine-T (0.4 mg/mL) was added to the reaction vial (total reaction volume = 50  $\mu\text{L}$ ) and mixed gently, and 1 min later, the reaction was terminated with sodium metabisulfite (0.2 mg/mL) and  $\text{NaI}^{125}$  (5 mM). The reaction mixture was immediately applied to a Sephadex G-10 column (1  $\times$  20 cm) previously washed with 10 mM phosphate buffer (pH 7.4).  $^{125}\text{I}$ -ScAMP-TME was eluted with 10 mM phosphate buffer containing 150 mM NaCl (pH 7.4) and stored at -20°C until further use. Immediately before it was used,  $^{125}\text{I}$ -ScAMP-TME was repurified by high-performance liquid chromatography (HPLC) on a reversed-phase  $\mu\text{Bondapak}$  column (Waters Associates, Milford, MA, USA) with a linear gradient (0–60% acetonitrile in 0.1% trifluoroacetic acid). Radioimmunoassay for cAMP was performed on the day of the experiments, and all samples from one experiment were analyzed in a single assay. Nonspecific binding was <2.0%. The 50% intercept was at  $16.50 \pm 0.79$  fmol/tube ( $n = 10$ ). The amount of cAMP was expressed as picomole per minute per gram of atrial tissue.

**2.6. Measurement of  $\text{K}^+$  Concentration in Perfusates.** Before and after the perfusion of beating rabbit atria with HEPES buffer, the  $\text{K}^+$  concentration in the perfusates was measured by using an electrolyte analyzer (NOVA 5, Biochemical, Waltham, MA, USA) and expressed as mmol/L.

**2.7. Reagents.** HEPES, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate, glucose, BSA, sodium acetate, aprotinin, glycine, lysozyme, theophylline, sodium azide, potassium phosphate monobasic, potassium phosphate dibasic, charcoal, diltiazem, verapamil, ouabain, and helveticoside were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The following reference materials were obtained from the sources specified: anti-cAMP (Merck Bioscience Calbiochem, USA), anti-ANP (Homemade, Korea), staurosporine (Biomol Research Laboratories Inc, USA), and  $^{125}\text{I}$ -Na (Amersham Biosciences, Sweden). Stock solutions of diltiazem, verapamil, staurosporine, and helveticoside were prepared in DMSO. Control experiments demonstrated that the highest DMSO level (0.2%) had no effect on beating rabbit atria.

**2.8. Statistical Analysis.** The results are shown as means  $\pm$  SE. Data was analyzed by repeated measures ANOVA followed by Bonferroni's multiple-comparison test. Student's  $t$ -test for unpaired data was also applied. Statistical significance was defined as  $P < 0.05$ .

### 3. Results

**3.1. Effect of ELA on the Atrial Dynamics, cAMP Efflux, and ANP Secretion.** In beating rabbit atria, treatment with ELA increased stroke volume and pulse pressure in a dose-dependent manner (Figures 1(a)(A) and 1(a)(B)). Treatment with ELA also increased cAMP efflux in beating rabbit atria (Figure 1(a)(C)). On the other hand, treatment with ELA markedly decreased ANP secretion in beating rabbit atria (Figure 1(a)(D)). Ouabain, which was used as a positive control, significantly increased stroke volume (Figure 1(b)(A)) and pulse pressure (Figure 1(b)(B)), with no change in cAMP efflux (Figure 1(b)(C)) and ANP secretion (Figure 1(b)(D)).

**3.2. Effect of Staurosporine on ELA-Induced Changes.** To define the role of protein kinases in the ELA-induced positive inotropic effect, the effects of staurosporine, a nonspecific PK inhibitor, on beating rabbit atria were tested. Treatment with ELA ( $5 \times 10^{-4}$  g/mL) induced an increase in stroke volume, pulse pressure, and cAMP efflux and a decrease in ANP secretion in beating rabbit atria (Figures 2(a)(A), 2(a)(B), 2(a)(C), and 2(a)(D)). Treatment of beating atria with staurosporine ( $1 \times 10^{-6}$  M) significantly decreased stroke volume and pulse pressure, in comparison with the corresponding levels in controls (Figures 2(b)(A) and 2(b)(B)). However, subsequent treatment with ELA ( $5 \times 10^{-4}$  g/mL) reverted the changes in atrial stroke volume and pulse pressure and increased the values to levels much higher than basal levels (Figures 2(b)(A) and 2(b)(B)). Staurosporine did not affect cAMP efflux in beating atria. However, ELA substantially increased cAMP efflux in the staurosporine-pretreated atrium (Figure 2(b)(C)).

In addition, staurosporine had no effect on ANP secretion in beating atria. However, ELA markedly decreased ANP secretion in the staurosporine-pretreated atrium (Figure 2(b)(D)).

**3.3. Effect of Diltiazem on ELA-Induced Changes.** To investigate whether  $\text{Ca}^{2+}$  channels are involved in the ELA-induced positive inotropic activity, diltiazem, an L-type  $\text{Ca}^{2+}$  channel blocker, was used to pretreat beating atria. Treatment of beating atrium with diltiazem ( $5 \times 10^{-6}$  M) markedly decreased stroke volume and pulse pressure (Figures 3(a)(A) and 3(a)(B)). However, the diltiazem-induced reductions in atrial stroke volume and pulse pressure were reverted to levels greater than the basal levels by subsequent treatment with ELA ( $5 \times 10^{-4}$  g/mL) (Figures 3(a)(A) and 3(a)(B)). As shown in Figure 3(a)(C), cAMP efflux level was not altered by treatment with diltiazem but increased by perfusion with ELA after the pretreatment with diltiazem (Figure 3(a)(C)). Diltiazem had no effect on ANP secretion in beating atria. However, ELA markedly decreased ANP secretion in the diltiazem-pretreated atrium (Figure 3(a)(D)).

**3.4. Effect of Verapamil on ELA-Induced Changes.** To confirm that L-type  $\text{Ca}^{2+}$  channels are involved in the ELA-induced positive inotropic effect, verapamil, another L-type  $\text{Ca}^{2+}$  channel blocker, was also tested. Treatment with

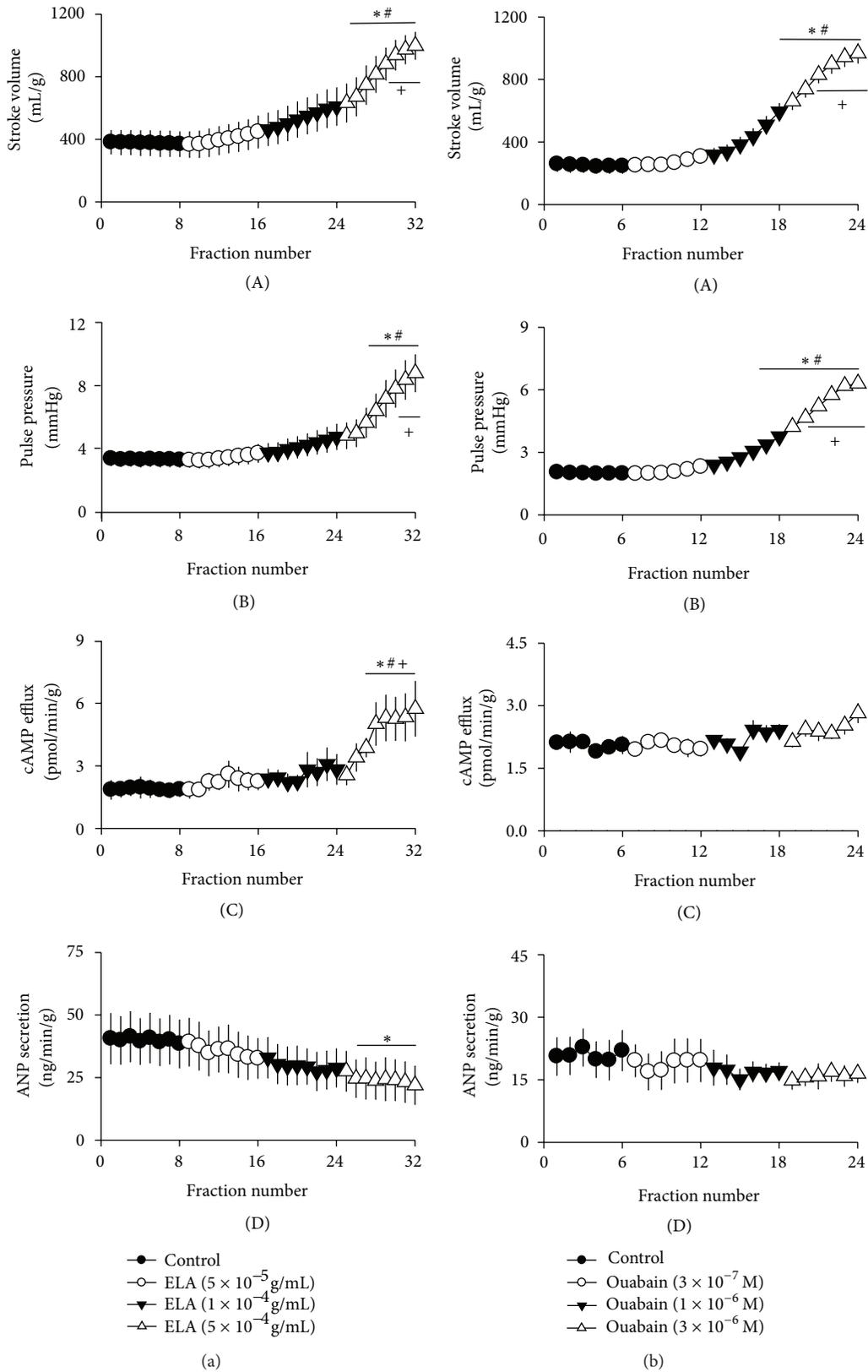


FIGURE 1: Dose-response curves of ELA (a) and ouabain (b) for stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values shown are mean  $\pm$  SE ( $n = 4$ );  $^+P < 0.05$  versus control;  $^{**}P < 0.01$  versus ELA ( $5 \times 10^{-5}$  g/mL) or ouabain ( $3 \times 10^{-7}$  M);  $^{###}P < 0.001$  versus ELA ( $1 \times 10^{-4}$  g/mL) or ouabain ( $1 \times 10^{-6}$  M) (compared with values for the last 3 fractions of control).

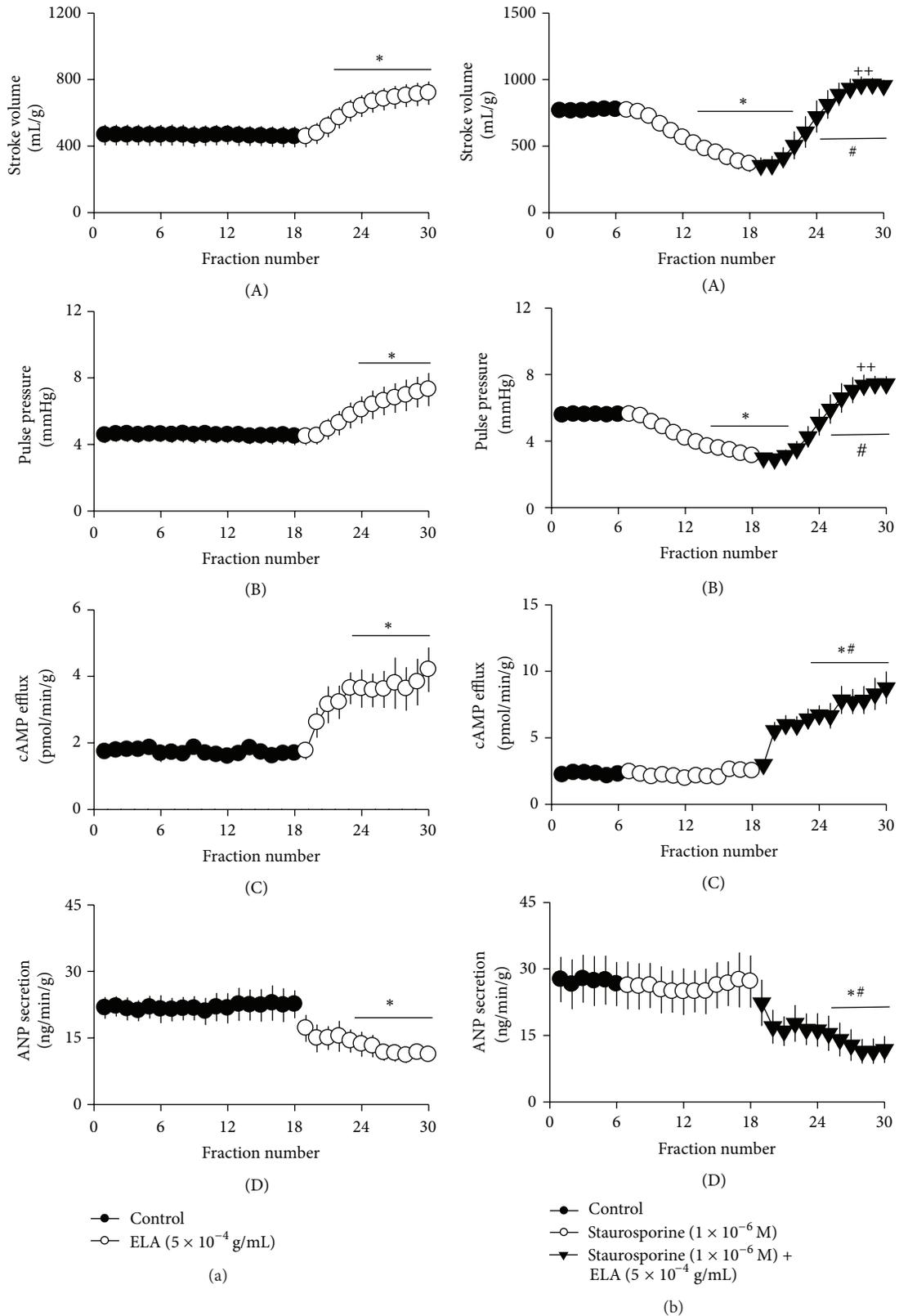


FIGURE 2: Effects of ELA (a) and staurosporine (b) on ELA-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria (1.3 Hz). Values shown are mean  $\pm$  SE ( $n = 4$ ); \*\*\* $P < 0.001$  versus control; ### $P < 0.001$  versus staurosporine (compared with values for the last 3 fractions of control or staurosporine).

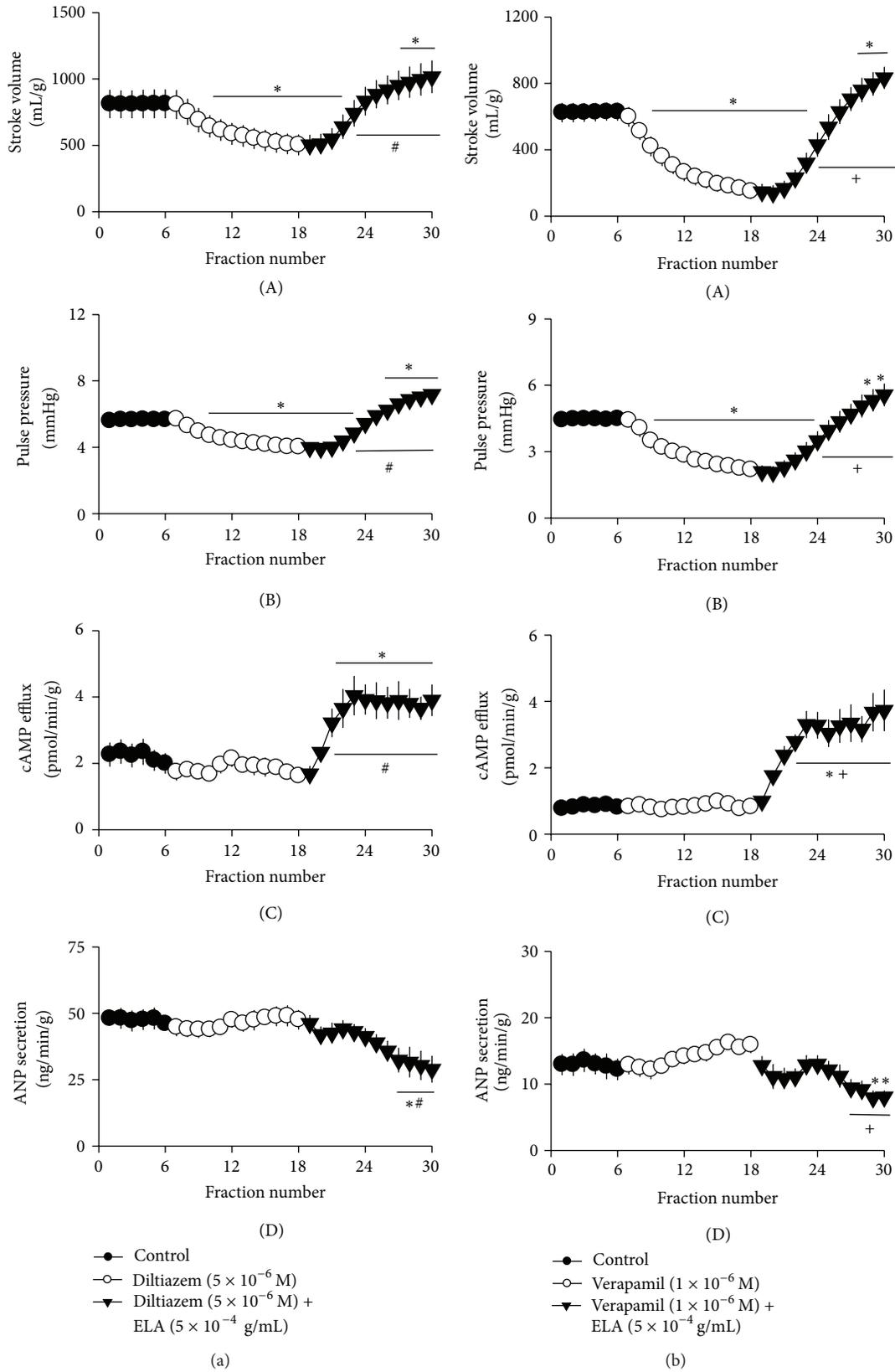


FIGURE 3: Effects of diltiazem (a) and verapamil (b) on ELA-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values shown are mean  $\pm$  SE ( $n = 4$ ); \*\*\* $P < 0.001$  versus control; ### $P < 0.001$  versus diltiazem or verapamil (compared with values for the last 3 fractions of control, diltiazem, or verapamil).

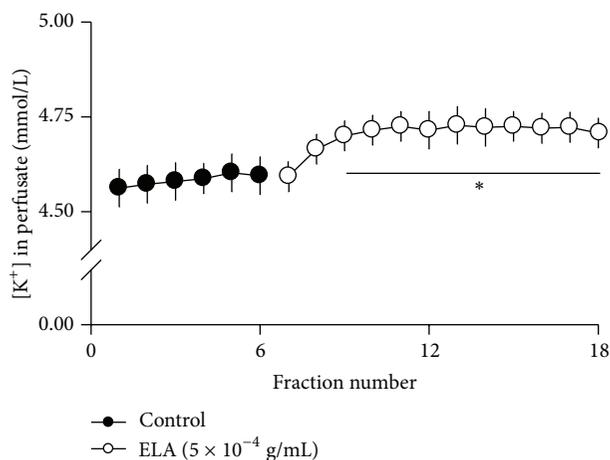


FIGURE 4: Change in ELA-induced  $K^+$  concentration in beating atria-derived perfusate. Values shown are mean  $\pm$  SE ( $n = 4$ ); \*\*\* $P < 0.001$ , versus control (compared with values for the last 3 fractions of control).

verapamil ( $1 \times 10^{-6}$  M) markedly decreased stroke volume and pulse pressure in beating rabbit atria (Figures 3(b)(A) and 3(b)(B)). However, subsequent treatment with ELA ( $5 \times 10^{-4}$  g/mL) reverted the verapamil-induced decreases in atrial stroke volume and pulse to values higher than basal levels (Figures 3(b)(A) and 3(b)(B)). The cAMP efflux level was not altered by treatment with verapamil but was increased by perfusion with ELA after the verapamil treatment (Figure 3(b)(C)). ELA also decreased ANP secretion in the verapamil-pretreated atrium (Figure 3(b)(D)).

**3.5. Change in the ELA-Induced  $K^+$  Concentration in Beating Atria-Derived Perfusate.** The  $K^+$  concentration in the beating atria-derived perfusate was examined to evaluate whether  $Na^+/K^+$ -ATPase is involved in the ELA-induced positive inotropic activity. As shown in Figure 4, the  $K^+$  concentration in the beating atria-derived perfusate was markedly increased by perfusion with ELA.

**3.6. Effect of Helveticoside on Atrial Stroke Volume, Pulse Pressure, cAMP Efflux, and ANP Secretion.** To determine whether the ELA-induced positive inotropic effects are due to helveticoside, which is a digitalis-like cardiac glycoside constituent of *Lepidium apetalum*, the effects of helveticoside on beating rabbit atria were determined. Similar to the ELA-induced pattern, helveticoside ( $2 \times 10^{-5}$  M) significantly increased stroke volume and pulse pressure (Figures 5(a)(A) and 5(a)(B)). However, there were no changes in cAMP efflux and ANP secretion after treatment with helveticoside (Figures 5(a)(C) and 5(a)(D)). The data were expressed  $\Delta\%$  changes of the mean values of fraction number 29/30 over the values of fraction number 17/18 (Figure 7).

**3.7. Effect of Staurosporine on Helveticoside-Induced Changes.** To define the roles of protein kinases in the helveticoside-induced positive inotropic activity, beating atria were treated

with staurosporine. The atrial stroke volume and pulse pressure after treatment of beating atrium with staurosporine ( $1 \times 10^{-6}$  M) were significantly lower than those in the controls (Figures 5(b)(A) and 5(b)(B)). When staurosporine-pretreated beating atria were treated with helveticoside ( $2 \times 10^{-5}$  M), atrial stroke volume and pulse pressure reverted to levels higher than those observed after the staurosporine treatment alone (Figures 5(b)(A) and 5(b)(B)). Staurosporine had no effect on cAMP efflux and ANP secretion in beating atria. Treatment of helveticoside with staurosporine also caused no changes in cAMP efflux and ANP secretion in beating atria (Figures 5(b)(C) and 5(b)(D)). The data were expressed  $\Delta\%$  changes of the mean values of fraction number 29/30 over the values of fraction number 17/18 (Figure 7).

**3.8. Effect of  $Ca^{2+}$  Channel Blockers on Helveticoside-Induced Changes.** To investigate whether  $Ca^{2+}$  channels are involved in the helveticoside-induced positive inotropic activity, beating rabbit atria were pretreated with diltiazem or verapamil. Treatment of beating atrium with diltiazem ( $5 \times 10^{-6}$  M) markedly decreased stroke volume and pulse pressure (Figures 6(a)(A) and 6(a)(B)). However, the diltiazem-induced reductions in atrial stroke volume and pulse pressure were significantly reverted by subsequent perfusion with helveticoside (Figures 6(a)(A) and 6(a)(B)). Verapamil ( $1 \times 10^{-6}$  M) also markedly decreased stroke volume and pulse pressure (Figures 6(b)(A) and 6(b)(B)), which recovered upon subsequent treatment with helveticoside ( $2 \times 10^{-5}$  M) (Figures 6(b)(A) and 6(b)(B)). Diltiazem and verapamil had no effect on cAMP efflux and ANP secretion in beating atria. Treatment of helveticoside with diltiazem or verapamil also caused no changes in cAMP efflux and ANP secretion in beating atria (Figures 6(a)(C) and 6(a)(D), 6(b)(C), and 6(b)(D)). The data were expressed  $\Delta\%$  changes of the mean values of fraction number 29/30 over the values of fraction number 17/18 (Figure 7).

## 4. Discussion

This study clearly shows that ELA increases stroke volume, pulse pressure, and cAMP efflux in beating rabbit atria. Because the cAMP-signaling pathway modulates the activation of L-type  $Ca^{2+}$  channels and PKs, leading to accentuation of cardiac contractility in beating atria [9, 16–18], it was expected that cAMP would be involved in the ELA-induced increase in atrial dynamics via the L-type  $Ca^{2+}$  channels and/or PKs. However, our results showed that blocking of L-type  $Ca^{2+}$  channels with diltiazem or verapamil had no effect on the ELA-induced increases in stroke volume, pulse pressure, and cAMP efflux in beating rabbit atria. Similarly, inhibition of PKs with staurosporine did not affect the ELA-induced increases in stroke volume, pulse pressure, and cAMP efflux. It has been reported that *Convallaria keiskei*, which contains the cardiac glycoside-like molecule convallatoxin, increases stroke volume and pulse pressure without an associated increase in cAMP efflux in the perfusate [19]. These results suggest that ELA-induced positive inotropic activity is

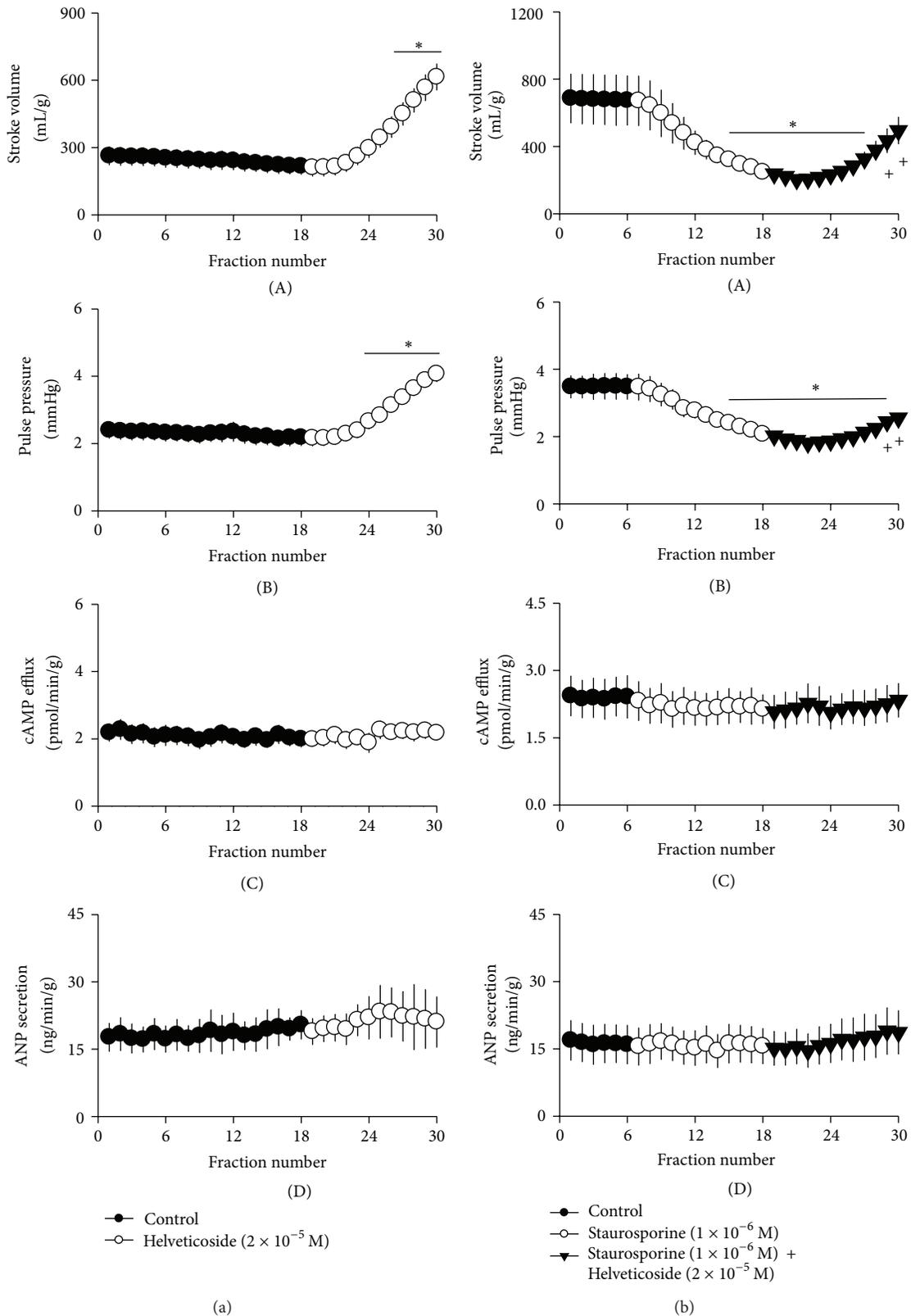


FIGURE 5: Effects of helveticoside (a) and staurosporine (b) on helveticoside-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values shown are mean  $\pm$  SE ( $n = 4$ ); \*\* $P < 0.01$ , \*\*\* $P < 0.01$  versus control; \*\*\* $P < 0.001$  versus staurosporine (compared with values for the last 3 fractions of control or staurosporine).

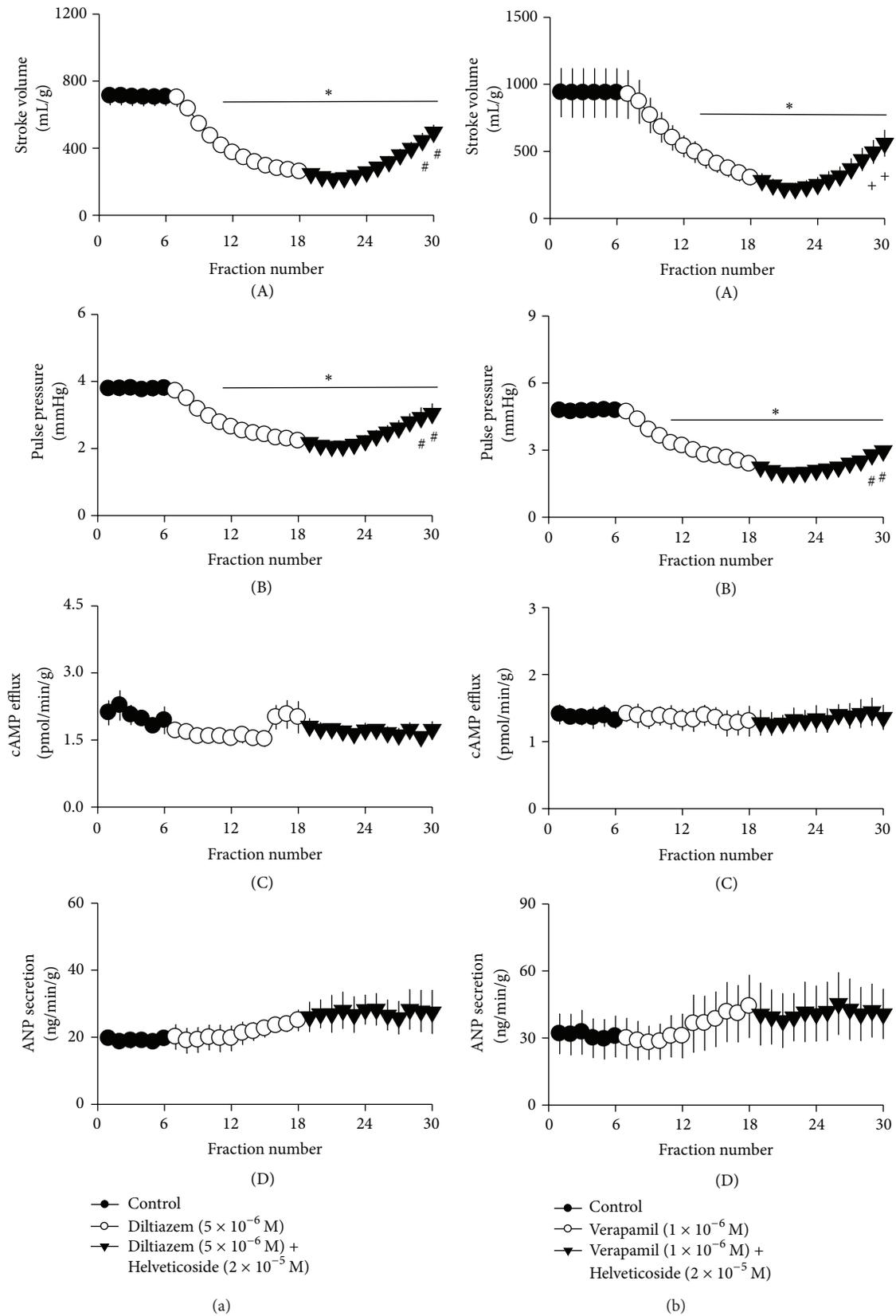


FIGURE 6: Effects of diltiazem (a) and verapamil (b) on helveticoside-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values are mean  $\pm$  SE ( $n = 4$ ); \*\*\* $P < 0.001$  versus control; ### $P < 0.001$  versus diltiazem, or verapamil (compared with values of the last 3 fractions of control, diltiazem, or verapamil).

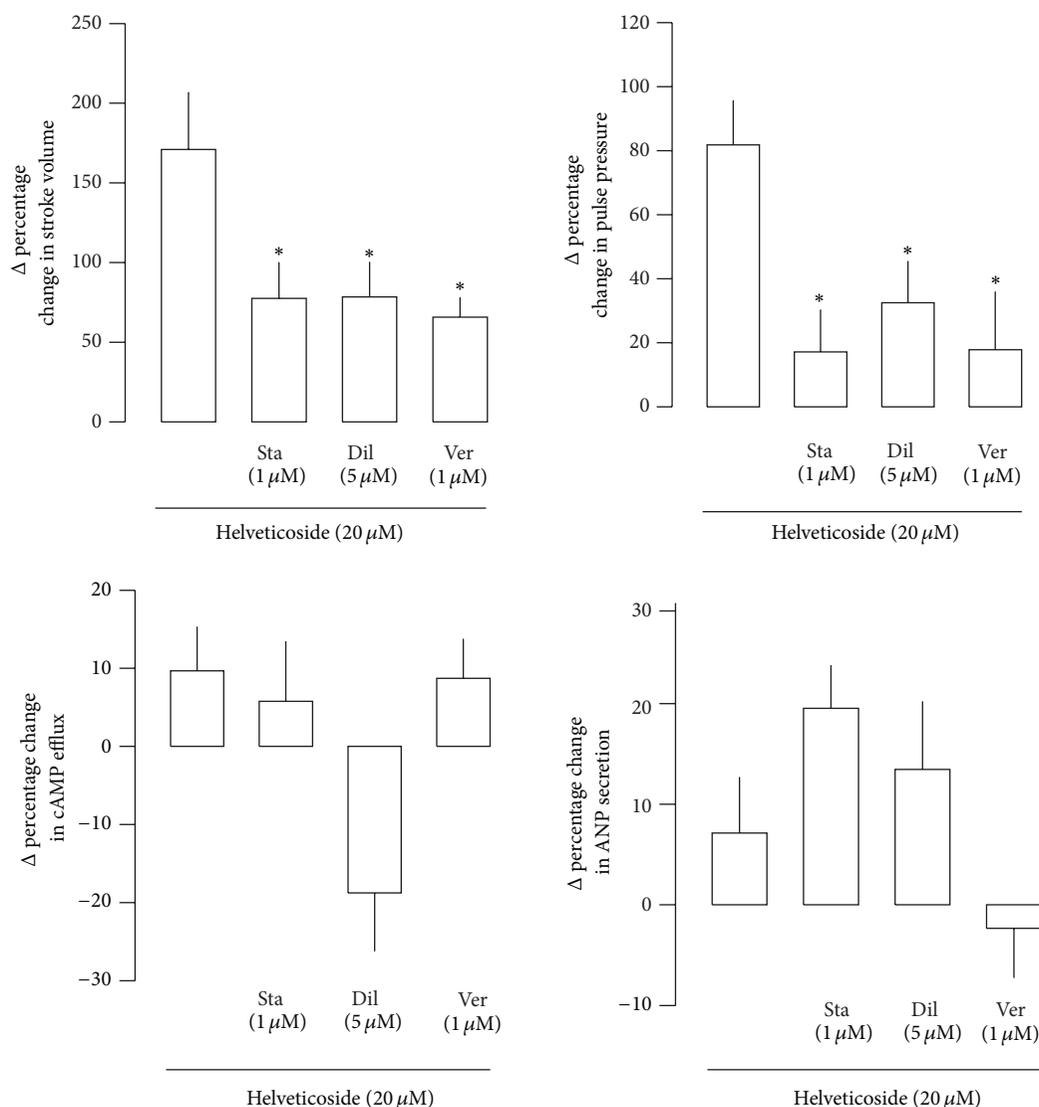


FIGURE 7: Effects of modulators on helveticoside-induced  $\Delta\%$  changes in pulse pressure, stroke volume, cAMP efflux, and ANP secretion. Data were derived from Figures 5 and 6. The values are  $\Delta\%$  changes of mean values of fraction number 29/30 over mean values of fraction number 17/18. Values are means  $\pm$  SE. \* $P < 0.05$  versus the mean values of two fractions before angles are changed (in modulators: staurosporine, diltiazem, and verapamil).

not caused by the pathway mediated by L-type  $\text{Ca}^{2+}$  channels and/or protein kinases.

Stimulation of  $\beta$ -adrenergic receptors with a sympathomimetic agent induces positive inotropic effects that are dependent on PKs and L-type  $\text{Ca}^{2+}$  channels [2, 3]. Stimulation of  $\beta$ -adrenergic receptors results primarily in an increase in cAMP production and consequent activation of PKs and phosphorylation of L-type  $\text{Ca}^{2+}$  channels, thereby further increasing the channel open time and/or the probability of opening of functional  $\text{Ca}^{2+}$  channels [20]. Increase in the cAMP level by PDE III inhibitors also accentuates cardiac contractility via activation of PKs and L-type  $\text{Ca}^{2+}$  channels [4]. In accordance with our hypothesis, the ELA-induced positive inotropic effect was not altered by pretreatment with L-type  $\text{Ca}^{2+}$  channel blockers and a protein kinase inhibitor.

These findings suggest that the activities of sympathomimetics and PDE III inhibitors could be excluded from the possible mechanism of the ELA-induced positive inotropic effect.

We also determined the effects of helveticoside on atrial dynamics, cAMP efflux, and ANP secretion in beating rabbit atria. Helveticoside, the main constituent of ELA, is a well-known digitalis-like compound. Similar to digitalis, helveticoside markedly increased the pulse pressure and stroke volume, without increasing cAMP efflux, in beating rabbit atria. Helveticoside also increased pulse pressure and stroke volume in the staurosporine-pretreated atria. Furthermore, helveticoside induced positive inotropic activity in diltiazem- and verapamil-pretreated atria. In cAMP and ANP regulation, we cannot rule out other component's possibility from ELA except for helveticoside. Thus, further study is needed to

clarify the effect of linoleic acid or olein on the cAMP efflux and ANP secretion.

A previous report suggested that helveticoside could inhibit  $\text{Na}^+/\text{K}^+$ -ATPase activity in an *in vitro* enzyme assay [21]. In this study, we show that treatment with ELA markedly increased  $\text{K}^+$  concentration in beating atria-derived perfusate. Many lines of evidence have demonstrated that digitalis-like cardiac glycosides increase cardiac contractility by elevating intracellular  $\text{Ca}^{2+}$  concentration via  $\text{Na}^+/\text{K}^+$ -ATPase inhibition-mediated activation of  $\text{Na}^+/\text{Ca}^{2+}$  exchanger [6, 22, 23]. In this case, the  $\text{K}^+$  efflux would be increased because  $\text{K}^+$  influx is inhibited due to the inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase in the myocardium. In our study, ouabain markedly increased the pulse pressure and stroke volume without increasing the cAMP efflux in beating rabbit atria, resulting in a positive inotropic effect via inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase activity. These findings suggest that the digitalis-like activity of helveticoside might be associated, at least in part, with ELA-induced positive inotropic activity.

The heart is also an endocrine gland, secreting ANP, which is involved in the regulation of body fluid and blood pressure [11, 24]. The present study shows that ELA significantly decreased ANP secretion in beating rabbit atria. Diltiazem or verapamil, but not staurosporine, slightly increased ANP secretion in beating rabbit atria. The ELA-induced reduction in ANP secretion was not affected by pretreatment with diltiazem, verapamil, or staurosporine. The potential roles of cyclic nucleotide and  $\text{Ca}^{2+}$  in the regulation of ANP release have been the subject of interest of many studies. cGMP and  $\text{Ca}^{2+}$  inhibit ANP secretion in perfused atria [25]. On the other hand, cAMP increases the ANP secretion in rat cardiomyocytes [26], isolated atrium [27], and perfused rat atria [28]. However, there are diverse reports on the effects of cAMP in the regulation of ANP secretion. Forskolin, an adenylyl cyclase activator, has been shown to decrease ANP secretion from cultured atrial myocytes [29, 30] and in perfuse beating rat hearts [31]. Likewise, 3-isobutyl-1-methylxanthine (IBMX) and 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP), a nonselective PDE inhibitor and a cAMP agonist, respectively, inhibit ANP secretion [30, 31]. Collectively, ELA significantly decreased the ANP secretion associated with increase in cAMP efflux in beating rabbit atria, consistent with other reports [26, 30, 31].

Taken together, the present study suggests that the ELA-induced positive inotropic activity may, at least in part, be due to inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase activity by helveticoside-like cardiac glycosides.

## Conflict of Interests

The authors report that they have no conflict of interests.

## Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (2008-0062484).

## References

- [1] E. Lonn and R. McKelvie, "Drug treatment in heart failure," *British Medical Journal*, vol. 320, no. 7243, pp. 1188–1192, 2000.
- [2] A. M. Katz, "Potential deleterious effects of inotropic agents in the therapy of chronic heart failure," *Circulation*, vol. 73, no. 3, pp. I-184–I-190, 1986.
- [3] J. A. Simaan, G. Fawaz, and K. Jabbour, "Comparison of the cardiodynamic and metabolic effects of dobutamine with those of norepinephrine and dopamine in the dog isolated heart," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 338, no. 2, pp. 174–179, 1988.
- [4] S. T. Rapundalo, R. J. Solaro, and E. G. Kranias, "Inotropic responses to isoproterenol and phosphodiesterase inhibitors in intact guinea pig hearts: comparison of cyclic AMP levels and phosphorylation of sarcoplasmic reticulum and myofibrillar proteins," *Circulation Research*, vol. 64, no. 1, pp. 104–111, 1989.
- [5] B. G. Katzung and W. W. Parmley, *Cardiac Glycosides & Other Drugs Used in Congestive Heart Failure*, McGraw-Hill, New York, NY, USA, 8th edition, 2001.
- [6] M. A. Konstam and D. L. Mann, "Contemporary medical options for treating patients with heart failure," *Circulation*, vol. 105, no. 19, pp. 2244–2246, 2002.
- [7] M. E. Diaz, H. K. Graham, S. C. O'Neill, A. W. Trafford, and D. A. Eisner, "The control of sarcoplasmic reticulum  $\text{Ca}^{2+}$  concentration in cardiac muscle," *Cell Calcium*, vol. 38, pp. 391–396, 2005.
- [8] G. Hasenfuss and B. Pieske, "Calcium cycling in congestive heart failure," *Journal of Molecular and Cellular Cardiology*, vol. 34, no. 8, pp. 951–969, 2002.
- [9] A. L. Bauman and J. D. Scott, "Kinase- and phosphatase-anchoring proteins: harnessing the dynamic duo," *Nature Cell Biology*, vol. 4, no. 8, pp. E203–E206, 2002.
- [10] A. J. De Bold, "Atrial natriuretic factor: a hormone produced by the heart," *Science*, vol. 230, no. 4727, pp. 767–770, 1985.
- [11] S. A. Atlas and J. H. Laragh, "Atrial natriuretic peptide: a new factor in hormonal control of blood pressure and electrolyte homeostasis," *Annual Review of Medicine*, vol. 37, pp. 397–414, 1986.
- [12] B. S. Jeung and M. K. Shin, *Dohae Hyangyak-Daesajeon, Younglimsa*, Seoul, Korea, 1st edition, 1990.
- [13] K. W. Cho, S. H. Kim, C. H. Kim, and K. H. Seul, "Mechanical basis of ANP secretion in beating atria: atrial stroke volume and ECF translocation," *American Journal of Physiology*, vol. 268, pp. 1129–1136, 1995.
- [14] X. Cui, S. J. Lee, S. Z. Kim, S. H. Kim, and K. W. Cho, "Effects of pituitary adenylyl cyclase activating polypeptide27 on cyclic AMP efflux and atrial dynamics in perfused beating atria," *European Journal of Pharmacology*, vol. 402, no. 1-2, pp. 129–137, 2000.
- [15] A. L. Steiner, C. W. Parker, and D. M. Kipnis, "Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides," *The Journal of Biological Chemistry*, vol. 247, no. 4, pp. 1106–1113, 1972.
- [16] M. A. Fink, D. R. Zakhary, J. A. Mackey et al., "AKAP-mediated targeting of protein kinase A regulates contractility in cardiac myocytes," *Circulation Research*, vol. 88, no. 3, pp. 291–297, 2001.
- [17] E. G. Kranias and R. J. Solaro, "Phosphorylation of troponin I and phospholamban during catecholamine stimulation of rabbit heart," *Nature*, vol. 298, no. 5870, pp. 182–184, 1982.

- [18] R. J. Lefkowitz, H. A. Rockman, and W. J. Koch, "Catecholamines, cardiac  $\beta$ -adrenergic receptors, and heart failure," *Circulation*, vol. 101, no. 14, pp. 1634–1637, 2000.
- [19] H. Choi, S. Ahn, B. G. Lee, I. Chang, and J. S. Hwang, "Inhibition of skin pigmentation by an extract of *Lepidium apetalum* and its possible implication in IL-6 mediated signaling," *Pigment Cell Research*, vol. 18, no. 6, pp. 439–446, 2005.
- [20] I. Verde, G. Vandecasteele, F. Lezoualc'h, and R. Fischmeister, "Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type  $\text{Ca}^{2+}$  current in rat ventricular myocytes," *British Journal of Pharmacology*, vol. 127, no. 1, pp. 65–74, 1999.
- [21] A. Babulova, L. Buran, and F. V. Selecky, "The cardiotoxic activity of helveticoside, a cardiac glycoside from *Erysimum canescens* Roth," *Arzneimittel-Forschung*, vol. 13, pp. 412–414, 1963.
- [22] T. W. Smith, E. M. Antman, and P. L. Friedman, "Digitalis glycosides: mechanisms and manifestations of toxicity. Part II," *Progress in Cardiovascular Diseases*, vol. 26, no. 6, pp. 495–540, 1984.
- [23] H. Reuter, S. A. Henderson, T. Han, R. S. Ross, J. I. Goldhaber, and K. D. Philipson, "The  $\text{Na}^+/\text{Ca}^{2+}$  exchanger is essential for the action of cardiac glycosides," *Journal of Cardiovascular Electrophysiology*, vol. 12, pp. 1295–1301, 2001.
- [24] M. Cantin and J. Genest, "The heart and the atrial natriuretic factor," *Endocrine Reviews*, vol. 6, no. 2, pp. 107–127, 1985.
- [25] S. J. Lee, S. Z. Kim, X. Cui et al., "C-type natriuretic peptide inhibits ANP secretion and atrial dynamics in perfused atria: NPR-B-cGMP signaling," *American Journal of Physiology*, vol. 278, no. 1, pp. H208–H221, 2000.
- [26] D. J. Church, V. van der Bent, M. B. Vallotton, A. M. Capponi, and U. Lang, "Calcium influx in platelet activating factor-induced atrial natriuretic peptide release in rat cardiomyocytes," *American Journal of Physiology*, vol. 266, no. 3, pp. E403–E409, 1994.
- [27] R. L. Schiebinger, "Mechanism of inhibition by methacholine of norepinephrine-stimulated ANP secretion," *American Journal of Physiology*, vol. 255, pp. 1429–1433, 1988.
- [28] C. Azizi, C. Barthelemy, F. Masson, G. Maistre, J. Eurin, and A. Carayon, "Myocardial production of prostaglandins: its role in atrial natriuretic peptide release," *European Journal of Endocrinology*, vol. 133, no. 2, pp. 255–259, 1995.
- [29] K. B. Seamon, W. Padgett, and J. W. Daly, "Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 6, pp. 3363–3367, 1981.
- [30] H. Iida and E. Page, "Inhibition of atrial natriuretic peptide secretion by forskolin in noncontracting cultured atrial myocytes," *Biochemical and Biophysical Research Communications*, vol. 157, no. 1, pp. 330–336, 1988.
- [31] H. Ruskoaho, O. Vuolteenaho, and J. Leppaluoto, "Phorbol esterase enhance stretch-induced atrial natriuretic peptide secretion," *Endocrinology*, vol. 127, no. 5, pp. 2445–2455, 1990.

## Research Article

# Effectiveness of *Panax ginseng* on Acute Myocardial Ischemia Reperfusion Injury Was Abolished by Flutamide via Endogenous Testosterone-Mediated Akt Pathway

Luo Pei,<sup>1</sup> Hou Shaozhen,<sup>2</sup> Dong Gengting,<sup>1</sup> Chen Tingbo,<sup>1</sup> Liu Liang,<sup>1</sup> and Zhou Hua<sup>1</sup>

<sup>1</sup> State Key Laboratory for Quality Research of Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau, China

<sup>2</sup> School of Chinese Pharmaceutical Science, Guangzhou University of Chinese Medicine, University Town, Guangzhou 510006, China

Correspondence should be addressed to Zhou Hua; [huazhou2009@gmail.com](mailto:huazhou2009@gmail.com)

Received 19 March 2013; Accepted 9 September 2013

Academic Editor: Keji Chen

Copyright © 2013 Luo Pei et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Mechanisms for *Panax ginseng*'s cardioprotective effect against ischemia reperfusion injury involve the estrogen-mediated pathway, but little is known about the role of androgen. A standardized *Panax ginseng* extract (RSE) was orally given with or without flutamide in a left anterior descending coronary artery ligation rat model. Infarct size, CK and LDH activities were measured. Time-related changes of NO, PI3K/Akt/eNOS signaling, and testosterone concentration were also investigated. RSE (80 mg/kg) significantly inhibited myocardial infarction and CK and LDH activities, while coadministration of flutamide abolished this effect of RSE. NO was increased by RSE and reached a peak after 15 min of ischemia; however, flutamide cotreatment suppressed this elevation. Western blot analysis showed that RSE significantly reversed the decreases of expression and activation of PI3K, Akt, and eNOS evoked by ischemia, whereas flutamide attenuated the effects of these protective mechanisms induced by RSE. RSE completely reversed the dropping of endogenous testosterone level induced by I/R injury. Flutamide plus RSE treatment not only abolished RSE's effect but also produced a dramatic change on endogenous testosterone level after pretreatment and ischemia. Our results for the first time indicate that blocking androgen receptor abolishes the ability of *Panax ginseng* to protect the heart from myocardial I/R injury.

## 1. Introduction

Ischemic heart disease (IHD) is one of the most common cardiovascular diseases, and it is often caused by occlusion and reperfusion of the coronary artery. Present studies have demonstrated that the prevention of damage induced by myocardial ischemia reperfusion (I/R) injury is the key to successful therapy for IHD [1]. In the past few years, gender disparity in myocardial response to acute I/R injury has been reported, and the difference is considered to be hormone(s) mediated [2]. Recent studies have demonstrated that estradiol and its metabolites are the major biologically active players in this action, and they protect the heart and blood vessels in multiple ways [3]. In contrast to estrogen, androgen's role in cardiac disease remains unclear [4, 5]. The relationship between androgens and cardiovascular disease is controversial due to numerous conflicting clinical and epidemiological

studies [6]. For example, androgen deprivation in prostate cancer therapy leads to higher mortality due to cardiovascular failure [7]. Administration of testosterone (a primary and most well-known androgen) can reduce myocardial ischemia in patients with coronary artery disease [8]. And low endogenous testosterone levels have been correlated with several risk factors for increased myocardial infarction [9]. It is therefore believed that androgen plays beneficial effects in cardiovascular diseases. However, testosterone had also been suggested to exhibit fewer antioxidant effects in cardiac muscle and to worsen cardiac function in mice suffering from myocardial infarction [10]. Zaugg et al. also carried out exogenous androgen supplementation in adult myocytes and demonstrated increase of apoptosis in rat ventricular cells [11]. This evidence nevertheless supports that androgen may have detrimental effects in cardiovascular diseases and have given rise to the popular belief that, after a myocardial

infarction, men are more likely than women to die on the way to the hospital [12]. Whether androgen supplement is beneficial or harmful is thus hard to determine. Consequently, more work is needed to elucidate the role of endogenous androgen in acute I/R injury which results in the most significant morbidity in cardiovascular diseases.

Ginseng is the root of *Panax ginseng* C.A. Meyer. It is now cultivated in China, Korea, Japan, and Russia. The name *Panax* means “all cure,” which describes the traditional conception that ginseng has power to heal all the disorders of the body [13]. Ginseng contains more than 30 types of ginsenosides, and it is these components to which most of the pharmacological actions of ginseng can be attributed. Recently, ginseng-derived herbal medicines and supplements have been studied for their ability to protect against myocardial I/R injury in various animal species and human objects. Studies by us and others suggest that these pharmaceutical effects are probably mediated by sex hormones and their related receptors, such as the glucocorticoid receptor (GR) or estrogen receptor (ER) [14]. While female hormones have been studied, male hormones have not, even though there is evidence for the role of androgen in the cardioprotective effect of ginseng. In this report, we investigated the role of endogenous androgen on the cardioprotective effect of RSE, a standardized ginseng extract, in an open-chest left anterior descending (LAD) coronary artery ligation rat model. Flutamide, an oral nonsteroidal antiandrogen drug by competing testosterone and its powerful metabolite with androgen receptors, was employed to show the potential role of androgen receptor in the cardioprotective effect of ginseng.

## 2. Materials and Methods

**2.1. Animals.** Male Sprague-Dawley rats weighing 280–340 g were purchased from the Laboratory Animal Services Center, the Chinese University of Hong Kong, Hong Kong. The animals were acclimated for 7 days under a cycle of 12 hours light and 12 hours dark at room temperature of  $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Chow diet and water were provided *ad libitum*. Animal care and treatment procedures were in accordance with the Institutional Guidelines and Animal Ordinance (Department of Health, Hong Kong Special Administrative Region).

**2.2. Materials.** Flutamide (F9397) was purchased from Sigma (St. Louis, MO, USA). 2,3,5-Triphenyltetrazolium chloride (1612634) was from International laboratory USA (SAN BRUNO, CA, USA). Assay kits of lactate dehydrogenase (LDH) and creatine kinase (CK-MB) were obtained from STANBIO (Texas, USA). Nitrate/Nitrite assay kit was purchased from Cayman Chemical (Michigan, USA). The root of *P. ginseng* C.A. Meyer was purchased in a wholesale market in Tonghua county of Jilin Province, China, and authenticated by Professor Ping Ding (an herbalist of Guangzhou University of Chinese Medicine) with quality conforming to the requirements of the Chinese Pharmacopoeia (CP) and Hong Kong Standard of Chinese Materia Medica (HKSCMM). The sample was stored in a desiccated condition in the laboratory until use. Voucher specimens in the form of the whole root were deposited in the State Key Laboratory for Quality

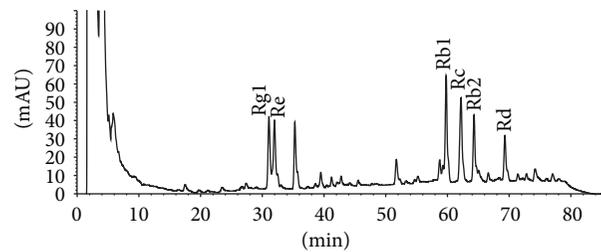


FIGURE 1: Chromatographic fingerprint of standardized ginseng extract (RSE).

Research of Chinese Medicine, Macau University of Science and Technology.

**2.3. Preparation of RSE.** The standardized ginseng extract RSE was prepared by ethanol extraction, a well-established and generally accepted method for preparing ginseng extract. The extraction parameters were optimized in our experiments to obtain a good recovery of major ginsenosides with consistent quality. In brief, the root was refluxed with 5 volumes (versus ginseng weight) of 70% ethanol 3 hr three times. The ethanol extracts were pooled and concentrated at  $60^{\circ}\text{C}$  under vacuum evaporation (0.08 MPa) to half the original volume. The concentrate was finally freeze-dried to powder. The extraction rate was 28%, meaning 1 kg ginseng produced 280 g RSE. To examine the chemical consistency of RSE, the chemical fingerprint (Figure 1) of RSE was established on a Phenomenex ODS column (250 mm  $\times$  4.6 mm i.d.; particle size 5  $\mu\text{m}$ ; Phenomenex Inc., USA) protected by a Security Guard Cartridge (C18, 4 mm  $\times$  3.0 mm i.d.; Phenomenex Inc., USA) by using high performance liquid chromatography (1100, Agilent Technologies, CA, USA) equipped with a G1312A binary pump, G1379A degasser, G1315B diode-array detector, and G1313A autosampler. The mobile phase was acetonitrile (A) and water (B), and the separation was conducted in a gradient manner, in which the ratio of A was 19%, 19%, 29%, 29%, and 40% at 0, 35, 55, 70, and 100 min, respectively. The flow rate was 1.0 mL/min. Detection was conducted at 203 nm. The HPLC fingerprint of RSE is shown in Figure 1. The contents of Rg1, Re, Rb1, Rc, Rb2, and Rd in RSE were 7.63, 6.90, 12.21, 10.65, 7.24, and 4.59 mg/g, respectively.

**2.4. Experimental Protocol.** Rats were randomly assigned to four experimental groups (shown in Figure 2), that is, Sham group (given vehicle by oral gavage at 60 min before sham surgery without I/R,  $n = 24$ ), I/RI group (given saline by oral gavage at 60 min before I/R,  $n = 31$ ), RSE group (given 80 mg/kg of RSE by oral gavage at 60 min before I/R,  $n = 29$ ), and RSE + Flutamide group (given 80 mg/kg of RSE by oral gavage and 10 mg/kg of flutamide by subcutaneous injection at 60 min before I/R, resp.,  $n = 31$ ). Sham group rats were subjected to all the procedures except that the LAD ligation was not tightened. The vehicle or drugs were given 60 min before sham operation or I/R procedure, which was designated as time 0 min. In each group, 3–5 rats were sacrificed at the following time points: 30 min after drug treatment,

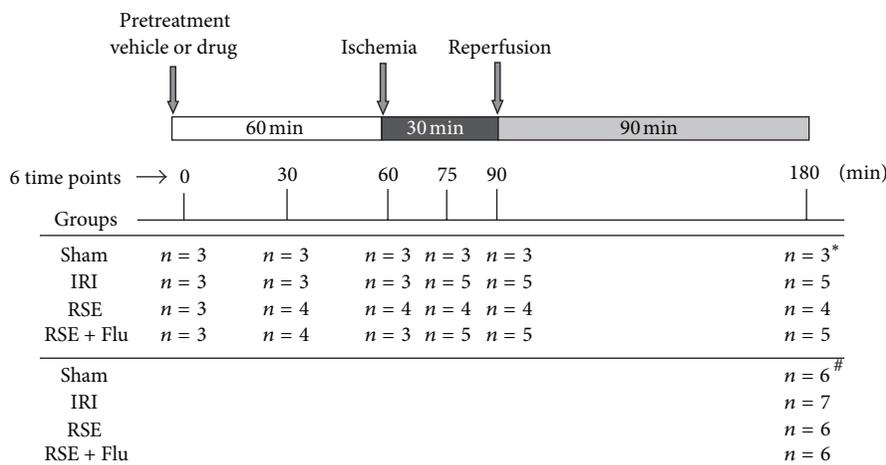


FIGURE 2: Outline of experimental protocol to evaluate the effects of flutamide on cardioprotection of RSE against myocardial I/R injury in rats. Sham: sham operation group; IRI: I/R injury group with vehicle treatment; RSE, I/R injury with RSE 80 mg/kg treatment; RSE + Flu: I/R injury group with RSE 80 mg/kg + flutamide 10 mg/kg treatment. 6 time points include 0 min, 30 min, and 60 min after pretreatment; 15 min after ischemia; 30 min after ischemia; 90 min after reperfusion. Heart tissue and blood sample were collected, respectively ( $n$ : number of rats at each time point). \*: heart and serum samples were collected for Western blot analysis, NO production and testosterone concentration assays. #: heart and serum samples were collected for TTC staining, CK and LDH activities determinations.

1 hr after drug treatment, 15 min after ischemia, 30 min after ischemia, and 90 min after reperfusion. At each time point, arterial blood samples were drawn from aorta immediately before animal sacrifice and then centrifuged at 1,000 g and 4°C for 15 min. All serum samples were stored at -80°C before the measurements of nitrate/nitrite, LDH, and CK. Heart was excised and weighed immediately. After collection of serum samples, 2 mL of 10% potassium chloride was injected via inferior vena cava to stop the heart in diastole, and the heart was excised for infarct size measurement by TTC staining or frozen in liquid nitrogen for Western blot analysis.

**2.5. Ischemia Reperfusion Protocol.** The ischemia reperfusion injury was produced in rat heart based on Burke's description with modifications [14]. In brief, rats were anesthetized with pentobarbital sodium (70 mg/kg body weight) by an i.p. injection of a mixture of 20% Dorminal (1 mL contains 200 mg pentobarbital sodium, Alfasan) and sterile saline at a ratio of 1:3 (v/v). Additional dose (40 mg/kg/h) was administered continuously via jugular vein by a microinjection pump at an interval of approximately 2 hr or at a rate as required to maintain anesthesia. After ensuring sufficient depth of anesthesia according to the sign of loss of palpebral reflex, the rats were placed on a warm board to control the body temperature at 37°C for surgery. The neck was opened with a ventral midline incision. The trachea was exposed and cannulated with a PE-90 catheter to establish an artificial respiration provided by a SAR-830/P ventilator (IITC, USA) with oxygen at a breath ratio of 1:1 and at a frequency of 70~80 breaths/min with tidal volume of 0.8~1.2 mL. The right carotid artery was isolated, and a Millar catheter (Millar Instruments, Inc., Houston, TX, USA) was inserted into the right carotid artery. Using a PowerLab (ADInstruments Pty Ltd., Castle Hill, Australia), mean aortic pressure (MAP) was recorded from the Millar catheter. Electrocardiogram (ECG) in lead

II was also recorded through the needle electrodes attached to the limbs. The heart rate and ST-segment elevation were calculated offline. The chest was opened at the left fourth intercostal space. The pericardium was incised, and the left atrium appendage was elevated to expose the LAD coronary artery. A 6-0 silk suture was passed around the LAD coronary artery, and the ends of the suture were threaded through a small vinyl tube to form a snare. The thoracic cavity was covered with saline-soaked gauze to prevent the heart from drying. At 1 hr after drug administration, ischemia was established by tightening the suture from both ends with fixed weight. The animals then underwent 30 min of ischemia, confirmed visually in situ by the appearance of regional epicardial cyanosis and ST-segment elevation. Reperfusion was introduced by releasing the snare gently for a period of 90 min. The sham control animals were subjected to the entire surgical procedures above except the introduction of LAD ligation and release.

**2.6. Infarct Size Measurement.** Measurement of heart infarct size was performed by TTC staining method as described previously [15]. Briefly, the left ventricle was cut perpendicular to the base-apex axis into six 2-3 mm slices. The slices were incubated in 1% TTC solution in PBS (pH 7.4) for 5 min at 37°C and then fixed in 10% formalin solution (pH 7.0) for 20~24 hr. TTC stains viable tissue a deep red color, and nonstained tissue is presumed to be infarcted. The images of the slice were captured by a LEICA digital camera 480, and infarct area in each slice was measured by computed planimetry with an image analyzing program ImageJ 1.26 (Wayne Rasband, National Institutes of Health, USA). Then the infarct area of each slice was determined by manual delineation of TTC-negative pale area of the image. The infarct weight of each slice was calculated by multiplying the ratio of infarct area within total area by the slice weight.

The infarct weight of each slice was summed to produce the total infarct weight of each left ventricle. Finally, the infarct size was calculated by dividing the total infarct weight of each left ventricle by the total weight of the ventricle [16].

**2.7. Measurement of Nitrate/Nitrite, LDH, and CK.** Total nitrate/nitrite concentrations of serum from six time points in I/R were measured in a simple two-step process by using a colorimetric assay. In brief, sera were ultrafiltered through a 30 KDa molecular weight cut-off filter using Amicon Ultra-4 Centrifugal Filter Units (Millipore, USA) that reduces background absorbance due to the presence of hemoglobin and improves color formation using the Griess reagents. The filtrates were then subjected to a nitrate/nitrite colorimetric assay (Cayman Chemical Company, USA) with a spectrophotometer (Bio-RAD, USA) at 540 nm according to the procedures recommended by the manufacturer. Serum activities of lactate dehydrogenase (LDH) and creatine kinase (CK-MB) were determined by using commercial assay kits according to manufacturer's recommendations.

**2.8. Western Blot Analysis.** Western blot analyses of total proteins and phosphorylated form of proteins in hearts were performed according to methods described previously with modifications. In brief, frozen heart tissue was homogenized using a homogenizer (IKA, Germany) in ice-cold lysis buffer (Sucrose 250 mM, Tris-HCl (pH 7.2) 50 mM, sodium EDTA 2 mM, beta-mercaptoethanol 2 mM, sodium fluoride 5 mM, sodium orthovanadate 1 mM, aprotinin 10  $\mu$ g/mL, leupeptin 10  $\mu$ g/mL) for 5 min. The homogenate was immediately centrifuged at 14,000 g for 20 min at 4°C; the supernatant was gently collected and stored at -80°C until use. The contents of total protein in the supernatants were determined using a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were boiled in sample loading buffer for 5 min, loaded on 10% SDS polyacrylamide gel, and finally transferred onto Immobilon-P membrane (Pore size: 0.45  $\mu$ m, Millipore, USA). The nonspecific binding sites on the membrane were blocked with 5% nonfat milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) at 4°C overnight. Then the membranes were incubated with specific primary antibodies at 18°C for 3 hours; the antibodies were anti-PI3 kinase p85, anti-phospho-PI3 kinase p85 (Tyr458), anti-Akt1/PKBa, anti-phospho-Akt1/PKBa (Ser473), anti-eNOS/NOSIII, anti-phospho-eNOS (Ser1177), and anti-GAPDH (Cell Signaling Technology or BD Transduction Laboratories, USA). Membranes were subsequently incubated with peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, USA) in 5% nonfat milk in TBST for 1 hr at room temperature. The membranes were then washed six times, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) method using hyperfilm and ECL reagent (Amersham, USA) according to the manufacturer's instructions. Band intensities were quantified using a densitometer analysis system (Quantity One software, Bio-Rad).

**2.9. Measurement of Testosterone in Serum.** Serum testosterone level was assayed using commercially available ELISA

kits according to the manufacturer's instructions (CALBIOTECH, INC., Austin Dr, Spring Valley, CA). One milliliter venous blood sample from each rat was drawn at four time points of 0, 60, 90, and 180 minutes. These blood samples were centrifuged at 1,000 g and 4°C for 20 minutes; serum stored was stored at -80°C for 2 weeks before assay.

**2.10. Statistical Analysis.** Data were expressed as mean  $\pm$  S.E.M. One-way ANOVA with multiple comparisons using the Student-Newman-Keuls test was used to analyze differences between groups. All statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Differences were considered significant if  $P < 0.05$ .

### 3. Results

**3.1. Myocardial Infarct Size after Myocardial I/R.** Occluding and releasing the LAD coronary artery of SD rats is a widely used model for antimyocardial I/R injury research. The severity of I/R injury can be assessed by measuring the size of infarct area (myocardial infarct size). As shown in Figure 3, the myocardial infarct size produced by 30 min ischemia and 90 min reperfusion in IRI group was  $10.5 \pm 2.20\%$ . Orally given 60 min before the acute ischemia, RSE at 80 mg/kg exhibited significant protection to rat hearts against I/R injury with an infarct size of  $0.3 \pm 0.32\%$  (compared with IRI group,  $P < 0.01$ ). Combined administration of RSE and flutamide (at 80 mg/kg and 10 mg/kg, resp.) did not induce any significant reduction in myocardial infarct size ( $10.7 \pm 3.56\%$ ,  $P < 0.05$  and  $P < 0.01$ , compared with IRI or RSE group, resp.). This clearly indicates that flutamide abolished the protective effect of RSE against I/R injury.

**3.2. Serum CK-MB and LDH Activities after Myocardial I/R.** Besides the evaluation of infarct size induced by I/R, we also measured myocardial enzymes in serum in order to further elucidate the effect of RSE with or without flutamide in rat open chest model. As shown in Figure 4, the activities of CK-MB and LDH in the serum of rats that underwent myocardial I/R (IRI group) were both significantly increased when compared with the sham group without I/R (CK-MB activity:  $1476 \pm 137$  U/L versus  $522 \pm 214$  U/L,  $P < 0.01$ ; LDH activity:  $435 \pm 85$  U/L versus  $67 \pm 16$  U/L,  $P < 0.01$ ). Compared with the IRI group, 80 mg/kg RSE treatment significantly inhibited the elevation of CK-MB ( $894 \pm 134$  U/L,  $P < 0.01$ ) or LDH ( $178 \pm 69$  U/L,  $P < 0.01$ ) activities induced by acute I/R injury. The joint use of RSE at 80 mg/kg and flutamide at 10 mg/kg did not significantly suppress the increase of serum CK-MB or LDH activity ( $1363 \pm 199$  U/L,  $378 \pm 45$  U/L, compared with IRI group  $P > 0.05$ ,  $P > 0.05$ , resp.). These results also revealed that flutamide significantly abolished the beneficial effect of RSE on the myocardial enzymes (compared with RSE group, CK-MB,  $P < 0.05$  and LDH,  $P < 0.05$ ) in I/R injury.

**3.3. Time Course Changes of Serum NO Production (Nitrate and Nitrite) during Pretreatment and I/R Period.** During acute myocardial I/R injury, nitric oxide (NO) plays important physiological and pathological roles in a time-dependent

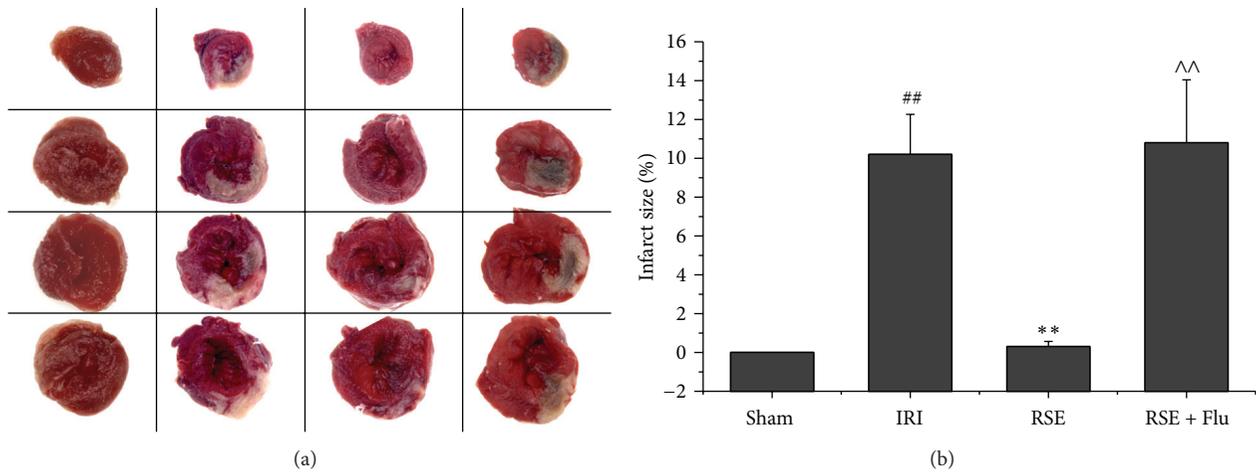


FIGURE 3: Infarct size after myocardial I/R injury. (a) Representative photos of TTC stained left ventricle slices. Deep red-staining areas indicate normal tissue, and unstained pale areas indicate infarct tissue. (b) Bar chart of myocardial infarct size determined by TTC staining. Data are shown as mean  $\pm$  S.E.M,  $n = 6\sim 7$ /group.  $^{##}P < 0.01$  versus Sham group,  $^{**}P < 0.01$  versus IRI group,  $^{\wedge}P < 0.01$  versus RSE group.

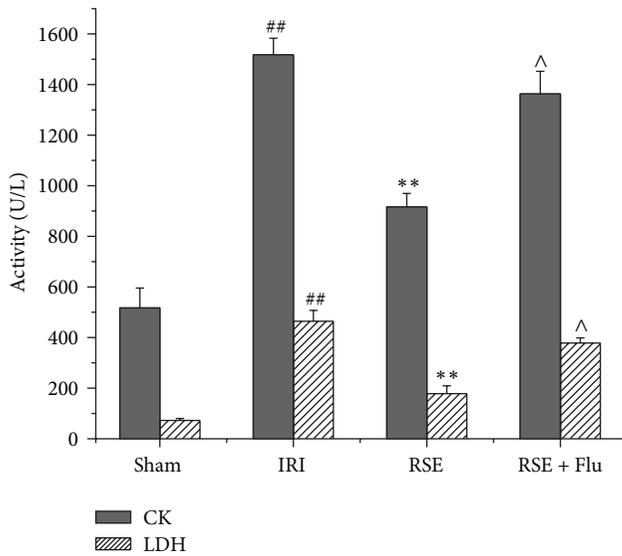


FIGURE 4: Effects of RSE and flutamide on CK and LDH activities in serum after myocardial I/R. The unit of enzymes was expressed as U/L. Data are shown as mean  $\pm$  S.E.M,  $n = 5$ /group.  $^{##}P < 0.01$ , versus Sham group;  $^{**}P < 0.01$  versus IRI group;  $^{\wedge}P < 0.05$  versus RSE group.

mechanism. We found that oral administration of RSE significantly protected rat hearts against I/R, and this effect was abolished by joint administration of flutamide. We wondered whether this protective effect of RSE involves an NO-related mechanism that is somehow affected by flutamide. We first measured the NO level in the serum of rats that underwent sham surgery or I/R process without drug pretreatment. The result showed no significant difference in the serum level of NO between the sham group and the IRI group. However, pretreatment of RSE in rats that underwent I/R process resulted in a remarkable elevation of serum NO production. NO production increased after 30 min RSE administration

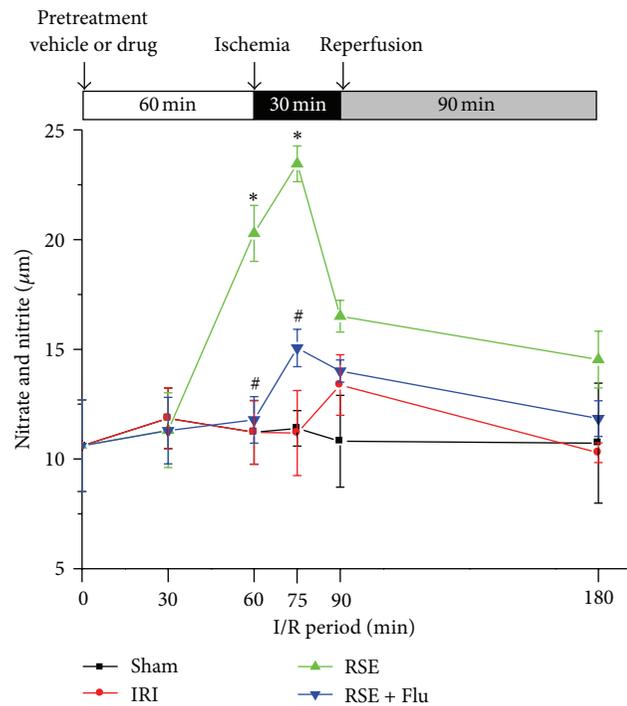


FIGURE 5: Effects of RSE and flutamide on time course changes of serum NO production. The concentrations of NO in serum were determined by measuring serum nitrite and nitrate levels. Data are shown as mean  $\pm$  S.E.M,  $n = 3\sim 5$ /group at each time point.  $^{*}P < 0.05$ , RSE group versus IRI group at the corresponding time point;  $^{\#}P < 0.05$ , RSE + Flu group versus RSE group at the corresponding time point.

and reached a peak at 15 min after ischemia (compared with IRI group,  $P < 0.05$ ). Figure 5 illustrates these time-dependent changes in serum NO production during pretreatment and I/R period. In the RSE + Flutamide group, the levels of NO production were significantly lower than those in RSE

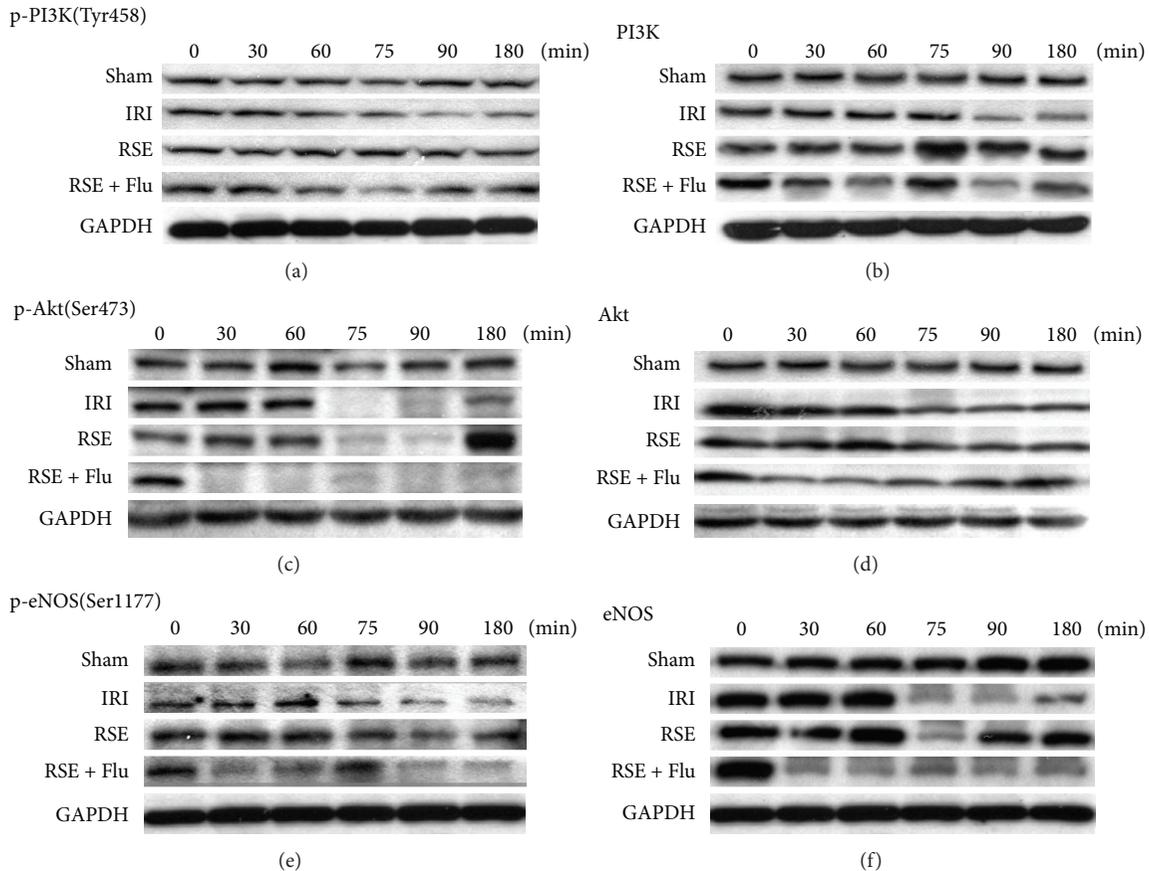


FIGURE 6: Effects of RSE and flutamide on time course changes of activation and expression of PI3K/Akt/eNOS signaling. PI3K/Akt/eNOS signaling in heart was investigated by Western blot at six time points. The blot was reprobed sequentially with antibodies special for PI3K or phospho-PI3K (Tyr458), Akt or phospho-Akt (Ser473), or eNOS or phospho-eNOS (Ser1177). Identical results were obtained in three separate rat hearts at each time point. Normalization of Western blot was ensured by GAPDH.

group ( $P < 0.05$  at 1 h after pretreatment and  $P < 0.05$  at 15 min after ischemia), suggesting that flutamide did indeed suppress NO production stimulated by RSE treatment.

**3.4. Time Course Changes of PI3K/AKT/eNOS Signaling Pathway in Heart Tissue during Pretreatment and I/R Period.** Since the NO production during I/R injury is closely regulated by the PI3K/AKT/eNOS signaling pathway, we further investigated the influence of RSE with or without joint administration of flutamide on this pathway during pretreatment and I/R period. Figure 6 illustrates representative results of the effects of RSE and flutamide on expression and activation of PI3K/Akt/eNOS pathway at the different time points during pretreatment and I/R period. Total and active PI3K, Akt, and eNOS were approximately equivalent in the sham group throughout the pretreatment and I/R periods. I/R significantly suppressed the expression and activation of PI3K after 30 min ischemia (Figures 6(a) and 6(b)). Expression of Akt in the IRI group was reduced slightly after ischemia, while its activation (p-Akt) was dramatically inhibited after ischemia (Figures 6(c) and 6(d)). Total eNOS in IRI group was decreased after ischemia and gradually returned to normal levels after reperfusion. However, the p-eNOS was constantly decreased compared with the sham group

(Figures 6(e) and 6(f)). RSE pretreatment returned PI3K back to a normal level and further upregulated activation of PI3K during the 30 min ischemia period. Although RSE did not significantly change the total form of Akt, it significantly elevated the p-Akt at 90 min after reperfusion. Meanwhile, in the presence of RSE pretreatment, active and total eNOS decreased remarkably at 15 min after ischemia and returned to normal level at 90 min after reperfusion. In the RSE and flutamide joint administration group, p-Akt dramatically and continuously decreased at 30 min after pretreatment. Interestingly, the expression and activation of eNOS in myocardial tissue was significantly reduced at 30 min after pretreatment. Combined with flutamide, RSE pretreatment did not show any obvious influence on returning total or active form of eNOS to normal levels during the I/R period. These results strongly suggest that the anti-ischemia reperfusion injury effect of RSE might result from, or at least involves, PI3K/Akt-activated eNOS activation in the early phase of ischemia (15 min after ischemia). This result is also partly in accordance with the results of NO production assay.

**3.5. Time Course Change of Serum Testosterone Level during Pretreatment and I/R Period.** As shown in Figure 7, the level of testosterone was stable in the sham group during the whole

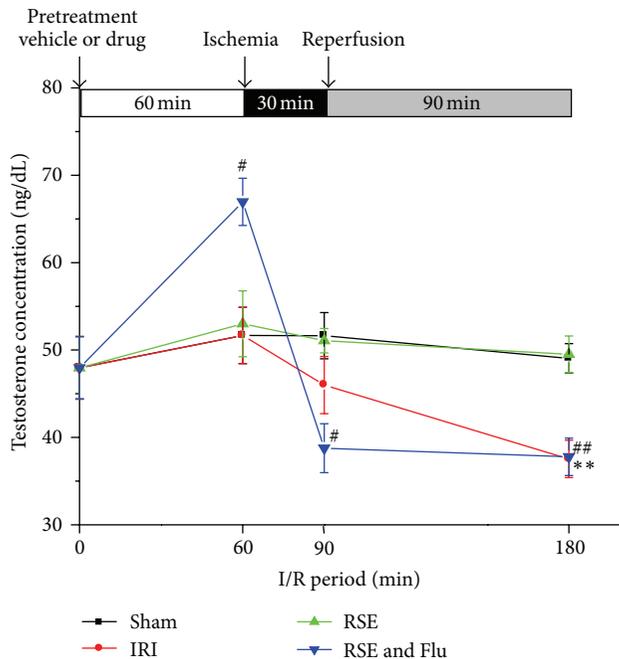


FIGURE 7: Effects of RSE and flutamide on time course changes of serum testosterone concentration. Data are shown as mean  $\pm$  S.E.M,  $n = 3-5$ /group at each time point. <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$ , RSE + Flu group compared with Sham group at the corresponding time point; <sup>\*\*</sup> $P < 0.01$  IRI group compared with Sham group at the corresponding time point.

pretreatment and I/R periods. Although 30 min ischemia did not cause a significant decrease in serum testosterone, additional 90 min reperfusion resulted in a significant decrease (23.5% drop compared with the sham group,  $P < 0.01$ ). Pretreatment with RSE completely suppressed the effect of I/R on serum testosterone levels. Interestingly, after 60 minutes pretreatment, the RSE + Flutamide group showed a significant increase ( $P < 0.05$ ) in serum testosterone, but this effect was dramatically reversed after 30 minutes of ischemia ( $P < 0.05$ ) and this reversion remained to the end of 90 min reperfusion.

#### 4. Discussion

In the rat open chest model, the hearts of rats that received RSE alone or both RSE and flutamide exhibited great difference in myocardial injury after acute ischemia reperfusion. We demonstrated for the first time an increased susceptibility to myocardial I/R injury after blocking androgen receptor in treatment of ginseng. Administering flutamide interrupted myocardial tissue recovery, as demonstrated by a significantly larger myocardial infarct size and higher myocardial enzyme activities. These effects could partly be explained by lower NO production, which leads to decreased cardiomyocytes' protective effect triggered in the early phase of ischemia as well as in the following reperfusion period. Furthermore, relationship between changes of PI3K/Akt/eNOS signal and endogenous testosterone level was time dependently associated with the contrasting effects on recovery from ischemia in ginseng with or without flutamide. These findings illustrate

that, during I/R, androgen receptor blockage by flutamide abolished the effectiveness of ginseng on acute myocardial I/R injury. This action was closely related to inhibition of PI3K/Akt/eNOS signaling and increase of endogenous androgen levels.

Sex differences exist in the response of myocardium to acute ischemia injury [17]. Clinical trials have found evidence of coronary heart disease in premenopausal women at much lower rate than in age-matched men [18]. Studies on experimental animals have demonstrated multiple beneficial effects of estrogens on the cardiovascular system, including amelioration of ischemia- and reperfusion-induced myocardial injury [15]. Therefore most previous studies have focused on the cardioprotective role of female hormones, whereas the role of male hormones in this significant gender difference in cardiovascular health has been largely neglected. Recently the role of androgen plays in the development of cardiovascular disease has attracted increasing interest. Testosterone, the most important endogenous androgen, has been considered as a vasoactive sex hormone steroid. Current results showed that testosterone may relax vascular smooth muscle by endothelium-dependent or -independent mechanisms, which may in part explain why testosterone replacement therapy improves myocardial ischemia in patients with coronary artery disease [16]. Thus, supplemental testosterone has been used to treat men with angina. The beneficial effects of supplemental testosterone on both ischemia and exercise tolerance have been demonstrated partially in several in vivo and in vitro studies. Administration of androgen was able to cause coronary dilatation and improve exercise-induced myocardial ischemia in male patients in men with established coronary artery disease [19]. This kind of androgen hormone supplement may be related to direct coronary vasodilation by NO-dependent and -independent pathways, and its activity does not seem to be dependent on conversion into estrogens. Most importantly, these possible mechanisms explain why the androgen hormone has been shown to improve angina pectoris in patients who received hormone replacement clinically. Therefore, these effects have led to the view that androgens are beneficial for the IHD.

On the contrary, more and more evidence shows that anabolic androgenic steroids are associated with myocardial ischemia, sudden cardiac death, and hypertension in athletes, suggesting that androgens may negatively affect myocardial tolerance of ischemia [20]. Androgenic steroids consist of a variety of different steroids with differing pharmacological properties. Among them, the incidence of cardiovascular morbidity closely associated with testosterone has been widely investigated [21]. Endogenous testosterone in men declines with age, and administering anabolic testosterone supplements to elderly men seems to improve their cardiovascular health [22]. And the use of testosterone esters like testosterone enanthate in males induces transient superphysiological androgen levels [23]. One study has shown that a single acute exposure to exogenous testosterone before ischemia worsened myocardial function recovery and increased activation death signaling [24]. Another study also supported that endogenous testosterone has a negative effect on myocardial function and Akt signaling [25]. These

findings imply that testosterone can have negative effects on the myocardium after acute injury. Thus, evaluation of the different roles of endogenous and supplemental testosterone for cardiovascular health is of growing importance.

Furthermore, many studies have shown that testosterone has an important role in cardiac injury because the heart can accumulate testosterone at higher concentrations than other androgen target organs, and functional androgen receptors are present in isolated cardiac myocytes [26]. Testosterone also has been demonstrated to modulate nuclear transcription by membrane receptor second messenger cascades, including L-type calcium channel, NO-dependent or NO-independent mechanisms [27], and apoptotic cell death of cardiomyocytes [28]. Another evidence also shows that the non-genomic pathway of androgen action possibly involves targeting of membrane hormone receptors to functional signaling modules in membrane caveolae, enabling the rapid activation of PI3K/Akt kinase pathways and endothelial nitric oxide synthase [29]. However, in these studies, the testosterone-regulated signaling of PI3K/Akt kinase remained unclear.

Recent reports on the cardioprotective effects of ginseng indicate that the upregulation of eNOS and NO production activity in I/R injury hearts by ginseng are possibly important mechanisms [30]. Our previous studies demonstrated that standardized ginseng extract protected rat hearts against ischemia and reperfusion injury in a dose-dependent manner. Oral treatment with RSE 1 h before ischemia significantly reduced myocardial I/R injury. This quick effect was possibly produced by increasing NO production. And this protection against I/R injury can be partially explained by active compounds of ginseng with sex hormone-like activities. Ginsenosides Rb1 and Rg1 have been shown to induce NO production and increase eNOS activation in aortic endothelial cells [31]. Activation of the steroid hormone receptors, such as ER and GR, through nongenomic and/or genomic pathways represents an important mechanism underlying the cardiovascular protective effect of ginseng therapy [32]. Therefore, we proposed to investigate whether there is possibly an association between the cardioprotective effect of ginseng and the androgen-mediated PI3K/Akt kinase signaling pathway. We applied flutamide, which is a known androgen-receptor blocker, and investigated whether blocking the androgen receptor might influence the protective effect of ginseng on the myocardial ischemia reperfusion injury in an *in vivo* rat heart model. In addition, because NO-dependent mechanism is of major importance for ischemia-reperfusion injury, we also used time-dependent relationship to assess the PI3K/Akt/eNOS signaling pathway during myocardial I/R.

The main findings of this study are that the androgen receptor blocker (flutamide) (1) abolished the antimyocardial I/R injury effect of *Panax ginseng*, (2) decreased NO production and enhanced CK-MB and LDH activities, (3) inhibited the expression and/or activation of PI3K/Akt/eNOS signaling pathway, and (4) exhibited a direct effect on endogenous testosterone level during myocardial I/R. Our present study provides new evidence that endogenous androgen-mediated action and downstream functional signaling modules are involved in the cardioprotective effect of *Panax ginseng*.

## Abbreviations

IHD:	Ischemic heart disease
I/R:	Ischemia reperfusion
NO:	Nitric oxide
TTC:	3,5-Triphenyltetrazolium chloride
RSE:	Standardized ginseng extract
LDH:	Lactate dehydrogenase
CK-MB:	Creatine kinase.

## Acknowledgment

This paper is supported by the Macao Science and Technology Development Fund (Project nos. 071/2011/A3 and 073/2011/A3).

## References

- [1] C. D. Collard and S. Gelman, "Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury," *Anesthesiology*, vol. 94, no. 6, pp. 1133–1138, 2001.
- [2] S. Bae and L. Zhang, "Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on akt and protein kinase C signaling," *Journal of Pharmacology and Experimental Therapeutics*, vol. 315, no. 3, pp. 1125–1135, 2005.
- [3] R. K. Dubey, S. P. Tofovic, and E. K. Jackson, "Cardiovascular pharmacology of estradiol metabolites," *Journal of Pharmacology and Experimental Therapeutics*, vol. 308, no. 2, pp. 403–409, 2004.
- [4] M. K. Ng, "New perspectives on mars and venus: unravelling the role of androgens in gender differences in cardiovascular biology and disease," *Heart Lung and Circulation*, vol. 16, no. 3, pp. 185–192, 2007.
- [5] G. M. C. Rosano, "Androgens and coronary artery disease. A sex-specific effect of sex hormones?" *European Heart Journal*, vol. 21, no. 11, pp. 868–871, 2000.
- [6] G. N. Levine, A. V. D'Amico, P. Berger et al., "Androgen-deprivation therapy in prostate cancer and cardiovascular risk: a science advisory from the American heart association, American cancer society, and American urological association: endorsed by the American society for radiation oncology," *Circulation*, vol. 121, no. 6, pp. 833–840, 2010.
- [7] S. E. . Gilbert, G. A. Tew, L. Bourke, E. M. Winter, and D. J. Rosario, "Assessment of endothelial dysfunction by flow-mediated dilatation in men on long-term androgen deprivation therapy for prostate cancer," *Experimental Physiology*, vol. 98, no. 9, pp. 1401–1410, 2013.
- [8] H. K. Tsai, A. V. D'Amico, N. Sadetsky, M. H. Chen, and P. R. Carroll, "Androgen deprivation therapy for localized prostate cancer and the risk of cardiovascular mortality," *Journal of the National Cancer Institute*, vol. 99, no. 20, pp. 1516–1524, 2007.
- [9] S. P. Zhao and X. P. Li, "The association of low plasma testosterone level with coronary artery disease in Chinese men," *International Journal of Cardiology*, vol. 63, no. 2, pp. 161–164, 1998.
- [10] J. Barp, A. S. Araújo, T. R. Fernandes et al., "Myocardial antioxidant and oxidative stress changes due to sex hormones," *Brazilian Journal of Medical and Biological Research*, vol. 35, no. 9, pp. 1075–1081, 2002.
- [11] M. Zaugg, N. Z. Jamali, E. Lucchinetti et al., "Anabolic-androgenic steroids induce apoptotic cell death in adult rat ventricular

- myocytes," *Journal of Cellular Physiology*, vol. 187, no. 1, pp. 90–95, 2001.
- [12] M. H. Lehmann, S. Hardy, D. Archibald, B. Quart, and D. J. MacNeil, "Sex difference in risk of torsade de pointes with d,l-sotalol," *Circulation*, vol. 94, no. 10, pp. 2534–2541, 1996.
- [13] L. J. Hofseth and M. J. Wargovich, "Inflammation, cancer, and targets of ginseng," *Journal of Nutrition*, vol. 137, supplement 1, pp. 183S–185S, 2007.
- [14] H. Zhou, S. Z. Hou, P. Luo et al., "Ginseng protects rodent hearts from acute myocardial ischemia-reperfusion injury through GR/ER-activated RISK pathway in an endothelial NOS-dependent mechanism," *Journal of Ethnopharmacology*, vol. 135, no. 2, pp. 287–298, 2011.
- [15] T. Furukawa, C. X. Bai, A. Kaihara et al., "Ginsenoside Re, a main phytosterol of *Panax ginseng*, activates cardiac potassium channels via a nongenomic pathway of sex hormones," *Molecular Pharmacology*, vol. 70, no. 6, pp. 1916–1924, 2006.
- [16] K. M. English, R. P. Steeds, T. H. Jones, M. J. Diver, and K. S. Channer, "Low-dose transdermal testosterone therapy improves angina threshold in men with chronic stable angina: a randomized, double-blind, placebo-controlled study," *Circulation*, vol. 102, no. 16, pp. 1906–1911, 2000.
- [17] J. Alfredsson and E. Swahn, "Management of acute coronary syndromes from a gender perspective," *Fundamental and Clinical Pharmacology*, vol. 24, no. 6, pp. 719–728, 2010.
- [18] T. Simoncini and A. R. Genazzani, "Non-genomic actions of sex steroid hormones," *European Journal of Endocrinology*, vol. 148, no. 3, pp. 281–292, 2003.
- [19] F. Callies, H. Strömer, R. H. Schwinger et al., "Administration of testosterone is associated with a reduced susceptibility to myocardial ischemia," *Endocrinology*, vol. 144, no. 10, pp. 4478–4483, 2003.
- [20] M. L. Sullivan, C. M. Martinez, P. Gennis, and E. J. Gallagher, "The cardiac toxicity of anabolic steroids," *Progress in Cardiovascular Diseases*, vol. 41, no. 1, pp. 1–15, 1998.
- [21] G. M. Rosano, A. Cornoldi, and M. Fini, "Effects of androgens on the cardiovascular system," *Journal of Endocrinological Investigation*, vol. 28, no. 3, supplement, pp. 32–38, 2005.
- [22] H. Sternbach, "Age-associated testosterone decline in men: clinical issues for psychiatry," *The American Journal of Psychiatry*, vol. 155, no. 10, pp. 1310–1318, 1998.
- [23] K. A. Abadilla and A. S. Dobs, "Topical testosterone supplementation for the treatment of male hypogonadism," *Drugs*, vol. 72, no. 12, pp. 1591–1603, 2012.
- [24] P. R. Crisostomo, M. Wang, G. M. Wairiuko, E. D. Morrell, and D. R. Meldrum, "Brief exposure to exogenous testosterone increases death signaling and adversely affects myocardial function after ischemia," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 290, no. 5, pp. R1168–R1174, 2006.
- [25] C. Huang, H. Gu, W. Zhang, J. L. Herrmann, and M. Wang, "Testosterone-down-regulated akt pathway during cardiac ischemia/ reperfusion: a mechanism involving BAD, Bcl-2 and FOXO3a," *Journal of Surgical Research*, vol. 164, no. 1, pp. e1–e11, 2010.
- [26] J. D. Marsh, M. H. Lehmann, R. H. Ritchie, J. K. Gwathmey, G. E. Green, and R. J. Schiebinger, "Androgen receptors mediate hypertrophy in cardiac myocytes," *Circulation*, vol. 98, no. 3, pp. 256–261, 1998.
- [27] G. Michels and U. C. Hoppe, "Rapid actions of androgens," *Frontiers in Neuroendocrinology*, vol. 29, no. 2, pp. 182–198, 2008.
- [28] F. Er, G. Michels, N. Gassanov, F. Rivero, and U. C. Hoppe, "Testosterone induces cytoprotection by activating ATP-sensitive  $K^+$  channels in the cardiac mitochondrial inner membrane," *Circulation*, vol. 110, no. 19, pp. 3100–3107, 2004.
- [29] E. Debing, E. Peeters, W. Duquet, K. Poppe, B. Velkeniers, and P. van den Brande, "Endogenous sex hormone levels in postmenopausal women undergoing carotid artery endarterectomy," *European Journal of Endocrinology*, vol. 156, no. 6, pp. 687–693, 2007.
- [30] E. A. Booth and B. R. Lucchesi, "Estrogen-mediated protection in myocardial ischemia-reperfusion injury," *Cardiovascular Toxicology*, vol. 8, no. 3, pp. 101–113, 2008.
- [31] L. Jia, Y. Zhao, and X. J. Liang, "Current evaluation of the millennium phytomedicine—ginseng (II): collected chemical entities, modern pharmacology, and clinical applications emanated from traditional Chinese medicine," *Current Medicinal Chemistry*, vol. 16, no. 22, pp. 2924–2942, 2009.
- [32] P. Y. Yue, N. K. Mak, Y. K. Cheng et al., "Pharmacogenomics and the Yin/Yang actions of ginseng: anti-tumor, angiomodulating and steroid-like activities of ginsenosides," *Chinese Medicine*, vol. 2, article 6, 2007.

## Research Article

# The Electrophysiological Effects of Qiliqiangxin on Cardiac Ventricular Myocytes of Rats

Yidong Wei,<sup>1</sup> Xiaoyu Liu,<sup>2</sup> Haidong Wei,<sup>3</sup> Lei Hou,<sup>1</sup> Wenliang Che,<sup>1</sup> Erlinda The,<sup>1</sup> Gang Li,<sup>1</sup> Muktanand Vikash Jhummon,<sup>1</sup> and Wanlin Wei<sup>4</sup>

<sup>1</sup> Department of Cardiology, Shanghai Tenth People's Hospital of Tongji University, 301 Yanchang Road, Shanghai 200072, China

<sup>2</sup> Department of Traditional Chinese Medicine, Shanghai Tenth People's Hospital of Tongji University, 301 Yanchang Road, Shanghai 200072, China

<sup>3</sup> Zhengzhou Second People's Hospital, 90 Hanghai Road, Zhengzhou, Henan 450015, China

<sup>4</sup> Department of Cardiology, General Hospital of Beijing Military Command, No. 5 Nanmencang, Dongsishitiao, Dongcheng District, Beijing 100007, China

Correspondence should be addressed to Wanlin Wei; [ywei@tongji.edu.cn](mailto:ywei@tongji.edu.cn)

Received 2 May 2013; Revised 24 June 2013; Accepted 2 July 2013

Academic Editor: Hao Xu

Copyright © 2013 Yidong Wei et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Qiliqiangxin, a Chinese herb, represents the affection in Ca channel function of cardiac myocytes. It is unknown whether Qiliqiangxin has an effect on Na current and K current because the pharmacological actions of this herb's compound are very complex. We investigated the rational usage of Qiliqiangxin on cardiac ventricular myocytes of rats. Ventricular myocytes were exposed acutely to 1, 10, and 50 mg/L Qiliqiangxin, and whole cell patch-clamp technique was used to study the acute effects of Qiliqiangxin on Sodium current ( $I_{Na}$ ), outward currents delayed rectifier outward  $K^+$  current ( $I_K$ ), slowly activating delayed rectifier outward  $K^+$  current ( $I_{Ks}$ ), transient outward  $K^+$  current ( $I_{to}$ ), and inward rectifier  $K^+$  current ( $I_{K1}$ ). Qiliqiangxin can decrease  $I_{Na}$  by  $28.53\% \pm 5.98\%$ , and its  $IC_{50}$  was 9.2 mg/L. 10 and 50 mg/L Qiliqiangxin decreased by  $37.2\% \pm 6.4\%$  and  $55.9\% \pm 5.5\%$  summit current density of  $I_{to}$ . 10 and 50 mg/L Qiliqiangxin decreased  $I_{Ks}$  by  $15.51\% \pm 4.03\%$  and  $21.6\% \pm 5.6\%$ . Qiliqiangxin represented a multifaceted pharmacological profile. The effects of Qiliqiangxin on Na and K currents of ventricular myocytes were more profitable in antiarrhythmic therapy in the clinic. We concluded that the relative efficacy of Qiliqiangxin was another choice for the existing antiarrhythmic therapy.

## 1. Introduction

The traditional Chinese herbs have been proven safe and efficient in the management of some diseases including arrhythmia since ancient times. Presently, there are four main classes of antiarrhythmic agents in clinical therapy. Three classes of them act on ion channels. As for the Chinese herb—Qiliqiangxin—few know the mechanism of antiarrhythmia. How does it impact on ion channels? Qiliqiangxin capsule is the developed Chinese herbs, which includes over 11 ingredients such as Ginseng, Radix Astragali, Aconite Root, *Salvia miltiorrhiza*, and Semen Lepidii Apetali. It was proved to be effective and safe by phase 3 clinic trial for the treatment of patients with chronic heart failure [1, 2]. Qiliqiangxin capsule

also can improve heart function and decrease serum level of TNF- $\alpha$  and relieve inflammatory cell infiltration of myocardium in rats with adriamycin induced cardiomyopathy [3]. In our previous study, Qiliqiangxin affected L-type  $Ca^{2+}$  channel and blocked  $I_{Ca-L}$ , as well as affected cardiac function finally. Qiliqiangxin has diphasic action that is either class IV antiarrhythmic agent or the agent for treating chronic heart failure [4, 5]. It is unknown whether Qiliqiangxin has an effect on Na current and K current because the pharmacological actions of this herb's compound are very complex. In this research, we compared the acute effects of Qiliqiangxin on the inward currents  $I_{Na}$  and outward currents  $I_K$ ,  $I_{Ks}$ ,  $I_{to}$ , and  $I_{K1}$  of ventricular myocytes and hoped to demonstrate the rational usage of Qiliqiangxin.

## 2. Material and Methods

**2.1. Vegetal Material.** Qiliqiangxin consists of Ginseng, Radix Astragali, Aconite Root, *Salvia miltiorrhiza*, Semen Lepidii Apetali, Cortex Periplocae Sepii Radicis, Rhizoma Alismatis, *Carthamus tinctorius*, Polygonatum Odorati, Seasoned Orange Peel, and Ramulus Cinnamomi [3] (Yiling Pharmaceutical Corporation, Shijiazhuang, China). The drug powder dissolved with sterile water at the concentration of 2.67 g/mL. 1 mg/L, 10 mg/L, and 50 mg/L Qiliqiangxin were prepared for the study.

**2.2. Study Models.** A total of 26 healthy Sprague-Dawley rats (9–11 weeks old, either sex, weight 210 to 300 g) were used in the study. All the rats used in the following experiments were subject to the Guiding Principles for the Care and Use of Laboratory Animals and the Recommendations from the Declaration of Tongji University. Cardiac ventricular myocytes were isolated from the hearts of rats using previous protocols [6]. Briefly, hearts were rapidly excised and cycloperfused with low calcium Tyrode's solution containing 0.08% collagenase, 0.006% protease, and then we get a single ventricular myocyte. The single ventricular myocyte selected for study is rod shaped and had clear striations and smooth and glossy surface.

**2.3. Whole Cell Patch Clamp.** We recorded that Na current in the external solution contained (mmol/L) Choline-Cl 120, NaCl 25, CsCl 4, CaCl<sub>2</sub> 1.8, CoCl<sub>2</sub> 2, MgCl<sub>2</sub> 1, HEPES 10, and Glucose 10; pH was adjusted to 7.4 with CsOH. The pipette solution contained (mmol/L) CsCl 140, NaCl 10, HEPES 5, EGTA 5, and Na<sub>2</sub>ATP 5; pH was adjusted to 7.3 with CsOH.  $I_{Na}$  was elicited from a potential -90 mV to +40 mV with 10 mV increments and by 200 ms pulses. For  $I_K$  recording, the external solution contained (mmol/L) Choline-Cl 145, MgCl<sub>2</sub> 2, EGTA 1, HEPES 5, and Glucose 5.5; pH was adjusted to 7.4 with LiOH. The pipette solution contained (mmol/L) KCl 140, MgCl<sub>2</sub> 1, K<sub>2</sub>ATP 5, HEPES 5, and EGTA 10; pH was adjusted to 7.3 with KOH. For  $I_{K1}$  recording, the external solution contained (mmol/L) Choline-Cl 145, KCl 5, MgCl<sub>2</sub> 1, EGTA 5, HEPES 10, and Glucose 10; pH was adjusted to 7.4 with KOH. The pipette solution contained (mmol/L) KCl 140, CaCl<sub>2</sub> 1, EGTA 10, HEPES 5, and K<sub>2</sub>ATP 5; pH was adjusted to 7.3 with KOH. For  $I_{to}$  recording, the external solution contained (mmol/L) NaCl 140, KCl 4, CaCl<sub>2</sub> 1.5, MgCl<sub>2</sub> 1, CdCl<sub>2</sub> 0.5, and HEPES 5, Glucose 10; pH was adjusted to 7.4 with NaOH. The pipette solution contained (mmol/L) KCl 140, MgCl<sub>2</sub> 1, K<sub>2</sub>ATP 5, EGTA 5, and HEPES 10; pH was adjusted to 7.3 with KOH. All recordings are at room temperature. The external solution was filled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.  $I_K$  was elicited from the holding potential of -50 mV to +60 mV and by 4500 ms pulses with 10 mV increments.  $I_{K1}$  was elicited from the holding potential of -120 mV to +60 mV and by 200 ms pulses with 10 mV increments.  $I_{to}$  was elicited from the holding potential of -80 mV and by 300 ms pulses with 10 mV increments from a potential -40 mV to +70 mV. The effects of Qiliqiangxin: we perfuse cell with Qiliqiangxin of 1, 10, 50 mg/L, and every cell was perfused 2-3 concentration

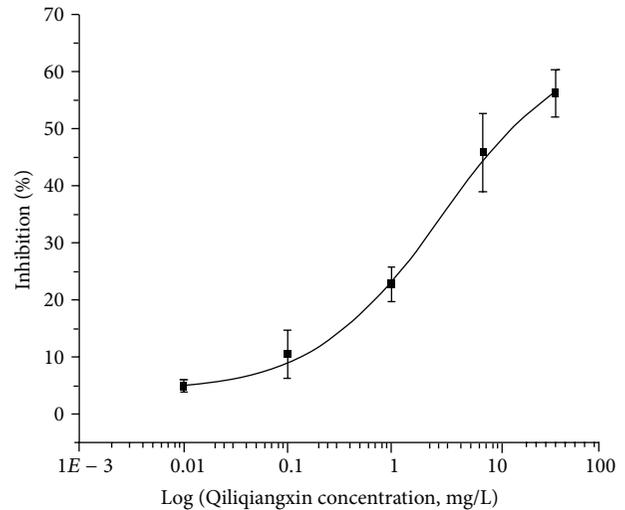


FIGURE 1: Qiliqiangxin's effect on  $I_{Na}$  of ventricular myocytes. Its  $IC_{50}$  was 9.2 mg/L.

steps. To normalize for differences in total membrane area, current densities (in pA/pF) were calculated by dividing the total current by the membrane capacitance of the cell. Data were sampled at 10 kHz and filtered at 2 kHz by using an Axopatch 200 A amplifier (Axon Instruments).

**2.4. Statistical Analysis.** pCLAMP 9.0 software was used for data acquisition, and analysis values are presented as means  $\pm$  S.D. Statistical comparisons between the different amiodarone concentrations groups were obtained by ANOVA. Comparisons between control and hypertrophied myocytes group means were performed with Student's *t*-test. Differences with  $P < 0.05$  were considered significant, completed by SPSS 11.5 Statistically package. Concentration response relationships were fit to the Hill equation to determine the concentration of drug required for 50% inhibition ( $IC_{50}$ ).

## 3. Result

**3.1. Current Density.** The inward currents density of  $I_{Na}$  in cardiac ventricular myocytes (pA/pF,  $n = 10$ ) was  $-56.46 \pm 4.88$ . The outward current densities of  $I_K$ ,  $I_{Ks}$ ,  $I_{to}$ , and  $I_{K1}$  (pA/pF,  $n = 8$ ) were  $7.27 \pm 0.95$ ,  $5.68 \pm 0.56$ ,  $34.71 \pm 2.83$ ,  $-22.82 \pm 5.34$  ( $I_{K1}$  inward), and  $10.16 \pm 0.20$  ( $I_{K1}$  outward).

**3.2. Effects of Qiliqiangxin on  $I_{Na}$ ,  $I_{to}$ ,  $I_{K1}$ , and  $I_K$ .** 1 Mg/L Qiliqiangxin can decrease  $I_{Na}$  of ventricular myocytes by  $28.53\% \pm 5.98\%$ , and its  $IC_{50}$  was 9.2 mg/L (Figure 1). Hypoconcentration (1 mg/L) Qiliqiangxin had no effects on  $I_{to}$  on ventricular myocytes, while hyperconcentration Qiliqiangxin had inhibition action. 10 and 50 mg/L Qiliqiangxin decreased by  $37.2\% \pm 6.4\%$  and  $55.9\% \pm 5.5\%$  summit current density of  $I_{to}$  (Figure 2). For  $I_{K1}$ , the difference concentration of Qiliqiangxin had no effects on  $I_{K1}$ .

Delayed rectifier  $K^+$  current ( $I_K$ ) is the major outward current responsible for ventricular repolarization. 1, 10, and

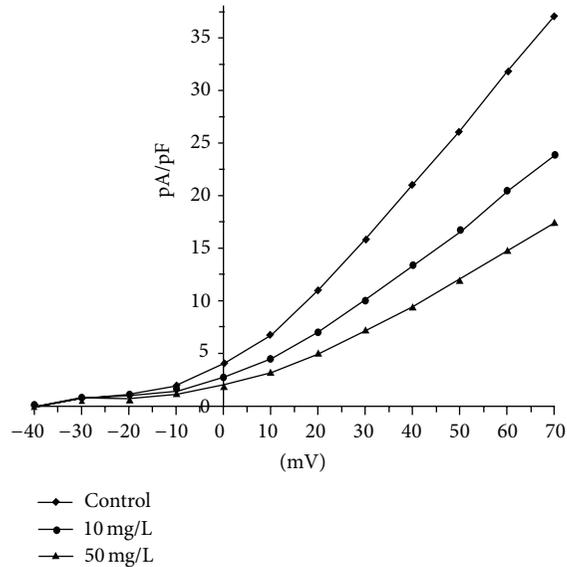


FIGURE 2: The effects of 10 and 50 mg/L Qiliqiangxin on current density-voltage curve of  $I_{to}$  in ventricular myocytes.

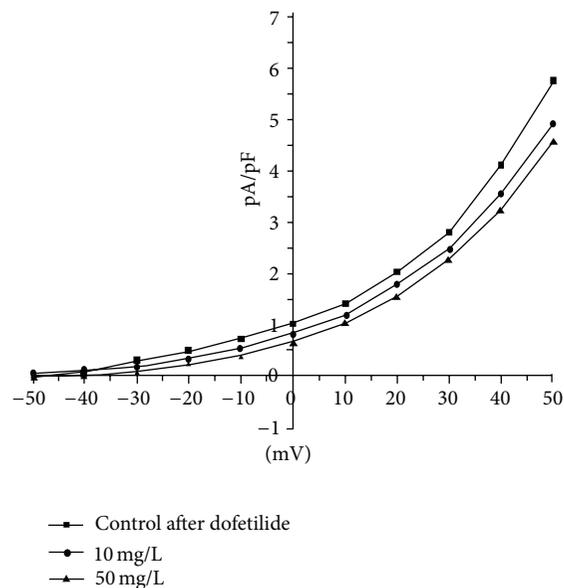


FIGURE 3: The effect of 10 and 50 mg/L Qiliqiangxin on current density-voltage curve of  $I_{Ks}$  in ventricular myocytes.

50 mg/L Qiliqiangxin decreased the current density of  $I_K$  by  $20.98\% \pm 3.97\%$ ,  $31.18\% \pm 5.1\%$ , and  $61.52\% \pm 5.97\%$ .  $I_K$  comprises two distinct current components slowly activating delayed rectifier outward  $K^+$  current ( $I_{Ks}$ ) and rapidly activating delayed rectifier outward  $K^+$  current ( $I_{Kr}$ ). We used dofetilide, a class III antiarrhythmic agent and a selective blocker of  $I_{Kr}$ , to inhibit  $I_{Kr}$  and then  $I_{Ks}$  was recorded alone. 10 and 50 mg/L Qiliqiangxin decreased by  $15.51\% \pm 4.03\%$  and  $21.6\% \pm 5.6\%$  current density of  $I_{Ks}$  on ventricular myocytes (Figure 3).

## 4. Discussion

From our previous study, Qiliqiangxin has biphasic action that is either class IV antiarrhythmic agent or the agent for treating chronic heart failure [5]. Qiliqiangxin includes over 11 ingredients. The mechanism of the antiarrhythmic action is complex and not completely understood. It should be contacted with Radix Astragal, Aconite Root, and Shensongyangxin. Radix Astragal effectively protected against cardiac dysfunction [7]. Aconite Root was proved to have positive inotropic and chronotropic action [8]. Shensongyangxin capsule was reported to effectively block  $I_{Ca-L}$  [9]. There were four main classes of antiarrhythmic agents in Vaughan Williams' classification. Qiliqiangxin affected L-type  $Ca^{2+}$  channel and blocked  $I_{Ca-L}$ . It should be a class IV agent. Does Qiliqiangxin have an effect on other currents, such as Na current and K currents, because of its antiarrhythmic action? We had introduced Qiliqiangxin to test Na current and K currents. Interestingly, 1 mg/L Qiliqiangxin can decrease  $I_{Na}$  of ventricular myocytes by  $28.53\% \pm 5.98\%$ , and its  $IC_{50}$  was 9.2 mg/L. K currents are outward currents that repolarize the ventricular myocyte are numerous and complex and there is substantial interspecies variation in the profile of repolarizing currents. Prominent  $I_{to}$  currents have been recorded in ventricular myocytes isolated from the hearts of many species, including mice, rats, rabbits, cows, cats, dogs, ferrets and humans. In ventricular myocytes of rat, 10 and 50 mg/L Qiliqiangxin decreased by  $37.2\% \pm 6.4\%$  and  $55.9\% \pm 5.5\%$  summit current density of  $I_{to}$ . 1, 10, and 50 mg/L Qiliqiangxin decreased current density of  $I_K$  by  $20.98\% \pm 3.97\%$ ,  $31.18\% \pm 5.1\%$  and  $61.52\% \pm 5.97\%$ . 10 and 50 mg/L Qiliqiangxin decreased by  $15.51\% \pm 4.03\%$  and  $21.6\% \pm 5.6\%$  current density of  $I_{Ks}$ . Acute Qiliqiangxin application can inhibit  $I_{to}$  and  $I_K$ . 10 and 50 mg/L Qiliqiangxin had little effects on  $I_{Kr}$ , and the resting membrane potential was not affected by it. Qiliqiangxin represented class I and IV antiarrhythmic agents. It can prolong QT interval, but it does not have a similar deleterious effect as pure class III compounds. Therefore, Qiliqiangxin represented a multifaceted pharmacological profile.

During the last decade, antiarrhythmic strategies have changed dramatically. In general, class I agents have the potential to increase mortality in patients with significant structural heart disease even though they may suppress cardiac arrhythmias. Class III and IV agents did not decrease the outcomes in clinic obviously. Qiliqiangxin is as one developed Chinese herbs, which expressed the inhibition of  $I_{Na}$ ,  $I_{Ca-L}$ , and multi  $K^+$  channels. The effects of Qiliqiangxin on ventricular myocytes were more profit in clinic efficacy. It is the most promising drug because of the relative efficacy and safety. Even though the mechanism of the antiarrhythmic action of Qiliqiangxin is complex and not completely understood, Qiliqiangxin is another choice for antiarrhythmic therapy at least. As for the further action in clinic of this antiarrhythmic agent, we need more research in the future.

## Authors' Contribution

Yidong Wei and Xiaoyu liu are co-first authors.

## Acknowledgment

This work was supported by the National Natural Science Funds of China (nos. 30800466, 81270193) for Yidong Wei.

## References

- [1] Y. L. Wu, C. H. Gu, G. C. Xu, C. Wei, and X. D. Gao, "Clinical observation of randomized double-blind and multicenter trial on Qiliqiangxin capsule in the treatment of chronic heart failure," *Chinese Journal of Difficult and Complicated Cases*, vol. 6, pp. 55–58, 2007.
- [2] J. H. Ling, P. B. Wu, W. Y. Cai, X. B. Xu, M. R. Zhuang, and C. L. Li, "Therapeutic effect of Qiliqiangxin capsule on the patients with chronic congestive heart failure," *Chinese Journal of Difficult and Complicated Cases*, vol. 5, pp. 49–55, 2008.
- [3] H. Xiao, Y. Song, Y. Li, Y. Liao, and J. Chen, "Qiliqiangxin regulates the balance between tumor necrosis factor- $\alpha$  and interleukin-10 and improves cardiac function in rats with myocardial infarction," *Cellular Immunology*, vol. 260, no. 1, pp. 51–55, 2009.
- [4] D. M. Bers, "Calcium cycling and signaling in cardiac myocytes," *Annual Review of Physiology*, vol. 70, pp. 23–49, 2008.
- [5] Y. Wei, X. Liu, L. Hou, W. Che, E. The, and M. V. Jhummon, "Qiliqiangxin affects L type  $\text{Ca}^{2+}$  current in the normal and hypertrophied rat heart," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 131830, 4 pages, 2012.
- [6] J.-P. Benitah, A. M. Gomez, and P. Bailly, "Heterogeneity of the early outward current in ventricular cells isolated from normal and hypertrophied rat hearts," *The Journal of Physiology*, vol. 469, pp. 111–138, 1993.
- [7] Z. Zhao, W. Wang, and F. Wang, "Effects of astragaloside IV on heart failure in rats," *Chinese Medicine*, vol. 4, article 6, 2009.
- [8] J. Lin, C. Chan, C. Yang, Y. Wang, H. Chiou, and Y. Su, "A cardiotoxic Chinese herb, a new medical treatment choice for portal hypertension?" *Experimental Biology and Medicine*, vol. 232, no. 4, pp. 557–564, 2007.
- [9] N. Li, Y. Huo, K. Ma, Q. Sun, and J. Pu, "Effects of solution of dry powder of ShenSongYangXin capsule on sodium current and L-type calcium current in ventricular myocytes: experiment with guinea pig," *Zhonghua Yi Xue Za Zhi*, vol. 87, no. 14, pp. 995–998, 2007.

## Research Article

# Ginseng Extracts Restore High-Glucose Induced Vascular Dysfunctions by Altering Triglyceride Metabolism and Downregulation of Atherosclerosis-Related Genes

Gabriel Hoi-huen Chan,<sup>1</sup> Betty Yuen-kwan Law,<sup>2,3</sup> John Man-tak Chu,<sup>2</sup>  
Kevin Kin-man Yue,<sup>2</sup> Zhi-hong Jiang,<sup>2</sup> Chi-wai Lau,<sup>4</sup> Yu Huang,<sup>4</sup> Shun-wan Chan,<sup>5</sup>  
Patrick Ying-kit Yue,<sup>1</sup> and Ricky Ngok-shun Wong<sup>1</sup>

<sup>1</sup> Department of Biology, Hong Kong Baptist University, Kowloon Tong, Kowloon, Hong Kong

<sup>2</sup> School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Kowloon, Hong Kong

<sup>3</sup> State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau

<sup>4</sup> School of Biomedical Science, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong

<sup>5</sup> Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

Correspondence should be addressed to Ricky Ngok-shun Wong; [rns Wong@hkbu.edu.hk](mailto:rns Wong@hkbu.edu.hk)

Received 21 March 2013; Revised 23 August 2013; Accepted 24 August 2013

Academic Editor: Hao Xu

Copyright © 2013 Gabriel Hoi-huen Chan et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The king of herbs, *Panax ginseng*, has been used widely as a therapeutic agent vis-à-vis its active pharmacological and physiological effects. Based on Chinese pharmacopeia *Ben Cao Gang Mu* and various pieces of literature, *Panax ginseng* was believed to exert active vascular protective effects through its antiobesity and anti-inflammation properties. We investigated the vascular protective effects of ginseng by administering ginseng extracts to rats after the induction of diabetes. We found that *Panax ginseng* can restore diabetes-induced impaired vasorelaxation and can reduce serum triglyceride but not cholesterol level in the diabetic rats. The ginseng extracts also suppressed the expression of atherosclerosis-related genes and altered the expression of lipid-related genes. The results provide evidence that *Panax ginseng* improves vascular dysfunction induced by diabetes and the protective effects may possibly be due to the downregulation of atherosclerosis-related genes and altered lipid metabolism, which help to restore normal endothelium functions.

## 1. Introduction

*Panax ginseng* is one of the most commonly used Chinese medicine and research targets. The major active components of *Panax ginseng* are ginsenosides which can be subdivided into three groups according to their basic structures: protopanaxadiol (PPD) type (e.g., Rb1, Rb2, Rc, Rd, Rg3, and Rh2), protopanaxatriol (PPT) type (e.g., Re, Rf, Rg1, Rg2, and Rh1), and oleanolic acid (e.g., Ro). Ginsenosides appear to be responsible for most of the activities of ginseng including antioxidation, anti-inflammation, and anticancer [1]. A review by Karmazyn et al. has found that the yearly

ginseng-related publication has been increasing exponentially from 1950 to 2010. They also reported that *Panax ginseng* played a protective role in the cardiovascular system [2]. This suggested the beneficial properties of ginseng on cardiovascular diseases in both experimental and clinical settings.

Atherosclerosis is one of the most common cardiovascular diseases and can remain asymptomatic for decades. In the mid 1970s, Russel Ross developed the popular “response to injury” theory by postulating that atherosclerosis begins with injuries on the endothelium, followed by adhesion and aggregation of platelets [3]. At about the same time,

Robert F. Furchgott, the Nobel Prize Laureate in Physiology or Medicine in 1998, discovered that acetylcholine induces endothelium-dependent relaxation in normal aortic tissue [4]. Upon early onset of atherosclerosis, endothelium can remain morphologically intact though inflammatory responses are triggered. Since then, numerous researches have been conducted to investigate the mechanisms of atherosclerosis to mitigate the associated diseases including adhesion of lipid-laden macrophages and smooth muscle cells which could finally result in endothelial denudation [5]. Besides, Hansson's research groups have reported that high level of total cholesterol and low density lipoprotein accumulated in the intima of the arteries, with the attack of myeloperoxidase and lipoxygenases, or by reactive oxygen species [6, 7] could also cause the early onset of atherosclerosis.

The primary objective of this study is to evaluate the protective effects of *Panax ginseng* on diabetes mellitus, a pathological condition which links to endothelial dysfunctions, through investigating the physiological parameters such as blood glucose, blood cholesterol, insulin, and advanced glycation end product in diabetic rat models. Furthermore, the changes of atherosclerosis-related genes expression in diabetic rats are also investigated after ginseng administration. The findings may help in the development of successful therapeutic interventions for atherosclerotic cardiovascular disease.

## 2. Materials and Methods

This study follows "The International Guiding Principles for Biomedical Research Involving Animals," The Hong Kong Code of Practice for Care and Use of Animals for Experimental Purposes (2004). All experimental procedures were conducted according to the Animals (Control of Experiments) Ordinance of the Department of Health, HKSAR (Animal Licenses ID: (11-6) DH/HA&P/8/2/6 Pt.2; (10-4) DH/HA&P/8/2/6 Pt.1; (10-9) DH/HA&P/8/2/6 Pt.1). All animal studies were performed in facilities approved by the Animal Ethics Committee of the Chinese University of Hong Kong (10/028/MIS).

**2.1. Animals.** Male Sprague-Dawley (SD) rats weighing 150–200 grams were housed in room under standard vivarium conditions with 12 hour light/dark cycle. Throughout the experimental period, animals were fed with standard rodent chow and water available *ad libitum*. The animals were acclimatized to the laboratory conditions for 10 days prior to the inception of experiments. Experimental diabetic condition was induced in rats by a single intraperitoneal injection (i.p.) of streptozotocin (75 mg/kg body weight) freshly dissolved in cold citrate buffer (0.1 M), while the normal control group was injected with citrate buffer only. Blood samples were collected from tail veins of overnight-fasted rats three days after streptozotocin administration. Rats with blood glucose level higher than 16.7 mmol/dL were selected for experiment.

The experimental rats were divided into seven groups: (1) normal control rats administered with water, (2) diabetic group of rats administered with water, (3) diabetic group administered with intraperitoneal injection of insulin, (4) diabetic group fed with PPT-type of ginseng (10 mg/kg/day), (5) diabetic group fed with PPT-type of ginseng (30 mg/kg/day), (6) diabetic group fed with PPD-type of ginseng (10 mg/kg/day), and (7) diabetic group fed with PPD-type of ginseng (30 mg/kg/day). The dosage of insulin followed a protocol developed by Kuo et al. [8], and water or drugs were administered for a total of 14 consecutive treatment days. Both PPD and PPT were administered orally in the form of aqueous suspension. Rats were anaesthetized by Ketamine-Rompun mixture (7.5:1), and blood was collected from the heart for further analysis. The animals were then sacrificed immediately by cervical dislocation. Aortae were removed and trimmed for tissue bath experiment. Other rat tissues including brain, heart, liver, spleen, eye, kidney, and aorta were immediately removed and instantly soaked in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  for further biochemical analysis.

**2.2. Ginseng Preparation.** *Panax ginseng* extract was prepared as described in Zhu et al. [9], which meets the requirement of the Chinese Pharmacopoeia and Hong Kong Standard of Chinese Materia Medica. Standardized ginseng extract (RSE) was prepared by ethanol extraction. The residue was then dissolved in water and partitioned successively with petroleum ether, EtOAc, and n-BuOH to give the petroleum-ether-soluble, EtOAc-soluble, and n-BuOH-soluble fractions. The n-BuOH extract was subjected to column chromatography eluted with a  $\text{CHCl}_3/\text{MeOH}$  gradient. Fractionated samples were combined and obtained according to the thin layer chromatography analysis. All samples were then stored in desiccated condition until further use. High performance liquid chromatography was used to confirm the identity of our samples with the standard ginsenosides (HPLC purity >98%) purchased from Chengdu Scholar Bio-Tech Co. Ltd. (Chengdu, China) or National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The contents of ginsenosides Rg1, Re, Rb1, Rc, Rb2, and Rd were 290.9, 339.6, 246.3, 231.3, 136.0, and 84.5 mg/g, respectively (Figure 1 and Table 1).

**2.3. Measurement of Contractile and Relaxant Responses in the Rat Aortic Rings.** Similar procedures were followed according to the protocol as described by Chan and Fiscus 2002 [10]. Briefly, thoracic aortae were isolated by cutting from the aortic arch to the diaphragm, resulting in a length of 30–40 mm tissue. In order to prevent physical damage of endothelium by forceps, the parts from the aortic arch were not used for experiment. Fat tissues were trimmed off from the aortae and before it was cut into 3 mm segments rings. The segments were then mounted carefully between two platinum hooks in 10 mL organ baths containing Krebs buffer (KRB) maintained at  $37^{\circ}\text{C}$  bubbled with 95%  $\text{O}_2$ –5%  $\text{CO}_2$  continuously. Following a 30 min equilibration period of resting tension of 1 gram, cumulative doses of phenylephrine

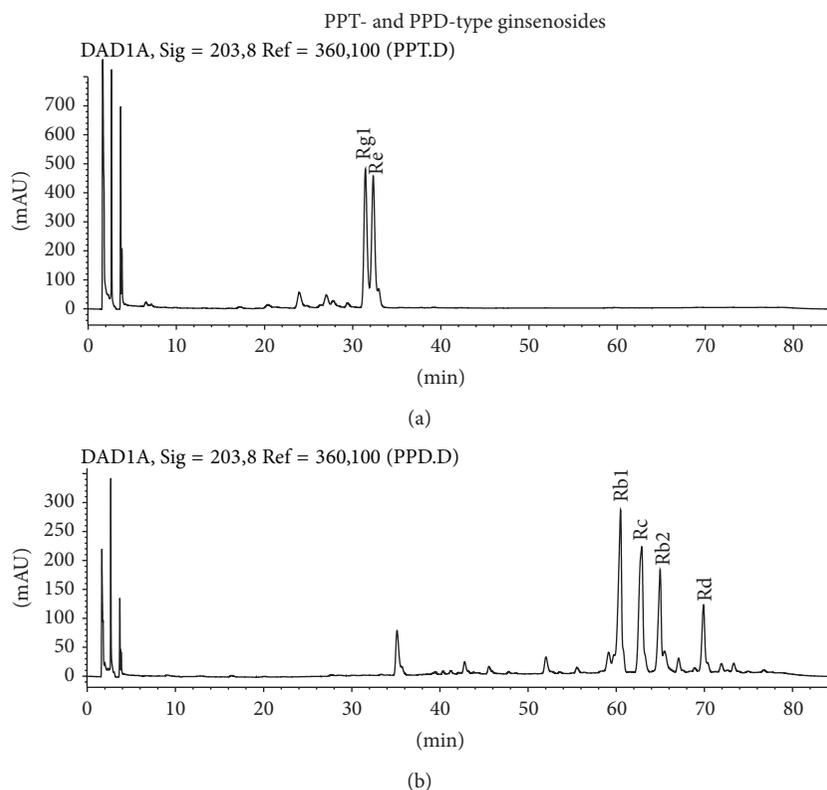


FIGURE 1: HPLC fingerprint of PPT-type (Panel (a)) and PPD-type (Panel (b)) ginseng extract used in current study. *Instruments.* An HP 1100 system (Hewlett-Packard, Wilmington, DE) consisting of a G1312A binary pump, a G1329A automatic sample injector, and a G1315A diode array detector was used to perform HPLC analysis. *Sample Preparation.* Approximately 0.20 g powdered ginseng was accurately weighed into a 50 mL conical flask, and 10 mL 70% methanol was added. The suspension was sonicated for 30 min, and the sample solution was filtered through a 0.45  $\mu\text{m}$  filter and used as the test solution for quantitative analysis of ginsenosides in Radix Ginseng. *Chromatographic Conditions.* HPLC analysis of Radix Ginseng was performed on an Alltima  $\text{C}_{18}$  HPLC column (4.6 mm  $\times$  250 mm, 5  $\mu\text{m}$ ) at 25°C with a sample injection volume of 20  $\mu\text{L}$ . The mobile phase was a gradient elution of  $\text{KH}_2\text{PO}_4$  buffer (2 mmol/L, pH 6.8) and acetonitrile, starting isocratically with 21% acetonitrile for 15 min and increasing to 38% acetonitrile over 55 min. The flow rate of the mobile phase was 1.0 mL/min, and the detector wavelength was 203 nm.

( $1 \times 10^{-9}$  M to  $1 \times 10^{-5}$  M) were added in each aortic ring. After the addition of phenylephrine, the aortic rings were washed with fresh and bubbled KRB solution every 10 minutes over a 30-minute period. A single dose of phenylephrine at  $1 \times 10^{-7}$  M was added until the aortic rings maintained 50 percent of maximum tension. Doses of acetylcholine ( $1 \times 10^{-9}$  M to  $1 \times 10^{-5}$  M) were added cumulatively to check the endothelial functions. All of the doses were added after the responses reached plateau.

**2.4. Blood Profile of the Experimental Rats.** Serum tests for total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), and high density lipoprotein (HDL) were conducted in The State Key laboratory for Chinese Medicine and Molecular Pharmacology of the Hong Kong Polytechnic University. Terminal colorimetric analysis method was used for quantification of TC, TG, LDL, and HDL, respectively. The tests were conducted with ECHO automatic biochemistry analyzer (Logotech, Italy) and UV2800 spectrophotometer (Unico, Shanghai).

Blood glucose level was measured using glucometer (Elite, Bayer Corporation, USA). Serum insulin and glycation end products were measured using kits purchased from Millipore (EZRMI-13K) and Cusabio (CSB-E08140r), respectively.

**2.5. RT<sup>2</sup> Profiler Rat Atherosclerosis PCR Array Analysis.** Aortic samples for PCR array analysis were used to obtain the total RNA by Qiagen RNeasy Mini Kit (Catalogue number: PARN-038A, Qiagen). This pathway specific RT-PCR array was used to evaluate the potential alterations of related genes after PPD-type and PPT-type treatments (30 mg/kg/day) in rats. The atherosclerosis array comprised of 87 genes selected based on their involvement in regulating vascular and endothelial cell homeostasis or inflammation. There were 5 housekeeping genes served as positive controls. Total RNA was reverse transcribed using the RT<sup>2</sup> First Strand Kit. Real-time PCR reactions were carried out on ABI 7500 (Applied Biosystems) using the RT<sup>2</sup> SYBR Green qPCR Mastermix (Qiagen) according to manufacturer's instructions. Data analysis was performed using the Qiagen's integrated

TABLE 1: Contents of ginsenosides in the prepared PPT-type and PPD-type ginsenosides.

Sample	Ginsenosides	Content (mg/g)	Percentage
PPT-type ginsenosides	Rg1	290.9	29.09%
	Re	339.6	33.96%
	Rb1	246.3	24.63%
PPD-type ginsenosides	Rc	231.30	23.13%
	Rb2	136.0	13.61%
	Rd	84.5	8.45%

web-based software package for the PCR Array System, which automatically performs all  $\Delta\Delta Ct$  based fold-change calculations from raw threshold cycle data.

**2.6. Statistical Analysis.** All values are expressed as mean  $\pm$  standard error of mean (SEM). The significant differences between the young and aged groups in the isolated tissue experiments were analyzed using one way ANOVA with Newman-Keuls multiple comparison as post hoc test in the statistical package (Graphpad prism v6.0). A  $P$  value less than 0.05 was considered to be significant. The mean values were obtained from at least 5 animals or 3 DNA samples per treatment group.

### 3. Results

**3.1. Ginseng Extracts Restore High Glucose-Induced Endothelial Dysfunction.** Acetylcholine (ACh) causes vasodilation by activation of endothelial nitric oxide synthase and prostaglandin production. The aortic tissue was challenged with acetylcholine ( $1 \times 10^{-9}$  M– $1 \times 10^{-5}$  M) and caused concentration-dependent relaxations in aortic rings from young rats. Normal rats showed 100% relaxation (restored the contracting state to resting state) at maximum dose  $1 \times 10^{-5}$  M, while the response was only 62.5% of the relaxation in the diabetic rats (Figure 2(a)), showing an impairment of the endothelium. For positive control, diabetic rats were injected with insulin and the normal vasorelaxation was maintained (Figure 2(b)). After feeding PPD-type and PPT-type ginseng extracts for two weeks, the impaired vasorelaxation due to high glucose level was restored (Figures 2(c) to 2(f)), indicating that the endothelial functions were maintained under the diabetic conditions for the ginseng-fed groups.

**3.2. Blood Profile, Body Weight, Distribution of Visceral Adipose Tissue, and Organs Weight of the Experimental Rats.** In this study, blood glucose, insulin, advanced glycation end products, serum total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), and triglyceride were examined. Except for normal and insulin injected positive control group, all of the diabetic groups were considered to be diabetic (blood glucose  $>16.7$  mmol/dL) and with a significant reduction of insulin level (Figure 3(a)). There are no statistical differences between the ginseng-fed or nonfed diabetic groups for blood glucose, insulin, serum total cholesterol, HDL, and LDL (Figures 3 and 5(a)–5(c)), indicating

the ginseng extracts have no improvement on hyperglycemic conditions or alternation of cholesterol levels. However, there is a slight decrease in the level of glycation end products (Figure 4), when the diabetic group was fed with PPT-type of ginseng at a dosage of 30 mg/kg/day. In addition, there was also significant decrease in serum triglyceride level for all ginseng-fed groups, which indicated that both PPD-type and PPT-type are effective in lowering serum triglyceride (Figure 5(d)). Visceral adipose tissue is associated with fatty acid metabolism. The distribution of visceral adipose tissue surrounding mesenteric arteries was shown in Figure 6. More visceral adipose tissue was found in control group when compared to the diabetic group. However, more visceral adipose tissue was observed in diabetic rats after feeding with PPD-type and PPT-type of ginseng extracts. The body mass and organ mass are the health indicators for the experimental rats. Figure 7 showed the body and organ weight of the experimental rats. The body weight of the insulin-injected diabetic groups is slightly larger than other groups. Among all organs measured (liver, pancreas, heart, adrenal gland, and kidneys), the PPD-type fed diabetic groups have significantly smaller adrenal glands than diabetic group ( $P < 0.05$ ).

**3.3. Ginseng Extract Suppresses the Expression of Atherosclerosis-Related Genes.** PCR array analysis showed the fold change of atherosclerosis-related gene expression (Figure 8 and Table 2) for different treatment groups. When compared to normal control group, diabetic groups showed an upregulation on several atherosclerosis-related gene expressions, which indicate an increased risk of atherosclerosis. Besides, the gene expressions related to inflammations including adhesion molecules such as selectin (platelet) and ICAM1 and macrophage activation including chemokine (C-C) motif ligand 2 (CCl-2), chemokine (C-X-C) motif ligand 1 (CxC1-1), interleukin 1 receptor 2 (IL1-R2), interleukins (IL3, IL4 and IL5), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were down-regulated. Apart from genes related to inflammation, other genes involved in the development of atherosclerosis were also checked. Apoptotic genes, such as Bid as well as genes responsible for vascular endothelial cells and vascular smooth muscle cell proliferation and migration (including von Willebrand factor homolog, heparin-binding EGF-like growth factor, and thrombospondin 4), were also downregulated in ginseng-fed diabetic groups. On the other hand, lipid-related genes expression including apolipoprotein E (ApoE), lipase, and peroxisome proliferator-activated receptor (PPAR)  $\gamma$  were increased in the ginseng-fed groups.

TABLE 2: PCR array analysis of expression change in selected atherosclerosis-related genes.

Gene name	Fold change <sup>#</sup>		
	Diabetes	PPD-fed diabetic group (30 mg/kg/day)	PPT-fed diabetic group (30 mg/kg/day)
<i>Adhesion molecules</i>			
Selectin (platelet)	+2.33	+1.18	+1.29
Intercellular adhesion molecule 1	+2.06	+1.46	+1.46
<i>Macrophages</i>			
Chemokine (C-C) motif ligand 2	+2.49	+0.56	+0.47
Chemokine (C-X-C) motif ligand 1	+3.19	+0.89	+0.58
Interleukin 1 receptor, type II	+3.28	+1.22	+1.38
Interleukin 3	+0.95	+0.48	+0.82
Interleukin 4	+1.23	+0.53	+0.83
Interleukin 5	+1.08	+0.78	+0.79
Tumor necrosis factor- $\alpha$	+1.56	+1.31	+1.03
<i>Lipid metabolism</i>			
Apolipoprotein E	+1.53	+2.95	+2.50
Lipase	+13.85	+24.93	+8.82
Peroxisome proliferator-activated receptor- $\gamma$	+2.54	+5.83	+2.06
<i>Cell growth and migration</i>			
Fibrinogen beta chain	+0.93	+0.43	+0.62
von Willebrand factor homolog	+4.45	+3.33	+2.99
Heparin-binding EGF-like growth factor	+2.53	+1.95	+2.04
Laminin $\alpha$ 1	+0.93	+0.64	+0.61
<i>Extracellular matrix (ECM)</i>			
Fibronectin	+2.91	+1.94	+1.66
<i>Apoptosis</i>			
Bcl2-like 1	+0.73	+0.75	+0.83
BH3 interacting domain death agonist	+1.45	+1.25	+1.23

<sup>#</sup>Fold changes (comparing to control group, fold change = 1) are calculated according to manufacturer's analysis software.

In general, the ginseng-fed diabetic groups showed a decreased expression on atherosclerosis-related genes, which indicates the decreased risk of atherosclerosis after ginseng treatments.

#### 4. Discussion

Endothelium controls vascular tone through the production of vasodilator mediators, endothelium-derived relaxing factors (EDRF), which act on vascular smooth muscle cells. The EDRF comprise nitric oxide (NO), prostacyclin, and an elusive endothelium-derived hyperpolarizing factor (EDHF). Multiple mechanisms lead to endothelial dysfunction [11, 12], and endothelial dysfunction plays a key role in the pathogenesis of vascular diseases. Hyperglycemia is linked to the pathogenesis of diabetic complications involving alternations of intracellular metabolism and formation of advanced glycation end products.

The attenuated endothelium-dependent vasodilations have been demonstrated in various vascular tissues of diabetic animal model [13]. In the present study, we examined

the endothelial functions using the physiological isolated tissue bath setup and found that the high glucose-impaired vasodilations were restored after ginseng extracts treatment (Figure 2). The result indicates that ginseng extract plays a protective role in restoring normal endothelial functions in diabetic models. Different molecular mechanisms have been demonstrated to cause the vascular dysfunctions. Reports have suggested that hyperglycaemia-induced endothelial dysfunction is due to activation of protein kinase C (PKC) [14], inhibition of endothelial nitric oxide synthase [15, 16], early and advanced nonenzymatic glycation, and oxidative stress [17–19]. In atherosclerotic conditions, up-regulation of adhesion molecules, increased cytokine secretion, apoptosis, enhanced low-density lipoprotein oxidation, platelet activation, and vascular smooth muscle cell proliferation and migration are always observed [20–22]. Therefore, compounds that are able to modulate atherosclerosis and maintain normal endothelial functions are highly desirable.

Although the levels of LDL, TC, HDL, and insulin in diabetic group were not significantly different when compared with the ginseng-fed groups, ginseng-fed diabetic

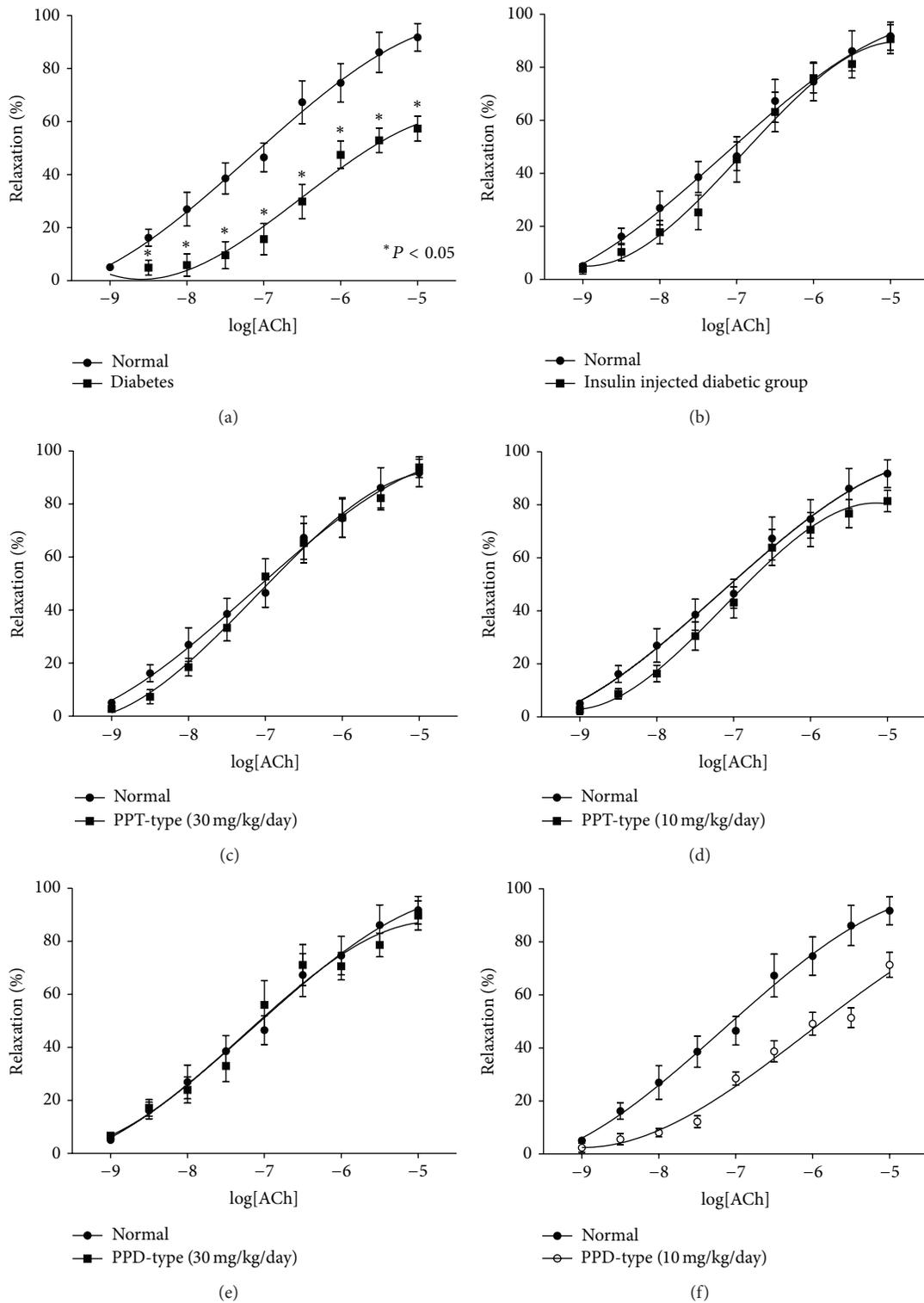


FIGURE 2: Ginseng extracts restore acetylcholine-induced endothelium dependent vasorelaxation. After the addition of phenylephrine, doses of acetylcholine ( $1 \times 10^{-9}$  M to  $1 \times 10^{-5}$  M) were added cumulatively to check the endothelial functions. Control group showed an attenuation of acetylcholine-induced vasorelaxation (Panel (a)). The insulin injected diabetic group (Panel (b)), PPT-type (30 mg/kg/day) fed diabetic group (Panel (c)), PPT-type (10 mg/kg/day) fed diabetic group (Panel (d)), PPD-type (30 mg/kg/day) fed diabetic group (Panel (e)), and PPD-type (10 mg/kg/day) (Panel (f)) fed diabetic group showed restoration of the attenuated vasorelaxation. Results were expressed as the mean  $\pm$  standard error; \*  $P < 0.05$  for the indicated comparisons.

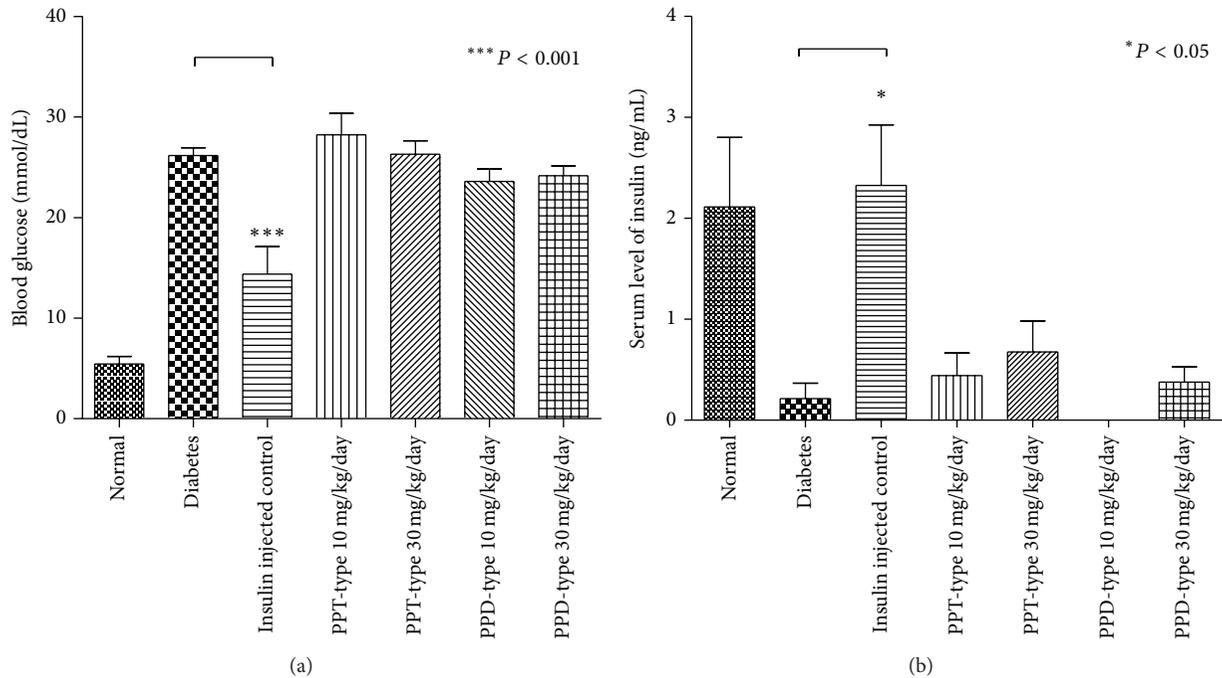


FIGURE 3: Blood glucose level (Panel (a)) and serum insulin level (Panel (b)) of control, diabetic, and ginseng extract-fed diabetic groups. The bar indicates standard error; \*  $P < 0.05$  for the indicated comparisons versus diabetic group.

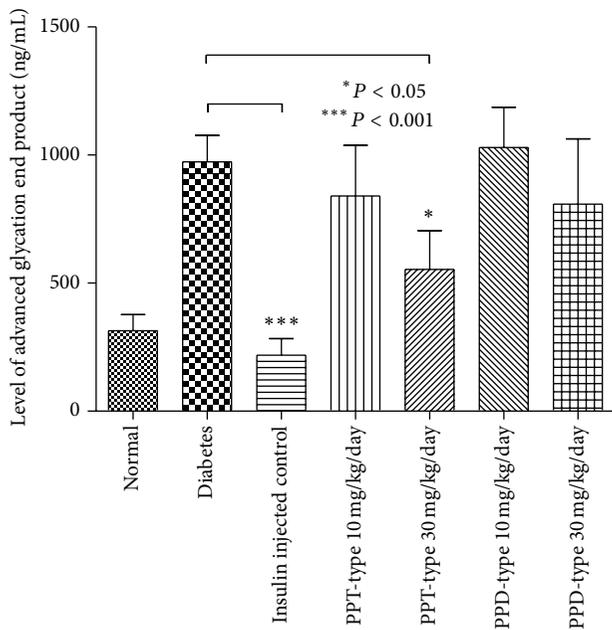


FIGURE 4: Level of advanced glycation end product in serum of control, diabetic, and ginseng extract-fed diabetic groups. Results were expressed as the mean  $\pm$  standard error; \*  $P < 0.05$  for the indicated comparisons versus diabetic group.

groups showed a decrease in serum triglyceride level after ginseng feeding (Figure 5(d)). Increased levels of serum triglyceride and free fatty acids are common features of

diabetic dyslipidemia [23]. There are direct correlations of serum triglyceride with triglyceride-associated nonalcoholic fatty liver disease (NAFLD), which is a multifactorial syndrome linked with cardiovascular diseases [24]. To further investigate the underlying mechanisms of the altered triglyceride metabolism, we performed PCR array analysis to examine the changes of gene expressions in rat aorta after ginseng treatment. By comparing normal, diabetic, and ginseng-fed diabetic groups, we have studied the change of expression in 87 different atherosclerosis or lipid metabolism related genes. Several lipid metabolism related genes such as ApoE, lipase, and PPAR- $\gamma$  are upregulated in the aorta of ginseng extract-fed groups when compared to diabetic control group, showing the beneficial effects of ginseng. ApoE is responsible for catabolism of triglyceride-rich lipoprotein and cardiovascular diseases and was found to be related to proinflammatory cytokines [25]. On the other hand, up-regulated gene expression of lipase leads to increase process of dietary lipids (e.g., triglyceride) which may explain the decreased triglyceride levels. PPAR- $\gamma$  is up-regulated by PPD, and it is the target of thiazolidinediones, the drugs used in treatment of diabetes mellitus. The upregulations of these genes provide possible explanation to the lowered triglyceride levels.

There is no statistically significant difference in body weight among the normal and the diabetic groups, possibly due to large variations of body weights of diabetic groups. Interestingly, the insulin-injected diabetic control group has significant weight gain (Figure 7(a)). The weight gain in the insulin-injected diabetic group has been reported by Jansen et al. in 2010 [26], which may be due to insulin therapy.

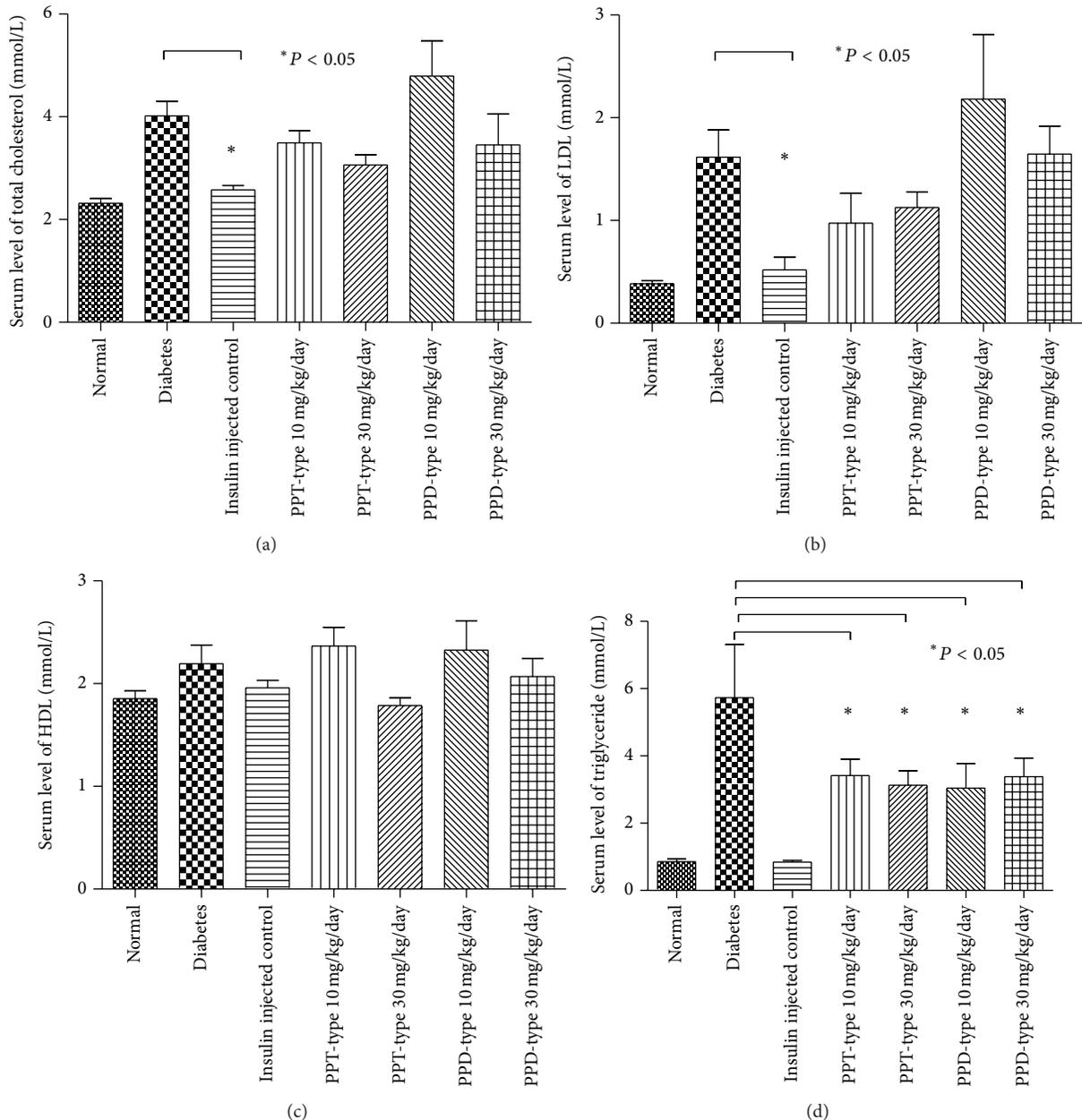


FIGURE 5: Serum levels of total cholesterol (Panel (a)), LDL (Panel (b)), HDL (Panel (c)), and triglyceride (Panel (d)) in control, diabetic, and ginseng extract-fed diabetic groups. Results were expressed as the mean  $\pm$  standard error;  $*P < 0.05$  for the indicated comparisons versus diabetic group.

The weight of adrenal glands (Figure 7(b)) is significantly smaller in the groups fed with PPD-type groups of ginseng extract except the liver (Figure 7(c)); other organs including pancreas, heart, and kidneys are not significantly different in weight among all diabetic groups (Figures 7(d)–7(f)).

Known to be responsible for “fight-or-flight” response, the size of adrenal glands reflects adrenocorticoid secretion [27], and adrenal enlargement is directly related to stress [28] like diabetes mellitus [29]. Interestingly, though insulin therapy is the known most effective method for diabetes, it cannot reverse adrenal gland enlargement. This may due to intensive

injection of insulin which imposed stress on the rats. However, PPD-type extract, at both dosages of 10 mg/kg/day and 30 mg/kg/day, can reduce the size of enlarged adrenal glands in diabetic groups significantly (Figure 7(b)).

It has been shown with evidence that endothelial apoptosis might be a major cause of plaque erosion [30]. If there is endothelial apoptosis, lipid-laden foam cells derived from macrophages produce phospholipid oxidation products (OX-PL) and play a role in atherosclerosis. There are two forms of atherosclerotic plaques, (1) stable plaque, which is made up of thick fibrous cap isolating small lipid core and

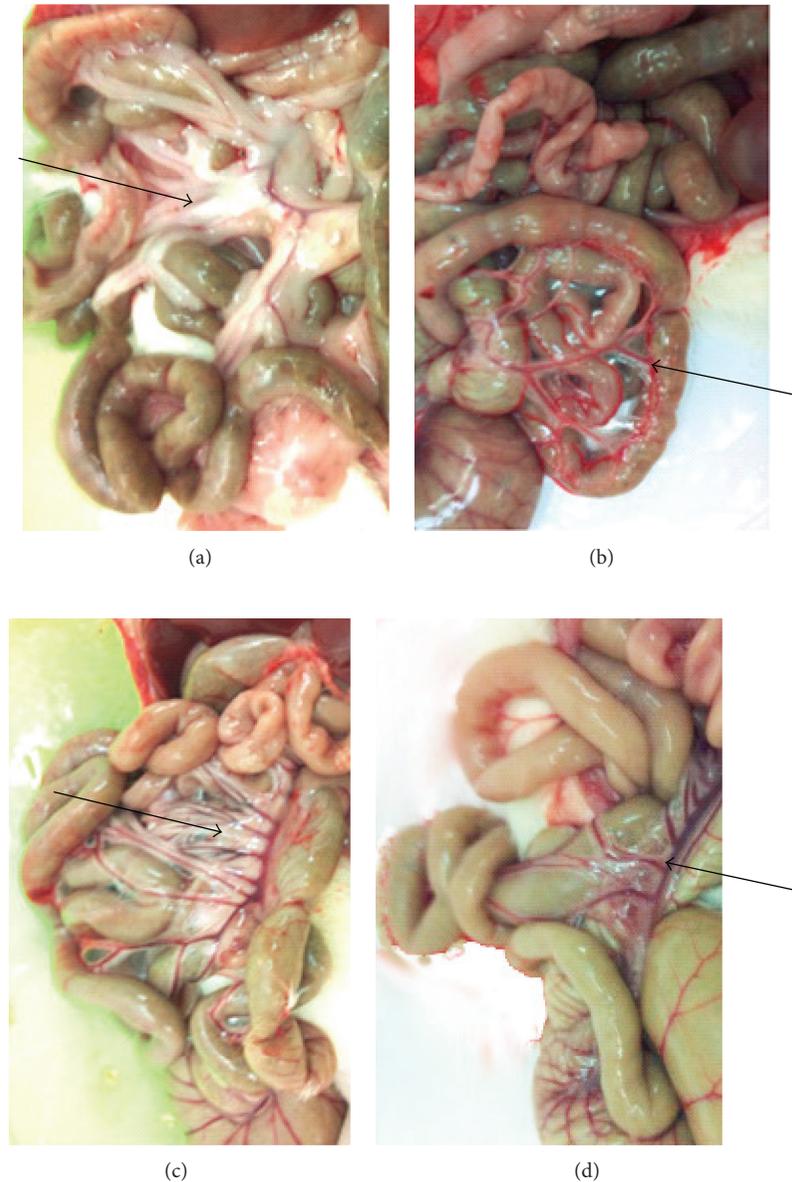


FIGURE 6: Distribution of visceral adipose tissue surrounding mesenteric arteries. The mesenteric bed from normal rats (Panel (a)) is surrounded by adipose tissue, whereas the mesenteric bed of diabetic rats (Panel (b)) is not surrounded by any adipose tissue. The PPD-type fed diabetic group (Panel (c)) and PPT-type fed diabetic group (Panel (d)) have comparatively more adipose tissue than the diabetic group.

associated with low risk of thromboembolic complications, and (2) unstable plaque, which is a large lipid core covered by thin fibrous cap and prone to rupture and thrombus formation and associated with high risk of thromboembolic complications [31]. Hence, in addition to the decrease in triglyceride levels and changes in lipid metabolism related genes expression, the decrease in apoptosis-related genes such as Bcl2-like 1 and Bid may also help to reduce risk of atherosclerosis and restore normal aorta vasorelaxation. Figure 6 shows that there is more visceral adipose tissue in the ginseng-fed groups, and the observation may be related to the altered lipid metabolism in diabetic conditions. It is known that visceral adipose tissue is linked to fatty

acid metabolism [32]. However, as most of the researches focus on the adverse effects of visceral adipose tissue which is a common observation in obesity, we cannot find any evidence to explain the current phenomenon. However, it has been found that ginsenoside Rb1 promotes adipogenesis in 3T3-L1 cells by enhancing PPAR- $\gamma$ 2 and C/EBP- $\alpha$  functions [33]. According to our present data on increased PPAR- $\gamma$  expression, we believe that ginseng extract can modulate lipid metabolism in the diabetic condition through altered gene expression, which may result in an increased amount of visceral adipose tissue.

Furthermore, similar observation has been reported by a recent paper published by Liu et al. [34], who showed that

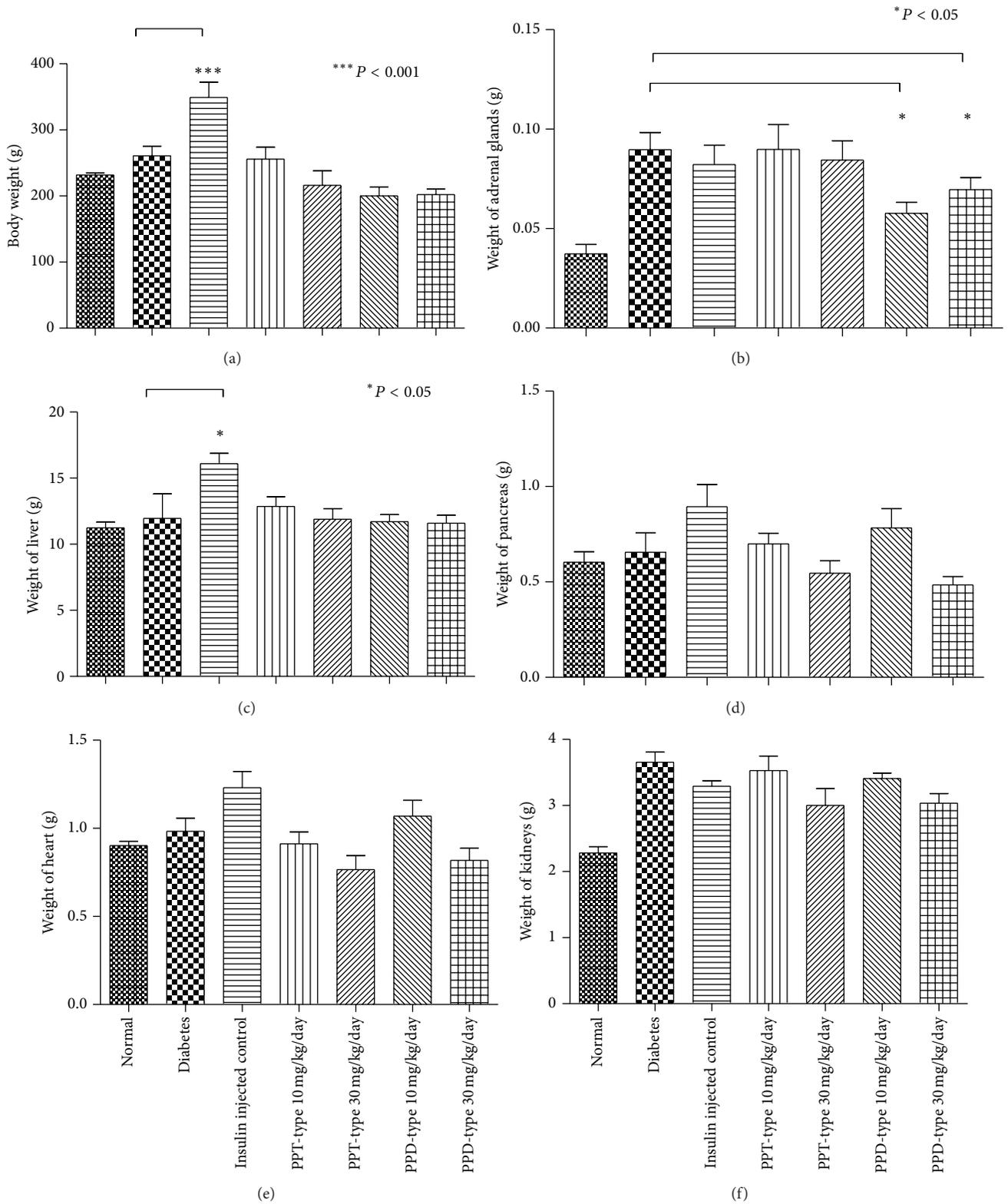


FIGURE 7: Weights of rats (Panel (a)) and weights of adrenal gland (Panel (b)), liver (Panel (c)), pancreas (Panel (d)), hearts (Panel (e)), and kidneys (Panel (f)). The insulin injected control group is slightly heavier than other groups. Results were expressed as the mean  $\pm$  standard error; \*  $P < 0.05$  for the indicated comparisons versus diabetic group.

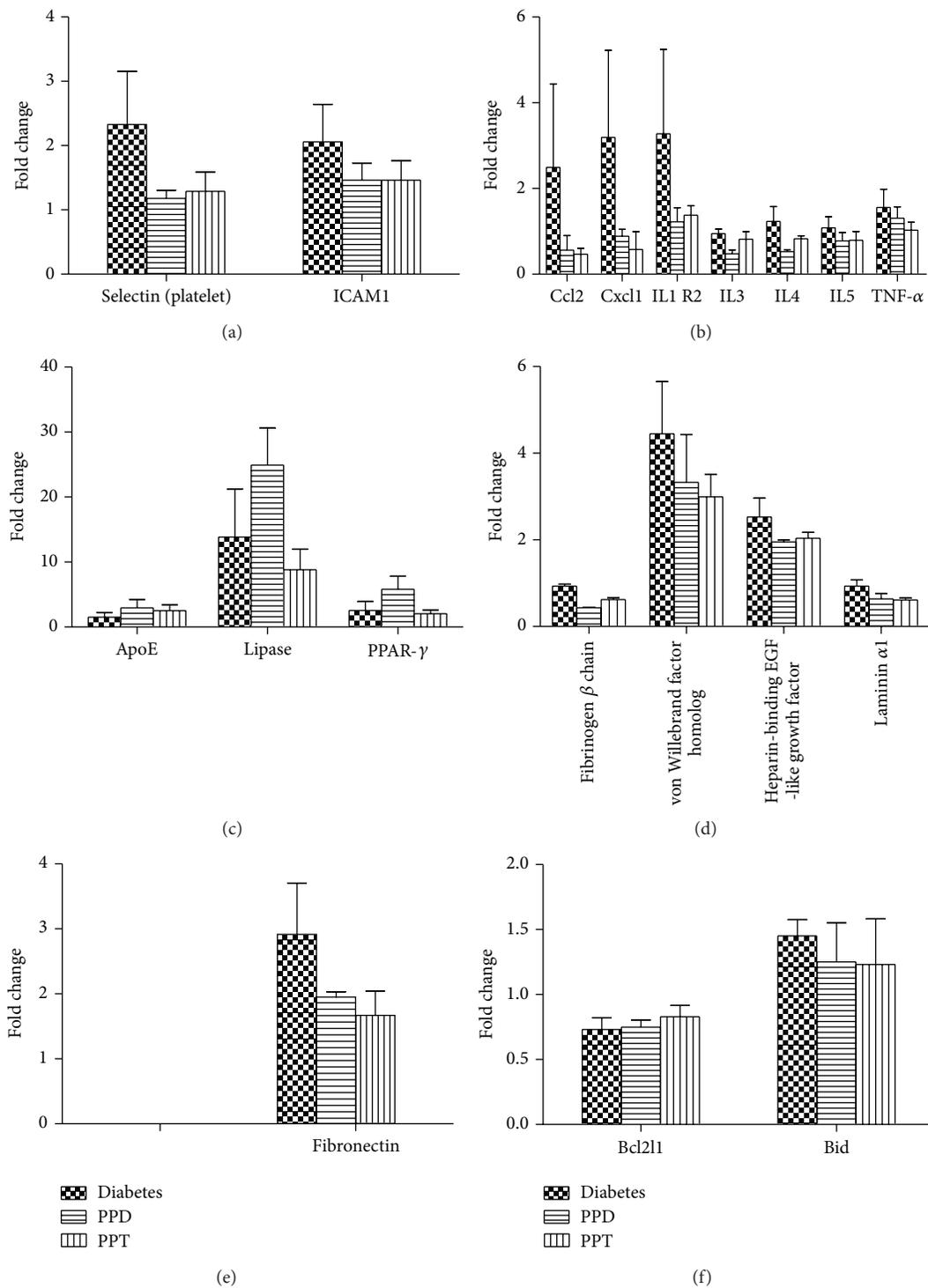


FIGURE 8: Comparison of different atherosclerosis related-gene expressions on adhesion molecules (Panel (a)), macrophages (Panel (b)), lipid metabolism (Panel (c)), smooth muscle cells proliferation and migration (Panel (d)), extracellular matrix (Panel (e)), and apoptosis (Panel (f)) by PCR array analysis. The PPD and PPT groups were fed with PPD-type and PPT-type of ginseng extract at dosage of 30/mg/kg/day, respectively. The fold change for normal control was set at 1.

ginsenosides can lower both triglyceride and total cholesterol level. In contrast, our results demonstrated a decrease in only the triglyceride level by using a lower dosage of ginseng extract. Therefore, we believe that ginseng may have potential therapeutic effects on elevated lipid levels and this effect may be dose dependent.

Although glucose level is not lowered in all ginseng-fed diabetic groups, PPT-type ginseng extract fed at a dose of 30 mg/kg/day decreased the level of glycation end products in diabetic rats (Figure 4). Glycation end-product can be formed exogenously by heating or cooking or endogenously through normal aging or accelerated formation under diabetic conditions. The glycation process yields two different products: early and advanced glycation endproducts (AGEs). Recent finding shows advanced glycation end products formed on haemoglobin and HbA1c, which is a well-established important indicator for glycaemia monitoring. The advanced glycation end products that accumulate in vascular tissues are likely related to alterations in the connective tissue composition of the microvascular wall, which results in increased tissue rigidity [11]. During the pathogenesis of diabetes, endothelial cells intake more glucose [35, 36] and in turn increase the proton gradient and eventually produce reactive oxygen species and damaged DNA and more glycation end products will be produced. In other words, lower levels of advanced glycation end products usually show less hyperglycemic damages.

Biomarkers for accurately predicting clinical outcome and assessing disease risk and progression would greatly facilitate cardiovascular disease diagnosis and therapy. Finding the right balance between safety and efficacy of therapeutic methods probably requires assessing a variety of anti-inflammatory mechanisms and so forth [37]. Figure 8 showed a panel of atherosclerosis genes (including genes related to adhesion molecules, inflammation, vascular cell proliferation, and migration) which are downregulated. The findings may bring beneficial therapeutic implications to the vascular complications in diabetes. However, the underlying mechanisms, for example, increased lipid metabolism but observable increased amount of visceral adipose tissue, remain to be determined.

## 5. Conclusion

Ancient pieces of literature *Shennong Ben Cao Jing* and *Kai Bao Ben Cao* have mentioned the potential antiobesity and antidiabetic effects of *Panax ginseng*. Here, our present study proves that the endothelium-dependent relaxation can be impaired by diabetes mellitus and the damage can be protected by feeding with ginseng extracts (both PPD-type and PPT-type). Furthermore, the PCR array result reveals that ginseng may exert endothelial protection effect by downregulating the gene expressions of adhesion molecules, inflammatory cytokines, and chemokines. The protective mechanisms may partially due to lowering of serum triglyceride levels or alternating atherosclerosis-related and lipid-related gene expressions, which may result in anti-inflammation and endothelial cell protection. Therefore, further studies would

be required to differentiate the protective mechanisms by individual ginsenosides in the future.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Gabriel Hoi-huen Chan and Betty Yuen-kwan Law contributed equally to this work and should be considered co-first authors.

## Acknowledgments

This work was supported by Strategic Development Fund of HKBU (SDF 11-0117-P05) awarded to Professor Ricky Ngokshun Wong. The authors would like to give special thanks to Dr. AE James, Director of the Laboratory Animal Services Center at The Chinese University of Hong Kong, Mr. L. W. Lam, and Dr. T. W. C. Lo for their technical assistance.

## References

- [1] J.-M. Lü, Q. Yao, and C. Chen, "Ginseng compounds: an update on their molecular mechanisms and medical applications," *Current Vascular Pharmacology*, vol. 7, no. 3, pp. 293–302, 2009.
- [2] M. Karmazyn, M. Moey, and X. T. Gan, "Therapeutic potential of ginseng in the management of cardiovascular disorders," *Drugs*, vol. 71, no. 15, pp. 1989–2008, 2011.
- [3] R. Ross and J. A. Glomset, "The pathogenesis of atherosclerosis," *New England Journal of Medicine*, vol. 295, no. 7, pp. 369–377, 1976.
- [4] R. F. Furchgott and J. V. Zawadzki, "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine," *Nature*, vol. 288, no. 5789, pp. 373–376, 1980.
- [5] A. Faggiotto, R. Ross, and L. Harker, "Studies of hypercholesterolemia in the nonhuman primate—I. Changes that lead to fatty streak formation," *Arteriosclerosis*, vol. 4, no. 4, pp. 323–340, 1984.
- [6] G. K. Hansson and A. Hermansson, "The immune system in atherosclerosis," *Nature Immunology*, vol. 12, no. 3, pp. 204–212, 2011.
- [7] P. Libby, P. M. Ridker, and G. K. Hansson, "Progress and challenges in translating the biology of atherosclerosis," *Nature*, vol. 473, no. 7347, pp. 317–325, 2011.
- [8] H.-K. Kuo, P.-C. Wu, C.-N. Kuo, and Y.-H. Chen, "Effect of insulin on the expression of intraocular vascular endothelial growth factor in diabetic rats," *Chang Gung Medical Journal*, vol. 29, no. 6, pp. 555–560, 2006.
- [9] G.-Y. Zhu, Y.-W. Li, D. K.-P. Hau, Z.-H. Jiang, Z.-L. Yu, and W.-F. Fong, "Protopanaxatriol-type ginsenosides from the root of *Panax ginseng*," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 1, pp. 200–205, 2011.
- [10] G. H. H. Chan and R. R. Fiscus, "Severe impairment of CGRP-induced hypotension in vivo and vasorelaxation in vitro in elderly rats," *European Journal of Pharmacology*, vol. 434, no. 3, pp. 133–139, 2002.

- [11] G. M. Rubanyi, "Endothelium-derived relaxing and contracting factors," *Journal of Cellular Biochemistry*, vol. 46, no. 1, pp. 27–36, 1991.
- [12] A. S. De Vriese, T. J. Verbeuren, J. Van De Voorde, N. H. Lameire, and P. M. Vanhoutte, "Endothelial dysfunction in diabetes," *British Journal of Pharmacology*, vol. 130, no. 5, pp. 963–974, 2000.
- [13] C. H. Leo, J. L. Hart, and O. L. Woodman, "Impairment of both nitric oxide-mediated and EDHF-type relaxation in small mesenteric arteries from rats with streptozotocin-induced diabetes," *British Journal of Pharmacology*, vol. 162, no. 2, pp. 365–377, 2011.
- [14] K. Taguchi, T. Kobayashi, T. Matsumoto, and K. Kamata, "Dysfunction of endothelium-dependent relaxation to insulin via PKC-mediated GRK2/Akt activation in aortas of ob/ob mice," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 301, no. 2, pp. H571–H583, 2011.
- [15] E. Linden, W. Cai, J. C. He et al., "Endothelial dysfunction in patients with chronic kidney disease results from advanced glycation end products (AGE)-mediated inhibition of endothelial nitric oxide synthase through RAGE activation," *Clinical Journal of the American Society of Nephrology*, vol. 3, no. 3, pp. 691–698, 2008.
- [16] D. N. Atochin and P. L. Huang, "Endothelial nitric oxide synthase transgenic models of endothelial dysfunction," *Pflugers Archiv European Journal of Physiology*, vol. 460, no. 6, pp. 965–974, 2010.
- [17] S.-I. Yamagishi and T. Matsui, "Advanced glycation end products, oxidative stress and diabetic nephropathy," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 2, pp. 101–108, 2010.
- [18] Z. Hegab, S. Gibbons, L. Neyses, and M. A. Mamas, "Role of advanced glycation end products in cardiovascular disease," *World Journal of Cardiology*, vol. 4, no. 4, pp. 90–102, 2012.
- [19] C. G. Schalkwijk and T. Miyata, "Early- and advanced non-enzymatic glycation in diabetic vascular complications: the search for therapeutics," *Amino Acids*, vol. 42, no. 4, pp. 1193–1204, 2012.
- [20] J. K. Liao, "Endothelium and acute coronary syndromes," *Clinical Chemistry*, vol. 44, no. 8, pp. 1799–1808, 1998.
- [21] A. N. N. Mertens and P. Holvoet, "Oxidized LDL and HDL: antagonists in atherothrombosis," *FASEB Journal*, vol. 15, no. 12, pp. 2073–2084, 2001.
- [22] P. L. Faries, D. I. Rohan, M. C. Wyers et al., "Vascular smooth muscle cells derived from atherosclerotic human arteries exhibit greater adhesion, migration, and proliferation than venous cells," *Journal of Surgical Research*, vol. 104, no. 1, pp. 22–28, 2002.
- [23] L. Rossetti and I. J. Goldberg, "A new piece in the diabetes puzzle," *Nature Medicine*, vol. 8, no. 2, pp. 112–114, 2002.
- [24] K. Hosoyamada, H. Uto, and Y. Imamura, "Fatty liver in men is associated with high serum levels of small, dense low-density lipoprotein cholesterol," *Diabetology & Metabolic Syndrome*, vol. 4, no. 1, article 34, 2012.
- [25] L. Liu, O. Aboud, R. A. Jones, R. E. Mrak, W. S. T. Griffin, and S. W. Barger, "Apolipoprotein E expression is elevated by interleukin 1 and other interleukin 1-induced factors," *Journal of Neuroinflammation*, vol. 8, article 175, 2011.
- [26] H. J. Jansen, G. Vervoort, M. Van der Graaf, and C. J. Tack, "Pronounced weight gain in insulin-treated patients with type 2 diabetes mellitus is associated with an unfavourable cardiometabolic risk profile," *Netherlands Journal of Medicine*, vol. 68, no. 11, pp. 359–366, 2010.
- [27] L. Adams and S. Hane, "Adrenal gland size as an index of adrenocortical secretion rate in the California ground squirrel," *Journal of Wildlife Diseases*, vol. 8, no. 1, pp. 19–23, 1972.
- [28] P. Du Ruisseau, Y. Taché, and H. Selye, "Effects of chronic stress on pituitary hormone release induced by combined hemi extirpation of the thyroid, adrenal and ovary in rats," *Neuroendocrinology*, vol. 24, no. 3-4, pp. 169–182, 1977.
- [29] P. Naeser, "Adrenal function in the diabetic mutant mouse (gene symbol dbm)," *Acta Physiologica Scandinavica*, vol. 98, no. 4, pp. 395–399, 1976.
- [30] E. Durand, A. Scoazec, A. Lafont et al., "In vivo induction of endothelial apoptosis leads to vessel thrombosis and endothelial denudation: a clue to the understanding of the mechanisms of thrombotic plaque erosion," *Circulation*, vol. 109, no. 21, pp. 2503–2506, 2004.
- [31] V. Fuster, Z. A. Fayad, and J. J. Badimon, "Acute coronary syndromes: biology," *The Lancet*, vol. 353, no. 2, pp. 5–9, 1999.
- [32] J. O. Ebbert and M. D. Jensen, "Fat depots, free fatty acids, and dyslipidemia," *Nutrients*, vol. 5, no. 2, pp. 498–508, 2013.
- [33] W. Shang, Y. Yang, B. Jiang et al., "Ginsenoside Rb1 promotes adipogenesis in 3T3-L1 cells by enhancing PPAR $\gamma$ 2 and C/EBP $\alpha$  gene expression," *Life Sciences*, vol. 80, no. 7, pp. 618–625, 2007.
- [34] Z. Liu, W. Li, X. Li et al., "Antidiabetic effects of malonyl ginsenosides from *Panax ginseng* on type 2 diabetic rats induced by high-fat diet and streptozotocin," *Journal of Ethnopharmacology*, vol. 145, no. 1, pp. 233–240, 2013.
- [35] M. H. Dominiczak, "Obesity, glucose intolerance and diabetes and their links to cardiovascular disease. Implications for laboratory medicine," *Clinical Chemistry and Laboratory Medicine*, vol. 41, no. 9, pp. 1266–1278, 2003.
- [36] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.
- [37] I. F. Charo and R. Taub, "Anti-inflammatory therapeutics for the treatment of atherosclerosis," *Nature Reviews Drug Discovery*, vol. 10, no. 5, pp. 365–376, 2011.

## Research Article

# Gene Expression Profiling on the Molecular Action of Danshen-Gegen Formula in a Randomized Placebo-Controlled Trial of Postmenopausal Women with Hypercholesterolemia

**Chi-Man Koon,<sup>1,2</sup> Chun-Hay Ko,<sup>1,2</sup> Xu-Xu Sun,<sup>1,2</sup> Sandy Wan-Heng Hoi,<sup>1,2,3</sup> Jacqueline Chor-Wing Tam,<sup>1,2</sup> David Wing-Shing Cheung,<sup>1,2</sup> King-Fai Cheng,<sup>1,2</sup> Suet-Yee Pang,<sup>1,2</sup> Wing-Man Lo,<sup>1,2</sup> Ping Chook,<sup>1,2</sup> Clara Bik-San Lau,<sup>1,2</sup> Wai-Yee Chan,<sup>3</sup> Ping-Chung Leung,<sup>1,2</sup> Timothy Chi-Yui Kwok,<sup>4</sup> and Kwok-Pui Fung<sup>1,2,3,5</sup>**

<sup>1</sup> Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

<sup>2</sup> State Key Laboratory of Phytochemistry & Plant Resources in West China, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

<sup>3</sup> School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

<sup>4</sup> Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

<sup>5</sup> The Chinese University of Hong Kong-Zhejiang University Joint Laboratory on Natural Products and Toxicology Research, Shatin, New Territories, Hong Kong

Correspondence should be addressed to Kwok-Pui Fung; [kpfung@cuhk.edu.hk](mailto:kpfung@cuhk.edu.hk)

Received 22 March 2013; Revised 28 May 2013; Accepted 16 June 2013

Academic Editor: Myeong S. Lee

Copyright © 2013 Chi-Man Koon et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The Danshen-Gegen formula (DG) is a traditional Chinese herbal formula which has long been used to treat cardiovascular disease. DG was found to be a cardiovascular tonic in our recent research. However, a comprehensive investigation of the molecular mechanism of DG in cardiovascular disease has not been performed. The aim of this study was to clarify the transcriptional profiling of genes modulated by DG on postmenopausal women by using DNA microarray technology. We obtained 29 whole blood samples both from DG-treated and placebo-treated subjects. Blood lipid profile and intima-media thickness (IMT) were measured. Affymetrix GeneChip was used to identify differentially expressed genes (DEGs), followed by validation by the real-time PCR method. The results showed that DG-treated group has a significant improvement in IMT and lipid profile as compared to placebo-treated group. For the genomic study, the DG-treated group has a higher number of DEGs identified as compared to the placebo-treated group. Two important biological processes of “regulation of systemic arterial blood pressure by hormone” and “regulation of smooth muscle proliferation” have been identified by GePS in the DG-treated group. No significant biological process and cellular components were identified in the placebo-treated group. This genomic study on the molecular action of DG in postmenopausal women gathered sufficient molecular targets and pathways to reveal that DG could improve neointima thickening and hypertension.

## 1. Introduction

The term “postmenopause” is applied to women who have not experienced menstruation for a minimum of 12 months. The increasing incidence of cardiovascular disease in postmenopausal women could be related to estrogen deprivation which has unfavorable effects on blood vessels, lipid profile, and blood pressure [1]. The most common postmenopause treatment is hormone replacement therapy (HRT). HRT

introduces synthetic hormones into a woman's body and helps to eliminate postmenopausal symptoms. However, HRT has many side-effects, such as heart disease, blood clots, and breast cancer, ovarian cancer [2]. Besides medical therapy, another option to help postmenopausal women to alleviate their symptoms is alternative medicine.

Traditional Chinese medicine (TCM) is one of the alternative medicines which originated in ancient China and

which has evolved over thousands of years ago. TCM is not only commonly used in Asian countries but also in America because it has fewer side effects as compared to prescription medicines. Prescription medicine always has a specific action with strong efficiency in treating a specific syndrome, but it can produce side-effects if there is long-term usage. However, most diseases, especially the chronic diseases such as cardiovascular diseases (CVDs), are multifactorial. CVDs have many risk factors, such as hyperlipidemia, hypertension, and vascular thickening (e.g., IMT) in which IMT is used to track the regression, arrest, or progression of atherosclerosis. Patients very often need to take a series of medications. TCM always comes in a combination of herbs with various chemical components that may act in an additive or synergistic fashion [3]. The biological action of the multiple chemical components of TCM may show multitargeting effects and act through multiple molecular pathways in order to achieve a balance of body functions.

Danshen and Gegen formula (DG) is a modified herbal formula used in traditional Chinese medicine; it consists of two herbs which exhibited prominent effects on hypertension [4–6], atherosclerosis [3, 7, 8], and cardioprotection [9–11] in our laboratory studies. In our previous clinical trial, this formula improved the cardiovascular functions in patients, such as producing a mild decrease in low-density lipoprotein (LDL), improved brachial flow-mediated dilation (FMD), and IMT; such patients having severe cardiovascular problems [12]. The current clinical study aimed at using DG to diminish the process of cardiovascular deterioration in postmenopausal women by measuring the IMT, lipid profile, and blood pressure. Secondly, this genomic study employed the use of whole blood samples to serve as a platform and it aimed to correlate the molecular action of DG with clinical outcomes by using the DNA microarray technology. Furthermore, the application of microarray technology and associated bioinformatic data mining tools could simultaneously allow the analysis of a large number of molecular targets which are possibly associated with the biological effects of DG.

## 2. Materials and Methods

**2.1. Herbal Preparation and Identification of Chemical Markers.** The procedures used to make the herbal preparation and the identification of chemical markers were the same as in our previous report [5]. In brief, the raw materials were washed to remove all trace particles and contaminants. The raw components were then cut into a uniform size and mixed in the weight ratio of Danshen to Gegen of 7:3 (DGW). The raw mixture was allowed to soak with 10-fold of water (v/w) for 1.5 hours followed by extraction at 100°C for 1 hour. The residue was then subjected to two more subsequent extractions for 1 hour and then for half an hour. The combined extracts were then dried at –660 mmHg and 60°C and stored in desiccators at room temperature for future use. The dried herbal extract was dissolved in a culture medium and sterilized by filtration before being added to the cells. The liquid chromatographic system which

we used was the HPLC system equipped with a photodiode array (PDA) UV detector. The chromatographic separation of the chemical markers was achieved by using an Agilent Eclipse XDB-C18 column. A gradient elution (1 mL/min) was achieved by using 0.5% acetic acid in acetonitrile (solvent A) and 0.5% acetic acid in water (solvent B) as the mobile phases.

**2.2. Study Design and Populations.** All subjects were 45–65-year-old postmenopausal women whose menstruation had ceased more than 12 months previously and whose fasting serum LDL was  $\geq 3.5$  mmol/L and  $< 4.9$  mmol/L. None of the subjects were taking hormones, statins, and nutritional supplements. Furthermore, women who have had children and who have a normal diet (nonvegetarian) were eligible. The detailed inclusion and exclusion criteria of this clinical trial are listed in the supplementary information available online at <http://dx.doi.org/10.1155/2013/703705>.

Eligible subjects were screened at the CUHK Chinese Medicine Research Centre. After signing written informed consents, they were clinically assessed. Then the subjects were randomized to take two capsules of DG (1 gram), or two capsules of an image-identical placebo (1 gram) daily, in double-blind and parallel fashion for 12 months. The placebo capsule contained medical starch and a natural sugar-processed pigment in a 2:1 ratio. Peripheral blood samples were collected from the subjects before and after DG or placebo treatment. Clinical visits for progress monitoring were arranged at 12-week intervals. Blood tests and ultrasound vascular assessments were repeated at 52 weeks. Ultrasound vascular assessment was performed by using B-mode ultrasonography: carotid artery scan (7–10 MHz probe, color Doppler). Centralized measurement of IMT of the common carotid artery was performed by using verified automatic measurement IMT software. The primary objective was to evaluate the efficacy of DG treatment for preventing atherosclerosis in postmenopausal women who have borderline hypercholesterolemia.

**2.3. Ethics Approval.** Prior to study commencement, the study protocol, the informed consent documents, and any other appropriate documents were submitted to the Clinical Research Ethics Committee (CREC), Joint The Chinese University of Hong Kong—New Territories East Cluster. CREC approval number is CRE-2008.342-T. The participants in the study had given written informed consent. Approval was granted on October 21, 2008.

**2.4. Blood Collection, RNA Purification, RNA Quantification, and RNA Integrity.** Three subjects from the DG group and three subjects from the placebo treatment group were selected for the microarray study. Whole blood samples were collected in PAXgene blood RNA tubes (Qiagen, USA) which contain a proprietary reagent composition to protect RNA molecules from degradation by RNases and to minimize *ex vivo* changes in the gene expression. The samples were gently inverted and stored at –80°C within two hours of collection.

RNA was extracted from whole blood by using the PAXgene Blood RNA Kit in accordance with the manufacturer's

TABLE 1: RT-PCR primer pairs for the gene identified in the microarray analysis.

	Primer sequence (5' → 3')	
	Forward	Reverse
ACE	TGGTGACTGATGAGGCTGAG	TCTTGCTGGTCTCTGTGGTG
AVPR1B	CCTGGCTATCTTCGTTCGTC	CCAGGCCTGTGTCTTGACTT
CTSB	TTGCCAACTCCTGGAACACTGACT	AGGCCACGGCAGATTAGATCTTT
DRD5	TCATCTATGCCCTCAACGCCGACT	ATGTAGGCAGCTGCGATTTCCCTTG
ECE1	ACCTGTCTTCCTCGCTGGAAGTTT	TCGGGTTTCCTCATCCATCCACTT
EDN1	GGGGATCTGAGTCTGTCCAA	CAACACACATGCTGGGAAAC
ELANE	CTGGGAGCCCATAACCTCTC	CACGATGTCGTTGAGCAAGT
ILK	GAGCCAGGCTGTGAAGTTTGCTTT	TCGGGCAGTCATGTCCTCATCAAT
MAPK1	ACGTTGGTACAGGGCTCCAGAAAT	TTCCTGGAAAGATGGGCCTGTTA
MAPK14	AGTCCTGAGCACCTGGTTTCTGTT	ACATGCACACACACTAACACGCAC
PTGS2	CAAATCCTTGCTGTCCCACCCAT	GTGCACTGTGTTTGGAGTGGGTTT
MFN2	AGCCCTGGTATTGATGTCACCACA	ATGAAGATGTTTGGCCGGGAGAGA
NOS3	CCCTTCAGTGGCTGGTACAT	TATCCAGTCCATGCAGACA
NPPA	GGGTCTCTGCTGCATTTGTGTCAT	AGAGGCGAGGAAGTCACCATCAAA
NR3C1	TGAAGGTTTCTGCGTCTTCACCCT	CTGCGCATTGCTTACTGAGCCTTT
PRKCB	ACCGCCTGTACTTTGTGATGGAGT	CCGATGGCAATTTCTGCAGCGTAA
PRKCD	TGCCGCTGAGATAATGTGTGGACT	TTGCACATCCCAAAGTCGGCAATC
RAF1	TGCCGAACAAGCAAAGAACAGTGG	AGTCTGAACACTGCACAGCACTCT
STAT3	ATGGAAGAATCCAACAACGGCAGC	AGGTCAATCTTGAGGCCTTGGTGA
STAT5B	AAATTCAAGGCCGAAGTGCAGAGC	CATCACACCGTCAAACCATTGCCA
GAPDH	ACAGTCAGCCGCATCTTCTT	ACGACCAAATCCGTTGACTC

guidelines. Briefly, the samples were removed from  $-80^{\circ}\text{C}$  and incubated at room temperature for two hours to ensure complete lysis. Following cell lysis, nucleic acids were pelleted and treated with a buffer containing proteinase-K. After digestion with RNase-free DNase (Qiagen), the RNA was subsequently purified on PAXgene spin columns and eluted in  $40\ \mu\text{L}$  of elution buffer.

Freshly extracted RNA was measured by using a NanoDrop 2000 UV-visible spectrophotometer (Thermo Scientific, USA). RNA integrity was additionally assessed by using a Eukaryote total RNA nanochip and the Agilent 2100 Bioanalyser (Agilent Technologies, Germany).

**2.5. Microarray Hybridization.** cRNA was transcribed *in vitro* by incorporating a biotinylated pseudouridine molecule by using GeneChip Expression 3'-Amplification Reagents for IVT Labeling, over 16 hours at  $37^{\circ}\text{C}$ . Hybridization was performed by using GeneChip Human Gene 1.0 ST Array (Affymetrix) containing 764,885 distinct probes corresponding to 28,869 well-annotated genes. After washing, the chips were stained with streptavidin-phycoerythrin in accordance with the Affymetrix EukGE-WS2v5 protocol by using a Fluidic FS450 station. The microarrays were read with the GeneChip Scanner 3000 (Affymetrix). The Affymetrix GeneChip Operating Software version 1.2 was used to manage the Affymetrix GeneChip array data and to automate the control of the GeneChip fluidics stations and scanners.

**2.6. Analyses for Genomic Study.** The acquisition and initial quantification of array images were conducted using the

AGCC software (Affymetrix). The subsequent data analyses were performed by using Partek Genomics Suite Version 6.4 (Partek, USA). We first performed a one-way ANOVA to identify the genes between the groups at a  $P$  value of less than 0.05 and then calculated the relative difference in fold change (FC) between the groups. Although these criteria were used with low stringency, this is expected to detect more genes truly associated with DG treatment at the expense of increasing the number of false positives to be validated by qPCR bioinformatics and experimental means. Cluster analyses and principal component analysis (PCA) were conducted with Partek default settings. Comprehensive data and literature mining was performed through Genomatix Pathway System (GePS) (<http://www.genomatix.de/>) for extracting gene relationships based on information extracted from public proprietary databases.

**2.7. Quantitative Real-Time PCR Analysis.** Quantitative Real-Time PCR (qPCR) confirmation of the selected genes was performed by using iScript one-step RT-PCR Kit with SYBR Green (Bio-rad) on CFX96 Real-Time PCR Detection System (Bio-Rad) in accordance with the manufacturer's instructions. The RNA from the remaining subjects in the treatment group ( $n = 18$ ) and the placebo group ( $n = 11$ ) was used for qPCR confirmation of the selected genes. The primer list for qPCR is shown in Table 1. The threshold cycle (Ct), the cycle number at which the amount of amplified genes of interest reached a fixed threshold, was determined. Relative expression of the RT-PCR product was calculated by using the comparative  $2^{-\Delta\Delta\text{Ct}}$  method. The endogenous control

GAPDH was used for normalization. The fold difference of posttreatment was then determined by normalizing all values to the mean of the relative expression for pretreatment. The differences in the gene expression level between the DG-treated and the placebo-treated group were compared by a student *t*-test; a probability of  $P < 0.05$  was considered to be statistically significant.

**2.8. Statistical Analyses.** Baseline characteristics are presented as descriptive statistics (frequency (%) or mean, standard deviation (SD), and standard error (SE) minimum, median, and maximum). The data were processed to give group mean values and standard error. Differences within a group were compared by a paired *t*-test. Differences between groups were compared by the Mann-Whitney test. Significance level was defined as  $\alpha = 0.05$ . Data were analyzed by using the SPSS software (version 16.00, SPSS Inc.).

### 3. Results

**3.1. Patient Characteristics.** There were no differences detected in any patient baseline characteristics between the DG-treated group and the placebo-treated group included in the primary outcome IMT (Table 2).

**3.2. Effect of DG on Carotid IMT.** When compared to the baseline, there was a significant improvement in IMT in the DG group after six months ( $P = 0.006$ ) and after 12 months ( $P < 0.001$ ). However, no such significant change was observed in the placebo group (Figure 1(b)). In the DG group, IMT was significantly improved 1.55% after 12-month treatment as compared to only 0.42% in the placebo group ( $P = 0.017$ ), as shown in Figure 1(a).

**3.3. Effect of DG on Blood Pressure.** At the end of the study, the blood pressure of all the subjects both in the DG-treated and the placebo-treated group was within normal range. There were no statistical differences of systolic and diastolic blood pressure between the two groups for the baseline measurement and during the study period (Table 3).

**3.4. Effect of DG on Blood Lipid Profile.** Blood lipid levels are shown in Figure 2(a). After 12-month treatment, a remarkable decrease in total cholesterol (TC) and low-density lipoprotein (LDL) in the DG-treated group was observed but not in the placebo-treated group. In the DG-treated group, TC and LDL were significantly decreased (6.2% and 7.3%) when compared to the corresponding baseline ( $P = 0.003$  and  $P = 0.009$ ). No such change was observed in the placebo-treated group ( $P = 0.186$  and  $P = 0.569$ ). However, the percentage change of TC and LDL in the DG-treated group was not significantly different from that in the placebo-treated group ( $P = 0.153$ ) (Figure 2(b)). However, there was no significant change in high-density lipoprotein (HDL) and triglyceride (TG) in both groups after 12-month treatment (Figure 2(b)).

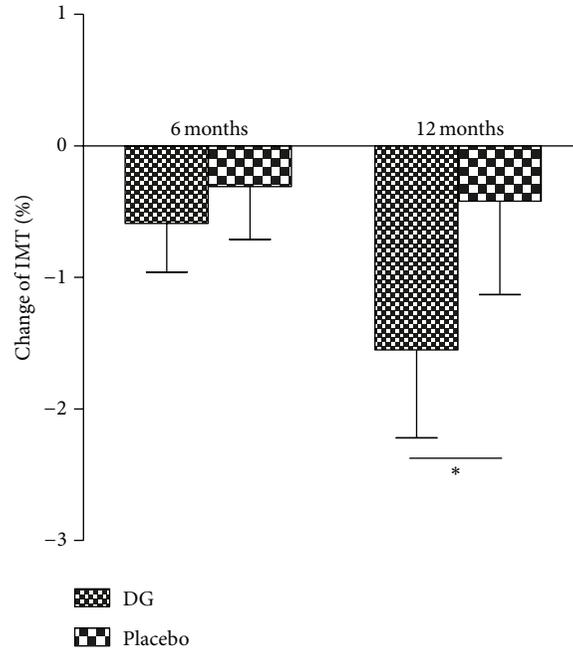
TABLE 2: Patient baseline characteristics.

Item	DG ( $n = 18$ )	Placebo ( $n = 11$ )	<i>P</i> value
Age (year)	56.7 $\pm$ 4.8	56.1 $\pm$ 3.8	0.663
Minimal	47.0	48.0	
Maximal	65.0	63.0	
Body weight (kg)	53.8 $\pm$ 5.4	54.3 $\pm$ 7.4	0.779
BMI (kg/m <sup>2</sup> )	22.3 $\pm$ 2.3	23.0 $\pm$ 2.7	0.400
SBP (mmHg)	125.3 $\pm$ 14.5	127.3 $\pm$ 10.5	0.635
DBP (mmHg)	72.6 $\pm$ 11.7	74.7 $\pm$ 8.7	0.525
TC (mmol/L)	6.6 $\pm$ 0.6	6.3 $\pm$ 0.5	0.091
LDL (mmol/L)	4.3 $\pm$ 0.5	4.0 $\pm$ 0.4	0.052
HDL (mmol/L)	1.8 $\pm$ 0.4	1.7 $\pm$ 0.3	0.258
TG (mmol/L)	1.1 $\pm$ 0.4	1.4 $\pm$ 0.8	0.229
Glucose (mmol/L)	5.1 $\pm$ 0.4	5.0 $\pm$ 0.4	0.483
Creatinine (umol/L)	64.9 $\pm$ 7.5	61.3 $\pm$ 6.1	0.106
Urea (mmol/L)	5.2 $\pm$ 1.0	4.7 $\pm$ 0.9	0.117
IMT (mm)	0.784 $\pm$ 0.125	0.763 $\pm$ 0.101	0.563
Plaque			
Yes	9	5	0.273
No	12	14	
Plaque index	1.56 $\pm$ 0.88	2.00 $\pm$ 1.73	0.530

**3.5. Differential Expressed Genes (DEGs).** The differential gene expression of two whole blood samples (Pre: before treatment; Post: after 12 months of treatment) was compared in the DG-treated group ( $n = 3$ ) and the placebo-treated group ( $n = 3$ ) ( $P < 0.05$ ). For the DG-treated group, there were 954 differentially expressed genes (DEGs) with 485 being upregulated and 469 being downregulated. For the placebo group, there were 457 DEGs with 290 upregulated and 167 downregulated. There were only 19 common DEGs shared between the DG-treated group and the placebo-treated group, as shown in the Venn diagram (Figure 3).

**3.6. Principal Component Analyses (PCA) and Cluster Analyses.** To evaluate intraexperimental technical variation, the gene expression profile of three repeated hybridizations of each sample was performed by using principal component analysis (PCA). The PCA results revealed that there was no overlapping in the clusters of three repeated hybridizations (red balls) of the Pre and Post samples of the DG-treated group and the placebo-treated group (Figure 4). The cluster analyses of Pre and Post whole blood samples of the DG-treated group and the placebo-treated group ( $n = 3$ ) are shown in Figure 5. The gene expression profile (as shown in cluster) of the Pre samples was well separated from that of the Post samples in the DG-treated group, whereas the cluster of the Pre sample was overlapped with that of the Post samples in the placebo-treated group (Figure 5).

**3.7. Data and Literature Mining by Genomatix Pathway System (GePS).** The biological processes were presented by



(a)

Group	Baseline	6 months	12 months	<i>P</i> value <sup>1</sup>	<i>P</i> value <sup>2</sup>
DG ( <i>n</i> = 18)	0.781 ± 0.124	0.776 ± 0.123 (-0.59%) <sup>#</sup>	0.769 ± 0.120 (-1.55%) <sup>#</sup>	0.006	0.000
Placebo ( <i>n</i> = 11)	0.760 ± 0.101	0.757 ± 0.101 (-0.31%) <sup>#</sup>	0.757 ± 0.102 (-0.42%) <sup>#</sup>	0.132	0.230
<i>P</i> value <sup>3</sup>		(0.24)	(0.017)		

<sup>1</sup>*P* value: comparison within group: 6 months versus baseline (paired *t*-test).  
<sup>2</sup>*P* value: comparison within group: 12 months versus baseline (paired *t*-test).  
<sup>3</sup>*P* value: comparison of % change in IMT between groups (Mann-Whitney test).  
<sup>#</sup>% change in IMT from baseline.

(b)

FIGURE 1: (a) Percentage changes from baseline in IMT at 6-month and 12-month DG or placebo treatment, \**P* < 0.05; (b) IMT of subjects at baseline, 6-month, and 12-month DG or placebo treatment (in mm).

TABLE 3: Blood pressure of subjects at baseline and 12 months after DG or placebo treatment.

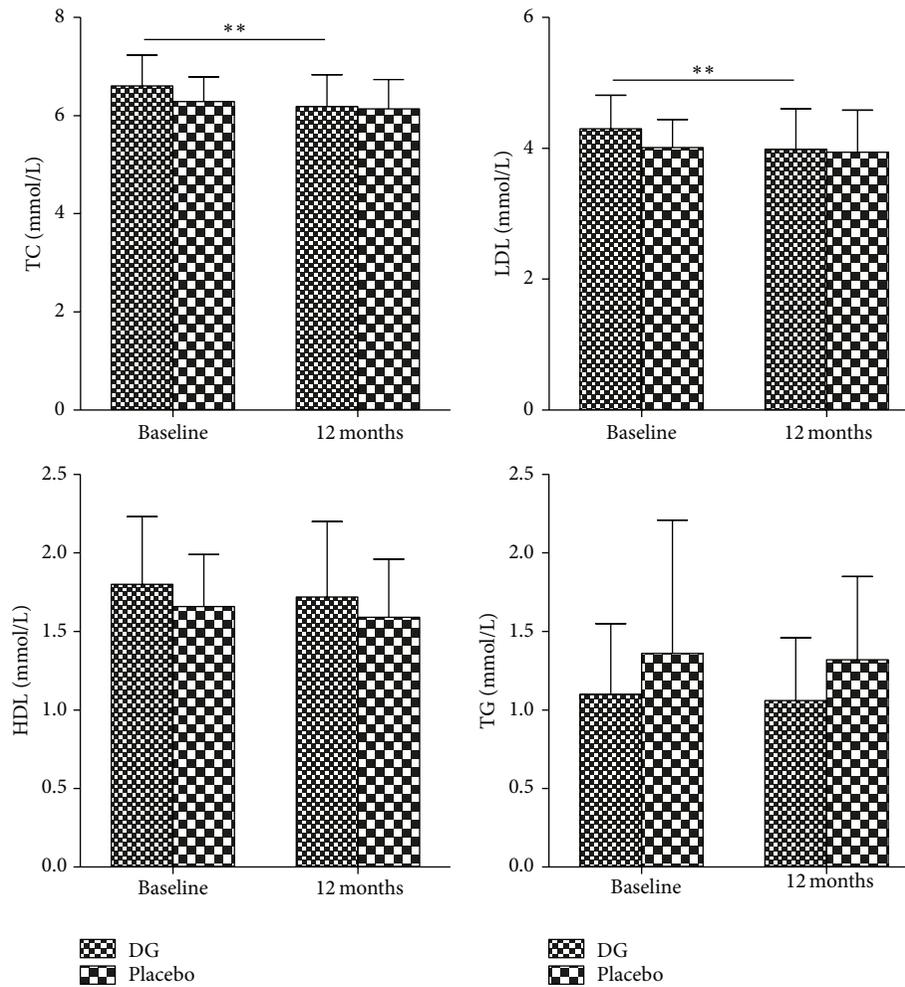
Item	Systolic BP (mmHg)		Diastolic BP (mmHg)	
	Baseline	12 months	Baseline	12 months
TCM ( <i>n</i> = 18)	125.3 ± 14.5	120.0 ± 16.8	72.6 ± 11.7	74.9 ± 10.8
Placebo ( <i>n</i> = 11)	127.3 ± 10.5	123.8 ± 9.8	74.7 ± 8.7	76.7 ± 8.9
<i>P</i> value <sup>#</sup>	0.635	0.552	0.525	0.869

<sup>#</sup>Compared between groups (Mann-Whitney test).

an AmiGo gene ontology (GO) database via the Genomatix Pathway System (GePS) for extracting DEGs relationships based on information extracted from public proprietary databases. The two most important biological processes of “regulation of systemic arterial blood pressure by hormone” and “regulation of smooth muscle proliferation” were identified by GePS in the DG-treated group. No significant biological process and cellular components were identified

in the placebo-treated group. For the biological process of “regulation of systemic arterial blood pressure by hormone,” six genes were identified with a *P* value of  $5.98 \times 10^{-5}$ . For the biological process of “regulation of smooth muscle proliferation,” seven genes were identified with a *P*-value of  $1.79 \times 10^{-3}$ . These identified genes are shown in orange color while the co-citation genes are shown in grey color in Figures 6 and 7.

The most significantly regulated “blood pressure-” related genes included endothelin-1 (EDN1), endothelin-converting enzyme-1 (ECE1), dopamine D5 receptor (DRD5), vasopressin V1b receptor (AVPR1B), angiotensin-converting enzyme (ACE), and endothelial nitric oxide synthase (NOS3) and its co-citation genes atrial natriuretic factors (NPPA), protein kinase C beta type (PRKCB), signal transducers and activators of transcription 3 (STAT3), glucocorticoid receptor alpha 2 (NR3C1), mitogen-activated protein kinase 1 (MAPK1), and mitogen-activated protein kinase 14 (MAPK14) (Table 4). The ratio indicated the upregulation (>1.00) or downregulation (<1.00). There were four upregulated genes and eight downregulated genes in those



(a)

	Group	Baseline	12 months	% change	$P$ value <sup>1</sup>
TC	DG	6.60 ± 0.63	6.18 ± 0.65	-6.2%	0.003
	Placebo	6.29 ± 0.50	6.14 ± 0.59	-2.3%	0.186
	$P$ value <sup>#</sup>	0.091	0.314	0.153	
LDL	DG	4.30 ± 0.51	3.98 ± 0.62	-7.33%	0.009
	Placebo	4.01 ± 0.43	3.94 ± 0.64	-1.69%	0.569
	$P$ value <sup>#</sup>	0.052	0.264	0.153	
HDL	DG	1.80 ± 0.43	1.72 ± 0.48	-4.9%	0.068
	Placebo	1.66 ± 0.33	1.59 ± 0.37	-3.9%	0.154
	$P$ value <sup>#</sup>	0.258	0.351	0.714	
TG	DG	1.10 ± 0.45	1.06 ± 0.40	1.2%	0.576
	Placebo	1.36 ± 0.85	1.32 ± 0.53	13.0%	0.817
	$P$ value <sup>#</sup>	0.229	0.211	0.390	

<sup>1</sup> $P$  value: within group 12 months versus baseline; <sup>#</sup>compared between groups (Mann-Whitney test).

(b)

FIGURE 2: (a) TC, LDL, HDL, and TG in mmol/L at 12 months compared with baseline,  $**P < 0.01$ ; (b) percentage change in blood lipid profile (TC, LDL, HDL, and TG) of subjects after 12-month DG or placebo treatment.

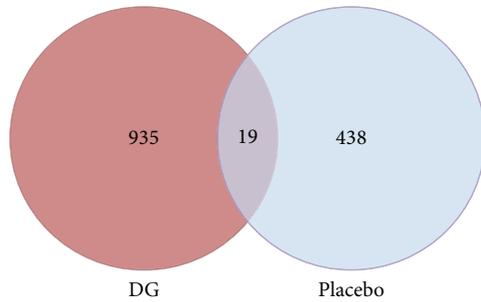


FIGURE 3: Venn diagram of differentially expressed genes (DEGs) in the DG-treated group and the placebo-treated group. Nonoverlapped area is the number of DEGs found only in corresponding group. Overlapped area is the number of common DEGs shared between the DG-treated group ( $n = 3$ ) and the placebo group ( $n = 3$ ).

pathways. The most significantly regulated “smooth muscle proliferation-” related genes included EDN1, NOS3, prostaglandin G/H synthase 2 (PTGS2), integrin-linked protein kinase (ILK), Elastase-2 (ELANE), mitofusin-2 (MFN2) and signal transducers and activators of transcription 5B (STAT5B) and its co-citation genes NPPA, protein kinase C delta (PRKCD), RAF1, cathepsin B (CTSB), MAPK1, NR3C1, mitogen-activated protein kinase 14 (MAPK14), and STAT3 (Table 5). There were five upregulated genes and ten downregulated genes in those pathways.

The gene expression level of the 11 DEGs, both in the “blood pressure” and “smooth muscle proliferation” pathways, is shown in Figure 8. The DG-treated group has a significant lower fold change in EDN1 (2.76 versus 1.04), PTGS2 (1.45 versus 1.04), ELANE (3.81 versus 0.80), STAT5B (1.66 versus 0.82), ECE1 (1.15 versus 0.88), AVPR1B (3.43 versus 0.83), and ACE (1.28 versus 0.89) gene expression as compared to the placebo-treated group. The DG-treated group has a significant higher fold change in NOS3 (1.24 versus 3.36), ILK (0.74 versus 1.33), MFN2 (0.86 versus 3.56), and DRD5 (0.85 versus 2.12) gene expression as compared to the placebo-treated group.

The gene expression level of the nine co-citation genes both in the “blood pressure” and “smooth muscle proliferation” pathways is shown in Figure 9. The DG-treated group has a significant lower fold change in all co-citation genes as compared to the placebo-treated group, NPPA (1.71 versus 1.03), PRKCD (2.08 versus 1.02), RAF1 (1.81 versus 0.82), CTSB (2.99 versus 0.99), MAPK1 (1.26 versus 1.03), NR3C1 (1.80 versus 1.03), MAPK14 (1.32 versus 1.01), STAT3 (1.14 versus 0.79), and PRKCB (1.31 versus 0.89).

By the integration of DEGs in these two biological processes and by performing network analysis using GePS, which dynamically generates functional association networks based on the curated literature information of protein-protein interaction, coexpression, and genetic regulation, core DEGs can be identified. Figure 10 shows the network with the basis of those DEGs. Both EDN1 and NOS3 are the core of the network, as highlighted.

TABLE 4: Genes regulated after DG treatment in microarray analysis. Blood pressure-related genes are shown in identified genes which are shown in orange color while co-citation genes are shown in grey color.

Number	Gene title	Gene symbol	Ratio
1	Endothelin 1	EDN1	0.86
2	Endothelin-converting enzyme 1	ECE1	0.87
3	Dopamine D5 receptor	DRD5	1.30
4	Vasopressin V1b receptor	AVPR1B	0.68
5	Angiotensin-converting enzyme	ACE	0.84
6	Nitric oxide synthase, endothelial	NOS3	1.13
7	Atrial natriuretic factor	NPPA	1.28
8	Protein kinase C beta type	PRKCB	0.83
9	Signal transducers and activators of transcription 3	STAT3	0.90
10	Glucocorticoid receptor alpha 2	NR3C1	1.28
11	Mitogen-activated protein kinase 1	MAPK1	0.85
12	Mitogen-activated protein kinase 14	MAPK14	0.72

TABLE 5: Genes regulated after DG treatment in microarray analysis. Smooth muscle proliferation-related genes are shown in identified genes which are shown in orange color while co-citation genes are shown in grey color.

Number	Gene title	Gene symbol	Ratio
1	Endothelin-1	EDN1	0.86
2	Nitric oxide synthase, endothelial	NOS3	1.13
3	Prostaglandin G/H synthase 2	PTGS2	0.94
4	Integrin-linked protein kinase	ILK	1.21
5	Elastase-2	ELANE	0.89
6	Mitofusin-2	MFN2	1.3
7	Signal transducer and activator of transcription 5B	STAT5B	0.84
8	Atrial natriuretic factor	NPPA	1.28
9	Protein kinase C delta	PRKCD	0.82
10	Oncogene RAF1	RAF1	0.88
11	Cathepsin B	CTSB	0.83
12	Mitogen-activated protein kinase 1	MAPK1	0.85
13	Glucocorticoid receptor alpha	NR3C1	1.28
14	Mitogen-activated protein kinase 14	MAPK14	0.72
15	Signal transducer and activator of transcription 3	STAT3	0.90

#### 4. Discussion

The cellular and biological basis of DG on the cardiovascular tonic effect has been demonstrated in our previous preclinical and clinical studies [3, 5–12]. However, the molecular mechanisms of DG which exert their effects via multiple targets in a complex network are difficult to identify. Many factors, such as ion channels, protein-influencing calcium homeostasis, cytokines, growth factor, metabolic, and inflammatory

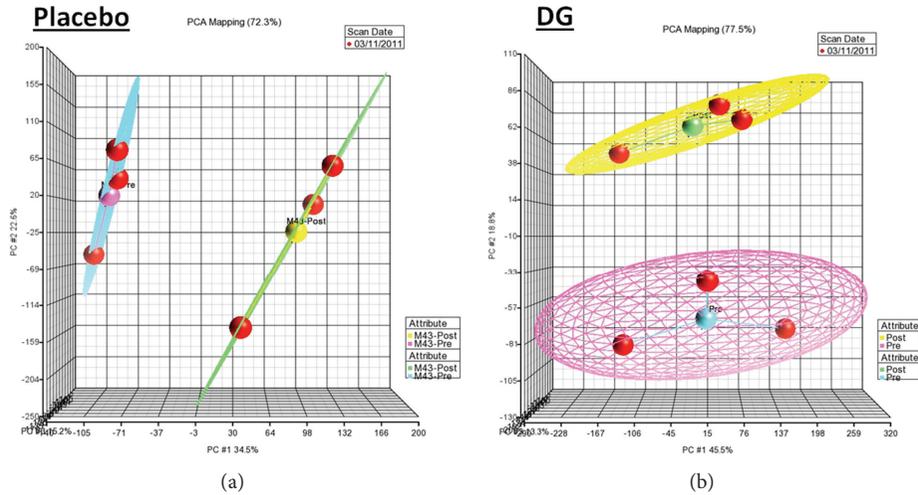


FIGURE 4: PCA analysis of Pre and Post samples of the DG-treated group and the placebo-treated group. Each Pre and Post sample of each group was repeated in three individual hybridizations. The cluster of three red balls shows the intraexperimental variation of each sample.

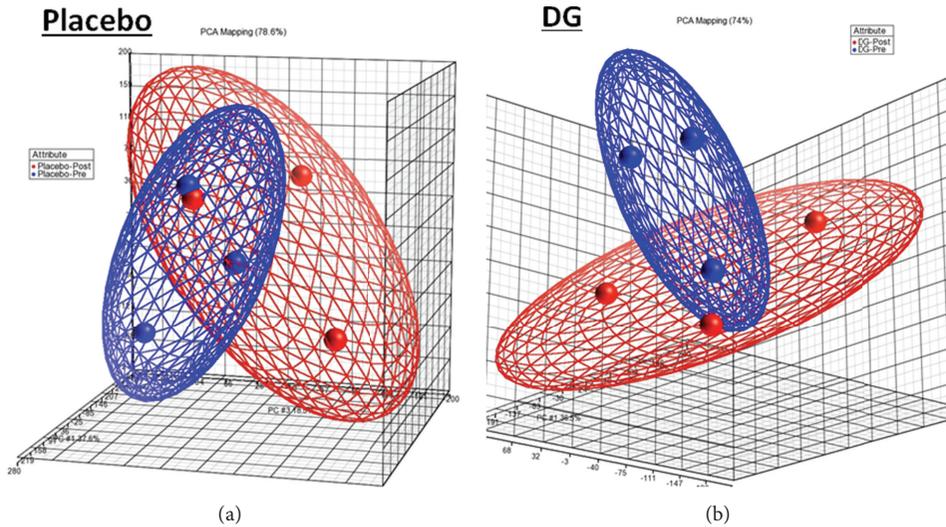


FIGURE 5: Cluster analyses of Pre and Post samples in three individual subjects of the DG-treated group and the placebo-treated group. The overlapped area of the clusters (red and blue) indicates the greater similarity of the gene expression profile.

stressors, may be involved in these molecular mechanisms [13]. With the help of the data and literature mining tool (GePS), it is now possible to extract gene relationships based on information extracted from public proprietary databases and therefore targeted network pathways. The present study using microarray analysis demonstrated that hundreds of differentially expressed genes (DEGs) were modulated in subjects with DG treatment. These findings suggest that these DEGs play critical roles in the pathways of hypertension and the pathophysiology of atherosclerosis (smooth muscle cell proliferation). In addition, the results of the molecular findings may correlate with the clinical outcomes.

The present clinical study was designed to evaluate the efficacy of DG treatment in the prevention of atherosclerosis in postmenopausal women who have borderline hypercholesterolemia. Postmenopausal women are associated with

changes in their metabolism together with new cardiovascular risk factors because of the changes of their hormonal status. The risk factors are the changes of lipid profile, obesity, hypertension, and atherosclerosis [14]. The results found that DG reduced the IMT significantly and that it decreased the TC and LDL. Although there was no change in blood pressure in the DG-treated group, it did not mean DG has no such effect; this is because all the subjects had normal blood pressure during the study period.

For the microarray study, a larger change in gene expression was found in the DG-treated group (Figure 3), and this was correlated with the number of DEGs (the DG-treated group has twofold more DEGs than the placebo-treated group, 954 versus 457). Hence, DG may exert certain effects from the genomic point of view. The PCA results indicated that the intraexperimental variations were well-controlled

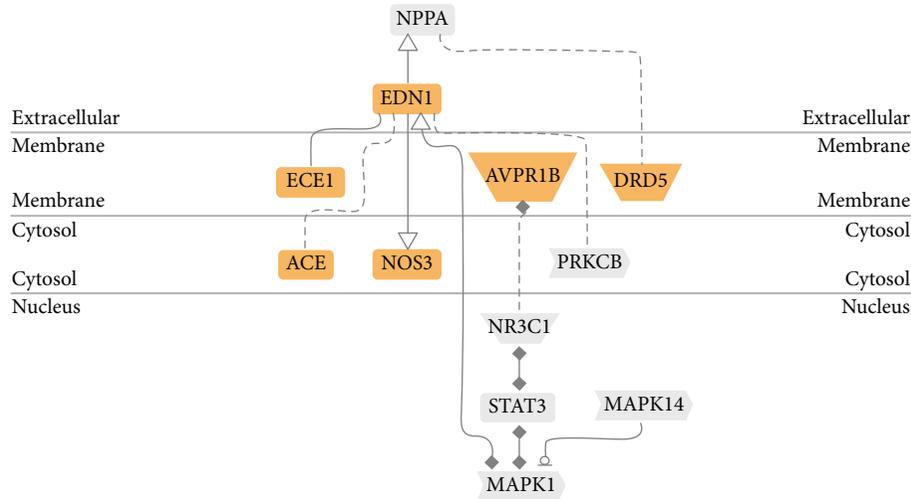


FIGURE 6: A pathway of “regulation of systemic blood pressure by hormone” was generated by the GePS program from Genomatix that connects the DEGs found after DG treatment for 12 months.

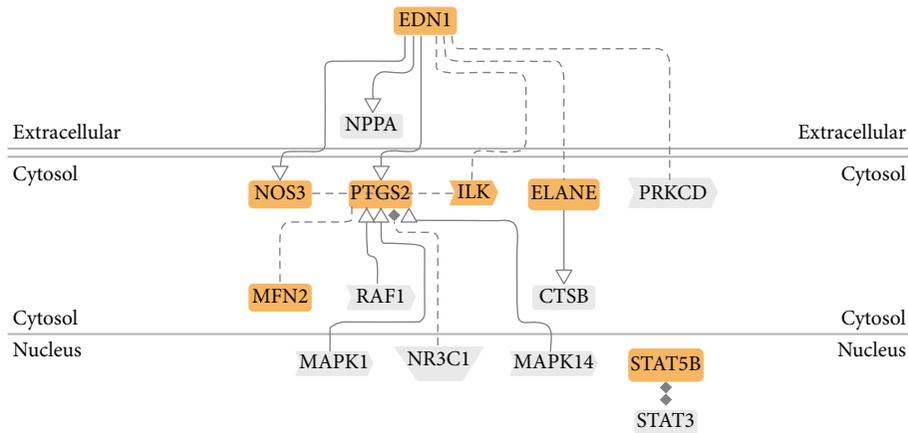


FIGURE 7: A pathway of “regulation of smooth muscle cell proliferation” was generated by the GePS program from Genomatix that connects the DEGs found after DG treatment for 12 months.

with no overlapping in the clusters of the three repeated hybridizations (red balls) of the each sample (Figure 4). The cluster analysis results indicated that the gene expression profile of the placebo-treated group, as compared to the baseline, showed greater similarity because there was overlapping in the two clusters (Figure 5). However, the two clusters are well separated in the DG-treated group. Therefore, the DG-treated group, has a greater change in the gene expression profile than the placebo-treated group. These results provide hints that DG might exert certain effects on postmenopausal subjects.

Two molecular pathways, (1) regulation of blood pressure (Figure 6) and (2) regulation of smooth muscle proliferation (Figure 7), were targeted by associating several hundreds of DEGs by using microarray and genome-wide association analysis. These two pathways were targeted only in the DG-treated group but not in the placebo-treated group. The functionally related genes EDN1, NOS3, PTGS2, ILK, ELANE, MFN2, ECE1, AVPR1B, DRD5, and ACE were either upregulated or downregulated in the DG-treated group and

this resulted in negative regulation both in blood pressure and smooth muscle proliferation.

Our microarray data showed that DG treatment resulted in a lower level of EDN1 and a higher level of NOS3 as compared to the placebo treatment. Endothelin-1 (EDN1), a member of the endothelin family, has been known to be the most potent and long-acting vasoconstrictor with mitogenic activity [15, 16]. It is released mainly from the basal surface of the endothelium and it is partially synthesized in and released from smooth muscle cells (SMCs). It acts on vascular SMC in a paracrine and autocrine fashion by controlling the basal vascular tone [17]. Upregulation of EDN1 has been correlated with hypertension. Proliferation of SMC is a key feature of several pathogenic processes, including atherosclerosis and restenosis after coronary angioplasty. Because EDN1 also possesses mitogenic properties, it plays a role in regulating the proliferation of intimal smooth muscle cells and inducing IMT in atherosclerosis [18]. Endothelial NOS (eNOS), also known as nitric oxide synthase 3 (NOS3), is an enzyme that

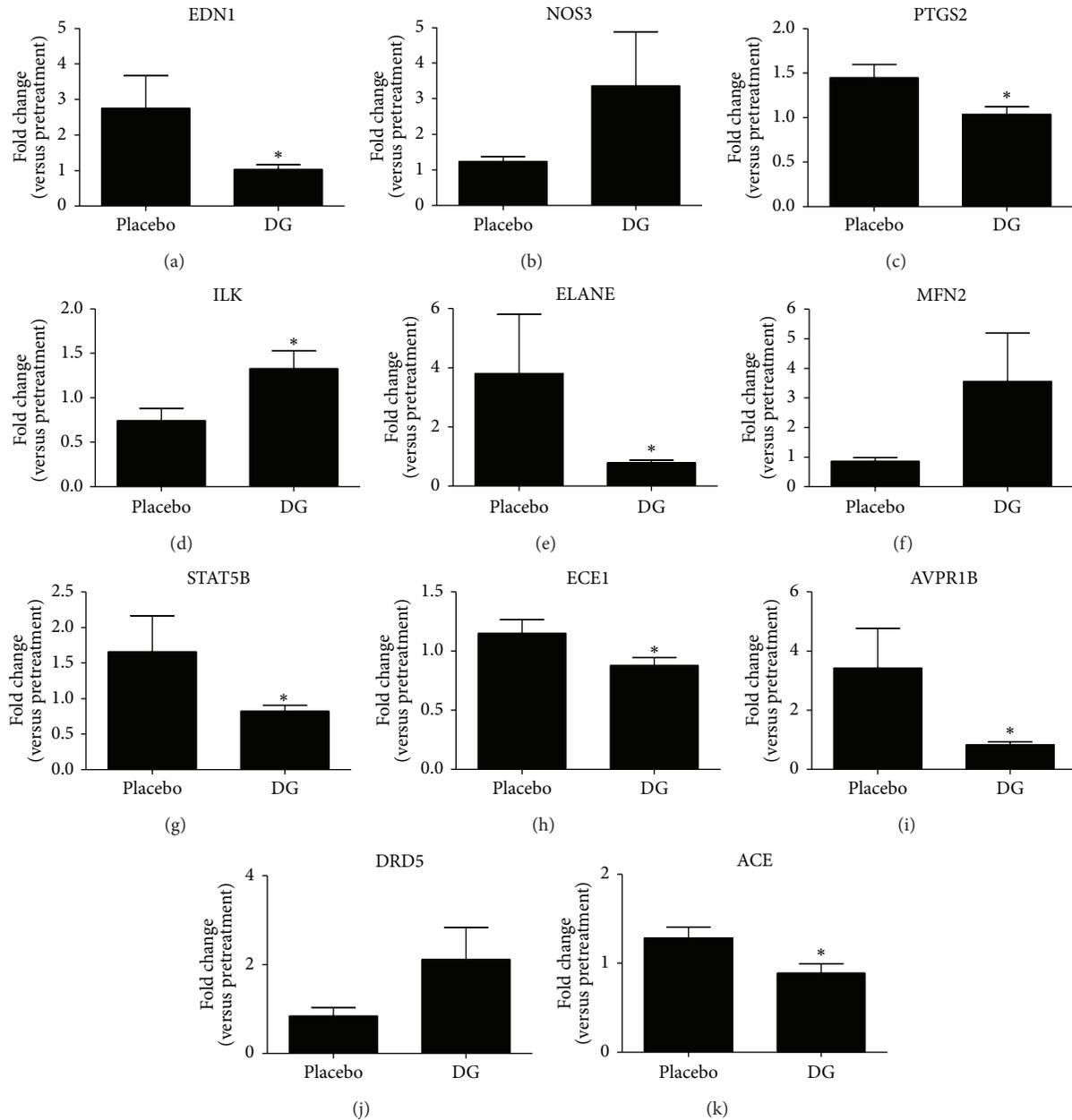


FIGURE 8: Gene expression level of the 11 DEGs after DG and placebo treatment. The RNA from the remaining subjects in the treatment group ( $n = 18$ ) and the placebo group ( $n = 11$ ) was used. The  $y$ -axis represents fold change (posttreatment versus pretreatment). The bar on the column stands for the mean  $\pm$  S.E.M., \* $P < 0.05$  as compared to the placebo group.

is encoded by the NOS3 gene. Nitric oxide (NO), a potent vasodilator constitutively produced by eNOS, is thought to be the endothelium-derived relaxing factor that mediates relaxation [19, 20] in blood vessels and is involved with regulating vascular tone by inhibiting the smooth muscle contraction which is an important element for the prevention of hypertension. NO, which was synthesized by eNOS, diffuses to underlying SMCs and stimulates one of its molecular targets, namely, soluble guanylate cyclase. Generated cGMP then mediates biological responses, including vasorelaxation, inhibition of cell proliferation and migration, and extracellular matrix production [21, 22]. Gene transfer studies have

proved that adenoviral eNOS delivery to balloon-injured rat carotid arteries restores vascular NO production and reduces neointima formation, at least in part because of the antiproliferative effect on SMC [23, 24]. Therefore, a lower level of EDN1 and a higher level of NOS3 after DG treatment suggest an antihypertensive effect and reduction in neointima formation in these two key DEGs.

Endothelin-1 (ET-1) is formed by the cleavage of the endothelin precursor, big endothelin (big-ET), by an endothelin-converting enzyme (ECE1). Hence, the inhibition of ECE1 leads to a reduction of physiologically active ET-1 and the associated vasoconstricting activity of this molecule. One

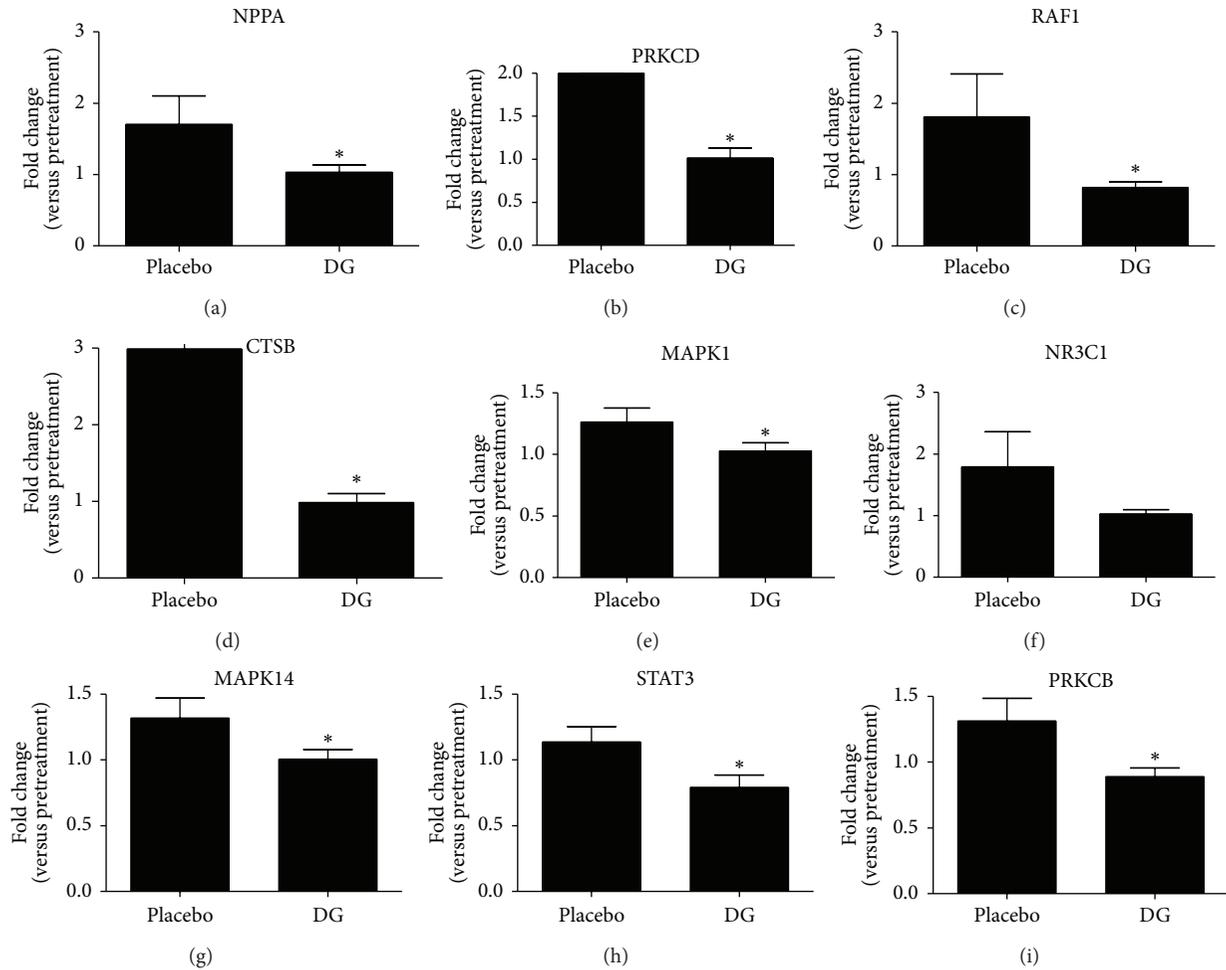


FIGURE 9: qPCR analyses of the nine co-citation genes after DG and placebo treatment. The RNA from the remaining subjects in the treatment group ( $n = 18$ ) and the placebo group ( $n = 11$ ) was used. The  $y$ -axis represents fold change (posttreatment versus pretreatment). The bar on the column stands for the mean  $\pm$  S.E.M., \*  $P < 0.05$  as compared to the placebo group.

clinical study found that the ECE1 C-339A polymorphism was genotyped in 698 women in which 669 women could also be genotyped for EDN1 K198N polymorphism. The results from this large association suggest that the genes ECE1 and EDN1 interact to modulate blood pressure levels in women [25]. Our study also found that DG treatment could significantly downregulate both ECE1 and EDN1 in postmenopausal women.

Arginine vasopressin receptor 1B (AVPR1B, previously known as vasopressin 3 receptor or antidiuretic hormone receptor 1b) is a protein that acts as a receptor for arginine vasopressin (AVP). A study of the role of AVP for the development of hypertension after constriction of the abdominal aorta proximal to the renal arteries concluded that AVP plays an important role in the development of hypertension and that the action is mediated via the vascular AVP-receptor (AVPR1B) [26]. Angiotensin I-converting enzyme (ACE), an exopeptidase, is a circulating enzyme that participates in the body's rennin-angiotensin system, which mediates extra-cellular volume and arterial vasoconstriction. This enzyme plays a key role in the production of angiotensin II and in

the catabolism of bradykinin, two peptides involved in the modulation of vascular tone and in the proliferation of SMC [27]. DRD5 gene encodes the D5 subtype of the dopamine receptor. The D5 receptor is important in blood pressure regulation. A study used D5 dopamine receptor knockout mice to try to correlate with hypertension, and it was found that disruption of the D5 gene increases blood pressure [28, 29]. Dopamine was found to interact with AVP, and the increase in blood pressure caused by AVP can be opposed by dopamine [30]. DRD5 upregulation could be beneficial to hypertension. Based on the downregulation of AVPR1B and ACE and the upregulation of DRD5, DG treatment may be involved in the rennin-angiotensin system and the receptor-mediated improvement of hypertension.

The gene PTGS2 encodes prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase (COX-2), and it is the key enzyme in prostaglandin biosynthesis. It is responsible for the prostanoid biosynthesis which involves inflammation and mitogenesis [31]. The activation of this gene is an early response to injury in vascular SMC. In an *in vivo* experimental model of balloon angioplasty of the

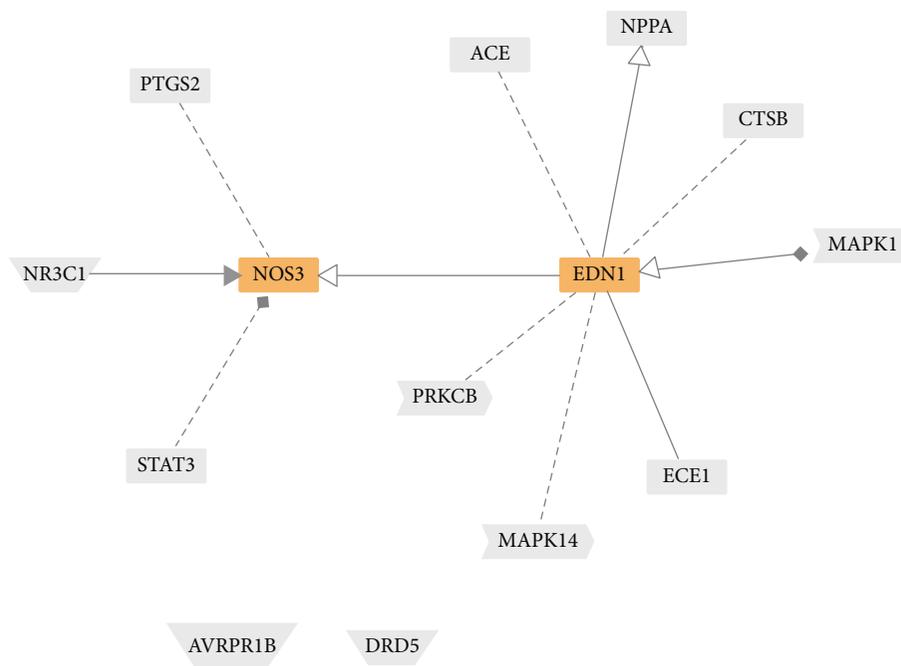


FIGURE 10: Integration of DEGs in two biological processes (blood pressure and smooth muscle proliferation) by using GePS. Two core DEGs (NOS and EDN1) are identified by highlighting in orange color.

carotid artery, it was shown that the COX-2 protein is induced in arterial SMC of the injury. SMC activation in arterial vessels walls after balloon injury is prolonged, with rounds of migration and division of SMC lasting up to two weeks [32, 33]. Integrin-linked kinase (ILK) is a 59 kDa serine-threonine kinase which has been associated with multiple cellular functions, including cell migration, cell proliferation, cell-adhesions, and signal transduction. Following a balloon catheter injury of the rat carotid artery *in vivo*, a dramatic decrease in ILK levels coincided with both the proliferation and the migration of SMCs, which leads to the formation of a thickened neointima. An increase in the ILK level may inhibit SMC migration, proliferation, and neointimal thickening [34]. The gene ELANE encodes serine proteases elastases that degrade the extracellular matrix, releasing growth factors and chemotactic peptides, inducing glycoproteins such as tenascin, and thereby promoting vascular cell proliferation and migration. It was found that elastase activity was induced by carotid arterial injury in mice together with inflammatory cells adherent to or within the vessels [35]. The inhibition of elastase could be a therapeutic target for neointimal thickening. The MFN2 gene provides instructions for making a protein called mitofusin 2. This protein helps to determine the shape and structure (morphology) of the mitochondria, the energy-producing centers within cells. In addition, MFN2 is an important suppressor of vascular SMC proliferation and it can trigger SMC apoptosis via a mitochondrial death pathway. Recent studies have demonstrated that MFN2 acts as an endogenous Ras inhibitor and that deregulation of MFN2 expression leads to vascular proliferative disorders in the settings of genetic hypertension, atherosclerosis, and

restenosis after vascular injury. In addition, the overexpression of MFN2 overtly suppresses the mitogenic stimulus-evoked vascular SMC proliferation in culture, and it also blocks balloon injury-induced restenosis *in vivo* via inhibiting the Ras-Raf-MEK-ERK/MAPK signaling pathway [36]. Based on the downregulation of PTGS2, ILK, and ELANE and the upregulation of MFN2, DG treatment may involve the multiple negative regulation of neointimal thickening by inhibiting vascular SMC proliferation and migration in mitogen-activated protein kinase pathways and signal transducers (MAPK) and be an activator of the transcription protein pathway (JAK-STAT). Our results also showed the downregulation of co-citation genes, MAPK1, MAPK14, STAT3, and STAT5B, in the abovementioned pathways and this may imply that DG treatment may improve neointimal thickening via the inhibition of SMC proliferation.

By integration of DEGs in these two biological processes and performing network analysis using GePS, both EDN1 and NOS3 were the core of the network (Figure 10). Endothelin-1 (EDN1) is a potent vasoconstrictor, it is overexpressed in the vasculature in hypertension, and it is able to increase vascular growth [37]. The endothelial nitric oxide synthase (NOS3) gene is positively associated with hypertension [38] and with inhibited vascular SMC proliferation and neointimal formation [23]. As a consequence, the downregulation of EDN1 and the upregulation of NOS3 could have antihypertension and inhibition of intimal smooth muscle proliferation [39].

The clinical outcome of carotid IMT reduction after DG treatment in the same study showed consistent observation with this molecular target study with the mechanism elucidated. The other finding from molecular targeting is the

antihypertensive effect of DG treatment, as shown in the patient's blood samples. However, there is no change in blood pressure of a patient in this clinical study, because the subjects are all in normotensive state. But our previous *in vivo* study showed the antihypertensive effect of DG in a spontaneous hypertensive rat [6]. Although no relationship can be found regarding the antihypertensive effect of DG between clinical and molecular studies, a molecular targeting study shows the power of revealing the hidden properties of DG treatment that could not be shown in the setting of a clinical study.

## 5. Conclusions

This genome-wide association study on the molecular action of DG in postmenopausal women with hypercholesterolemia using 18 treatments and 11 placebo samples before and after treatment has gathered sufficient molecular targets and pathways to reveal that herbal formula DG could improve neointima thickening and hypertension.

## Acknowledgments

This study was supported by grants on the State Key Laboratory of Phytochemistry and Plant Resources in West China (CUHK) from HKSAR and CUHK, grants on UGC-Area of Excellence Project AoE/B-10/01 from HKSAR, Focused Innovations Scheme (Major Area Scheme A- Phase 2) of The Chinese University of Hong Kong, Si Yuan Foundation on project titled "The Chinese University of Hong Kong-Zhejiang University Joint Laboratory on Natural Products and Toxicology Research," and Hop Wai Short-Term Research Grant. The authors would also like to thank Dr. Wilmshurst David John of the Research Administration Office of The Chinese University of Hong Kong for proofreading the paper.

## References

- [1] G. M. C. Rosano, C. Vitale, G. Marazzi, and M. Volterrani, "Menopause and cardiovascular disease: the evidence," *Climacteric*, vol. 10, supplement 1, pp. 19–24, 2007.
- [2] S. Rozenberg, J. Vandromme, and C. Antoine, "Postmenopausal hormone therapy: risks and benefits," *Nature Reviews Endocrinology*, vol. 9, no. 4, pp. 216–227, 2013.
- [3] D. W. Cheung, C. M. Koon, E. Wat et al., "A herbal formula containing roots of *Salvia miltiorrhiza* (Danshen) and *Pueraria lobata* (Gegen) inhibits inflammatory mediators in LPS-stimulated RAW 264.7 macrophages through inhibition of nuclear factor  $\kappa$ B (NF $\kappa$ B) pathway," *Journal of Ethnopharmacology*, vol. 145, no. 3, pp. 776–783, 2013.
- [4] Y. Deng, E. S. K. Ng, J. H. K. Yeung et al., "Mechanisms of the cerebral vasodilator actions of isoflavonoids of Gegen on rat isolated basilar artery," *Journal of Ethnopharmacology*, vol. 139, no. 1, pp. 294–304, 2012.
- [5] F. F. Y. Lam, S. Y. Deng, E. S. K. Ng et al., "Mechanisms of the relaxant effect of a Danshen and Gegen formulation on rat isolated cerebral basilar artery," *Journal of Ethnopharmacology*, vol. 132, no. 1, pp. 186–192, 2010.
- [6] C. F. Ng, C. M. Koon, D. W. S. Cheung et al., "The antihypertensive effect of Danshen (*Salvia miltiorrhiza*) and Gegen (*Pueraria lobata*) formula in rats and its underlying mechanisms of vasorelaxation," *Journal of Ethnopharmacology*, vol. 137, no. 3, pp. 1366–1372, 2011.
- [7] C. M. Koon, K. S. Woo, P. C. Leung, and K. P. Fung, "*Salvia miltiorrhizae* Radix and *Puerariae lobatae* Radix herbal formula mediates anti-atherosclerosis by modulating key atherogenic events both in vascular smooth muscle cells and endothelial cells," *Journal of Ethnopharmacology*, vol. 138, no. 1, pp. 175–183, 2011.
- [8] D. Wing-Shing Cheung, C. M. Koon, C. F. Ng et al., "The roots of *Salvia miltiorrhiza* (Danshen) and *Pueraria lobata* (Gegen) inhibit atherogenic events: a study of the combination effects of the 2-herb formula," *Journal of Ethnopharmacology*, vol. 143, no. 3, pp. 859–866, 2012.
- [9] F. Hu, C. M. Koon, J. Y. Chan, K. M. Lau, Y. W. Kwan, and K. P. Fung, "Involvements of calcium channel and potassium channel in Danshen and Gegen decoction induced vasodilation in porcine coronary LAD artery," *Phytomedicine*, vol. 19, no. 12, pp. 1051–1058, 2012.
- [10] F. Hu, C. M. Koon, J. Y. Chan, K. M. Lau, and K. P. Fung, "The cardioprotective effect of Danshen and Gegen decoction on rat hearts and cardiomyocytes with post-ischemia reperfusion injury," *BMC Complementary and Alternative Medicine*, vol. 10, no. 12, p. 249, 2012.
- [11] C. C. Fong, F. Wei, Y. Chen et al., "Danshen-Gegen decoction exerts proliferative effect on rat cardiac myoblasts H9c2 via MAPK and insulin pathways," *Journal of Ethnopharmacology*, vol. 138, no. 1, pp. 60–66, 2011.
- [12] W. Y. Tam, P. Chook, M. Qiao et al., "The efficacy and tolerability of adjunctive alternative herbal medicine (*Salvia miltiorrhiza* and *Pueraria lobata*) on vascular function and structure in coronary patients," *Journal of Alternative and Complementary Medicine*, vol. 15, no. 4, pp. 415–421, 2009.
- [13] F. Censi, G. Calcagnini, P. Bartolini, and A. Giuliani, "Principal component analysis of gene expression data: the case of atrial fibrillation," in *Proceedings of the 3rd International Symposium on Applied Sciences in Biomedical and Communication Technologies (ISABEL '10)*, pp. 1–4, IEEE, Roma, Italy, November 2010.
- [14] R. Rossi, T. Grimaldi, G. Origliani, G. Fantini, F. Coppi, and M. G. Modena, "Menopause and cardiovascular risk," *Pathophysiology of Haemostasis and Thrombosis*, vol. 32, no. 5–6, pp. 325–328, 2002.
- [15] M. Beghetti, S. M. Black, and J. R. Fineman, "Endothelin-1 in congenital heart disease," *Pediatric Research*, vol. 57, no. 5, pp. 16R–20R, 2005.
- [16] G. P. Diller and M. A. Gatzoulis, "Pulmonary vascular disease in adults with congenital heart disease," *Circulation*, vol. 115, no. 8, pp. 1039–1050, 2007.
- [17] S. J. Wort, M. Woods, T. D. Warner, T. W. Evans, and J. A. Mitchell, "Endogenously released endothelin-1 from human pulmonary artery smooth muscle promotes cellular proliferation: relevance to pathogenesis of pulmonary hypertension and vascular remodeling," *American Journal of Respiratory Cell and Molecular Biology*, vol. 25, no. 1, pp. 104–110, 2001.
- [18] Y. Hirata, Y. Takagi, Y. Fukuda, and F. Marumo, "Endothelin is a potent mitogen for rat vascular smooth muscle cells," *Atherosclerosis*, vol. 78, no. 2–3, pp. 225–228, 1989.
- [19] D. D. Rees, R. M. Palmer, and S. Moncada, "Role of endothelium-derived nitric oxide in the regulation of blood pressure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 9, pp. 3375–3378, 1989.

- [20] C. X. Zhao, X. Xu, Y. Cui et al., "Increased endothelial nitric-oxide synthase expression reduces hypertension and hyperinsulinemia in fructose-treated rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 328, no. 2, pp. 610–620, 2009.
- [21] S. Moncada, R. M. Palmer, and E. A. Higgs, "Nitric oxide: physiology, pathophysiology, and pharmacology," *Pharmacological Reviews*, vol. 43, no. 2, pp. 109–142, 1991.
- [22] C. Nathan and Q. W. Xie, "Nitric oxide synthases: roles, tolls, and controls," *Cell*, vol. 78, no. 6, pp. 915–918, 1994.
- [23] S. Janssens, D. Flaherty, Z. Nong et al., "Human endothelial nitric oxide synthase gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation after balloon injury in rats," *Circulation*, vol. 97, no. 13, pp. 1274–1281, 1998.
- [24] F. Sharif, S. O. Hynes, K. J. A. McCullagh et al., "Gene-eluting stents: non-viral, liposome-based gene delivery of eNOS to the blood vessel wall in vivo results in enhanced endothelialization but does not reduce restenosis in a hypercholesterolemic model," *Gene Therapy*, vol. 19, no. 3, pp. 321–328, 2012.
- [25] B. Funalot, D. Courbon, T. Brousseau et al., "Genes encoding endothelin-converting enzyme-1 and endothelin-1 interact to influence blood pressure in women: the EVA study," *Journal of Hypertension*, vol. 22, no. 4, pp. 739–743, 2004.
- [26] A. Aperia, B. Sahlgren, A. C. Eklöf, S. Lundin, and P. Melin, "Role of arginine-vasopressin for the development of hypertension following aortic constriction," *Acta Physiologica Scandinavica*, vol. 128, no. 4, pp. 495–499, 1986.
- [27] F. Cambien, O. Poirier, L. Lecerf et al., "Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction," *Nature*, vol. 359, no. 6396, pp. 641–644, 1992.
- [28] Z. Yang, D. R. Sibley, and P. A. Jose, "D5 dopamine receptor knockout mice and hypertension," *Journal of Receptors and Signal Transduction*, vol. 24, no. 3, pp. 149–164, 2004.
- [29] C. Zeng, Z. Yang, L. D. Asico, and P. A. Jose, "Regulation of blood pressure by D5 dopamine receptors," *Cardiovascular & Hematological Agents in Medicinal Chemistry*, vol. 5, no. 3, pp. 241–248, 2007.
- [30] C. Zeng, G. M. Eisner, R. A. Felder, and P. A. Jose, "Dopamine receptor and hypertension," *Current Medicinal Chemistry*, vol. 3, no. 1, pp. 69–77, 2005.
- [31] C. H. Lee, J. W. Roh, J. S. Choi et al., "Cyclooxygenase-2 is an independent predictor of poor prognosis in uterine leiomyosarcomas," *International Journal of Gynecological Cancer*, vol. 21, no. 4, pp. 668–672, 2011.
- [32] C. L. Jackson and S. M. Schwartz, "Pharmacology of smooth muscle cell replication," *Hypertension*, vol. 20, no. 6, pp. 713–736, 1992.
- [33] A. W. Clowes, M. M. Clowes, and M. A. Reidy, "Kinetics of cellular proliferation after arterial injury. III. Endothelial and smooth muscle growth in chronically denuded vessels," *Laboratory Investigation*, vol. 54, no. 3, pp. 295–303, 1986.
- [34] B. Ho, G. Hou, J. G. Pickering, G. Hannigan, B. L. Langille, and M. P. Bendeck, "Integrin-linked kinase in the vascular smooth muscle cell response to injury," *American Journal of Pathology*, vol. 173, no. 1, pp. 278–288, 2008.
- [35] S. H. Zaidi, X. M. You, S. Ciura, S. O'Blenes, M. Husain, and M. Rabinovitch, "Suppressed smooth muscle proliferation and inflammatory cell invasion after arterial injury in elafin-overexpressing mice," *The Journal of Clinical Investigation*, vol. 105, no. 12, pp. 1687–1695, 2000.
- [36] X. Guo, K. H. Chen, Y. Guo, H. Liao, J. Tang, and R. P. Xiao, "Mitofusin 2 triggers vascular smooth muscle cell apoptosis via mitochondrial death pathway," *Circulation Research*, vol. 101, no. 11, pp. 1113–1122, 2007.
- [37] M. Iglarz and E. L. Schiffrin, "Role of endothelin-1 in hypertension," *Current Hypertension Reports*, vol. 5, no. 2, pp. 144–148, 2003.
- [38] Y. Miyamoto, Y. Saito, N. Kajiyama et al., "Endothelial nitric oxide synthase gene is positively associated with essential hypertension," *Hypertension*, vol. 32, no. 1, pp. 3–8, 1998.
- [39] K. Hasegawa, H. Fujiwara, K. Doyama et al., "Endothelin-1-selective receptor in the arterial intima of patients with hypertension," *Hypertension*, vol. 23, no. 3, pp. 288–293, 1994.

## Research Article

# Sang-qi Granula Reduces Blood Pressure and Myocardial Fibrosis by Suppressing Inflammatory Responses Associated with the Peroxisome Proliferator-Activated Receptors and Nuclear Factor $\kappa$ B Protein in Spontaneously Hypertensive Rats

Lan-Yu Chen,<sup>1</sup> Chun-Shui Pan,<sup>2</sup> Xiao-Hong Wei,<sup>2</sup> Lin Li,<sup>1</sup> Jing-Yan Han,<sup>2</sup> and Li Huang<sup>1</sup>

<sup>1</sup> China-Japan Friendship Hospital, China

<sup>2</sup> Tasy Microcirculation Research Center, Peking University Health Science Center, Beijing, China

Correspondence should be addressed to Li Huang; lihstrong@163.com

Received 22 March 2013; Revised 8 August 2013; Accepted 18 August 2013

Academic Editor: Hao Xu

Copyright © 2013 Lan-Yu Chen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Aim.** Sang-qi Granula (SQ) is a compound prepared from Chinese herbs and is currently used for treatment of hypertension in China. Given its protective effects on cardiac function in decreasing blood pressure, we investigated the mechanism of protective effects of SQ on myocardium. **Methods.** 16 male normal Wistar-Kyoto rats and 16 spontaneous hypertension rats (SHR) were employed without medical treatment. 16 SHR were employed with SQ treatment. Rats in each group were sacrificed at two time points (8-week treatment and 16-week treatment). Blood pressure (BP), and heart weight/body weight (HW/BW) were measured. The expression of myeloperoxidase (MCP-1), ICAM-1, TNF- $\alpha$ , and CD68-positive cells was assessed. The interstitial collagen volume fraction (CVF), perivascular collagen volume area (PVCA), and the expression of TGF- $\beta$ , Smad-3, PPAR $\alpha$ ,  $\gamma$ , and NF- $\kappa$ B (P65 and P50) were observed. **Results.** SQ significantly inhibited the elevation of the blood pressure and HW/BW of SHR. Next, SQ prevented myocardial fibrosis. Finally, a proinflammatory mediator associated with NF- $\kappa$ B (TNF- $\alpha$ , ICAM-1, MCP-1, CD68), TGF- $\beta$ , and Smad-3 related to collagen deposition, which is upregulated in SHR group, was significantly suppressed by SQ. Expression of NF- $\kappa$ B was decreased in SHQ+SQ group compared to PPAR $\alpha$ , and  $\gamma$  expression was increased by SQ. **Conclusion.** Treatment with SQ ameliorates cardiac fibrosis induced by hypertension by attenuating the upregulation of ICAM-1, TNF- $\alpha$ , MCP-1, TGF- $\beta$ , Smad-3, P65, and P50 expression and improving PPAR $\alpha$  and PPAR $\gamma$  expression level. The results suggest that SQ may be an option for preventing cardiac fibrosis through PPAR signalling pathway.

## 1. Introduction

The World Health Statistics 2012 report indicated that the global average prevalence of hypertension is around 10%, and up to one-third of population in some Pacific Island countries is in hypertension. Even in Africa, however, more than 40% (and up to 50%) of adults in many countries are estimated to have high blood pressure. Long-term hypertension is an important and a prevalent contributor to morbidity and mortality from cardiovascular disease, and prolonged hypertension is accompanied by continuous vasoconstriction

which can finally result in target organ damage, such as heart failure, stroke, and renal failure.

Myocardial fibrosis is the result of chronic arterial hypertension and induces abnormality of cardiac function and arrhythmia. On the other hand, it is well accepted that vascular inflammation plays a major role in the cardiac fibrosis. Therefore, besides lowering blood pressure, attenuating of vascular inflammation is considered as an essential goal for the treatment of cardiac fibrosis following hypertension.

Sang-qi Granula (SQ) is a compound prepared from tradition Chinese herbs used for treating hypertension.

The main pharmacological components of it are herba *Taxillus chinensis*, barberry wolfberry fruit, eucommia bark, cassiae torae, chrysanthemi indicis, danshen root, kudzu vine root, and *Alisma L. orientale* Juzep. SQ has an anti-inflammatory action by alleviating the myocardial inflammation reaction in our previous study [1]. SQ's potent multiple functions and long history without adverse health effects and side effects make it a possible substitute for therapeutic treatment for myocardial fibrosis following hypertension.

The current project focuses on cardiac cell signaling related to transcription factors peroxisome proliferator-activated receptor (PPAR) and nuclear factor  $\kappa$ B (NF- $\kappa$ B). PPARs belong to a superfamily of nuclear ligand-activated transcription factors that impact cell metabolism, cell differentiation, and inflammation. The nuclear hormone receptor superfamily consisting of 3 isoforms of PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$ . PPAR $\alpha$  also exerts direct anti-inflammatory activity [2, 3]. Moreover, PPAR $\alpha$  is deactivated during cardiac hypertrophic growth [4] suggesting a role of PPAR $\alpha$  in regulating cardiac remodeling. In addition, systemic activation of PPAR $\gamma$  by its agonist has been shown to prevent the progression of multiple cardiovascular diseases, such as hypertension, atherosclerosis, and chronic kidney disease by reducing inflammation and downregulating angiotensin II (AngII)-induced Ang II type 1 receptor (AT<sub>1</sub>R) expression [5–8]. Active NF- $\kappa$ B promotes inflammation by promoting the transcription of various proinflammatory genes, including cell adhesion molecules, inflammatory cytokines, and chemokines. Cardiac NF- $\kappa$ B activity is positively correlated with myocardial fibrosis [9, 10], and inhibition of NF- $\kappa$ B activity limits myocardial fibrosis progression. PPAR and NF- $\kappa$ B have been described as physiological antagonists: PPAR activation reduces NF- $\kappa$ B/DNA binding [11]. In heart failure models, PPAR agonists reduce cardiac NF- $\kappa$ B activity and reduce morbidity and mortality [12, 13].

In our study, SQ may alter cardiac PPAR and/or NF- $\kappa$ B activity. If the SQ altered cardiac PPAR expression, it could also limit cardiac NF- $\kappa$ B expression and associated cardiac inflammation and fibrosis. We then tested the hypothesis that SQ is also associated with increasing cardiac PPAR expression, decreasing NF- $\kappa$ B expression, and reducing cardiac expression of cytokines and growth factors relevant to myocardial fibrosis pathogenesis.

## 2. Method

**2.1. Animals.** Thirty-two four-week-age male spontaneously hypertensive rats (SHR) were purchased from Beijing Vital River Animal Technique Limited Corporation (certificate no. SCXK 2006-0008) and sixteen four-week-age male Wistar-Kyoto (WKY) rats were obtained from SLAS Laboratory Animal (Shanghai, certificate no. SCXK 2007-0005). The animals were housed in cages at  $22 \pm 2^\circ\text{C}$  and humidity of  $40 \pm 5\%$  under a 12-hour light/dark cycle and received standard diet and water ad libitum. The experimental procedures were in accordance with the European commission guidelines (2010/63/EU). All animals were handled according to the guidelines of the Peking University Animal Research

Committee. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University (LA2011-38).

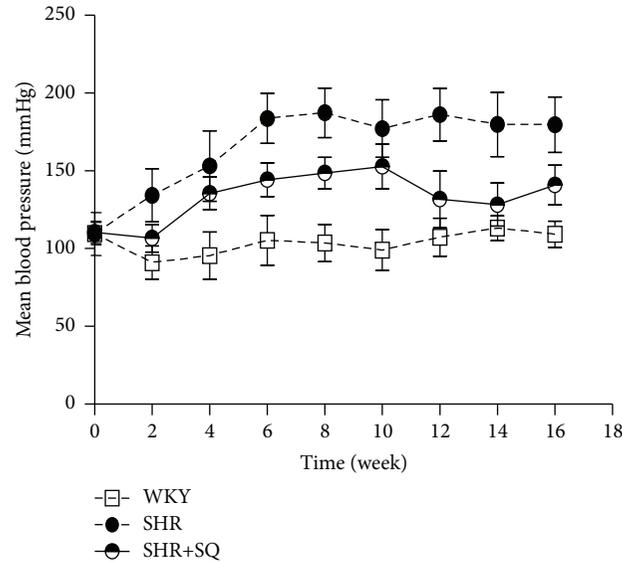
**2.2. Animal Grouping and Medicine.** SHRs were randomized into a SHR group ( $n = 16$ ) and a SHR+SQ group ( $n = 16$ ), with given 0.9% NaCl and Sang-Qi Granula (produced by China-Japan Friendship Hospital) at a dose of  $100 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  treatment separately. WKY rats were fed with 0.9% NaCl and served as the control group (WKY group). The drug and 0.9% NaCl administration were performed via gastric gavage twice a day until the end of 16 weeks.

**2.3. Blood Pressure Measurement.** Systolic blood pressure (BP) was monitored once every two weeks at 8 Am in a quiet room. After staying in a box at  $29 \pm 1^\circ\text{C}$  for 10 min, the tail systolic blood pressure was measured using a blood pressure monitor (BP-98A, U0130163, Tokyo, Japan). Body weight was measured once a week.

**2.4. Tissue Preparation for Histology.** Half of the animals in each group ( $n = 6$ ) were anesthetized with pentobarbital sodium ( $0.1 \text{ g/kg}$  body weight) intraperitoneally at the 8th week. The rest of the animals were sacrificed at the 16th week. The hearts were rapidly excised and washed with saline on ice. The hearts were accurately weighed after the excess water on the surface was removed with filter paper. The ratio of the heart weight to body weight (HW/BW) was calculated. Then the left ventricular (LV) was divided into two parts: a section from the LV free wall was fixed in phosphate buffered 10% formalin overnight and embedded in paraffin for histopathological examination. The remaining part of LV was snap frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for subsequent protein. Blood samples were taken from abdominal aorta and the plasma was stored at  $-20^\circ\text{C}$  until assay.

**2.5. Masson Staining.** The sections were stained with Masson and examined with a light microscope (BX512DP70, Olympus, Tokyo, Japan), according to the standard procedure [14]. Five fields in the ventricles of each animal were randomly selected, and the interstitial collagen volume fraction (CVF) and perivascular collagen volume area (PVCA) were quantified in the slides, in which the collagen fibers were visualized in blue.

**2.6. Immunohistochemistry.** The sections were incubated with antibody against CD68 after blocking with goat serum albumin. Incubation with PBS instead of the primary antibody served as a negative control. The samples were then incubated with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (Zhongshan Goldenbridge Biotechnology Co., Ltd., China; dilution 1 : 3000). The images were captured by a digital camera connected to a microscope (BX512DP70, Olympus, Tokyo, Japan) and analyzed with Image-Pro Plus 5.0 software (IPP, Media Cybernetic,



Group	n	0W	8W	16W
WKY	16	87.26 ± 4.96	103.54 ± 11.96	109.17 ± 8.34
SHR	16	109.82 ± 13.71*	162.19 ± 17.65*	154.88 ± 16.41*
SHR+SQ	15	110.52 ± 7.10*	148.56 ± 10.04**	141.44 ± 12.81**

FIGURE 1: WKY: Wistar-Kyoto rats without treatments. SHR: spontaneous hypertensive rats without treatments. SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatments. Data were expressed as mean ± SD of 12 animals.

Bethesda, MD, USA). Five fields of left ventricle were examined for each animal.

**2.7. ELISA.** At weeks of 8 and 16, animals from each group were anesthetized and the hearts were removed and homogenized in lysis buffer including protease inhibitor on ice. After being centrifuged at 20000 rcf for 60 minutes, the supernatant was collected for determination of MCP-1 content in heart tissues by ELISA, according to the manufacturer's instruction. TNF- $\alpha$  and ICAM-1 content in rat plasma were evaluated by Elisa.

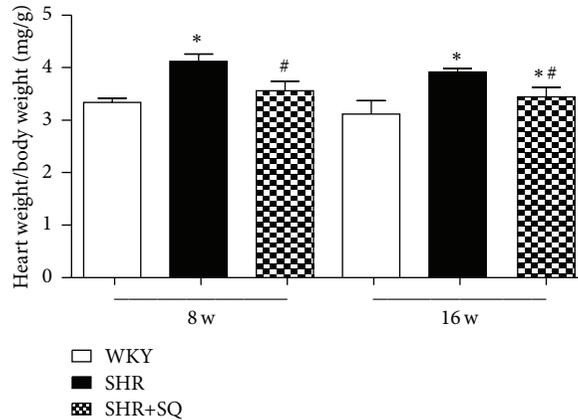
**2.8. Western Blot Analysis.** Western blot was performed as described previously [15]. Briefly, the heart was removed at weeks 8 and 16 and then was homogenized in lysis buffer including protease inhibitors. About 100  $\mu$ g of the supernatant was mixed with 4 $\times$  sample buffer. The protein samples were separated on Tris-glycine SDS-PAGE in a reducing condition. The nuclear proteins were extracted using Nuclear and Cytoplasmic Extraction Reagents (NEPER) kits (Thermo Scientific) according to the manual provided by the manufacturer. About 100  $\mu$ g of protein from each sample was separated by 12% SDS-PAGE. The primary antibodies used included those that directed against PPAR $\alpha$  (1:1000, Abcam, Cambridge, UK), PPAR $\gamma$  (1:1000, Abcam, Cambridge, UK), NF- $\kappa$ B P50 (1:1000, Abcam, Cambridge, UK), NF- $\kappa$ B P65 (1:800, Cell Signaling Technology, Boston,

MA, USA), ICAM-1 (1:200, Santa Cruz Biotechnology, Santa Cruz, USA), TGF- $\beta$  (1:1000 Abcam, Cambridge, UK), Smad 3 (1:1000 Abcam, Cambridge, UK), GAPDH (1:2000, Cell Signaling Technology, Boston, MA, USA), and H3 (Histone3, 1:1000 Cell Signaling Technology, Boston, MA, USA). After washing with Tris-buffered saline containing 0.05% Tween-20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:3000, Cell Signaling Technology, Boston, MA, USA) at room temperature for 60 min. The membranes were analyzed using the enhanced chemiluminescence system, according to the manufacturer's protocol and exposed in a dark box. The protein signal was quantized by scanning densitometry in the X-film by bioimage analysis system (Image-Proplus 5.0, Media Cybermetrics, Bethesda, MD, USA).

**2.9. Statistical Analysis.** All parameters are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA, followed by Turkey test for multiple comparisons. A probability of less than 0.05 was considered to be statistically significant.

### 3. Result

**3.1. Effects of SQ on Systolic Blood Pressure (SBP) and Heart Weight/Body Weight (HW/BW) in SHR.** Time-related changes in SBP for the three groups are shown in Figure 1.



Group	<i>n</i>	BW (g)	HW (mg)	HW/BW (mg·g <sup>-1</sup> )
WKY	12	8 w 307 ± 17.26	8 w 1079 ± 101.9	3.5 ± 0.29
		16 w 361.75 ± 21.22	16 w 1127 ± 71.6	3.12 ± 0.24
SHR	12	8 w 264.75 ± 18.13	8 w 1046 ± 59.2	3.96 ± 0.24*
		16 w 295.25 ± 15.52	16 w 1093 ± 85.5	3.92 ± 0.06*
SHR+SQ	12	8 w 248 ± 15.07	8 w 927 ± 86.4	3.69 ± 0.21#
		16 w 285 ± 21.66	16 w 1055 ± 68.3	3.61 ± 0.04*#

FIGURE 2: WKY: Wistar-Kyoto rats without treatment. SHR: spontaneous hypertensive rats without treatment. SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. Data were expressed as mean ± SD of 12 animals. \* $P < 0.05$  versus WKY # $P < 0.05$  versus SHR.

After 8 weeks of treatment, SBP in SHR group was significantly higher than that in WKY group and SHR+SQ group while SBP in WKY group was lower than that in SHR+SQ group. After 16 weeks, the trend is still the same, but SBP in SHR+SQ was much lower than before. We now observed the effect of SQ on HW/BW (Figure 2). The result showed that the ratios of HW/BW in SHR group were increased compared with WKY group and SHR+SQ group at weeks 8 and 16. In SHR+SQ group, HW/BW was a little higher than that in WKY group. But there was no difference between WKY and SHR+SQ group.

**3.2. Effects of SQ on Interstitial and Perivascular Fibrosis in Left Ventricle of SHR.** The SHR group showed a significant increase in the CVF compared to WKY group and SHR+SQ group (Figure 3). The collagen deposit immediately surrounding the vascular was also increased in SHR group compared to WKY group and SHR+SQ group (Figure 4). Treatment with SQ for 8 weeks and 16 weeks decreases both interstitial and perivascular collagen accumulation in SHR, and the effect of long-term treatment is more excellent. All of those factors indicated that SQ inhibited myocardial fibrosis by suppressing fibril deposition.

**3.3. SQ Increasing the Downregulated Expression of PPARs.** In SHR group, both PPAR $\alpha$  and PPAR $\gamma$  expression were sharply decreased ( $P < 0.05$ ) as compared with SHR+SQ

group, which were statistically significant (Figures 5(a) and 5(b)). The expression level of PPAR $\alpha$  and PPAR $\gamma$  in SHR+SQ group is similar to WKY group. The conserved upregulated expression in PPAR $\alpha$  and PPAR $\gamma$  (relative to SHR group) could suggest a specific effect of SQ on PPAR $\alpha$  and PPAR $\gamma$  expression.

**3.4. SQ Reducing NF- $\kappa$ B Expression.** The protein levels of P65 and P50 proteins in the nucleus were significantly increased in SHR group but reduced in the SHR+SQ group. There was very weak expression of P50 and P65 in the WKY group (Figure 6). In contrast, the cytoplasmic levels of P65 and P50 in SHR group were significantly lower than those in WKY and SHR+SQ groups. In line with the alteration of the NF- $\kappa$ B signaling, NF- $\kappa$ B targeted cytokines, such as TNF- $\alpha$  and MCP-1 expression, were significantly elevated in SHR group.

**3.5. SQ Attenuating Proinflammatory Mediators, Infiltration of Monocytes, and Inhibiting Collagen Deposition.** There was significantly increased expression of TNF- $\alpha$ , ICAM-1, and MCP-1 in SHR group whereas the upregulated expression was reduced in SHR+SQ group (Figures 7(a), 7(b), and 7(c)). TNF- $\alpha$  is a useful index of the level of cardiac inflammation and collagen. After 8 and 16 weeks of treatment with SQ, TNF- $\alpha$  in SHR+SQ group was less than that in SHR group, suggesting that SQ facilitated the degradation of collagen and decreased inflammation. Immunohistochemistry staining of CD68 is shown in Figure 8 to display monocyte infiltration

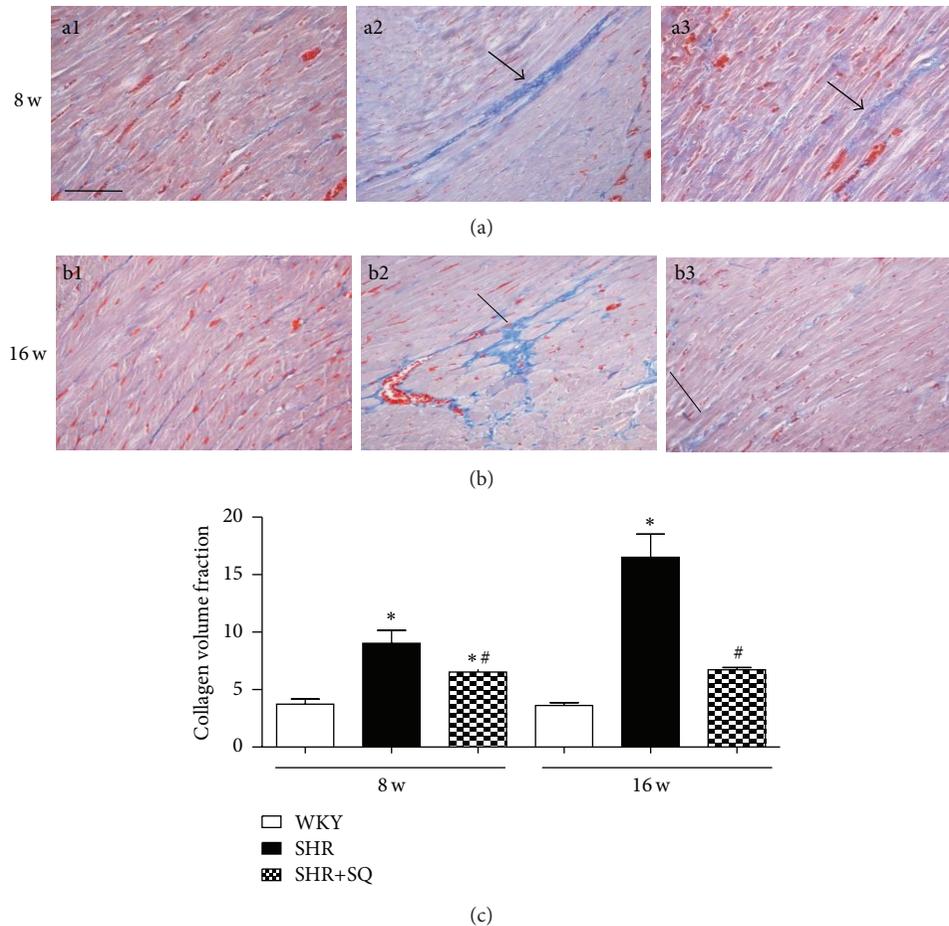


FIGURE 3: (a1) WKY: Wistar-Kyoto rats without treatment. (a2) SHR: spontaneous hypertensive rats without treatment. (a3) SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. (b1) WKY: Wistar-Kyoto rats without treatment. (b2) SHR: spontaneous hypertensive rats without treatment. (b3) SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. Data were expressed as mean  $\pm$  SD of 3 animals. \* $P < 0.05$  versus WKY # $P < 0.05$  versus SHR.

and myocardial damage. The number of CD68 positive cells increased prominently in SHR group whereas only few CD68 positive cells exhibited in WKY group and SHR+SQ group. This result was in line with an increase in the expression of MCP-1 in SHR group.

**3.6. SQ Inhibiting Collagen Deposition and Expression of TGF- $\beta$ 1 and Smad-3.** Results showed that, compared with SHR group, SQ had reduced TGF- $\beta$ 1 and Smad-3 expression, which were statistically significant. In contrast, the expression of TGF- $\beta$ 1 and Smad-3 in SHR group was sharply increased (Figures 9(a) and 9(b)).

#### 4. Discussion

Uncontrolled and prolonged elevation of BP pressure will lead to a variety of changes in the myocardial structure, coronary vasculature function, and the function of the cardiac conductive system. These changes in turn would induce the development of left ventricular hypertrophy

(LVH), coronary artery disease (CAD), and systolic and diastolic dysfunction of the myocardium, as well as complications that manifest clinically as angina or myocardial infarction, cardiac arrhythmias (especially atrial fibrillation), and congestive heart failure (CHF). Although these diseases generally develop in response to chronically elevated BP, marked and acute elevation of BP can lead to accentuation of an underlying predisposition to any of the symptoms traditionally associated with chronic hypertension.

Hypertension produces collagen deposition, changes referred to as myocardial fibrosis, which leads to depressed cardiac performance. Myocardial fibrosis is characterized by both quantitative and qualitative alterations of cardiac extracellular matrix (ECM) and hypertrophy of cardiocytes [16]. Cardiac fibroblasts phenotypically transformed myofibroblasts play a crucial role in the regulation of the ECM composition of the heart by synthesizing collagen and other matrix proteins [15, 17]. Myocardial fibrosis is a complex phenomenon reflecting the loss of the physiological reciprocal regulation between stimulatory (e.g., angiotensin II, endothelin I, catecholamines, aldosterone, basic fibroblast

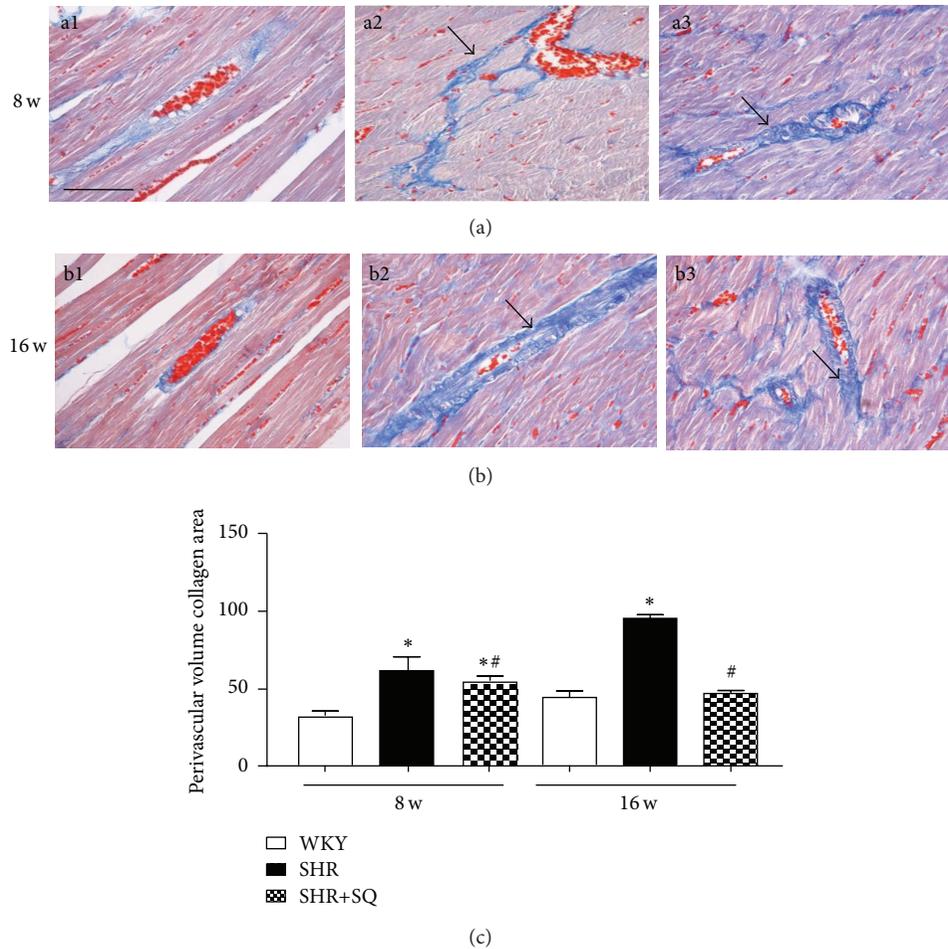


FIGURE 4: (a1) WKY: Wistar-Kyoto rats without treatment. (a2) SHR: spontaneous hypertensive rats without treatment. (a3) SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. (b1) WKY: Wistar-Kyoto rats without treatment. (b2) SHR: spontaneous hypertensive rats without treatment. (b3) SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. Data were expressed as mean  $\pm$  SD of 3 animals. \* $P < 0.05$  versus WKY # $P < 0.05$  versus SHR.

growth factor, insulin-like growth factor, etc.) and inhibitory factors (prostaglandins, nitric oxide, natriuretic peptides, etc.) acting on the turnover of fibrillar collagen [18]. In this study, the systolic blood pressure was decreased in SHR+SQ group compared with SHR group. The CVF and PVCA, important indexes of cardiac fibrosis, were obviously lower in SHR+SQ group. The results showed that the degree of fibrosis was significantly lower when treated with SQ.

In several fibrotic processes, the role of inflammation has been clearly demonstrated. Several hypertension models revealed that perivascular fibrosis was often associated with inflammation cell around small arteries in the myocardium [19]. Profibrogenic cytokines are indeed released by inflammation cells [20]. Increased wall tension is involved in the extravasation of inflammatory cells around vessels, and then various cytokines from infiltrating cells, such as macrophages, become a trigger for perivascular and interstitial fibrosis [21]. Since Shahar [22] demonstrated that fibroblast proliferation in human interstitial lung disease was related to inflammatory cells, such as macrophages and lymphocytes,

which can release cytokines that can act on cardiac resident interstitial fibroblasts.

It provides extensive pharmacological effect on cardiovascular system. It is verified that Sang-qi Granula plays an important role in inhibiting ventricular hypertrophy in animal experiments in the past [5]. In traditional Chinese medicine (TCM), hypertension is classified as “dizziness”. Its basic pathogenesis is asthenia in origin and asthenia in superficiality. Asthenia in origin is the impairment of the liver and kidney. Asthenia in superficiality is the hyperactivity of liver-Yang, retention of phlegmatic dampness, and obstruction of collaterals by blood stasis. Consequently, we should apply, therapy strategies like nourishing the liver and kidney, calming the liver and suppressing Yang, eliminating dampness, resolving phlegm, and activating blood circulation to remove blood stasis. So herba taxilli, and eucommia bark were used to tonify the liver and kidney; barberry wolfberry fruit and *Alisma L. orientale* Juzep were used to nourish kidney Yin and clear deficient fire. *Cassiae torae* and *chrysanthemi indicis* were used to clear liver heat and suppress liver Yang. *Salvia miltiorrhiza* root was used to

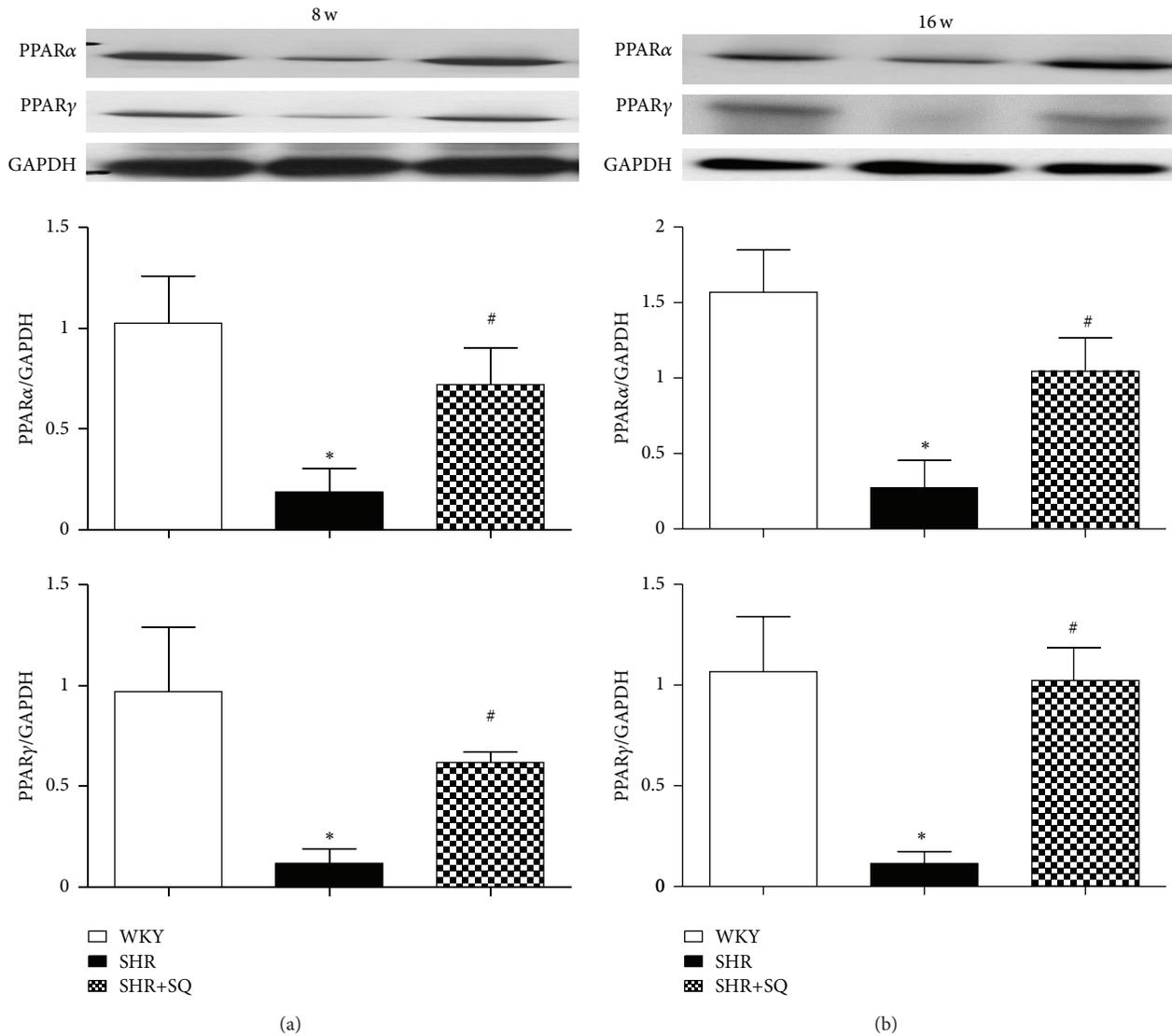


FIGURE 5: WKY: Wistar-Kyoto rats without treatment. SHR: spontaneous hypertensive rats without treatment. SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. Data were expressed as mean  $\pm$  SD of 4 animals. \* $P < 0.05$  versus WKY # $P < 0.05$  versus SHR.

nourish blood and promote blood circulation as well as communication between the heart and kidney. These herbs played an important role in harmonizing Yin and Yang, calming liver, suppressing endogenous wind, and promoting blood circulation. They can treat principal and subordinate symptoms simultaneously.

However, as a therapeutic agent, the mechanism of SQ in preventing myocardial fibrosis still need to be investigated furtherly. The major process may be the inhibition of NF- $\kappa$ B, a nuclear transcription factor that transactivates promoters of many inflammation infection and stress genes, including cytokines, and elicits a hypertrophic response in cardiac myocytes [23]. However, in resting cells, NF- $\kappa$ B proteins are present in the cytoplasm as inactive heterodimers composed of two subunits, P50 and P65, and are bound to the inhibitory protein I $\kappa$ Ba, which prevents it from translocating into the nucleus of the cell [24]. I $\kappa$ Ba, the intrinsic inhibitor of NF- $\kappa$ B,

is phosphorylated and proteolytically degraded through a 26S proteasome. On stimulation, I $\kappa$ Ba can facilitate NF- $\kappa$ B translocation into the nucleus and regulates gene transcription [25]. NF- $\kappa$ B translocates to the nucleus and binds to the I-kappa-B motif of the target gene, which causes activation of several factors involved in inflammatory responses. Various stimuli, including ischemia, free radicals, and cytokines, activate NF- $\kappa$ B by inducing I $\kappa$ Ba phosphorylation [11].

NF- $\kappa$ B also plays an important role in myocardial fibrosis. NF- $\kappa$ B contributes to myocardial fibrosis pathogenesis because it regulates genes/proteins important for disease progression, including cytokines (e.g., TNF- $\alpha$ ), interleukins (e.g., IL-6), growth factors (e.g., TGF- $\beta$ ), and adhesion molecules (e.g., intercellular adhesion molecule) [11]. It is postulated that after MI, activation of NF- $\kappa$ B resulted in the expression of proinflammatory cytokines such as TNF- $\alpha$  and MCP-1 in cardiomyocytes, which promoted the infiltration

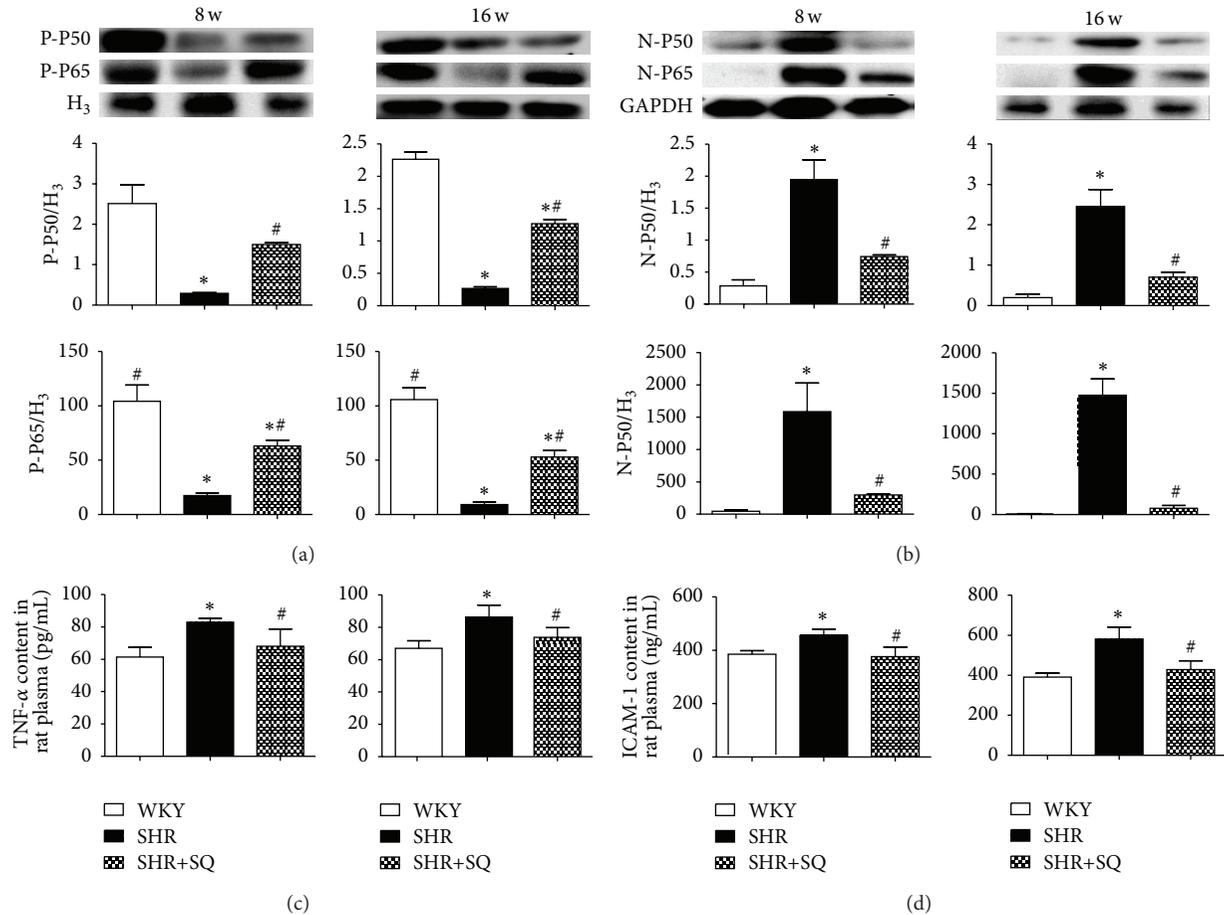


FIGURE 6: WKY: Wistar-Kyoto rats without treatment. SHR: spontaneous hypertensive rats without treatment. SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. Data were expressed as mean  $\pm$  SD of 4 animals. \* $P < 0.05$  versus WKY # $P < 0.05$  versus SHR.

of inflammatory cells, contributing to myocardial fibrosis [26]. TNF- $\alpha$  plays an important role in myocardial damage [27]. TNF- $\alpha$  stimulates the release of various inflammatory factors through autocrine and paracrine and induces cardiac myocyte apoptosis [28, 29]. Myocardial damage and apoptosis result in considerable infiltration of monocytes through MCP-1. MCP-1 is mainly released from apoptotic cells and recruits monocyte from the circulation to the apoptotic lesion. Infiltration of monocytes is a significant episode in the initiation of myocardial fibrosis, because the monocytes may differentiate into macrophages and participate in the healing process through production of growth factors, such as TGF- $\beta$  and Smad-3 [30]. TGF- $\beta$ /Smad 3 pathway plays an important role in cardiac remodeling [31]. In cardiovascular system, TGF- $\beta$  is implicated in the development and progression of hypertension, heart failure, and other cardiovascular diseases [32, 33]. TGF- $\beta$  is a cytokine with a broad range of regulatory effects on inflammation and cell proliferation, and it can regulate these processes through signaling pathway proteins called Smads [34]. In particular, the TGF- $\beta$ /Smad 3 pathway can regulate inflammatory response. The pathway suppresses cytokine and chemokine expression in immune and endothelial cells and reduces macrophage chemotaxis

[34]. In the process of ventricular remodeling, another aspect of the TGF- $\beta$ /Smad 3 pathway is the regulation of fibroblast activity TGF- $\beta$  induces phenotypic changes in fibroblasts to increase the expression of extracellular matrix protein [34]. As such, cardiac-specific deletion of NF- $\kappa$ B activation inhibits inflammatory response which leads to a reduction in myocardial fibrosis via TGF- $\beta$ /Smad 3 pathway. Given the critical role of NF- $\kappa$ B signaling in cardiac fibrosis [27, 28], NF- $\kappa$ B activation may represent an important mechanism for myocardial fibrosis.

The present study demonstrated that SQ has direct beneficial effects on cardiac inflammation and collagen deposition of SHR. The protective effects are associated with decreasing infiltration of monocyte, NF- $\kappa$ B, ICAM-1, TNF- $\alpha$ , MCP-1, and TGF- $\beta$ /Smads signaling molecules expression and increasing in PPAR $\alpha$  and PPAR $\gamma$  expression. These findings indicated that the favorable cardiac effects of SQ on SHR are at least partly dependent on PPAR $\alpha$  and PPAR $\gamma$  inhibiting inflammation through NF- $\kappa$ B signaling.

Inhibiting of NF- $\kappa$ B signaling may occur through different mechanisms and one of these mechanisms may be inhibiting the activation of PPAR $\alpha$  and PPAR $\gamma$ . PPAR opposes NF- $\kappa$ B activity in several ways. On one hand, PPAR improves

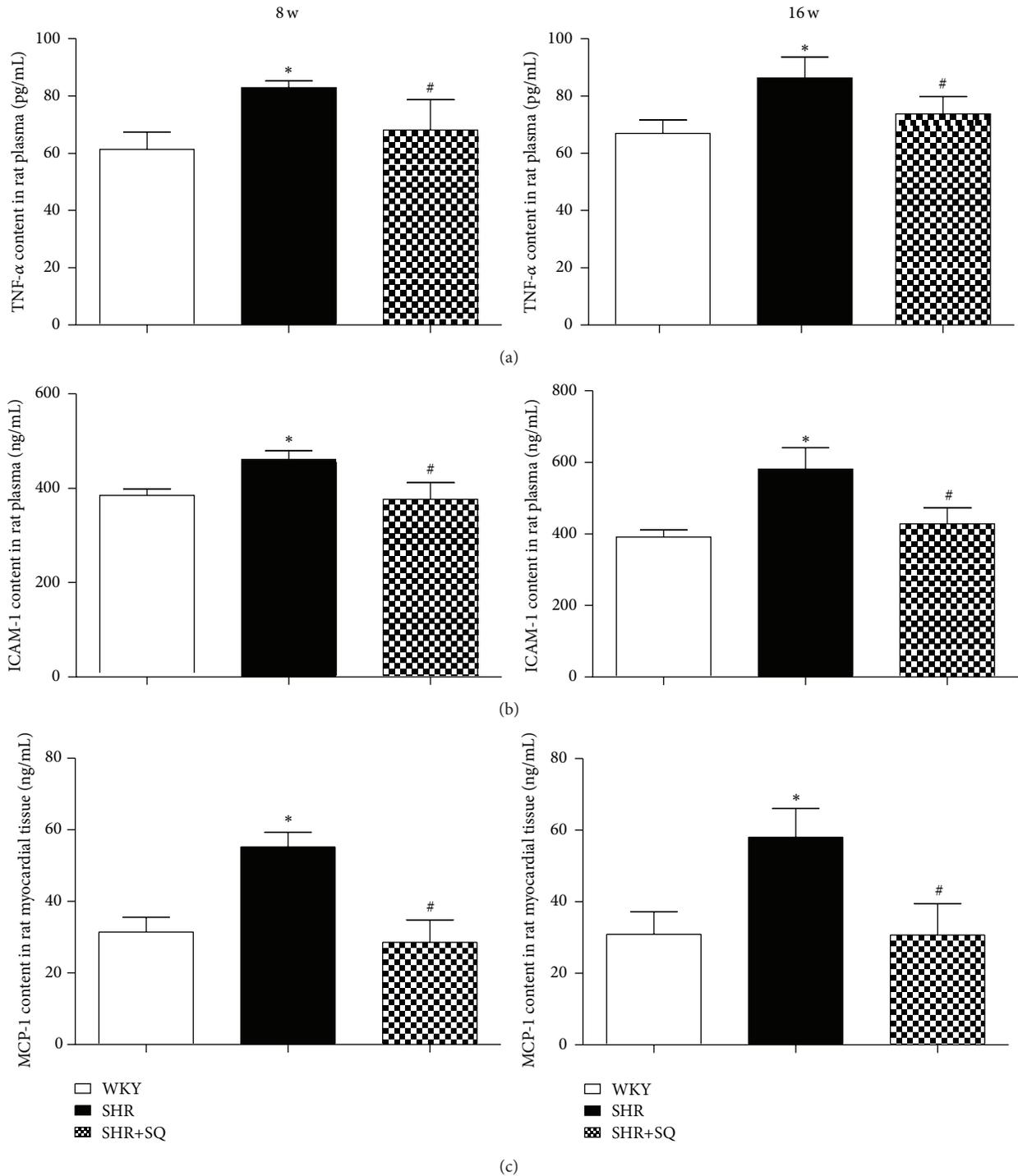


FIGURE 7: WKY: Wistar-Kyoto rats without treatment. SHR: spontaneous hypertensive rats without treatment. SHR+SQ: spontaneous hypertensive rats with Sang-qi Granula treatment. Data were expressed as mean  $\pm$  SD of 7 animals. \* $P < 0.05$  versus WKY # $P < 0.05$  versus SHR.

I $\kappa$ B $\alpha$  transcription [29]. Delerive [35, 36] found that PPAR $\gamma$  activators directed protein-protein-induced hepatic expression of I $\kappa$ B, thereby preventing the P50 and P65 translocation into the nucleus. On the other hand, NF- $\kappa$ B can inhibit PPAR binding to genomic PPAR response elements, thereby reducing PPAR transcriptional activity and the expression

level of PPAR-related transcripts [37]. The PPAR receptors are expressed by multiple cell types in the cardiovascular system, including cardiac myocytes and fibroblasts.

Studies with PPAR $\alpha$  and PPAR $\gamma$  confirm the inverse association of PPARs activity with NF- $\kappa$ B activity. Diep also reported in human aortic smooth muscle cells that

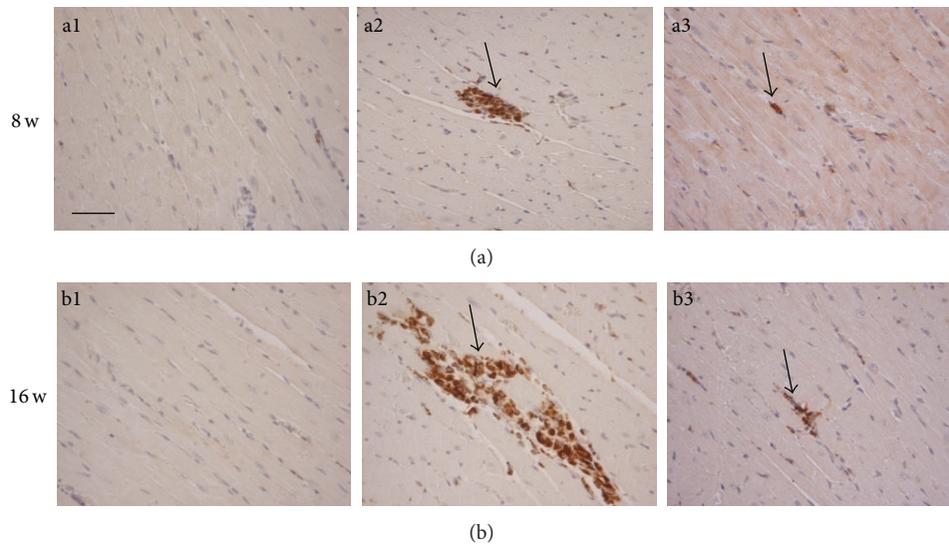


FIGURE 8: (a1) Wistar-Kyoto rats without treatment. (a2) spontaneous hypertensive rats without treatment. (a3) spontaneous hypertensive rats with Sang-qì Granula treatment. (b1) Wistar-Kyoto rats without treatment. (b2) spontaneous hypertensive rats without treatment. (b3) Spontaneous hypertensive rats with Sang-qì Granula treatment.

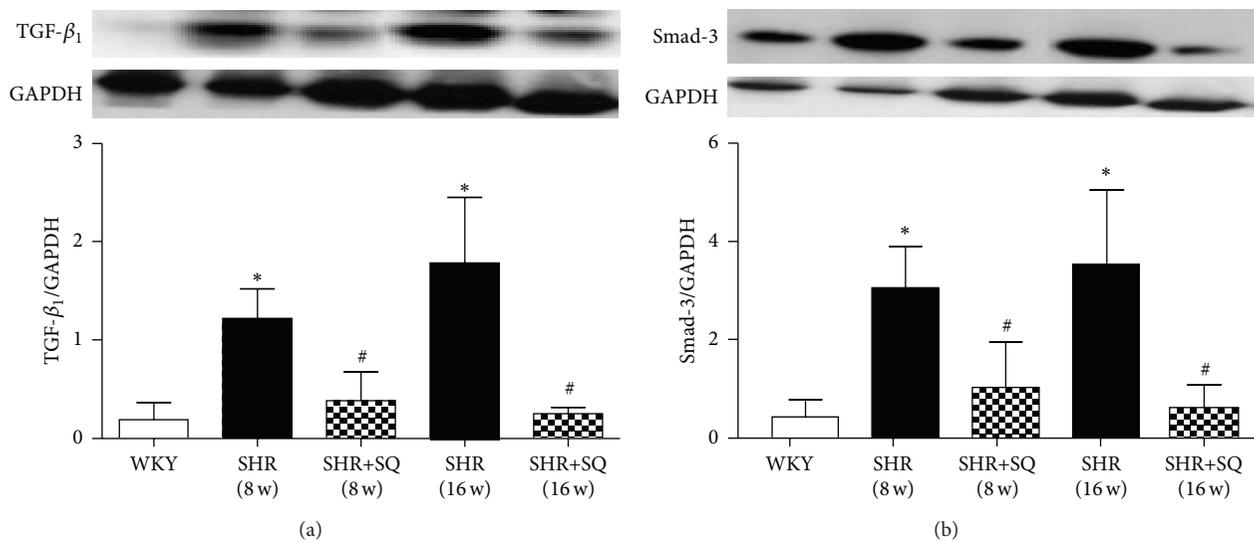


FIGURE 9: WKY: Wistar-Kyoto rats without treatment. SHR: spontaneous hypertensive rats without treatment. SHR+SQ: spontaneous hypertensive rats with Sang-qì Granula treatment. Data were expressed as mean  $\pm$  SD of 4 animals. \* $P < 0.05$  versus WKY # $P < 0.05$  versus SHR.

PPAR $\alpha$  activation inhibits cytokine-induced activation of a number of inflammatory genes, such as VCAM-1, COX-2, and IL-6 by negatively interfering with NF- $\kappa$ B transcriptional activity [38]. Schiffrin demonstrated that activation of PPAR $\alpha$  resulted in inhibition of NF- $\kappa$ B pathways that regulate expression of adhesion molecules ICAM-1 and VCAM-1 [39]. Recent studies have focused on ligands of PPAR $\gamma$ , for its actions on the myocardium [40], and other studies have suggested a role for PPAR $\gamma$  as an inhibitor of cardiac hypertrophy [36], that can decrease the NF- $\kappa$ B binding activity. PPAR $\gamma$  activators also directed protein-protein induced hepatic expression of I $\kappa$ B, thereby preventing the P50 and P65 translocation into the nucleus [35, 36].

In conclusion, the protective effect of SQ on myocardium may partly account for a decrease of NF- $\kappa$ B expression, inflammatory factors expression, myocardial damage, and MCP-1 expression which may diminish monocyte migration and infiltration and then inhibit cardiac fibrosis.

## 5. Limitation

As a limitation of this study, it should be pointed out that another mechanism exists in myocardial fibrosis besides inflammatory responses through the NF- $\kappa$ B signaling pathway. Furthermore, studies are necessary to determine the

upstream and downstream pathways of NF- $\kappa$ B and PPARs in order to elucidate the underlying molecular mechanism.

## 6. Conclusion

We demonstrated that prolonged hypertension-induced myocardial fibrosis was clearly prevented by treatment with SQ. From molecular analyses, we concluded that the reverse process of myocardial fibrosis was dependent on upregulation of PPAR $\alpha$  and PPAR $\gamma$  expression, downregulation of NF- $\kappa$ B expression, and suppressing TNF- $\alpha$ , ICAM-1, and MCP-1 production through the NF- $\kappa$ B signaling pathway. However, as a therapeutic agent, the effect of SQ on restraining myocardial fibrosis still needs further investigation.

## References

- [1] L. Li and L. Huang, "SQ granule inhibits myocardial fibrosis in spontaneously hypertension rats," *Chinese Journal of Microcirculation*, vol. 20, 2010.
- [2] P. Delerive, J. C. Fruchart, and B. Staels, "Peroxisome proliferator-activated receptors in inflammation control," *Journal of Endocrinology*, vol. 169, no. 3, pp. 453–459, 2001.
- [3] N. Marx, G. K. Sukhova, T. Collins, P. Libby, and J. Plutzky, "PPAR $\alpha$  activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells," *Circulation*, vol. 99, no. 24, pp. 3125–3131, 1999.
- [4] P. M. Barger, J. M. Brandt, T. C. Leone, C. J. Weinheimer, and D. P. Kelly, "Deactivation of peroxisome proliferator-activated receptor- $\alpha$  during cardiac hypertrophic growth," *The Journal of Clinical Investigation*, vol. 105, no. 12, pp. 1723–1730, 2000.
- [5] A. Jones, R. Deb, E. Torsney et al., "Rosiglitazone reduces the development and rupture of experimental aortic aneurysms," *Circulation*, vol. 119, no. 24, pp. 3125–3132, 2009.
- [6] K. Takeda, T. Ichiki, T. Tokunou et al., "Peroxisome proliferator-activated receptor  $\gamma$  activators downregulate angiotensin II type 1 receptor in vascular smooth muscle cells," *Circulation*, vol. 102, no. 15, pp. 1834–1839, 2000.
- [7] A. Sugawara, A. Uruno, M. Kudo, K. Matsuda, C. W. Yang, and S. Ito, "Effects of PPAR $\gamma$  on hypertension, atherosclerosis, and chronic kidney disease," *Endocrine Journal*, vol. 57, no. 10, pp. 847–852, 2010.
- [8] K. Benkirane, É. C. Viel, F. Amiri, and E. L. Schiffrin, "Peroxisome proliferator-activated receptor  $\gamma$  regulates angiotensin II-stimulated phosphatidylinositol 3-kinase and mitogen-activated protein kinase in blood vessels in vivo," *Hypertension*, vol. 47, no. 1, pp. 102–108, 2006.
- [9] J. Ma, M. Wei, Q. Wang, and T. Peng, "Deficiency of capn4 gene inhibits nuclear factor- $\kappa$ B (NF- $\kappa$ B) protein signaling/inflammation and reduces remodeling after myocardial infarction," *Journal of Biological Chemistry*, vol. 287, pp. 27480–27489, 2012.
- [10] H. J. Maier, T. G. Schips, A. Wietelmann, and T. Wirth, "Cardiomyocyte-specific  $\kappa$ B kinase (IKK)/NF- $\kappa$ B activation induces reversible inflammatory cardiomyopathy and heart failure," *PNAS*, vol. 109, pp. 11794–11799, 2012.
- [11] E. M. Seymour, M. R. Bennink, S. W. Watts, and S. F. Bolling, "Whole grape intake impacts cardiac peroxisome proliferator-activated receptor and nuclear factor  $\kappa$ B activity and cytokine expression in rats with diastolic dysfunction," *Hypertension*, vol. 55, no. 5, pp. 1179–1185, 2010.
- [12] T. Ogata, T. Miyauchi, S. Sakai, M. Takanashi, Y. Irukayama-Tomobe, and I. Yamaguchi, "Myocardial fibrosis and diastolic dysfunction in deoxycorticosterone acetate-salt hypertensive rats is ameliorated by the peroxisome proliferator-activated receptor- $\alpha$  activator fenofibrate, partly by suppressing inflammatory responses associated with the nuclear factor- $\kappa$ -B pathway," *Journal of the American College of Cardiology*, vol. 43, no. 8, pp. 1481–1488, 2004.
- [13] P. J. H. Smeets, B. E. J. Teunissen, A. Planavila et al., "Inflammatory pathways are activated during cardiomyocyte hypertrophy and attenuated by peroxisome proliferator-activated receptors PPAR $\alpha$  and PPAR $\gamma$ ," *The Journal of Biological Chemistry*, vol. 283, no. 43, pp. 29109–29118, 2008.
- [14] K. Sun, Q. Hu, C. M. Zhou et al., "Cerebralcare granule<sup>®</sup>, a chinese herb compound preparation, improves cerebral micro-circulatory disorder and hippocampal CA1 neuron injury in gerbils after ischemia-reperfusion," *Journal of Ethnopharmacology*, vol. 130, no. 2, pp. 398–406, 2010.
- [15] J. S. Swaney, D. M. Roth, E. R. Olson, J. E. Naugle, J. G. Meszaros, and P. A. Insel, "Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 2, pp. 437–442, 2005.
- [16] P. L. Bu, X. Q. Zhao, L. L. Wang, Y. X. Zhao, C. B. Li, and Y. Zhang, "Tong-xin-luo capsule inhibits left ventricular remodeling in spontaneously hypertensive rats by enhancing PPAR- $\gamma$  expression and suppressing NF- $\kappa$ B activity," *Chinese Medical Journal*, vol. 121, no. 2, pp. 147–154, 2008.
- [17] G. A. Walker, K. S. Masters, D. N. Shah, K. S. Anseth, and L. A. Leinwand, "Valvular myofibroblast activation by transforming growth factor- $\beta$ : implications for pathological extracellular matrix remodeling in heart valve disease," *Circulation Research*, vol. 95, no. 3, pp. 253–260, 2004.
- [18] K. T. Weber, "Fibrosis and hypertensive heart disease," *Current Opinion in Cardiology*, vol. 15, pp. 264–272, 2000.
- [19] A. Nicoletti and J. B. Michel, "Cardiac fibrosis and inflammation: interaction with hemodynamic and hormonal factors," *Cardiovascular Research*, vol. 41, no. 3, pp. 532–543, 1999.
- [20] W. A. Border and N. A. Noble, "Transforming growth factor  $\beta$  in tissue fibrosis," *The New England Journal of Medicine*, vol. 331, no. 19, pp. 1286–1292, 1994.
- [21] T. Ogata, T. Miyauchi, S. Sakai, M. Takanashi, Y. Irukayama-Tomobe, and I. Yamaguchi, "Myocardial fibrosis and diastolic dysfunction in deoxycorticosterone acetate-salt hypertensive rats is ameliorated by the peroxisome proliferator-activated receptor- $\alpha$  activator fenofibrate, partly by suppressing inflammatory responses associated with the nuclear factor- $\kappa$ -B pathway," *Journal of the American College of Cardiology*, vol. 43, no. 8, pp. 1481–1488, 2004.
- [22] I. Shahar, E. Fireman, M. Topilsky et al., "Effect of IL-6 on alveolar fibroblast proliferation in interstitial lung diseases," *Clinical Immunology and Immunopathology*, vol. 79, no. 3, pp. 244–251, 1996.
- [23] S. Gupta, N. H. Purcell, A. Lin, and S. Sen, "Activation of nuclear factor- $\kappa$ B is necessary for myotrophin-induced cardiac hypertrophy," *The Journal of Cell Biology*, vol. 159, no. 6, pp. 1019–1028, 2002.
- [24] P. F. Gomez, M. H. Pillinger, M. Attur et al., "Resolution of inflammation: prostaglandin E2 dissociates nuclear trafficking

- of individual NF- $\kappa$ B subunits (p65, p50) in stimulated rheumatoid synovial fibroblasts," *Journal of Immunology*, vol. 175, no. 10, pp. 6924–6930, 2005.
- [25] W. D. Strayhorn and B. E. Wadzinski, "A novel in vitro assay for deubiquitination of I $\kappa$ B $\alpha$ ," *Archives of Biochemistry and Biophysics*, vol. 400, no. 1, pp. 76–84, 2002.
- [26] S. Frantz, J. Bauersachs, and G. Ertl, "Post-infarct remodelling: contribution of wound healing and inflammation," *Cardiovascular Research*, vol. 81, no. 3, pp. 474–481, 2009.
- [27] L. Zelarayan, A. Renger, and C. Noack, "NF- $\kappa$ B activation is required for adaptive cardiac hypertrophy," *Cardiovascular Research*, vol. 84, no. 3, pp. 416–424, 2009.
- [28] S. M. O. 'Donnell, M. W. Hansberger, and J. L. Connolly, "Organ-specific roles for transcription factor NF- $\kappa$ B in reovirus-induced apoptosis and disease," *The Journal of Clinical Investigation*, vol. 115, no. 9, pp. 2341–2350, 2005.
- [29] O. Yokoseki, J. I. Suzuki, H. Kitabayashi et al., "Cis element decoy against nuclear factor- $\kappa$ B attenuates development of experimental autoimmune myocarditis in rats," *Circulation Research*, vol. 89, no. 10, pp. 899–906, 2001.
- [30] R. Kleemann, P. P. Gervois, L. Verschuren, B. Staels, H. M. G. Princen, and T. Kooistra, "Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NF $\kappa$ B-C/EBP- $\beta$  complex formation," *Blood*, vol. 101, no. 2, pp. 545–551, 2003.
- [31] M. Bujak, G. Ren, H. J. Kweon et al., "Essential role of Smad3 in infarct healing and in the pathogenesis of cardiac remodeling," *Circulation*, vol. 116, no. 19, pp. 2127–2138, 2007.
- [32] A. Bobik, "Transforming growth factor-betas and vascular disorders," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, pp. 1712–1720, 2006.
- [33] R. A. Rahimi and E. B. Leof, "TGF- $\beta$  signaling: a tale of two responses," *Journal of Cellular Biochemistry*, vol. 102, no. 3, pp. 593–608, 2007.
- [34] D. Seo and J. M. Hare, "The transforming growth factor- $\beta$ /Smad3 pathway: coming of age as a key participant in cardiac remodeling," *Circulation*, vol. 116, no. 19, pp. 2096–2098, 2007.
- [35] P. Delerive, P. Gervois, J. C. Fruchart, and B. Staels, "Induction of I $\kappa$ B $\alpha$  expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferator-activated receptor- $\alpha$  activators," *The Journal of Biological Chemistry*, vol. 275, no. 47, pp. 36703–36707, 2000.
- [36] P. Delerive, K. de Bosscher, S. Besnard et al., "Peroxisome proliferator-activated receptor  $\alpha$  negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF- $\kappa$ B and AP-1," *The Journal of Biological Chemistry*, vol. 274, no. 45, pp. 32048–32054, 1999.
- [37] N. Marx, G. K. Sukhova, T. Collins, P. Libby, and J. Plutzky, "PPAR $\alpha$  activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells," *Circulation*, vol. 99, no. 24, pp. 3125–3131, 1999.
- [38] Q. N. Diep, K. Benkirane, F. Amiri, J. S. Cohn, D. Endemann, and E. L. Schiffrin, "PPAR $\alpha$  activator fenofibrate inhibits myocardial inflammation and fibrosis in angiotensin II-infused rats," *Journal of Molecular and Cellular Cardiology*, vol. 36, no. 2, pp. 295–304, 2004.
- [39] E. L. Schiffrin, F. Amiri, K. Benkirane, M. Iglarz, and Q. N. Diep, "Peroxisome proliferator-activated receptors: vascular and cardiac effects in hypertension," *Hypertension*, vol. 42, no. 4, pp. 664–668, 2003.
- [40] M. Asakawa, H. Takano, T. Nagai et al., "Peroxisome proliferator-activated receptor  $\gamma$  plays a critical role in inhibition of cardiac hypertrophy in vitro and in vivo," *Circulation*, vol. 105, no. 10, pp. 1240–1246, 2002.

## Research Article

# Anti-Proliferative Effect of an Aqueous Extract of *Prunella vulgaris* in Vascular Smooth Muscle Cells

Sun Mi Hwang,<sup>1,2</sup> Yun Jung Lee,<sup>1,3</sup> Yong Pyo Lee,<sup>1,3</sup> Jung Joo Yoon,<sup>1,3</sup> So Min Lee,<sup>1,3</sup> Jeong Dan Cha,<sup>4</sup> Kyung Min Choi,<sup>4</sup> Dae Gill Kang,<sup>1,3</sup> and Ho Sub Lee<sup>1,3</sup>

<sup>1</sup> College of Oriental Medicine and Professional Graduate School of Oriental Medicine, Wonkwang University, Shinyong-dong, Iksan, Jeonbuk 570-749, Republic of Korea

<sup>2</sup> Center for Bioanalysis, Division of Metrology for Quality of Life, Korea Research Institute of Standards and Science, Doryong-dong, Yuseong-gu, Daejeon 305-340, Republic of Korea

<sup>3</sup> Hanbang Body-Fluid Research Center, Wonkwang University, Shinyong-dong, Iksan, Jeonbuk 570-749, Republic of Korea

<sup>4</sup> Department of Research Development, Institute of Jinan Red Ginseng, Jinan, Jeonbuk 567-801, Republic of Korea

Correspondence should be addressed to Dae Gill Kang; dgkang@wku.ac.kr

Received 21 March 2013; Accepted 10 August 2013

Academic Editor: Myeong S. Lee

Copyright © 2013 Sun Mi Hwang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The abnormal proliferation of vascular smooth muscle cells (VSMCs) in arterial walls is an important pathogenic factor of vascular disorders such as diabetic atherosclerosis. We have reported the anti-inflammatory effect of an aqueous extract from *Prunella vulgaris* (APV) in vascular endothelial cell. In the present study, APV exhibited inhibitory effects on high glucose-stimulated VSMC proliferation, migration, and invasion activities, inducing G<sub>1</sub> cell cycle arrest with downregulation of cyclins and CDKs and upregulation of the CKIs, p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup>. Furthermore, APV dose dependently suppressed the high glucose-induced matrix metalloproteinase activity. High glucose-induced phosphorylation of ERK, p38 MAPK, was decreased by the pretreatment of APV. NF- $\kappa$ B activation by high glucose was attenuated by APV, as an antioxidant. APV attenuated the high glucose-induced decrease of nuclear factor E2-related factor-2 (Nrf2) translocation and heme oxygenase-1 (HO-1) expression. Intracellular cGMP level was also increased by APV treatment. These results demonstrate that APV may inhibit VSMC proliferation via downregulating ROS/NF- $\kappa$ B/ERK/p38 MAPK pathways. In addition, APV has a beneficial effect by the interaction of Nrf2-mediated NO/cGMP with HO-1, suggesting that *Prunella vulgaris* may be useful in preventing diabetic atherosclerosis.

## 1. Introduction

Atherosclerosis is one of the most important pathogenic mechanisms in vascular dysfunction. Chronic hyperglycemia-induced atherosclerosis involves a complex series of events, including abnormal vascular smooth muscle cells (VSMCs) proliferation and migration, which contribute importantly to the formation of organized atherosclerotic plaque [1, 2]. VSMCs are the main cellular component of the arterial wall and fundamental for the maintenance of normal vascular function and structure. Within the arterial media, VSMCs are normally arrested at G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle and are thus quiescent [3]. After vessel injury by inflammation and oxidative stress, VSMCs migrate into the intima,

where they reenter the cell cycle [4]. Transition through G<sub>1</sub> phase and entry into S phase require activation of cyclin-dependent kinases (CDKs) such as CDK2 and CDK4 through the formation of cyclin/CDK complexes, a process in which cyclin D1 and cyclin E play major roles. The kinase activities of the cyclin/CDK complexes are negatively regulated by CDK inhibitors (CKIs) such as p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> [5, 6]. High glucose activates the expression of several genes involved in mitogen-activated protein kinase- (MAPK-) dependent mitogenic response, contributing to VSMC proliferation and migration, as a result, to the development of atherosclerosis [7]. VSMC migration, which depends on an alteration of the proteolytic balance within the arterial wall toward matrix breakdown, is partly mediated by matrix metalloproteinase

(MMP) [8–10]. The promoter region of MMP-2 contains various cis-acting elements, including potential binding sites for the transcription factors: nuclear factor-kappa B (NF- $\kappa$ B), activator protein-1 (AP-1), and stimulatory protein-1 (SP-1). Various growth factors, cytokines, and hormones stimulate the expression of MMP-2 via activation of the NF- $\kappa$ B pathway [11, 12].

Heme oxygenase- (HO-) 1 is a stress-inducible protein well known for playing a role of important components in intracellular antioxidant, anti-inflammatory, and antiapoptotic effects [13, 14]. HO-1, which is highly expressed in vascular tissues, protects against vasculopathy and confers a cytoprotective function in the circulation. HO-1 deficiency results in cytokines production-mediated endothelial damage [15]. Therefore, HO-1 may play an important role in sustaining the health of the vascular system. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that normally resides in the cytoplasm bound to Kelch-like ECH-associated protein- (Keap-) 1 [16]. Upon activation by oxidative stress, it binds to the antioxidant response element (ARE) and activates transcription of ARE-regulated genes. ARE-regulated genes may contribute to the maintenance of redox homeostasis by serving as endogenous antioxidant systems through the action of proteins such as heme oxygenase-1 (HO-1), ferritin, glutathione peroxidase (GPx), and NAD(P)H: quinone oxidoreductase [17].

*Prunella vulgaris* var. lilacina (herbal name: *Prunellae Spica*) is a perennial herb that is widely distributed around Far East Asia countries throughout Korea, China, and Japan. *Prunella vulgaris* has been used as a traditional medicine to reduce sore throat, alleviate fever, and accelerate wound healing. In addition to dried flower, fruit-spike of *Prunella vulgaris* has been used in oriental medicine to treat hypertension and tuberculosis [18, 19]. A variety of components (campherol, rutin, triterpenoids, and phenolic acids; rosmarinic acid, caffeic acid, and tannins) have been identified [20]. We previously reported the anti-inflammatory and antidiabetic effects of *Prunella vulgaris* [19, 21]. Now, we investigated the anti-proliferative effect of an aqueous extract from *Prunella vulgaris* (APV) on high glucose stimulating human aortic smooth muscle cells (HASMCs).

## 2. Methods and Materials

**2.1. Preparation of an Aqueous Extract of *Prunella vulgaris*.** The *Prunella vulgaris* var. lilacina (Herba) was purchased from Herbal Medicine Cooperative Association in Jeollabuk Province, Republic of Korea, in January 2010. A voucher specimen (no. HBN161) has been deposited in the Herbarium of the Professional Graduate School of Oriental Medicine, Wonkang University (Korea). The dried *Prunella vulgaris* var. lilacina (100 g) was soaked for 2 hr in water (1L) and then boiled in distilled water at 100°C for 2 hr. The yield of aqueous extract of *Prunella vulgaris* (APV) was approximately 16.27% of the plant powder. The extract was subsequently concentrated using rotary evaporator and then used in the present study.

**2.2. Cell Culture.** HASMCs were obtained from Cascade Biologics (Van Allen Way, Carlsbad, CA), as cryopreserved primary cultures, and grew in culture flask in vascular smooth muscle cell growth medium; M231 was supplemented with 5% smooth muscle growth supplement (SMGS) and 0.2% gentamicin/amphotericin solution according to Cascade Biologics' recommended protocol. Cells of passages 5 and 6 were grown in monolayers at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air, and used for experiments at >80% confluence.

**2.3. Determination of Cell Proliferation.** [<sup>3</sup>H]-thymidine incorporation was performed to determine effect of APV on high glucose-induced cell proliferation in HASMCs [22]. Briefly, HASMCs on the 24-well culture plates were pretreated with or without APV for 1 h, which was followed by treatment with high glucose at 37°C for 24 h. After incubation, 1  $\mu$ Ci of [<sup>3</sup>H]-thymidine (PerkinElmer, Boston, MA) was added for 24 h. The medium was removed, and the cells were washed with ice-cold PBS. Next, plates were incubated with 10% TCA at room temperature for 5 min and then solubilized at room temperature for 30 min in 0.3 N NaOH, 1% SDS. [<sup>3</sup>H]-thymidine activity was measured in liquid scintillation counter (LS 6500 Multipurpose Scintillation Counter, Beckman, Fullerton, CA).

**2.4. Cell Migration Assay.** The cell migration assay was evaluated by wound healing assay [23]. Briefly, HASMCs on the 12-well culture plates were incubated approximately 80% confluent and then made a scratch with yellow tip. Next, HASMCs were pretreated with APV for 1 h, which was followed by treatment with high glucose at 37°C for 24 h. After incubation, the microscopic photographs of migrated cells were measured by a fluorescence microscopy (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

**2.5. Cell Invasion Assay.** The cell invasion assay [24] was cultured on matrigel-coated filter inserts that fit into 24-well matrigel invasion chamber obtained from BD (Two Oak Park, Bedford, MA). Briefly, HASMCs on the 24-well invasion chambers were incubated with APV for 1 h, which was followed by treatment with high glucose at 37°C for 24 h. After incubation, the cells on the upper side of the filter were removed using cotton swabs. The migrated cells were fixed by methyl alcohol for 5 min and then stained with hematoxylin. The microscopic photographs of invaded cells were measured by a fluorescence microscopy.

**2.6. Western Blot Analysis.** Cell homogenates (40  $\mu$ g of protein) were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Blots were then washed with H<sub>2</sub>O, blocked with 5% skimmed milk powder in Tris-Buffered Saline Tween-20 (TBST) (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) for 1 h, and incubated with the appropriate primary antibody at dilutions recommended by the supplier. Then the membrane was washed, and primary antibodies were detected with goat anti-rabbit-IgG or rabbit anti-mouse-IgG conjugated

to horseradish peroxidase, and the bands were visualized with enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK). Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using the ChemiDoc image analyzer (Bio-Rad Laboratories, Hercules, CA).

**2.7. Gelatin Zymography.** HASMCs were pretreated with APV for 1 h and stimulated with high glucose for 24 h. The supernatant, conditioned medium was collected for zymography [25]. SDS-PAGE for measurement of MMP-2 activity was added with 0.1% gelatin in the 10% separated gel. The gel was washed with renaturation buffer (2.5% Triton X-100 in DW) at room temperature for 1 h and then incubated with development buffer (Invitrogen Corporation, Carlsbad, CA) at 37°C for overnight. Next, the gel was stained with 0.2% Coomassie brilliant blue R stain reagent at room temperature for 1 h and then washed with destain buffer for look to visualize the clear area using a ChemiDoc image analyzer.

**2.8. Transient Transfection and Luciferase Assay.** The smooth muscle cells were grown to 60–80% confluence, and the cells were transiently cotransfected with the plasmids using Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol [26]. Briefly, the transfection mixture containing 0.5 µg of either the pGL3-4κB-Luc or pGL4.MMP-9-Luc2 and 0.1 µg of pCMV-β-gal was mixed with the Lipofectamine LTX reagent and added to the cells. After 24 h, the cells were treated with APV for 30 min and stimulated with high glucose for 24 h and then lysed. The luciferase and β-galactosidase activities were determined as described elsewhere using Luciferase assay kit (Promega, Madison, WI). The luciferase activity was normalized with respect to the β-galactosidase activity and expressed as a percentage of the activity of the high glucose group.

**2.9. Radioimmunoassay (RIA) for Determination of cGMP Production.** The cGMP production was evaluated by RIA [27]. Briefly, HASMCs on the 6 cm culture dish were incubated approximately 80% confluent and then pretreated with APV for 1 h, which was followed by treatment with high glucose at 37°C for 24 h. After incubation, the cGMP production was measured by a γ-counter (1480 Automatic Gamma Counter, PerkinElmer, Turku, Finland).

**2.10. Intracellular Reactive Oxygen Species (ROS) Assay.** The fluorescent probe, 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCFDA), was used to determine the intracellular generation of ROS by stimulation of high glucose. Briefly, the confluent HASMCs in the 24-well culture plates were pretreated with or without APV for 1 h. After removing the APV from the wells, the cells were incubated with 20 µM CM-H<sub>2</sub>DCFDA for 1 h. The cells were stimulated with high glucose, and the fluorescence intensity was measured at an excitation and emission wavelength of 485 nm and 530 nm, respectively, using a flow cytometry on FACScalibur (BD, San Diego, CA).

**2.11. Statistical Analysis.** All the experiments were repeated at least three times. The results were expressed as a mean ± S.E., and the data were analyzed using one-way ANOVA followed by Dunnett's test or Student's *t*-test using Sigma Plot (Sigma plot for Windows, version 10.0, USA) to determine any significant differences.  $P < 0.05$  was considered as statistically significant.

### 3. Results

**3.1. Effect of APV on High Glucose-Induced HASMCs Proliferation.** In the [<sup>3</sup>H]-thymidine incorporation assay (Figure 1(a)), stimulation with high glucose (25 mM) increased DNA synthesis. Pretreatment with APV significantly decreased high glucose-induced increase of DNA synthesis in a dose-dependent manner ( $P < 0.01$ ). To explore the anti-proliferative effect of APV, protein levels of the cyclins and CDKs were examined by Western blot analysis. As a result, pretreatment with APV decreased high glucose-induced protein expressions of cyclin D1 and cyclin E as well as CDK2 and CDK4 (Figure 2(a)). In contrast, pretreatment with APV increased high glucose-induced protein expressions of CKIs, p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> (Figure 2(b)). In this study, APV (1–10 µg/mL) did not alter any cytotoxicity (data not shown).

**3.2. Effect of APV on High Glucose-Induced HASMCs Migration and Invasion.** As shown in Figure 1(b), the HASMCs that migrated to the empty space were also visualized under a microscope. The HASMCs migration was increased by treatment with high glucose compared with control. However, pretreatment of APV inhibited cell migration. In addition, effect of APV in measurement of high glucose-induced HASMCs invasion was detected using a BD BioCoat Matrigel Invasion Chamber. As shown in Figures 1(c) and 1(d), the cells which invaded the lower chamber were also visualized under a fluorescence microscope. The cell invasion was increased by treatment with high glucose compared with control. However, pretreatment of APV significantly inhibited HASMCs invasion ( $P < 0.01$ ).

**3.3. Effect of APV on High Glucose-Induced MMP-9 Activity.** The results of gelatin zymography revealed that high glucose increased MMP-9 activity as zymogen secretion without change of MMP-2 (Figure 3(a)). To determine whether APV inhibits high glucose-induced increase of MMP-9 expression, Western blot analysis was performed (Figure 3(b)). Pretreatment with APV decreased MMP-9 activation and expression induced by high glucose. Thus, transient transfections were performed using the MMP-9-dependent luciferase reporter plasmid in order to further examine the effect of APV on the MMP-9 transcription activity. As shown in Figure 3(c), high glucose increased MMP-9 transcription activity, and the concentration over 0.1 µg/mL of APV significantly inhibited high glucose-induced MMP-9 transcriptional activity ( $P < 0.05$ ). However, activation and expression of MMP-2 have no significant difference by APV treatment.

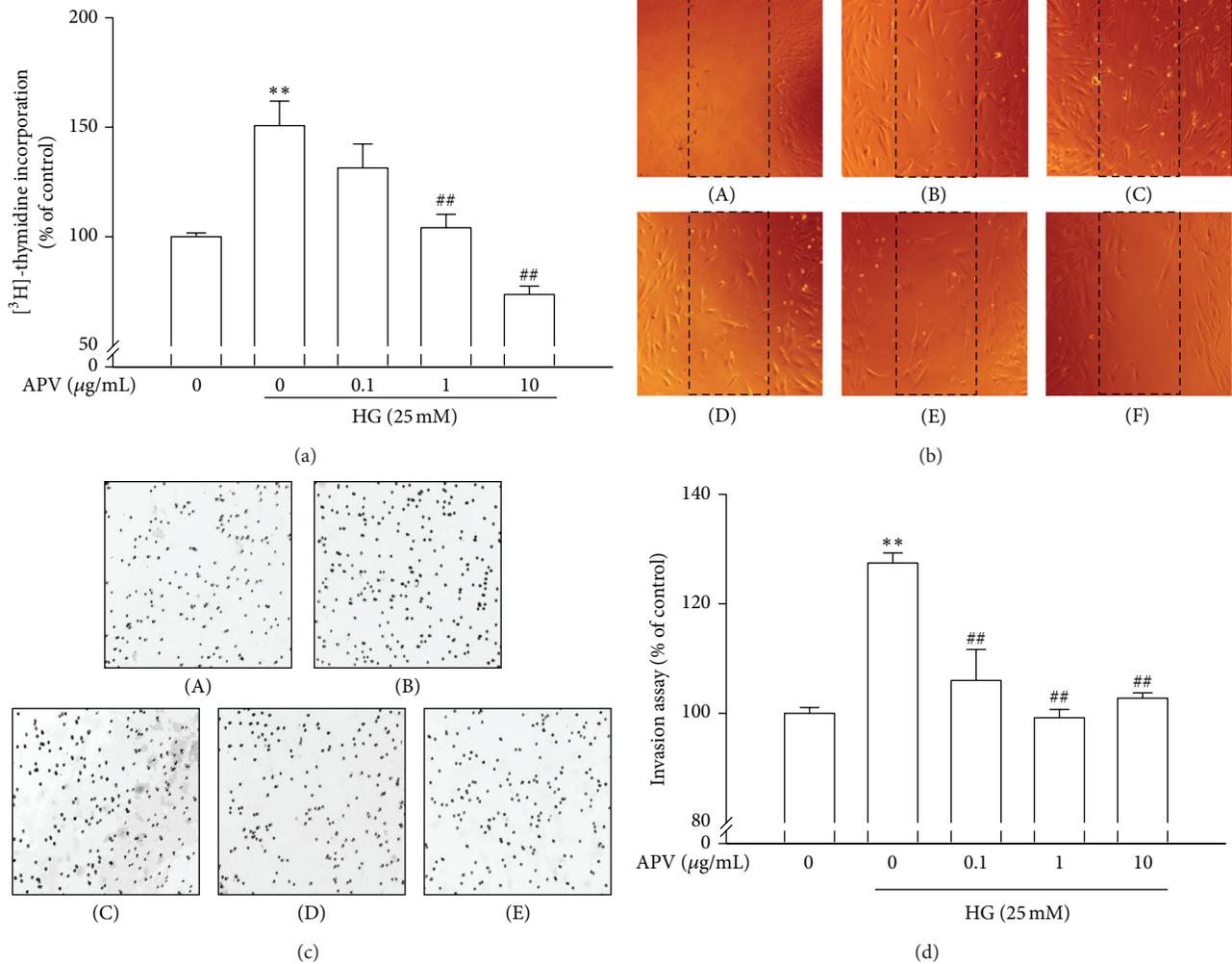


FIGURE 1: Effect of APV on high glucose-induced HASMCs proliferation, migration, and invasion. (a) Confluent HASMCs were incubated with or without APV and high glucose for 24 h and then treated with [<sup>3</sup>H]-thymidine for 24 h. (b) HASMCs were incubated approximately 80% confluent and then made a scratch with yellow tip. HASMCs were pretreated with APV for 1 h, which was followed by treatment with high glucose at 37°C for 24 h. ((A): wound, (B): control, (C): HG (25 mM), (D): HG + APV 0.1 μg/mL, (E): HG + APV 1 μg/mL, (F): HG + APV 10 μg/mL). (c) HASMCs on the 24-well invasion chambers were incubated with APV for 1 h, which was followed by treatment with high glucose at 37°C for 24 h. ((A): control, (B): HG (25 mM), (C): HG + APV 0.1 μg/mL, (D): HG + APV 1 μg/mL, (E): HG + APV 10 μg/mL). Values were expressed as mean ± S.E. of five independent experiments. (d) Each electrophoretogram is representative of the results from three individual experiments. \*\**P* < 0.01 versus control; ##*P* < 0.01 versus high glucose alone.

**3.4. Effect of APV on High Glucose-Induced NF-KappaB Activation.** *NF-kappaB* promoter activity measured whether APV could suppress *NF-kappaB* promoter in HASMCs. Thus, transient transfections were performed using the *NF-kappaB*-dependent luciferase reporter plasmid in order to further examine the effects of APV on the *NF-kappaB* transcription activity. As shown in Figure 4(a), high glucose increased *NF-kappaB* transcription activity, and the concentration over 1 μg/mL of APV significantly inhibited high glucose-induced *NF-kappaB* transcriptional activity.

**3.5. Effect of APV on High Glucose-Induced p-ERK and p38 MAPKs Activation.** To confirm the involvement with MAPKs family in regulation of HASMCs proliferation by

APV, it was performed by Western blotting. As a result, high glucose induced phosphorylations of p44/42 ERK and p38 MAPKs in HASMCs. However, APV decreased the high glucose-induced phosphorylations of ERK and p38 MAPKs. On the other hand, JNK has no significant difference in its phosphorylation (Figure 4(c)).

**3.6. Effect of APV on Nrf2-Mediated HO-1 and cGMP Signaling Pathways.** To determine the effect of APV on the HO-1 formation, nuclear translocation of nuclear factor E2-related factor 2 (Nrf2) in HASMCs was examined. Figure 5(a) showed that high glucose significantly decreased the translocation of Nrf2 in nuclei, whereas pretreatment with APV increased Nrf2 translocation in nuclei (Figure 5(a)). Likewise, high

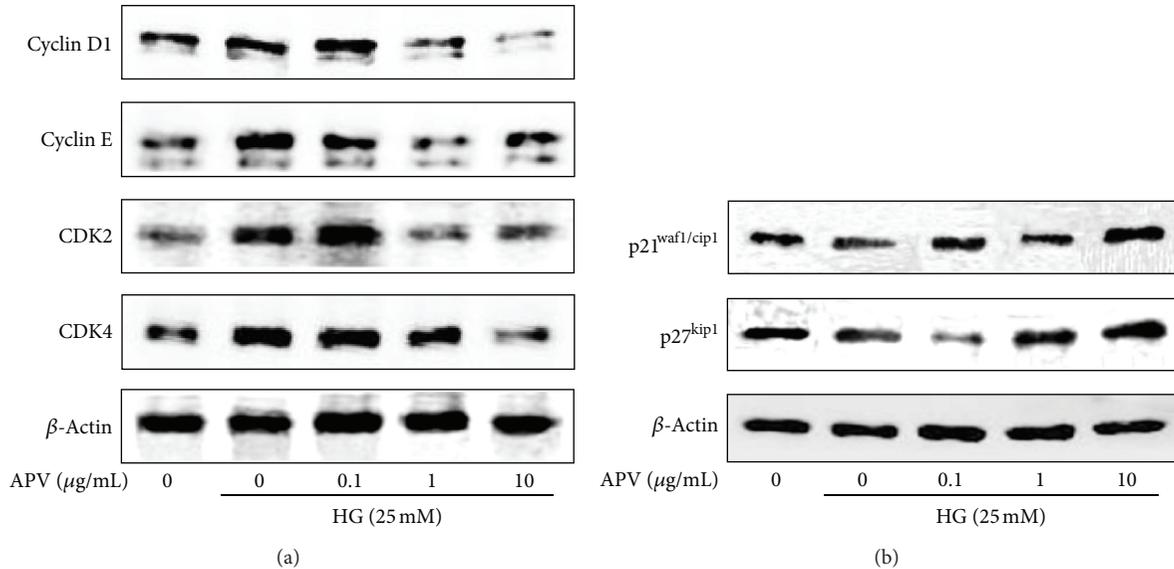


FIGURE 2: Effects of APV on high glucose-induced cell cycle regulator (a) cyclins, CDKs, and (b) CKIs proteins. The total cellular protein (40 µg) extracts were prepared and separated on 10% SDS-PAGE and blotted with the antibodies specific for cyclin D1, cyclin E, CDK2, CDK4, p21<sup>waf1/cip1</sup>, p27<sup>kip1</sup>, and β-actin. Each electrophoretogram is representative of the results from three individual experiments.

glucose decreased expression of heme oxygenase-1 (HO-1), but it was increased by treatment of APV (Figure 5(b)). In the experiment to determine whether APV inhibits high glucose-induced decrease of cGMP, various APV concentrations ranging from 0.1 to 10 µg/mL were added to HASMCs. As shown in Figure 6, pretreatment with APV increased high glucose-induced suppression of cGMP in a dose-dependent manner ( $P < 0.01$ ).

**3.7. Effect of APV on High Glucose-Induced ROS Formation.** ROS has been implicated as a common second messenger in various pathways leading to NF-κB activation [21]. Thus, the level of intracellular ROS production was assessed by monitoring the fluorescence in order to determine whether APV can reduce the level of high glucose-induced oxidative stress in HASMCs. APV was compared with NAC (10 µM) as a positive control. As a result, DCF fluorescence level showed a marked increase after treatment with high glucose. However, pretreatment with APV and NAC significantly inhibited high glucose-induced DCF-sensitive cellular ROS levels (Figure 7).

#### 4. Discussion

In arterial media, VSMCs are normally quiescent and remain in the G<sub>0</sub>/G<sub>1</sub> phase of cell cycle. VSMC proliferation and migration play a key role in the atherosclerosis which are induced by high blood plasma concentration [1]. Thus, the aim of the present study was to determine whether APV exerts an antiatherogenic property throughout the inhibition of HASMCs proliferation. We previously reported an anti-inflammatory effect of APV on high glucose-stimulated vascular endothelial cells, suggesting a possibility of anti-proliferative effect [19, 21]. Consistent with the previous

report, APV inhibited high glucose-induced HASMC proliferation in a dose-dependent manner.

The action of MMPs has recently emerged as an important component of the natural history of atherosclerosis and of the vascular response to injury [28, 29]. Especially, gelatinase, MMP-2, and MMP-9 play pivotal roles in HASMC proliferation, and the inhibitory effect on their expression is important in the search for therapeutic natural herbs [30]. Considerable interest in this study was the marked decrease in the secretions of MMP-9 activity from high glucose-induced HASMCs in response to APV. APV decreased not MMP-2 but MMP-9 in measurement of zymography or immunoblot analysis. This result suggests that an anti-proliferative effect of APV is specific to the inhibition of MMP-9 in HASMC. Some in vitro research reported that inhibition of both cell proliferation and MMP-9 activity has appeared by various oriental herbs, which is used for the treatment of atherosclerosis [19, 31]. MMP-9 gene expression can be activated via a number of signaling pathways including those involving MAPKs family such as ERK1/2, p38 MAPK, JNK, and PI3K/Akt, which are the upstream modulators of AP-1 or NF-κB [32]. Our results showed that high concentrations of glucose stimulated phosphorylation of p38 MAPKs and ERK, whereas p38 MAPKs and ERK were attenuated by a pretreatment with APV. In contrast to these two kinases, JNK is not activated in the present condition, and it was also reported in the diabetic rats or mesangial cells upon exposure to high glucose [33, 34]. However, other studies suggested the involvement of MAPKs family such as ERK, p-38, and JNK in VSMC proliferation [6, 35]. We suspect that this discrepancy is dependent on the various conditions including origin, time, and stimuli. Thus, a further study is needed to clarify a possible role of MAPKs family in response to high glucose. These observations led

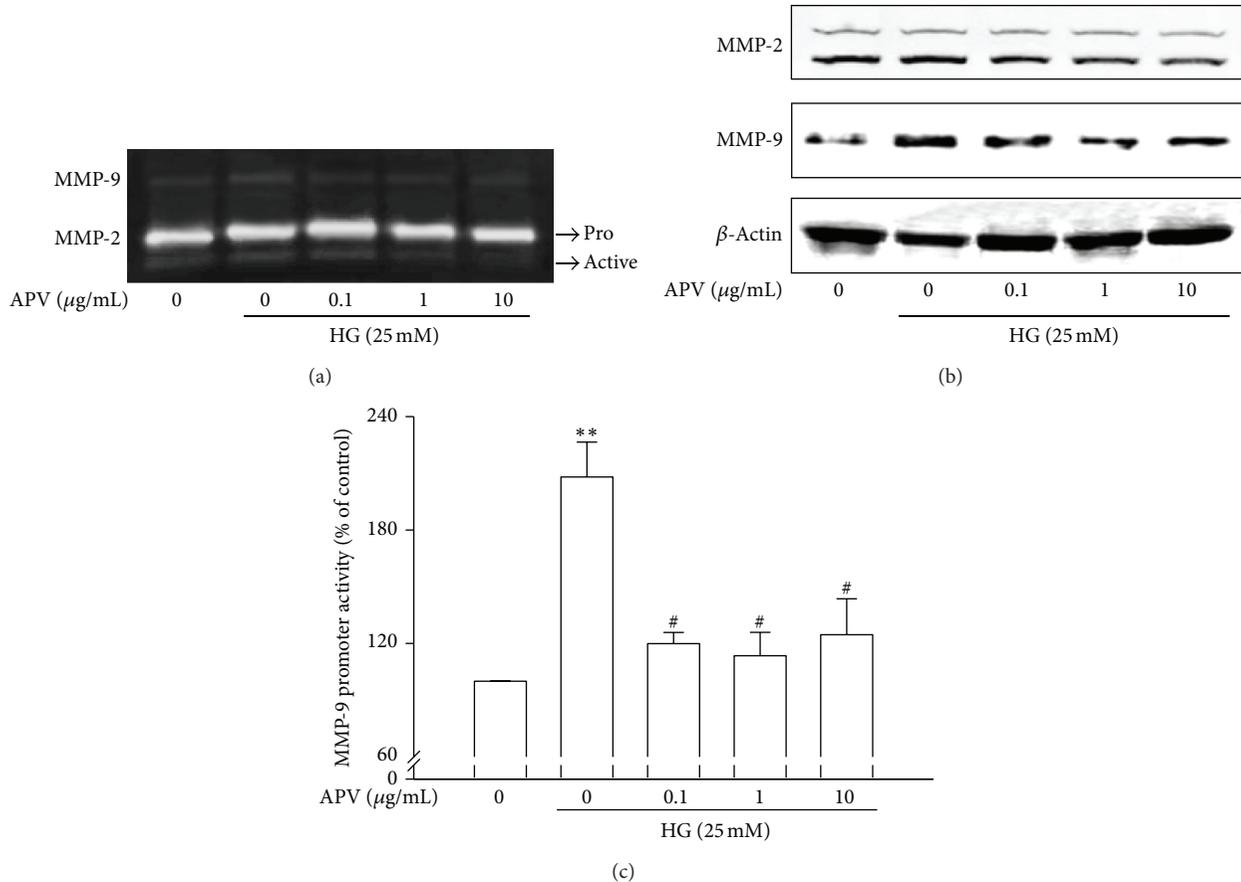


FIGURE 3: Effects of APV on high glucose-induced MMP-2 or -9 activation and expression. (a) Conditioned supernatant was prepared, and the levels of MMP-2/-9 were measured by gelatin zymography, as described in Section 2. Electrophoretogram is representative of the results from three individual experiments. (b) The total cellular protein (40  $\mu$ g) extracts were prepared and separated on 10% SDS-PAGE and blotted with the antibodies specific for MMP-2, MMP-9, and  $\beta$ -actin. Each electrophoretogram is representative of the results from three individual experiments. (c) The HASMCs were transiently transfected with pGL4.MMP-9-Luc2 and pCMV- $\beta$ -gal. This was followed by harvesting, and their luciferase activities were determined. Values were expressed as mean  $\pm$  S.E. of three individual experiments. \*\* $P < 0.01$  versus control; # $P < 0.05$  versus high glucose alone.

us to suggest the participation of p38 MAPKs and ERK in HASMC proliferation. Thus, we demonstrate that APV could ameliorate HASMC proliferation via inhibiting the activities of MMP-9, p-p38, and p-ERK.

It has been well established that high glucose-induced oxidative stress may be a key factor in the development of diabetic vascular complications [36]. Recent studies have demonstrated that the increased level of ROS is one of the most important contributors to diabetic vascular complications, which is produced as a result of longstanding hyperglycemic stress [37, 38]. Therefore, blocking the generation of excess ROS will significantly improve vascular injury in diabetics. In the present study, APV attenuated high glucose-induced ROS production as well as the activations of ERK and p38 MAPK and NF- $\kappa$ B signaling pathways, implying that APV-mediated inhibitory effects on high glucose-stimulated HASMCs proliferation are due to blocking ROS-dependent ERK and p38 MAPK signaling cascades. These findings suggest that APV may have beneficial effect in protecting against vascular complications in diabetics.

HO, a cytoprotective enzyme, is the rate-limiting enzyme in the degradation of heme. Among the three HO isoforms reported, HO-1 is highly inducible by heme and by a vast array of nonheme substances such as endotoxin and hydrogen peroxide, suggesting that HO-1 may play a significant role in protection of tissues from oxidative injuries [39]. It is confirmed that APV increased Nrf2 activation and HO-1 expression against high glucose condition in vascular endothelial cells [21]. The Nrf2/antioxidant response element (ARE) pathway plays an important role in regulating cellular antioxidants, including HO-1, which is a cytoprotective enzyme [40]. Thus, our findings suggest that Nrf2-mediated HO-1 upregulation was mediated by APV in adaptive survival response to high glucose-induced oxidative stress.

Since reduced NO-cGMP signaling contributes to vascular inflammation, firstly, endothelial-derived NO production has attracted increasing attention in this study [41]. It was reported that oriental herb-mediated NO signaling is involved in endothelial protection against the HG-induced

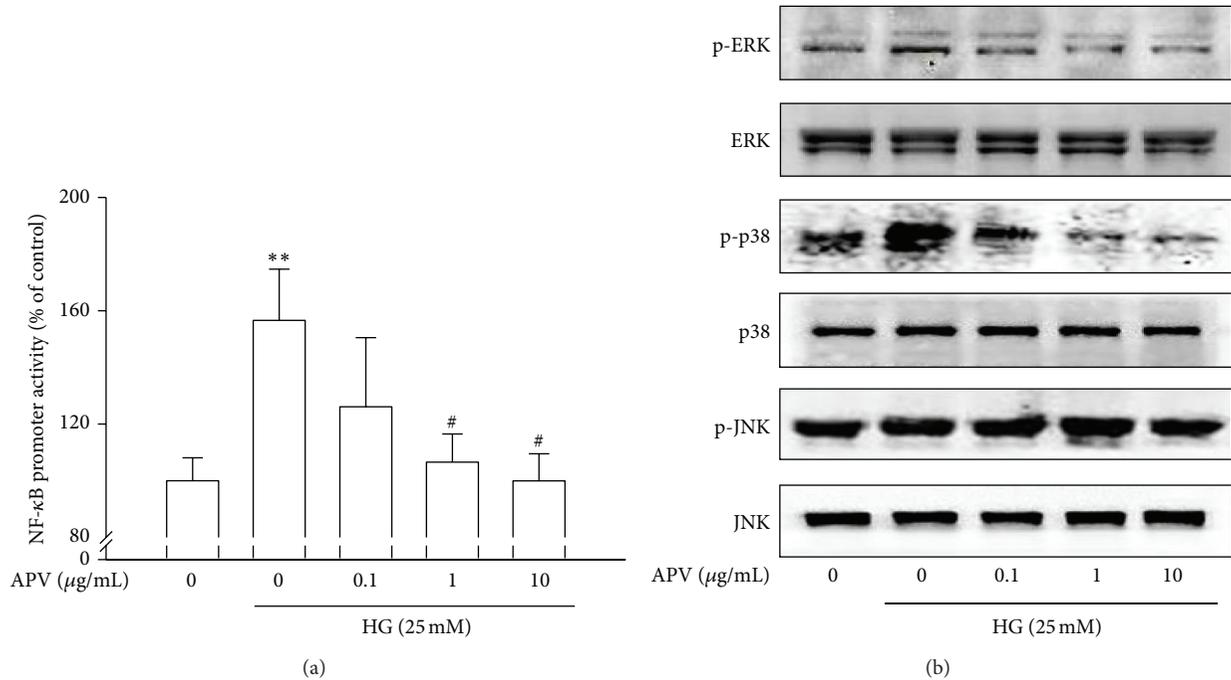


FIGURE 4: (a) Effect of APV on high glucose-induced *NF-kappaB* activation. The HASMCs were transiently transfected with pGL3-4κB-Luc and pCMV-β-gal. This was followed by harvesting, and their luciferase activities were determined. Values were expressed as mean ± S.E. of three individual experiments. \*\* $P < 0.01$  versus control; # $P < 0.05$  versus high glucose alone. (b) Effect of APV on high glucose-induced MAPKs activation. The total cellular protein (40 μg) extracts were prepared and separated on 10% SDS-PAGE and blotted with the antibodies specific for p-ERK, p-p38, and p-JNK. Each electrophoretogram is representative of the results from three individual experiments.

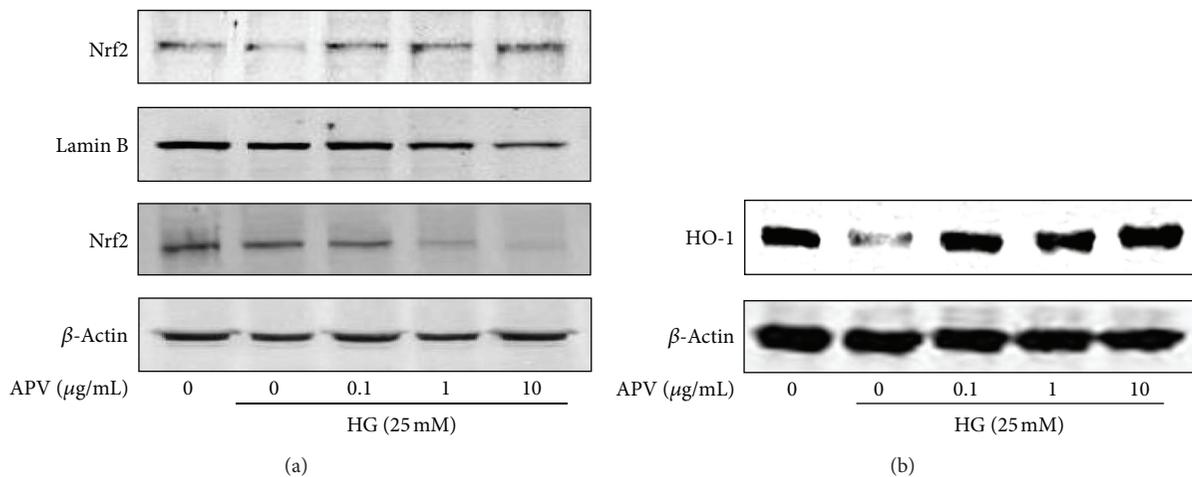


FIGURE 5: (a) Effect of APV on high glucose-induced expression of Nrf2 and translocation into nucleus in HASMCs. Cytoplasm and nuclei fractions were extracted, and protein levels were determined by Western blot analysis. Values were expressed as mean ± S.E. of three individual experiments. (b) Effect of APV on high glucose-induced expression of HO-1. The total cellular protein (40 μg) extracts were prepared and separated on 10% SDS-PAGE and blotted with the antibody specific for HO-1 and β-actin. Each electrophoretogram is representative of the results from three individual experiments.

vascular inflammation [42]. Despite high glucose stimulation, APV induced intracellular cGMP production, truly having the powerful vascular relaxation effect in VSMCs. However, it could not be concluded that APV-induced cGMP production is dependent on or independent of endothelium, because there is no direct evidence that APV induced cGMP

production without NO donor in the present study. Thus, a further study is required to clarify the mechanisms of oriental herb-activated endogenous defense system against vascular inflammation.

Nitrosative stress caused by reactive nitrogen species such as NO and peroxynitrite overproduced during inflammation

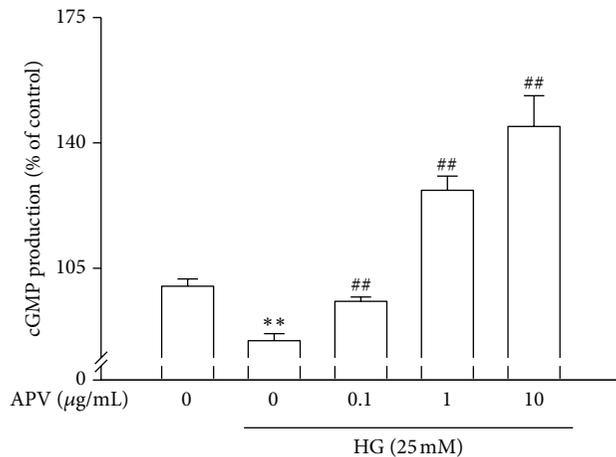


FIGURE 6: Effect of APV on high glucose-induced suppression of cGMP production in HASMCs. Values were expressed as mean  $\pm$  S.E. of three individual experiments. \*\* $P < 0.01$  versus control; ## $P < 0.01$  versus high glucose alone.

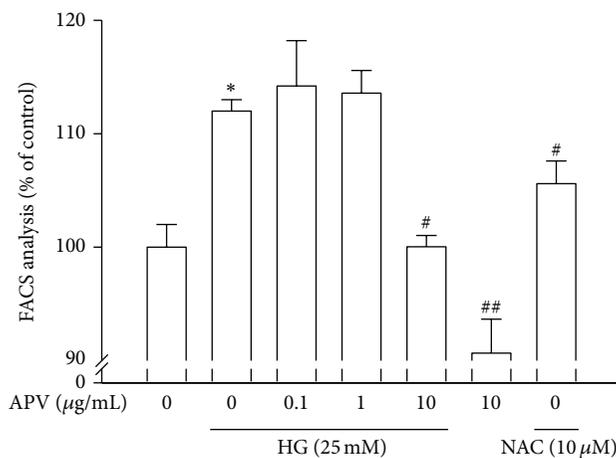


FIGURE 7: Effect of APV on high glucose-induced ROS formation. The fluorescence intensity of cells was measured using a flow cytometry on FACScalibur. Values were expressed as mean  $\pm$  S.E. of five independent experiments. \* $P < 0.05$  versus control; # $P < 0.05$ , ## $P < 0.01$  versus high glucose alone.

leads to cell death and has been implicated in the pathogenesis of many human ailments. However, relatively mild nitrosative stress may improve cellular defense capacities, rendering cells tolerant or adaptive to ongoing and subsequent cytotoxic challenges [43]. On the other hand, the fact that activated Akt-Nrf2 signaling plays a critical role in the regulation of the vasoprotective effect of APV is supported by our results. In addition, it is clear that HO-1 has been shown to be an important biological target of NO [44]. Thus, we suggest that APV-induced HO-1 activation in smooth muscle and bioactive NO in endothelium contribute to induce defense against HG-induced vascular inflammation.

In conclusion, APV showed potent antidiabetic atherosclerosis effects on high glucose-induced vascular smooth muscle cell proliferation, possibly through the

inhibition of ROS/NF- $\kappa$ B and activation of Nrf2/HO-1 pathways. Our data provide a possible molecular mechanism mediating the inhibitive effect of APV on HASMCs proliferation. In the light of results, *Prunella vulgaris* could be an effective therapeutic candidate against diabetes-associated cardiovascular diseases.

## Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korean government (2008-0062484) and the Ministry of Knowledge Economy (MKE-R0002038).

## References

- [1] V. J. Dzau, R. C. Braun-Dullaeus, and D. G. Sedding, "Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies," *Nature Medicine*, vol. 8, no. 11, pp. 1249–1256, 2002.
- [2] K. E. Bornfeldt and I. Tabas, "Insulin resistance, hyperglycemia, and atherosclerosis," *Cell Metabolism*, vol. 14, no. 5, pp. 575–585, 2011.
- [3] J. E. Kim and H. C. Choi, "Losartan inhibits vascular smooth muscle cell proliferation through activation of AMP-activated protein kinase," *Korean Journal of Physiology and Pharmacology*, vol. 14, no. 5, pp. 299–304, 2010.
- [4] M. Bond, G. B. Sala-Newby, Y.-J. Wu, and A. C. Newby, "Biphasic effect of p21<sup>cip1</sup> on smooth muscle cell proliferation: role of PI 3-kinase and Skp2-mediated degradation," *Cardiovascular Research*, vol. 69, no. 1, pp. 198–206, 2006.
- [5] C. Denicourt and S. F. Dowdy, "Cip/Kip proteins: more than just CDKs inhibitors," *Genes and Development*, vol. 18, no. 8, pp. 851–855, 2004.
- [6] Y. Zhan, S. Kim, Y. Izumi et al., "Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 5, pp. 795–801, 2003.
- [7] L. Zhu, G. Sun, H. Zhang et al., "PGC-1 $\alpha$  is a key regulator of glucose-induced proliferation and migration in vascular smooth muscle cells," *PLoS ONE*, vol. 4, no. 1, Article ID e4182, 2009.
- [8] A. Cho and M. A. Reidy, "Matrix metalloproteinase-9 is necessary for the regulation of smooth muscle cell replication and migration after arterial injury," *Circulation Research*, vol. 91, no. 9, pp. 845–851, 2002.
- [9] A. K. Death, E. J. Fisher, K. C. Y. McGrath, and D. K. Yue, "High glucose alters matrix metalloproteinase expression in two key vascular cells: potential impact on atherosclerosis in diabetes," *Atherosclerosis*, vol. 168, no. 2, pp. 263–269, 2003.
- [10] S. S. Signorelli, G. Malaponte, M. Libra et al., "Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease," *Vascular Medicine*, vol. 10, no. 1, pp. 1–6, 2005.
- [11] A. M. Deschamps and F. G. Spinale, "Pathways of matrix metalloproteinase induction in heart failure: bioactive molecules and transcriptional regulation," *Cardiovascular Research*, vol. 69, no. 3, pp. 666–676, 2006.
- [12] B. B. Aggarwal, "Nuclear factor- $\kappa$ B: the enemy within," *Cancer Cell*, vol. 6, no. 3, pp. 203–208, 2004.

- [13] N. K. Idriss, A. D. Blann, and G. Y. H. Lip, "Hemoxygenase-1 in cardiovascular disease," *Journal of the American College of Cardiology*, vol. 52, no. 12, pp. 971–978, 2008.
- [14] H.-O. Pae, Y. C. Lee, and H.-T. Chung, "Heme oxygenase-1 and carbon monoxide: emerging therapeutic targets in inflammation and allergy," *Recent Patents on Inflammation and Allergy Drug Discovery*, vol. 2, no. 3, pp. 159–165, 2008.
- [15] A. R. Kinderlerer, I. P. Gregoire, S. S. Hamdulay et al., "Heme oxygenase-1 expression enhances vascular endothelial resistance to complement-mediated injury through induction of decay-accelerating factor: a role for increased bilirubin and ferritin," *Blood*, vol. 113, no. 7, pp. 1598–1607, 2009.
- [16] Z.-W. Yu, D. Li, W.-H. Ling, and T.-R. Jin, "Role of nuclear factor (erythroid-derived 2)-like 2 in metabolic homeostasis and insulin action: a novel opportunity for diabetes treatment?" *World Journal of Diabetes*, vol. 15, pp. 19–28, 2012.
- [17] K.-A. Jung and M.-K. Kwak, "The Nrf2 system as a potential target for the development of indirect antioxidants," *Molecules*, vol. 15, no. 10, pp. 7266–7291, 2010.
- [18] Y.-P. Zhu, "Chinese material medica-chemistry, pharmacology and application," *Phytochemistry*, vol. 54, pp. 111–112, 2000.
- [19] S. M. Hwang, J. S. Kim, Y. J. Lee et al., "Anti-diabetic atherosclerosis effect of *Prunella vulgaris* in db/db mice with type 2 diabetes," *American Journal of Chinese Medicine*, vol. 50, pp. 937–951, 2012.
- [20] J. Zheng, J. He, B. Ji, Y. Li, and X. Zhang, "Antihyperglycemic activity of *Prunella vulgaris* L. in streptozotocin-induced diabetic mice," *Asia Pacific Journal of Clinical Nutrition*, vol. 16, no. 1, pp. 427–431, 2007.
- [21] S. M. Hwang, Y. J. Lee, J. J. Yoon et al., "*Prunella vulgaris* suppresses HG-induced vascular inflammation via Nrf2/HO-1/eNOS activation," *International Journal of Molecular Sciences*, vol. 13, no. 1, pp. 1258–1268, 2012.
- [22] S. M. Hwang, Y. J. Lee, J. J. Yoon, S. M. Lee, D. G. Kang, and H. S. Lee, "Gardenia jasminoides inhibits tumor necrosis factor- $\alpha$ -induced vascular inflammation in endothelial cells," *Phytotherapy Research*, vol. 24, no. 2, pp. S214–S219, 2010.
- [23] T.-J. Kim, H.-J. Han, S.-S. Hong et al., "Cudraticrusxanthone A isolated from the root bark of *Cudrania tricuspidata* inhibits the proliferation of vascular smooth muscle cells through the suppression of PDGF-receptor beta tyrosine kinase," *Biological and Pharmaceutical Bulletin*, vol. 30, no. 4, pp. 805–809, 2007.
- [24] N. Ramos-DeSimone, E. Hahn-Dantona, J. Siple, H. Nagase, D. L. French, and J. P. Quigley, "Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion," *Journal of Biological Chemistry*, vol. 274, no. 19, pp. 13066–13076, 1999.
- [25] S.-O. Lee, Y.-C. Chang, K. Whang, C.-H. Kim, and I.-S. Lee, "Role of NAD(P)H:quinone oxidoreductase 1 on tumor necrosis factor- $\alpha$ -induced migration of human vascular smooth muscle cells," *Cardiovascular Research*, vol. 76, no. 2, pp. 331–339, 2007.
- [26] S.-K. Moon, S.-H. Cho, K.-W. Kim et al., "Overexpression of membrane sialic acid-specific sialidase Neu3 inhibits matrix metalloproteinase-9 expression in vascular smooth muscle cells," *Biochemical and Biophysical Research Communications*, vol. 356, no. 3, pp. 542–547, 2007.
- [27] S. Z. Kim, S. H. Kim, J. K. Park, G. Y. Koh, and K. W. Cho, "Presence and biological activity of C-type natriuretic peptide-dependent guanylate cyclase-coupled receptor in the penile corpus cavernosum," *Journal of Urology*, vol. 159, no. 5, pp. 1741–1746, 1998.
- [28] R. Ross, "Atherosclerosis—an inflammatory disease," *New England Journal of Medicine*, vol. 340, no. 2, pp. 115–126, 1999.
- [29] A. C. Newby and A. B. Zaltsman, "Molecular mechanisms in intimal hyperplasia," *Journal of Pathology*, vol. 190, pp. 300–309, 2000.
- [30] J.-G. Yang, Y.-H. Shen, Y. Hong et al., "Stir-baked *Fructus gardeniae* (L.) extracts inhibit matrix metalloproteinases and alter cell morphology," *Journal of Ethnopharmacology*, vol. 117, no. 2, pp. 285–289, 2008.
- [31] Y. J. Lee, J. S. Kim, D. G. Kang, and H. S. Lee, "Buddleja officinalis suppresses high glucose-induced vascular smooth muscle cell proliferation: role of mitogen-activated protein kinases, nuclear factor- $\kappa$ B and matrix metalloproteinases," *Experimental Biology and Medicine*, vol. 235, no. 2, pp. 247–255, 2010.
- [32] Y. P. Hwang, H. J. Yun, H. G. Kim, E. H. Han, G. W. Lee, and H. G. Jeong, "Suppression of PMA-induced tumor cell invasion by dihydroartemisinin via inhibition of PKC $\alpha$ /Raf/MAPKs and NF- $\kappa$ B/AP-1-dependent mechanisms," *Biochemical Pharmacology*, vol. 79, no. 12, pp. 1714–1726, 2010.
- [33] M. J. Kang, W. Xiaoyan, L. Hao, K. Thai, and J. W. Scholey, "Effect of glucose on stress-activated protein kinase activity in mesangial cells and diabetic glomeruli," *Kidney International*, vol. 55, no. 6, pp. 2203–2214, 1999.
- [34] H. Jia, X. Qi, S. Fang et al., "Carnosine inhibits high glucose-induced mesangial cell proliferation through mediating cell cycle progression," *Regulatory Peptides*, vol. 154, no. 1–3, pp. 69–76, 2009.
- [35] H. L. Li, W. H. Peng, J. H. Zhuang et al., "Vaspin attenuates high glucose-induced vascular smooth muscle cells proliferation and chemokinesis by inhibiting the MAPK, PI3K/Akt, and NF- $\kappa$ B signaling pathways," *Atherosclerosis*, vol. 228, pp. 61–68, 2013.
- [36] F. Giacco and M. Brownlee, "Oxidative stress and diabetic complications," *Circulation Research*, vol. 107, no. 9, pp. 1058–1070, 2010.
- [37] S.-O. Lee, Y.-J. Jeong, M. H. Yu et al., "Wogonin suppresses TNF- $\alpha$ -induced MMP-9 expression by blocking the NF- $\kappa$ B activation via MAPK signaling pathways in human aortic smooth muscle cells," *Biochemical and Biophysical Research Communications*, vol. 351, no. 1, pp. 118–125, 2006.
- [38] S. Uemura, H. Matsushita, W. Li et al., "Diabetes mellitus enhances vascular matrix metalloproteinase activity role of oxidative stress," *Circulation Research*, vol. 88, no. 12, pp. 1291–1298, 2001.
- [39] T. Inoguchi and H. Nawata, "NAD(P)H oxidase activation: a potential target mechanism for diabetic vascular complications, progressive  $\beta$ -cell dysfunction and metabolic syndrome," *Current Drug Targets*, vol. 6, no. 4, pp. 495–501, 2005.
- [40] K. R. Brunt, M. R. Tsuji, J. H. Lai et al., "Heme oxygenase-1 inhibits pro-oxidant induced hypertrophy in HL-1 cardiomyocytes," *Experimental Biology and Medicine*, vol. 234, no. 5, pp. 582–594, 2009.
- [41] S. A. Rushworth and D. J. MacEwan, "The role of Nrf2 and cytoprotection in regulating chemotherapy resistance of human leukemia cells," *Cancers*, vol. 3, no. 2, pp. 1605–1621, 2011.
- [42] N. O. Rizzo, E. Maloney, M. Pham et al., "Reduced NO-cGMP signaling contributes to vascular inflammation and insulin resistance induced by high-fat feeding," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 4, pp. 758–765, 2010.

- [43] X. Cui, M. Chopp, A. Zacharek, C. Zhang, C. Roberts, and J. Chen, "Role of endothelial nitric oxide synthetase in arterio-genesis after stroke in mice," *Neuroscience*, vol. 159, no. 2, pp. 744–750, 2009.
- [44] H.-O. Pae, G.-S. Oh, B.-M. Choi, Y.-M. Kim, and H.-T. Chung, "A molecular cascade showing nitric oxide-heme oxygenase-1-vascular endothelial growth factor-interleukin-8 sequence in human endothelial cells," *Endocrinology*, vol. 146, no. 5, pp. 2229–2238, 2005.

## Research Article

# Optimizing Prescription of Chinese Herbal Medicine for Unstable Angina Based on Partially Observable Markov Decision Process

Yan Feng,<sup>1</sup> Yu Qiu,<sup>2</sup> Xuezhong Zhou,<sup>3</sup> Yixin Wang,<sup>1</sup> Hao Xu,<sup>2</sup> and Baoyan Liu<sup>4</sup>

<sup>1</sup> Department of General Practice, Anzhen Hospital, Capital Medical University, Beijing 100029, China

<sup>2</sup> Department of Cardiology, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>3</sup> School of Computer and Information Technology, Beijing Jiaotong University, Beijing 100044, China

<sup>4</sup> China Academy of Chinese Medical Sciences, Beijing 100700, China

Correspondence should be addressed to Hao Xu; xuhaotcm@hotmail.com and Baoyan Liu; liuby@mail.cintcm.ac.cn

Received 5 May 2013; Accepted 4 July 2013

Academic Editor: Keji Chen

Copyright © 2013 Yan Feng et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Objective.** Initial optimized prescription of Chinese herb medicine for unstable angina (UA). **Methods.** Based on partially observable Markov decision process model (POMDP), we choose hospitalized patients of 3 syndrome elements, such as *qi* deficiency, blood stasis, and turbid phlegm for the data mining, analysis, and objective evaluation of the diagnosis and treatment of UA at a deep level in order to optimize the prescription of Chinese herb medicine for UA. **Results.** The recommended treatment options of UA for *qi* deficiency, blood stasis, and phlegm syndrome patients were as follows: Milkvetch Root + Tangshen + Indian Bread + Largehead Atractylodes Rhizome (ADR = 0.96630); Danshen Root + Chinese Angelica + Safflower + Red Peony Root + Szechwan Lovage Rhizome Orange Fruit (ADR = 0.76); Snakegourd Fruit + Longstamen Onion Bulb + Pinellia Tuber + Dried Tangerine peel + Largehead Atractylodes Rhizome + Platycodon Root (ADR = 0.658568). **Conclusion.** This study initially optimized prescriptions for UA based on POMDP, which can be used as a reference for further development of UA prescription in Chinese herb medicine.

## 1. Introduction

Unstable angina (UA) is the clinical status between exertional stable angina and acute myocardial infarction [1]. In recent years, traditional Chinese medicine (TCM) got wealthy experience and remarkable achievements in the treatment of UA. However, there are still problems in how to scientifically evaluate the clinical efficacy of different clinical treatment recommendations and how to convert many personalized experiences into certain standardized treatment plans to follow.

Treatment regimen optimization based on partially observable Markov decision process (POMDP) is the course of using scientific methods of computation to find the most economical and convenient treatment program with best clinical efficacy among many treatment options [2–4]. The certain amount of knowledge accumulated and discovered from the massive clinical data and TCM treatment experience can not only verify the existing experience and theory of

TCM that may also find some new treatment experience. In this study, we will try to use data mining methods based on large-scale, nonexternal control observational clinical data in practice to seek and find the optimized TCM treatment prescription.

## 2. Materials and Methods

**2.1. Subjects.** From September 2009 to February 2011, a total of 2212 hospitalized subjects were enrolled from China-Japan Friendship Hospital affiliated to National Health and Family Planning Commission (Beijing, China) (589 cases), Xiyuan Hospital affiliated to China Academy of Chinese Medical Science (Beijing, China) (362 cases), Guang Anmen Hospital affiliated to China Academy of Chinese Medical Science (Beijing, China) (298 cases), Wangjing Hospital affiliated to China Academy of Chinese Medical Science (Beijing, China) (97 cases), Dongzhimen Hospital affiliated to Beijing

University of Chinese Medicine (Beijing, China) (193 cases), Beijing Integrative Medicine Hospital (Beijing, China) (121 cases), Beijing Chinese Medicine Hospital affiliated to Capital Medical University (Beijing, China) (186 cases), Beijing Anzhen Hospital affiliated to Capital Medical University (Beijing, China) (42 cases), Beijing Tongren Hospital affiliated to Capital Medical University (Beijing, China) (43 cases), People's Hospital affiliated to Peking University (Beijing, China) (41 cases), Huairou District Hospital of Traditional Chinese Medicine (Beijing, China) (138 cases), and Tongzhou District Hospital of Traditional Chinese Medicine (Beijing, China) (102 cases). All selected subjects fulfilled the diagnosis of unstable angina. Ethical approval was granted by the Ethics Committee of China-Japan Friendship Hospital (Beijing, China). Informed consent was obtained by each patient participating in this study.

**2.2. Diagnostic Standard.** UA diagnostic standard referred to the UA diagnosis and treatment recommendations by the China Cardiovascular Association released in 2007 [5]. According to the characteristics of angina pectoris, typical ECG changes, exercise treadmill ECG, Holter, cardiac scintigraphy, coronary angiography and risk elements to make the judgment in order to improve the accuracy of diagnosis. Diagnostic standards of TCM syndromes referred to the following references: (1) the TCM standards of coronary heart disease by China Association of Integrative Medicine Association for cardiovascular diseases [6] and (2) the chest stuffiness and pains dialectical standards in Chinese internal medicine [7].

**2.3. Inclusion Criteria.** The inclusion criteria were as follows: (1) meet the diagnostic criteria; (2) previous history of old myocardial infarction or coronary angiography confirmed at least one coronary stenosis  $\geq 50\%$ , (3) hospitalized patients with UA as first western medical diagnosis, (4) age, sex, drug use, and concomitant diseases are not limited, and (5) a signed informed consent.

**2.4. Exclusion Criteria.** The exclusion criteria were composed of five conditions: (1) occurred end-point events in 1-year fellow, (2) cancer and immune system diseases, (3) pregnant or lactating women, (4) serious diseases of liver, kidney, and hematopoietic system, and (5) patients with allergies or psychosis.

**2.5. End-Point Events Criteria.** (1) The primary end-point events are cardiovascular death, nonfatal myocardial infarction, revascularization (including intervention, coronary artery bypass grafting); (2) secondary end-point events are stroke, rehospitalization for ACS, heart failure, and other thrombotic complications.

**2.6. Western Medical Treatment.** According to the UA diagnosis and treatment recommendations by China Cardiovascular Association released in 2007 [5], patients were given conventional western drug therapy including anti-ischemic (nitrates, beta-blockers, calcium antagonists, and

ACE inhibitors), antiplatelet (aspirin and/or clopidogrel), anticoagulation (heparin or low molecular weight heparin), and statin drugs.

**2.7. Observed Indicators and Methods.** Doing clinical information collection, verification, supplementary, pre-processing and data mining, and analysis for data acquisition process in accordance with the unified design of UA. Using TCM clinical research data platform for individualized treatment, qualified clinical researchers after training and examination took patients' hospital stay information and entered them into the database through clinical information collection system; then the professionals of the school of Computer Science in Beijing Jiaotong University convert the data for extraction, washing, and analysis. To specify the TCM syndrome following the rules of "Diagnostics of Chinese Medicine" [8] and "TCM syndrome differential diagnosis" [9], such as "phlegm stagnation syndrome," "phlegm dampness syndrome," "phlegm stasis syndrome" unified as "turbid phlegm syndrome," break down the combined TCM syndromes into basic TCM syndrome elements. For example, break down "qi and yin deficiency syndrome" into syndrome elements as "qi deficiency syndrome" and "yin deficiency syndrome." When encountering difficulty in distinguishing and differentiating TCM syndromes, to discuss and resolve it under the guidance of experts and references, telephone followups were enrolled one year after the end of the event.

In order to simplify the data and find common rules, we elected the core prescription medicine as the object of analysis, and the extraction of core Chinese herbal medicine applied the complex network mining method. To divide five main symptoms of patients with UA: "chest tightness, chest pain, heart palpitations, shortness of breath, and fatigue" into "no, light, medium, and heavy" four grades and to observe the changes of the main symptoms for patients during hospitalization every 1-2 days after admission time with 5 times for each, use cluster analysis method to unify symptoms.

**2.8. Statistical Analysis.** Using Oracle 9.0 g to converse the demographic data, clinical features, syndromes, and drug treatment data, while general material use frequency statistical analysis. Getting use of the data mining platform built in the major project of the Beijing Municipal Science and Technology Commission for the prevention and treatment of major diseases to optimize the prescription of Chinese herbal medicine and doing POMDP mining analysis by Beijing Jiaotong University researchers with data mining tools. The level of average discounted reward (ADR) will take the degree of main symptoms improvement as a standard to evaluate the clinical efficacy of different treatment options.

### 3. Results

**3.1. The Demographic Information of the Subjects.** A total of 2212 (1341 males and 871 females) cases of subjects, aged more than 40 years, were included in this study. This group of subjects included 1061 cases of patients complicated with

TABLE 1: The demographic information of the subjects.

Items	Demographic information
Gender	
Males (case (%))	1341 (60.6%)
Females (case (%))	871 (39.4%)
Age (years)	61.2 ± 13.6
Hypertension (case (%))	1061 (48.0%)
Diabetes	514 (23.2%)
Hyperlipemia	513 (23.1%)
Old myocardial infarction	328 (14.8%)
Cerebral infarctions	219 (9.9%)

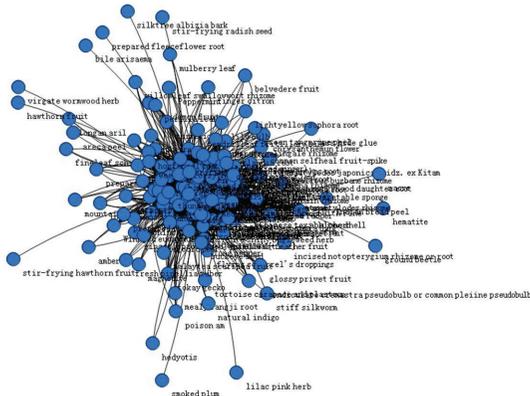


FIGURE 1: Overall situation of Chinese herbal prescription medicine for TCM syndrome element “qi Deficiency.”

hypertension, 514 cases of patients complicated with diabetes, 513 cases of patients complicated with hyperlipemia, 328 cases of patients complicated with old myocardial infarction, and 219 cases of patients complicated with cerebral infarctions. The demographic data is shown in Table 1.

3.2. TCM Syndrome Distribution in Patients with UA. Among the 2212 UA patients, the common Chinese syndrome elements were as follows: blood stasis (1931 cases, 87.3%), qi deficiency (1140 cases, 51.5%), turbid phlegm (1059 cases, 47.9%), yin deficiency (412 cases, 18.6%), qi stagnation (148 cases, 6.7%), yang deficiency (65 cases, 2.9%), heat (60 cases, 2.7%), and blood deficiency (23 cases, 1.0%).

3.3. Core Medicine of Chinese Herbal Prescription (Figures 1, 2, 3, and Table 2). Due to the requirements in the number of patients for model, we study the treatment options for patients with UA in three TCM syndrome elements as “qi deficiency,” “blood stasis,” and “turbid phlegm.” Use complex network clustering method to screen the core Chinese herbal medicine of three TCM syndrome elements. With all the Chinese herbal medicine used in the treatment options of a syndrome element as the nodes, the medicine in compatibility has “interconnected” feature, and the number of “interconnected” is the medicine “related frequency”. The most frequently related nodes with other medicine have the most critical role in all the medication of this syndrome

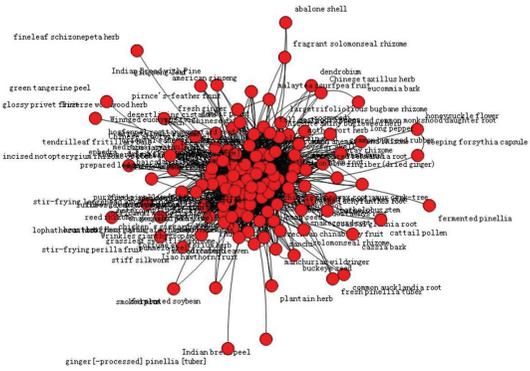


FIGURE 2: Overall situation of Chinese herbal prescription medicine for TCM syndrome element “blood stasis.”

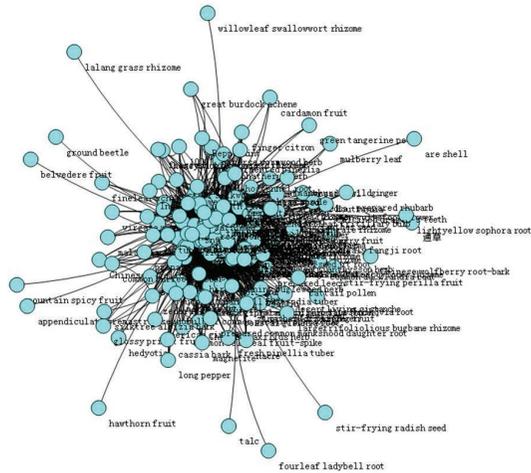


FIGURE 3: Overall situation of Chinese herbal prescription medicine for TCM syndrome element “turbid phlegm.”

element. Thus, the densest nodes are the core prescription medicine.

3.4. Optimization and Efficacy Evaluation of Prescriptions for Different TCM Syndrome Elements in Patients with UA (Table 3). Applying POMDP model to calculate the ADR of different prescriptions, the largest ADR prescription of TCM syndrome element can get the maximum benefit in symptom improvement and long-term efficacy. Among the prescriptions for 3 different TCM syndrome elements, the combination of “Milkvetch Root + Tangshen + Indian Bread + Largehead Atractylodes Rhizome” is the optimizing prescription of TCM syndrome element “qi deficiency” for patients with UA, the combination of “Danshen Root + Chinese Angelica + Safflower + Red Peony Root + Szechwan Lovage Rhizome, Orange Fruit” is the optimizing prescription of TCM syndrome element “blood stasis” for patients with UA, and the combination of “Snakegourd Fruit + Longstamen Onion Bulb + Pinellia Tuber + Dried Tangerine peel + Largehead Atractylodes Rhizome + Platycodon Root”

TABLE 2: Core medicine of Chinese herbal prescription.

Types of TCM syndrome element	Core medicine of Chinese herbal prescription
Qi deficiency	Tangshen, Heterophylly Falsestarwort Root, Largehead Atractylodes Rhizome, Milkvetch Root, Radix Glycyrrhizae, Indian Bread, and Chinese Date
Turbid phlegm	Pinellia Tuber, Snakegourd Fruit, Indian Bread, Dried Tangerine peel, Longstamen Onion Bulb, Largehead Atractylodes Rhizome, Platycodon Root, Golden Thread, Bamboo Shavings, Wrinkled Giant Hyssop + Herba Eupatorii, Wrinkled Giant Hyssop, Orange Fruit + Pinellia Tuber
Blood stasis	Szechwan Lovage Rhizome, Yanhusuo, Danshen Root, Peach Seed, Safflower, Chinese Angelica, Turmeric Root Tuber, Red Peony Root, Turmeric Root Tuber + Red Peony Root, Peach Seed + Radix bupleuri, Peach Seed + Platycodon Root, Szechwan Lovage Rhizome + Orange Fruit

TABLE 3: Optimizing prescription of different TCM syndrome elements for UA patients.

Types of TCM syndrome element	Optimizing prescription	ADR
Qi deficiency	Milkvetch Root + Tangshen + Indian Bread + Largehead Atractylodes Rhizome	0.96630
Blood stasis	Danshen Root + Chinese Angelica + Safflower + Red Peony Root + Szechwan Lovage Rhizome Orange Fruit	0.76
Turbid phlegm	Snakegourd Fruit + Longstamen Onion Bulb + Pinellia Tuber + Dried Tangerine peel + Largehead Atractylodes Rhizome + Platycodon Root	0.658568

is the optimizing prescription of TCM syndrome element “turbid phlegm” for patients with UA.

For commonly used names of Chinese herbal medicine, see Table 4.

#### 4. Discussion

With the development of TCM, researches in optimizing prescription of Chinese herbal medicine are concerned by more and more scholars. Clinical formulation optimization is the process of using certain methods to improve clinical medicine prescriptions and make them applied in clinical practice. The commonly used methods at present are mainly two approaches as generalizing empirical formulation after optimization and applying data mining model to promote the optimized formulation [10].

The target of empirical formulation optimization is to study the experienced prescriptions promoted by some expert or clinician by modern medical research methods such as randomized control trail (RCT) in order to prove the reliability and practicality, providing evidence for related field of formulation optimization. This method is more stringent and convincing in formulation optimization design, and the optimizing prescriptions for the study are derived from the established treatment ones. However, cases collecting, observing, and other series of clinical research process need a large amount of human and material resources, which increases the cost of study; these many prescriptions with very good clinical applications are difficult to discover and research. In addition, for the more rigorous research process, the applicable conditions of the results received strict limits. In clinical practice, the efficacy in patients is affected by a variety of complex factors, which should be the results of a variety of drugs or interventions, making the extrapolation of experience prescriptions subject to certain restrictions.

Applying data mining model to optimize the prescriptions has got manifold attempts in clinical practice [11–14]; the most common method is a dynamic programming strategy. Its purpose is to seek the best solution in many prescriptions applying optimization techniques. Its data are from the clinical data of real world, and the data collection is carried out in parallel with the clinical course. As long as establishing the regulation of data entry, the entire data acquisition does not require large-scale acquisition process, and the time is mainly spent in data computing, which greatly saves the cost of the study to facilitate the continuous optimization of the prescriptions. Furthermore, this dynamic process is a combination of man and machine model; the strict mathematical operation is carried out at the same time when doing empirical evaluation. The actual process can be combined with expert consensus and evaluation to select the best treatment prescriptions given by computer for the most appropriate patient. This process can also be used for the discovery of clinical prescriptions or provide a scientific and rational arithmetic verification process for the summary of experience prescriptions, which may be very meaningful in the future.

POMDP model [15] is a dynamic decision model based on Markov process promoted by the Russian mathematician Markov after some improvements. Hauskrecht and Fraser [16] used POMDP in treatment prescription of ischemic heart disease and did a decision successfully in many cases. Maillart et al. [17] made analysis in the frequency of X-rays and treatment options for breast cancer patients from the cost-benefit viewpoint with this model. Zhang et al. [18] established POMDP model for prostate biopsy decisions. These explorations all provide practical basis for solving problems of sequence decision with POMDP model in the medical field. This study selected this research strategy to optimize prescriptions of Chinese herbal medicine for patients with UA, which is an exploration on the methodology.

TABLE 4: Commonly used names of Chinese herbal medicine.

Chinese name	English name	Latin name
Dangshen	Tangshen	Radix Codonopsis
Huangqi	Milkvetch Root	Radix Astragali
Fuling	Indian Bread	Poria
Baizhu	Largehead Atractylodes Rhizome	Rhizoma Atractylodis Macrocephalae
Danshen	Danshen Root	Radix Salviae Miltiorrhiae
Danggui	Chinese Angelica	Radix Angelicae Sinensis
Honghua	Safflower	Flos Carthami
Chishao	Red Peony Root	Radix Paeoniae Rubra
Chuanxiong	Szechwan Lovage Rhizome	Rhizoma Chuanxiong
Zhiqiao	Orange Fruit	Fructus Aurantii
Gualou	Snakegourd Fruit	Fructus Trichosanthis
Xiebai	Longstamen Onion Bulb	Bulbus Allii Macrostemi
Banxia	Pinellia Tuber	Rhizoma Pinelliae
Chenpi	Dried Tangerine peel	Pericarpium Citri Reticulatae
Jiegeng	Platycodon Root	Radix Platycodi
Taizishen	Heterophylly Falsestarwort Root	Radix Pseudostellariae
Gancao	Radix Glycyrrhizae	Radix Glycythizae
Dazao	Chinese Date	Fructus Jujubae
Huanglian	Golden Thread	Rhizoma Coptidis
Zhuru	Bamboo Shavings	Caulis Bambusae in Taeniam
Huoxiang	Wrinkled Giant Hyssop	Agastache rugosa
Peilan	Herba Eupatorii	Eupatorium fortunei
Yanhusuo	Yanhusuo	Rhizoma
Taoren	Peach Seed	Semen Persicae
Yujin	Turmeric Root Tuber	Radix Curcumae
Baishao	White peony Alba	Radix Paeoniae Alba
Chaihu	Radix bupleuri	Bupleurum chinense
Shengdi	Rehmanniae radix	Rehmannia glutinosa Libosch

Concerning POMDP modeling requirements, this study was modeled following the dynamic clinical decision-making process to make the degree of remission in the symptoms for patients with UA and long-term end-point events as efficacy evaluation indicators for the choice of the optimal prescriptions; at the same time, it evaluated the original core prescription and extracted optimal treatment prescriptions as optimization recommendations of patients with UA in a certain TCM syndrome element for clinical reference.

Based on existing data, applying POMDP to compare the prescriptions of patients with same TCM syndrome element and no long-term end-point event, we found that optimizing prescription recommendation for “*qi* deficiency” patients is “Milkvetch Root + Si junzi decoction without Radix Glycyrrhizae,” prescription of “blood stasis” recommended “Danshen Root + Tao Hong Siwu decoction plus Orange Fruit without rehmanniae radix,” prescription of “turbid phlegm” recommended “Gualou xiebai banxia decoction plus Dried Tangerine peel, Largehead Atractylodes Rhizome, Platycodon Root”. Those are recommendations from strict mathematical model stimulating dynamic TCM prescription process, which are in line with conventional clinical thinking and put forward proposals worthy of strict clinical research.

Besides that, the prescriptions derived from real clinical data are experiences and summaries of clinical practice

with considerable clinical significance. The proposals are in conformity with the clinical normal circumstances and prove the reliability and operability of optimizing prescription method in efficacy evaluation on the other hand.

It should be noted that the rigorous mathematical comparison method using in this study to observe short-term and long-term efficacy of prescriptions in order to discover preliminary optimizing recommendations is limited to the number of patients, indicators, follow-up time, and so on that the extension value should apply modern medical research methods such as case-control trail to do retrospectively summarized comparison or large-scale, multicenter, and large sample RCT for further verification to increase the level of evidence-based medicine. However, in complex data of clinical practice, the efficacy of the patients is affected by many complicated factors; this formulation optimization idea still has great significance in efficacy comparison and screening treatment plan, and it also provides us an optimizing prescription method for clinical practice-based data worthy of further study.

## Acknowledgments

The current work was partially supported by Beijing Committee of Science and Technology (no. D08050703020801),

the TCM Public Welfare Scientific Research Project, State Administration of TCM of People's Republic of China (no. 201007001), the National Key Basic Research Prescription of China (no. 2006CB504803), the Twelve Five-year Plan of China (nos. 2013BAI02B01 and 2013BAI13B01), and Dean's Fund of Beijing Anzhen Hospital affiliated to Capital Medical University (2013Z05).

## References

- [1] Y. Z. Chen, *Practical Internal Medicine*, Beijing People's Health Publishing House, 11th edition, 2001.
- [2] E. J. Sondik, *The Optimal Control of Partially Observable Markov Processes*, Department of Electrical Engineering, Stanford University, Stanford, Calif, USA, 1971.
- [3] R. D. Smallwood and E. J. Sondik, "The optimal control of partially observable Markov processes over a finite horizon," *Operations Research*, vol. 21, no. 5, pp. 1071–1088, 1973.
- [4] E. J. Sondik, "The optimal control of partially observable Markov processes over the infinite horizon: discounted costs," *Operations Research*, vol. 26, no. 2, pp. 282–304, 1978.
- [5] China Cardiovascular Association, "Diagnosis and treatment guidelines of Unstable angina and non-ST-segment elevation myocardial infarction," *Chinese Medical Journal*, vol. 35, no. 4, pp. 295–304, 2007.
- [6] China Association of Integrative Medicine Association for Cardiovascular Diseases, "TCM standards of coronary heart disease," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 11, no. 5, p. 257, 1991.
- [7] Y. Y. Wang, *Chinese Medical Science*, Shanghai Science and Technology Press, Shanghai, China, 6th edition, 2001.
- [8] T. T. Deng, *Diagnostics of Chinese Medicine (Revised Edition)*, Shanghai Science and Technology Press, Shanghai, China, 2007.
- [9] N. L. Yao, *Syndromes Differential Diagnosis of Traditional Chinese Medicine*, People's Health Publishing House, Beijing, China, 2nd edition, 2002.
- [10] Y. M. Xie, Y. Y. Wang, and W. L. Wen, "Ideas and methods of clinical medical prescription optimization," *World Science and Technology: Modernization of Traditional Chinese Medicine*, vol. 10, no. 1, pp. 22–26, 2008.
- [11] J. Poon, Z. Luo, and R. Zhang, "Feature representation in the biclustering of symptom-herb relationship in Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 17, no. 9, pp. 663–668, 2011.
- [12] X. Z. Zhou, R. S. Zhang, J. Shah et al., "Patterns of herbal combination for the treatment of insomnia commonly employed by highly experienced Chinese medicine physicians," *Chinese Journal of Integrative Medicine*, vol. 17, no. 9, pp. 655–662, 2011.
- [13] L. Zhang, D. L. Yu, Y. G. Wang, and Q. Zhang, "Selecting an appropriate interestingness measure to evaluate the correlation between Chinese medicine syndrome elements and symptoms," *Chinese Journal of Integrative Medicine*, vol. 18, no. 2, pp. 93–99, 2012.
- [14] T. Chen, X. Z. Zhou, R. S. Zhang, and L. W. Zhang, "Discovery of regularities in the use of herbs in Chinese medicine prescriptions," *Chinese Journal of Integrative Medicine*, vol. 18, no. 2, pp. 88–92, 2012.
- [15] X. M. An and L. Lin, "Markov model research progress in the vital statistics," *Chinese Journal of Health Statistics*, vol. 24, no. 4, pp. 436–439, 2007.
- [16] M. Hauskrecht and H. Fraser, "Planning treatment of ischemic heart disease with partially observable Markov decision processes," *Artificial Intelligence in Medicine*, vol. 18, no. 3, pp. 221–244, 2000.
- [17] L. M. Maillart, J. S. Ivy, S. Ransom, and K. Diehl, "Assessing dynamic breast cancer screening policies," *Operations Research*, vol. 56, no. 6, pp. 1411–1427, 2008.
- [18] J. Zhang, B. T. Denton, H. Balasubramanian, N. D. Shah, and B. A. Inman, "Optimization of PSA screening policies: a comparison of the patient and societal perspectives," *Medical Decision Making*, vol. 32, no. 2, pp. 337–349, 2012.

## Research Article

# Protective Effect of Qiliqiangxin Capsule on Energy Metabolism and Myocardial Mitochondria in Pressure Overload Heart Failure Rats

Junfang Zhang,<sup>1</sup> Cong Wei,<sup>2,3</sup> Hongtao Wang,<sup>2,4</sup> Siwen Tang,<sup>3,4</sup>  
Zhenhua Jia,<sup>3,5</sup> Lei Wang,<sup>1,5</sup> Dengfeng Xu,<sup>3,4</sup> and Yiling Wu<sup>1,5</sup>

<sup>1</sup> Integrative Medicine Department, Hebei Medical University, 361 Zhongshan Road, Shijiazhuang 050017, China

<sup>2</sup> Hebei Yiling Medical Research Institute, Shijiazhuang 050035, China

<sup>3</sup> Key Research Centre of State Administration of Traditional Chinese Medicine (Collateral Disease of Cardiovascular), Shijiazhuang 050035, China

<sup>4</sup> Key Laboratory of Collateral Disease of Hebei Province, Shijiazhuang 050035, China

<sup>5</sup> Yiling Hospital, Hebei Medical University, Shijiazhuang 050091, China

Correspondence should be addressed to Yiling Wu; [weitcm@163.com](mailto:weitcm@163.com)

Received 17 November 2012; Revised 8 June 2013; Accepted 25 June 2013

Academic Editor: Keji Chen

Copyright © 2013 Junfang Zhang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Qiliqiangxin capsule (QL) was developed under the guidance of TCM theory of collateral disease and had been shown to be effective and safe for the treatment of heart failure. The present study explored the role of and mechanism by which the herbal compounds QL act on energy metabolism, *in vivo*, in pressure overload heart failure. SD rats received ascending aorta constriction (TAC) to establish a model of myocardial hypertrophy. The animals were treated orally for a period of six weeks. QL significantly inhibited cardiac hypertrophy due to ascending aortic constriction and improved hemodynamics. This effect was linked to the expression levels of the signaling factors in connection with upregulated energy and the regulation of glucose and lipid substrate metabolism and with a decrease in metabolic intermediate products and the protection of mitochondrial function. It is concluded that QL may regulate the glycolipid substrate metabolism by activating AMPK/PGC-1 $\alpha$  axis and reduce the accumulation of free fatty acids and lactic acid, to protect cardiac myocytes and mitochondrial function.

## 1. Introduction

Hypertension is one of the most common cardiovascular diseases and a major cause of heart failure. Increase of pressure load due to long-term hypertension will cause myocardial fibrosis and left ventricular compensatory hypertrophy. The accompanying neurohumoral factors, endocrine and metabolic abnormalities further promote ventricular remodeling; structural changes would result in the decline in coronary flow reserve, which eventually leads to heart diastolic and systolic dysfunction [1, 2]. At present, the overall prognosis of heart failure remains less optimistic; thus, further research on the pathogenesis of heart failure and finding new therapeutic targets and measures has become an urgent need in heart failure research. Qiliqiangxin capsule (QL) was

developed under the guidance of TCM theory of collateral disease [3]. It is a drug of Chinese medicine for the treatment of heart failure, registered in the Chinese State Food and Drug Administration (SFDA) in 1996, and had been used in clinical practice for more than a decade. Previous studies have shown that QL was able to inhibit Ang II and ALD levels, improve hemodynamics and cardiac function, inhibit ventricular remodeling [4], and reduce the concentration of plasma vasopressin (AVP) and cardiac stress [5]. QL is composed of a number of herbal plants. Previous studies have shown that some of the plants in the QL formula impacted energy metabolism. For example, Astragalus has been shown to inhibit myocardial hypertrophy and improve energy metabolism by increasing the membrane potential [6]. Previous studies have also shown that QL interfered with the

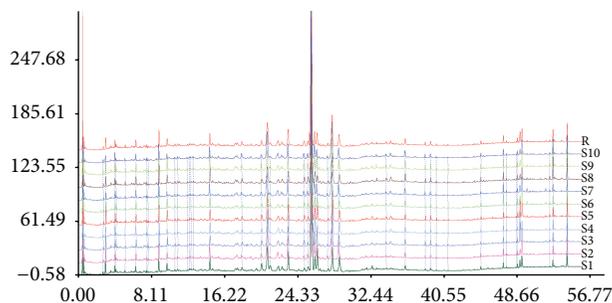


FIGURE 1: UPLC fingerprints of ten batches of QL capsule.

energy metabolism related proteins, particularly in increasing the expression of acetyl coenzyme A dehydrogenase and acetal enzyme, indicating that the improvement of heart failure by QL may be related to energy metabolism [3, 7]. However, the relationship between the improvement, by QL, of cardiac dysfunction caused by the chronic pressure overload and energy metabolism and mitochondrial protection remains to be further studied. The present study focused on the cardiac energy metabolism and mitochondrial function and conducted experiments in order to determine the intervention effect of QL on the energy of pressure overload heart failure rats and to further explore its possible mechanism of action by QL.

## 2. Materials and Methods

**2.1. Drugs and Reagents.** Qiliqiangxin capsule powder was provided by Shijiazhuang Yiling Pharmaceutical Co. Ltd. The main active pharmaceutical ingredients of QL included Astragalus, Ginseng, *Salvia miltiorrhiza*, Pepperweed Seed, Rhizoma Alismatis, *Polygonatum odoratum*, Ramulus Cinnamomi, *Carthamus tinctorius*, Cortex Periplocae, Tangerine Peel, and other herbs, see Supplementary Material available online at <http://dx.doi.org/10.1155/2013/378298>. The origin, harvest time, medicinal composites, and processing technology of the herbs were strictly normalized and standardized for different batches of QL capsules. The labeled compounds were verified and standardized according to the Chinese Pharmacopoeia (2005) to achieve quality control of the QL capsule. The aqueous extract of 10 different batches QL was randomly sampled and analysed for the chemical fingerprints by using ultrahigh performance liquid chromatography (UPLC). The similarity analysis of the fingerprints was performed; the similarities were found to be within the range of 0.978 to 1.000, showing that the overall quality of QL had good reproducibility (Figure 1).

Captopril (batch number 11061511) was manufactured by Changzhou Pharmaceutical Factory. Lactic acid detection kit (Jiancheng, Nanjing; batch number 20120710); free fatty acid detection kit (Landau, UK; lot number 234892); adenosine triphosphate ATP (Lot no. 100111202), adenosine diphosphate ADP (Lot no. 10090203190), and rhodamine 123 (Rhodamine-123) were purchased from Sigma Chemical Co., adenosine monophosphate AMP (China National Institutes for Food and Drug Control, batch number 140719-200501),

mitochondrial extraction kit (Beijing Solarbio Science & Technology Co., batch number 20120528). Trizol Reagent (Invitrogen); reverse transcriptase (M-MLV), ribonuclease inhibitor (RNasin), dNTP, Taq DNA polymerase, and random primers were purchased from Promega Corporation, USA; Hot Start Fluorescent PCR Core of Reagent Kits (SYBR Green I) was obtained from BBI.

**2.2. Animal Model and Administration.** Male Sprague-Dawley rats (body weight 250–270 g), 150, were provided by the Beijing Vital River Laboratory Animal Technology Co., Ltd (Animal license number: SCXK (Beijing) 2012-0001). The animals were housed at five/cage, fed with standard diet and water ad libitum, and were subject to a 12 h light and 12 h dark cycle. All animal experimental protocols were approved by Animal Care and Use Committee of Hebei Medical University and complied with laboratory animal management and use regulations. Chronic heart failure model was established by thoracic aortic coarctation (TAC) [8], including 135 rats. Rats were intraperitoneal anaesthetized with chloral hydrate (0.35 g/kg) before supine position fixed, intubated, and artificially ventilated. After thoracotomy, descending thoracic aortae were isolated, cleared of fat and connective tissue. Thoracic aortic and polyethylene plastic pipe were tied together securely using a silk string, and then pull out the pipe quickly. The degree of constricting was about 50%. The thorax incision was closed and then stitched with disinfection. The Sham group was with 15 rats which underwent a procedure with thoracic aorta isolation but without coarctation. Four weeks after operation, 75 rats were chosen from the 83 survived ones according to transthoracic echocardiography and ECG and randomly assigned to the following groups: Captopril group (Captopril), orally administered captopril 6.25 mg/kg/d; QL high-dose group (QL-H,  $n = 15$ ), middle-dose group (QL-M,  $n = 15$ ), low-dose group (QL-L,  $n = 15$ ), and the doses of administered QL powder were 1.0, 0.5, and 0.25 g/kg/d, respectively; the volume was 10 mL/kg/d; Model group (TAC,  $n = 15$ ); Sham group (Sham,  $n = 15$ ); Sham group and the test group were administered with an equal volume of 0.5% sodium carboxymethyl cellulose (CMC-Na), orally administered for 6 weeks.

**2.3. Heart Weight Index and Hemodynamic Measurement.** After treatment for six weeks, the animals were anesthetized to perform the separation of the right carotid artery and cannulation. Left ventricular systolic pressure (LVSP) and left ventricular pressure (LVP) were measured by using BL-420E polygraph (Chengdu TaiMeng Technology Corp., LTD). After collecting blood from the abdominal aorta, the heart was rapidly removed, rinsed with cold physiological saline, and water adsorbed by filter paper. The excess tissues around the hearts were removed, and hearts were weighed on a balance and then immediately put into liquid nitrogen for storage. Heart weight index was calculated using the formula: index = heart weight (HW)/body weight (BW).

**2.4. Content of Serum Lactic Acid (LA) and Free Fatty Acid (FFA).** Blood was stored at room temperature for 2 h and centrifuged for 10 min at 3500 rpm; the serum was then

TABLE 1: Primer for RT-PCR.

Gene	bp	GenBank ID	Primer Sequence (5' to 3')
GAPDH	120	NM_017008	S: TGAACGGGAAGCTCACTGG A: GCTTCACCACCTTCTTGTATGTC
CPT-I	87	NM_013200.1	S: CCA GGC AAA GAG ACA GAC TTG A: GCCAAACCTTGAAGAAGCGA
GLUT4	62	NM_012751	S: CCC ACA AGG CAC CCT CAC TA A: TGC CAC CCA CAG AGA AGA TG
AMPK	133	NM_019142.1	S: ACA GAA GCC AAA TCA GGG ACT A: CAC GGA TGA GGT AAG AGA GAC T
PGC-1 $\alpha$	168	NM_031347	S: AGC CAC TAC AGA CAC CGC AC A: CCT TTC AGA CTC CCG CTT C

GAPDH: glyceraldehydes 3-phosphate dehydrogenase; CPT-I: carnitine palmitoyl transferase I; GLUT4: Glucose transporter 4; AMPK: AMP-activated protein kinase; PGC-1 $\alpha$ : peroxisome proliferators-activated receptor- $\gamma$  coactivator-1 $\alpha$ ; MW: molecular weight.

loaded into EP tube. The optical density value was measured by using the XD711 ELISA analyzer (Shanghai Xun-Da Medical Instrument Corporation Ltd.) according to the experimental method specified in the instruction of lactic acid detection kit; serum free fatty acids were measured by using Hitachi 7080 biochemical analyzer.

**2.5. Mitochondrial Transmembrane Potential (MMP).** About 0.3 g of fresh myocardial tissue was prepared and rinsed by physiological saline. Myocardial tissue was sheared and washed with PBS buffer. Cell mass was filtered with 300 mesh metal mesh and single cell suspension was collected. The supernatant was discarded after centrifugation (1000 rpm, 5 min). Single cell suspension was adjusted to  $1 \times 10^5$ /mL in the culture medium. Rhodamine 123 was used as fluorescent probe (final concentration of 0.5  $\mu$ mol/L). The suspension was incubated for 15 min in the dark place, and then the changes in the myocardium mitochondria MMP were measured using flow cytometer (EPICS-XL II, Beckman Coulter, USA) with excitation wavelength and emission wavelength at 480 nm and 530 nm, respectively.

**2.6. Mitochondria Extract and Determination of Oxidative Respiratory Function.** Fresh myocardial tissue was collected at the end of the experiment and extracted with mitochondrial extraction kit to prepare mitochondrial suspension by differential centrifugation at low temperature. Protein content in myocardial mitochondrial suspension was determined by Coomassie brilliant blue. 2.5 mL of GENMED medium liquid was added into the reaction glass tank, mixed, and sealed, and the mitochondria, State IV substrate solution and State III substrate solution were added in proper sequence as instructed by the manufacturer. Mitochondrial State III and State IV respiration rate in the closed reaction system were determined using dissolved oxygen electrolytic analyzer (ORION 4 STAR, the U.S. Thermo Electron), and mitochondrial respiratory control ratio (RCR) was calculated according to the following formula:  $RCR = \text{State III respiration rate} / \text{State IV respiration rate}$ .

**2.7. Adenine Nucleotide ATP, ADP and AMP Levels and Energy Charge in Myocardial Tissue.** The cryopreserved myocardial

tissues were weighed and added 0.4 mol/L precooling perchloric acid at 5 mL/g ratio, homogenized on the ice bath, and then centrifuged at 4000 r/min for 10 min at 4°C. Supernatant (400  $\mu$ L) was collected and added 25  $\mu$ L 2 mol/L to adjust pH to 6.5, centrifuged again at 4000 r/min for 10 min at 4°C, and the supernatant was filtered with a 0.45 micron membrane. The supernatant preparation was loaded onto a 10 A high performance liquid chromatography detector (Shimadzu Corporation, Japan), followed by separation and detection at wavelength of 254 nm. ATP, ADP, and AMP levels were calculated according to the elution peak area and standard concentrations. The results were expressed at  $\mu$ mol/g tissue. Total adenylate pool (ATP + ADP + AMP) and the energy charge  $[EC = (ATP + 0.5 \times ADP) / (ATP + ADP + AMP)]$  were calculated.

**2.8. Real-Time RT-PCR.** Total RNA of myocardial tissue was extracted using Trizol, which was then reverse transcribed into double stranded cDNA. Real-time RT-PCR was carried out using a thermocycler (ABI 7300 Real-Time PCR System, USA). PCR thermal cycling parameters were as follows: the denaturing step at 96°C for 4 min, followed by 40 cycles annealing step at 94°C 30 s, 58°C 30 s, and 72°C 30 s. Fluorescence signal was collected in each cycle of the third step 72°C 30 s. Using GAPDH gene as an internal reference and by comparing target gene expression and with Control group, the relative quantitative value (RQ value) was calculated and used for statistical analysis. The primer sequences were shown in Table 1.

**2.9. Western Blot Analysis.** Myocardial tissue about 100 mg and 1ml precooled lysate were homogenised in ice bath. Supernatant was collected after centrifugation (4°C, 8000 rpm and 10 min), and the protein concentration of supernatant, measured by Nanodrop 2000 spectrophotometer, was adjusted to the final concentration of 1%. The samples were mixed with equal volumes of 5  $\times$  SDS sample buffer, and then boiled for 5 min, centrifuged, and loaded onto 12% SDS-PAGE for electrophoresis. After being transferred onto a polyvinylidene difluoride membrane (PVDF), the membranes were blocked with 5% milk tris-buffered saline-tween 20 and probed with primary antibody (1:1000) and

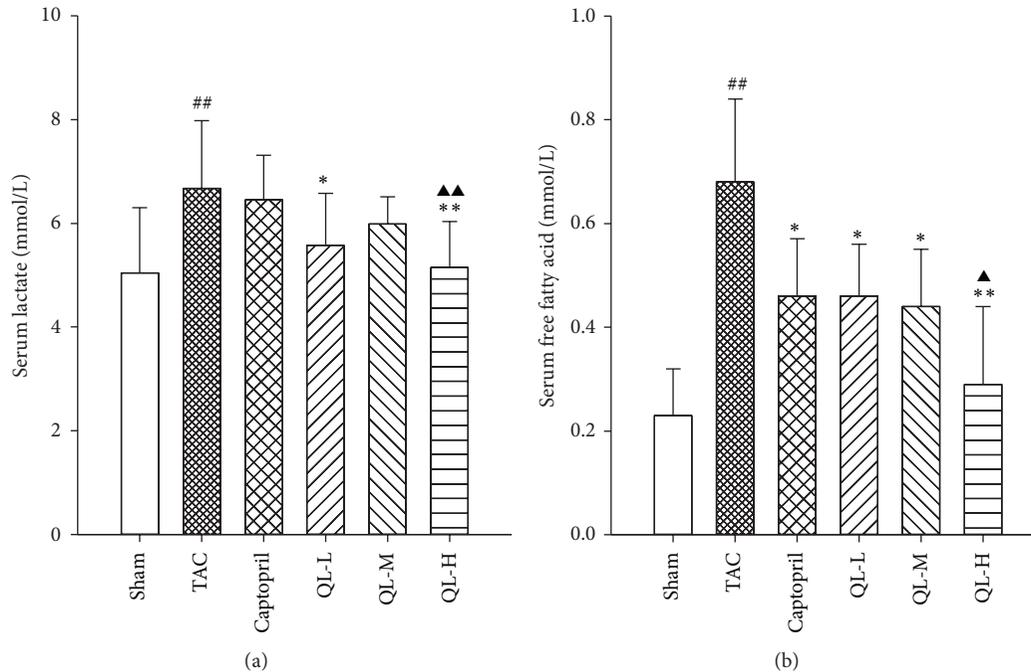


FIGURE 2: Serum lactate and free fatty acid content in all the six groups. (a) Serum lactate concentrations in the myocardium. (b) The content of serum free fatty acid in the myocardium. Rats were administered with vehicle (0.5% CMC-Na), QL (0.25 g/kg/d, 0.5 g/kg/d, 1 g/kg/d), or Captopril (6.25 mg/kg/d). ##  $P < 0.01$ , compared TAC group with Sham group; \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared respective dose group with TAC group; ▲  $P < 0.05$ , ▲▲  $P < 0.01$ , compared QL respective dose group with Captopril group.

TABLE 2: The changes of body weight, heart weight/body weight ratio (HW/BW), LVP, and LVSP.

Group	Heart weight (mg)	Body weight (g)	HW/BW (mg/g)	LVP (mmHg)	LVSP (mmHg)
Sham	1.32 ± 0.12	499 ± 49.3	2.40 ± 0.11	110 ± 21.0	105 ± 16.7
TAC	1.40 ± 0.14	490 ± 41.2	3.02 ± 0.47 <sup>##</sup>	144 ± 23.9 <sup>##</sup>	133 ± 23.2 <sup>##</sup>
Captopril	1.36 ± 0.10	492 ± 35.2	2.64 ± 0.25 <sup>*</sup>	127 ± 18.6 <sup>**</sup>	116 ± 19.1 <sup>*</sup>
QL-L	1.33 ± 0.14	500 ± 52.4	2.67 ± 0.31 <sup>*</sup>	127 ± 13.9 <sup>*</sup>	117 ± 18.6
QL-M	1.31 ± 0.20	503 ± 46.2	2.61 ± 0.35 <sup>*</sup>	125 ± 18.7 <sup>**</sup>	115 ± 13.9 <sup>*</sup>
QL-H	1.28 ± 0.13	490 ± 42.0	2.54 ± 0.29 <sup>**</sup>	119 ± 13.9 <sup>**</sup>	103 ± 16.0 <sup>**</sup>

Sham: control group; TAC: model group; QL-L: low-dose QL group; QL-M: medium-dose QL group; QL-H: high-dose QL group; The Sham rats and TAC rats were treated with vehicle (0.5%CMC-Na, 10 mL/kg/d); the low-dose QL group was treated with 0.25 g/kg/day QL; the medium-dose QL group was treated with 0.5 g/kg/day QL; the high-dose QL group was treated with 1 g/kg/day QL. Captopril group was treated with 6.25 mg/kg/day Captopril. Values were expressed as mean ± standard deviation. Compared with TAC, #  $P < 0.05$ , ##  $P < 0.01$ ; compared with Sham, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

HRP conjugated secondary antibody (1:10000), separated by extensive washings. The membrane was treated with enhanced chemiluminescence substrate and the bands on the membrane were visualized and analyzed using UVP Bio-Imaging System.

**2.10. Statistical Methods.** All data were presented as mean ± standard deviation, single factor analysis of variance (ANOVA) was performed with the statistical software SPSS 17.0, Dunnert'sT3 was used for unequal variances, and the difference was statistically significant at  $P < 0.05$ .

### 3. Results

**3.1. Changes in LVP, LVSP and Heart Weight Index.** The rats in each operated group manifested low activity, short of breath,

poor appetite, listlessness and other symptoms of heart failure. Compared with Sham group, LVP, LVSP and heart weight indices of the rats in Model group were significantly increased ( $P < 0.01$ ). Compared with Model group, the intervention of six weeks' administration with QL reduced the LVP and LVSP levels to some extent. Model group increased significantly compared with Sham group. Each treatment group had significantly lower heart weight index ( $P < 0.05$ ), especially in the QL-H and Captopril group in which the heart weight was significantly lower ( $P < 0.01$ ) (Table 2).

**3.2. Serum LA and FFA Levels in Each Group.** As shown in Figures 2(a) and 2(b), serum lactate and free fatty acids levels in Model group were higher compared with Sham group ( $P < 0.01$ ). Low dose and high dose groups of QL reduced serum lactate level in the pressure overload rat ( $P < 0.05$ );

TABLE 3: The p-AMPK, AMPK, PGC-1 $\alpha$ , CPT-I, and GLUT 4 protein expressions in myocardial tissue of different groups.

Group	p-AMPK	AMPK	PGC-1 $\alpha$	CPT-I	GLUT 4
Sham	0.50 $\pm$ 0.12	0.67 $\pm$ 0.15	0.80 $\pm$ 0.12	1.15 $\pm$ 0.20	0.70 $\pm$ 0.14
Model	0.31 $\pm$ 0.14	0.45 $\pm$ 0.17	0.29 $\pm$ 0.07 <sup>#</sup>	0.57 $\pm$ 0.10 <sup>#</sup>	0.39 $\pm$ 0.18 <sup>#</sup>
Captopril	0.79 $\pm$ 0.22 <sup>**</sup>	0.57 $\pm$ 0.36	0.76 $\pm$ 0.09 <sup>**</sup>	1.03 $\pm$ 0.32 <sup>*</sup>	0.71 $\pm$ 0.27 <sup>*</sup>
QL-L	0.68 $\pm$ 0.24 <sup>*</sup>	0.49 $\pm$ 0.16	0.35 $\pm$ 0.03	0.65 $\pm$ 0.17	0.62 $\pm$ 0.02
QL-M	0.79 $\pm$ 0.11 <sup>**</sup>	0.44 $\pm$ 0.14	0.53 $\pm$ 0.06 <sup>**</sup>	0.93 $\pm$ 0.27	0.75 $\pm$ 0.15 <sup>*</sup>
QL-H	0.94 $\pm$ 0.13 <sup>**</sup>	0.63 $\pm$ 0.22	0.75 $\pm$ 0.03 <sup>**</sup>	1.07 $\pm$ 0.36 <sup>*</sup>	0.81 $\pm$ 0.14 <sup>**</sup>

Sham: control group; TAC: model group; QL-L: low-dose QL group; QL-M: medium-dose QL group; QL-H: high-dose QL group; values were expressed as mean  $\pm$  standard deviation. Compared with TAC, <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$ ; compared with Sham, <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ .

the high-dose group significantly lowered serum lactate level ( $P < 0.01$ ). Although the positive group also reduced the lactic acid level, the difference was not significant. Compared with Model group, free fatty acid level was significantly lower in Captopril group and each dose group of QL ( $P < 0.05$ ). The high dose group had a highly significant difference ( $P < 0.01$ ).

**3.3. Changes in Mitochondrial Membrane Potential in Cardiac Myocytes and Respiratory Function.** Compared with Sham group, myocardial mitochondrial membrane potential was declined after model establishment. After drug intervention for six weeks, myocardial mitochondrial membrane potential was significantly elevated compared with Model group ( $P < 0.05$ ) (Figures 3(a) and 3(b)). Each treatment group showed an improved mitochondrial respiratory function and increased respiratory control ratio ( $P < 0.05$ ). The effects of Captopril group and QL-H group were highly significant ( $P < 0.01$ ), as shown in Figure 3(c).

**3.4. Total Adenylate Pool and Energy Charge Level in Myocardial Tissue.** Compared with Sham group, total adenylate and energy charge values in Model group were significantly lower ( $P < 0.01$ ), showing a disturbance of myocardial energy supply occurred in postoperative rats; the ventricular reconstruction was accompanied by metabolic remodeling. Compared with Model group, the drug intervention group significantly increased the adenosine pool and energy charge value; total adenosine pool and energy charge value were higher in QL-L group than Model group ( $P < 0.05$ ,  $P < 0.01$ , resp.). The increase of total adenylate and energy charge value in myocardial tissue was most notable in Captopril group and QL-H group (Figure 4).

**3.5. Real-Time PCR Results in Myocardial Tissue.** Compared with Sham group, the expression levels of AMPK, PGC-1 $\alpha$ , CPT-I, and GLUT4 mRNA were significantly lower in Model group ( $P < 0.01$ ); the relative expression levels of each group indicator were increased to some extent after drug intervention. The expression levels of AMPK, PGC-1 $\alpha$ , CPT-I, and GLUT4 mRNA were significantly different in QL-H group and Captopril group compared with the Model group ( $P < 0.05$ ,  $P < 0.01$ , resp.) (Figure 5).

**3.6. Western Blot Results in Myocardial Tissue.** Compared with Sham group, the protein expression of PGC-1 $\alpha$ , CPT-I, and GLUT4 in Model group decreased significantly ( $P < 0.05$ ,  $P < 0.01$ , resp.). Compared with Model group, the above protein expression in treatment group increased at varying degrees ( $P < 0.05$ ,  $P < 0.01$ ). Compared with Sham group, the protein expression of p-AMPK and AMPK in Model group showed no statistical difference ( $P > 0.05$ ). Compared with Model group, the expression level of p-AMPK in treatment group increased significantly, in which the QL-M and QL-H group increased obviously ( $P < 0.01$ ). The total amount of AMPK in each group did not show significant difference ( $P > 0.05$ ) (Table 3, Figure 6).

## 4. Discussion

QL capsules are traditional Chinese medicine (TCM) formula, which was developed according to TCM theory. The QL has extracts obtained from 11 herbs. Pharmacological studies have found that QL contains a number of active substances such as ginseng saponin, astragalus saponin, flavonoid, cardenolide, and phenolic acid. There is a significant effect in the clinical treatment of chronic heart failure as shown in previous studies [9, 10].

Failure or hypertrophic myocardium caused imbalance between demand increase and production decrease of ATP, which was the root cause of decrease in myocardial metabolic reserve and cardiac function degradation [11, 12]. The condition is reflected by the decrease of myocardial ATP level in the failing heart, mitochondrial dysfunction, and imbalance of carbohydrate and fatty acid oxidation [13].

Myocardial remodeling can decrease oxygen level in the myocardial tissue and affects substrate oxidation, thus resulting in the reducing of aerobic metabolic efficiency and the shift of energy supply to anaerobic glycolysis. In this case, free fatty acids (FFA) and glucose aerobic oxidation are decreased, and insulin resistance may occur [14, 15]. The increase in lactic acid and FFA levels within the cells may result in cardiac toxicity and exacerbation of energy metabolism [16]. Changes in myocardial substrate metabolism in heart failure reduce the ATP level and also cause the increase of lactic acid and the accumulation of free fatty acids, leading to intracellular acidosis and increasing cardiac myocytes injury. This is consistent with the experimental results from the present study.

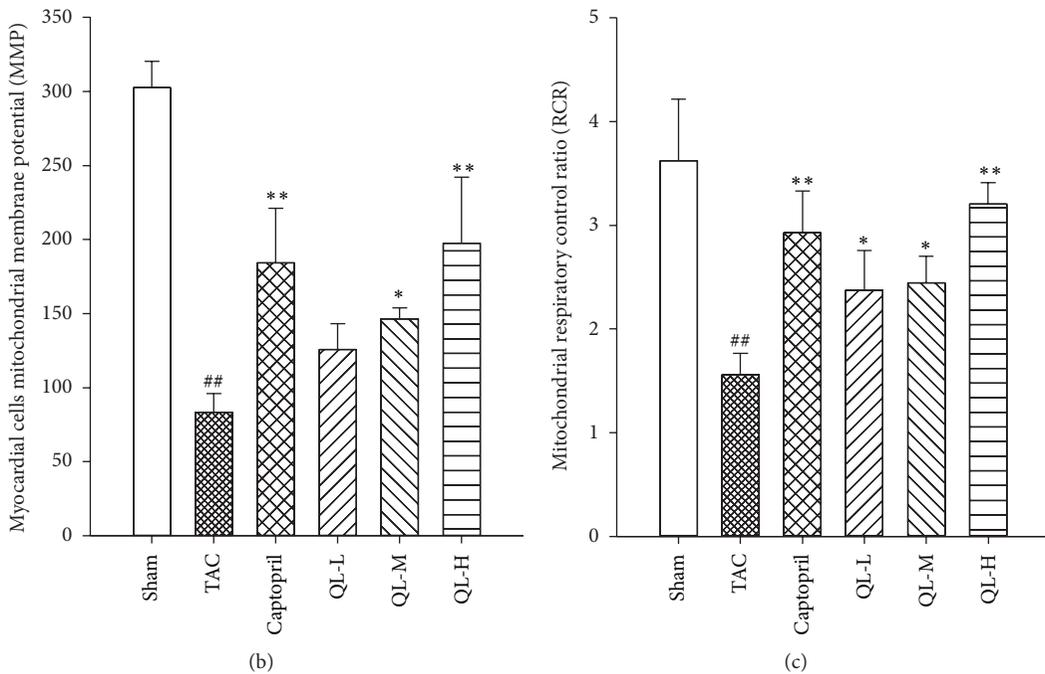
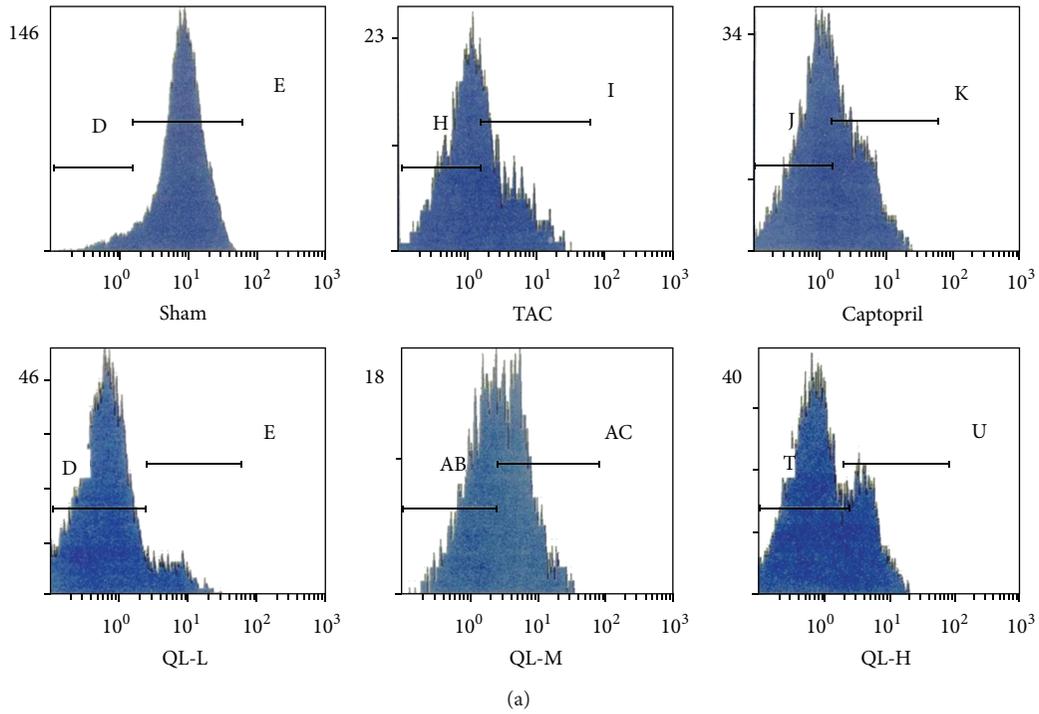


FIGURE 3: The measurement of myocardial cells mitochondrial membrane potential (MMP) and respiratory control ratio (RCR). (a) The measurement of myocardial cells mitochondrial membrane potential. (b) Comparison of MMP in different groups. (c) Comparison of mitochondria respiratory control ratio (RCR) in different groups. <sup>##</sup>  $P < 0.01$ , compared TAC group with Sham group; <sup>\*</sup>  $P < 0.05$ , <sup>\*\*</sup>  $P < 0.01$ , compared respective dose group with TAC group.

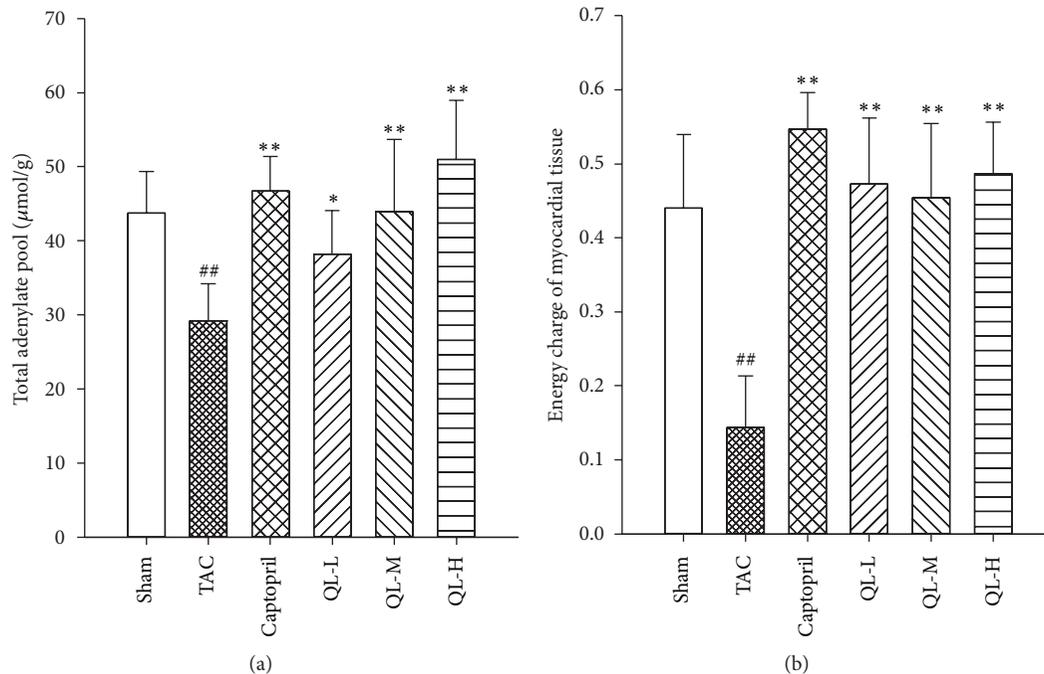


FIGURE 4: Total adenylate pool and energy charge of myocardial tissue in rats. (a) Effects of QL on the total adenylate pool in myocardial tissue. (b) Effects of QL on energy charge of myocardial tissue in rats. <sup>##</sup> $P < 0.01$ , versus TAC with Sham; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ , versus QL respective dose group with TAC.

QL lowered fatty acids and free fatty acids levels, which was closely related to substrate metabolism. We further detected the expression levels of carnitine acyltransferase enzyme-I (CPT-I) and glucose transporter protein 4 (GLUT4), which were key factors for fatty acids and glucose oxidation, respectively. Thus QL improved the expression levels of CPT-I and GLUT4. These data illustrated that QL promoted the oxidation of fatty acids and glucose by optimizing and regulating the glycolipid substrate metabolism, and decreasing the accumulation of lactic acid and free fatty acids, thereby protecting the cardiac myocytes.

The energy metabolism in cardiac myocytes is closely related to mitochondrial function status. Mitochondrial damage and significant reduction of oxidative phosphorylation efficiency can be caused by ischemia and hypoxia and the intermediate metabolite accumulation in failing heart. There is an internal negative and external positive transmembrane potential difference in mitochondrial inner-membrane. When the membrane potential decreases uncoupling of oxidative phosphorylation, ATP depletion and increase in oxygen radicals take place, thereby inducing cardiac myocytes into the irreversible process of apoptosis. High-energy phosphate is a direct source of energy for maintaining normal life activities of myocardial cells. The adenylate pool reflected the energy reserve state and the energy metabolic state. The results from the present study showed that, compared with Sham group, mitochondrial adenylate pool and energy charge value, RCR, and membrane potential were significantly lower in Model group, which were the same as the results of Ingwall [11], indicating that mitochondria and substrate oxidation

were damaged, and energy reserves and utilization were reduced. However, the administered group improved the above indicators, particularly for the high QL dose group which had improved obviously energy metabolism. Taken together, those data indicate that the effect of QL on protecting mitochondria and improving energy metabolism is one of the important mechanisms of improving cardiac function and delaying heart failure development process.

AMP-activated protein kinase (AMPK) is a key modulator of lipid and glucose metabolism and energy balance. When ATP level is reduced, AMPK is rapidly activated, and becomes involved in the cell energy regulation [17]. AMPK antagonized lipotoxicity in endothelial cells due to FFA by promoting the oxidation of endothelial cell on FFA [18], while the myocardial glucose uptake and GLUT4 translocation are increased. Activated AMPK not only inhibits fatty acid synthesis, but it also enhances fatty acid oxidation by reducing the levels of malonylCoA, which is an allosteric inhibitor of CPT-I [19]. Furthermore, AMPK stimulates GLUT4 translocation and increasing whole-body utilization of glucose [20]. PGC-1 $\alpha$  is a key nuclear receptor coactivator that can induce mitochondrial biogenesis, modulate number and mass of mitochondrial, fatty acid oxidation, and thermogenesis [21]. The current studies confirmed that QL activated AMPK and upregulated the expression of PGC-1 $\alpha$ , compared with the Model group. Thereby, QL regulated energy metabolism by activating AMPK/PGC-1 $\alpha$  signal factor. Specifically, QL regulated fatty acid and glucose metabolism, reduced the levels of FFA and LA through activation of AMPK. The improved mitochondrial respiratory function by QL has a relationship

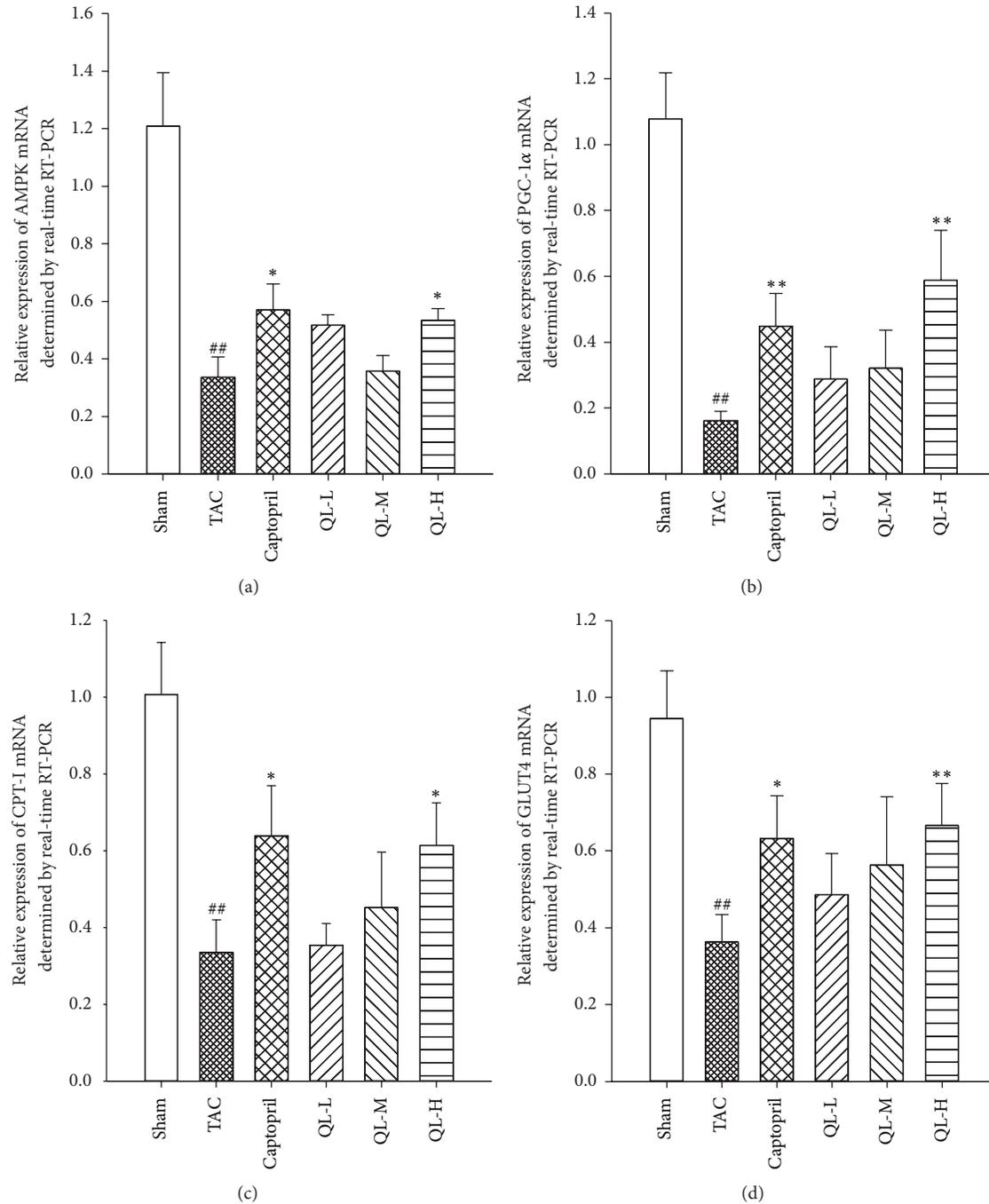


FIGURE 5: Real-time reverse transcription RT-PCR results. (a) The expression of AMPK mRNA in the cardiac tissue in the six groups was determined by real time RT-PCR. (b) The relative expression of PGC-1 $\alpha$ mRNA in the myocardium of the six groups was determined by real time RT-PCR. (c) Compared the relative expression of CPT-1 mRNA determined by real time RT-PCR. (d) The expression of GLUT4 in the myocardium was determined by real time RT-PCR. <sup>##</sup> $p < 0.01$  versus TAC with Sham; <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ , versus respective dose group with TAC.

with its regulation of PGC-1 $\alpha$  and the PGC-1 $\alpha$  downstream. PGC-1 $\alpha$  has many biological effects, for example, through the TR $\beta$ 1, NRFS, and ERRS it affects mitochondrial biogenesis [22]. Previous studies have shown that AICAR, AMPK-activating compound, could induce upregulation of PGC-1 $\alpha$  mRNA [23]. AMPK/PGC-1 $\alpha$ , a signaling pathway involved in

energy metabolism, was therefore the pathway regulated by QL.

In summary, the improvement of cardiac energy metabolism is an important part of delaying heart failure progression. QL reduced heart weight index of rat model of TAC-induced pressure overload, improved hemodynamic parameters, and

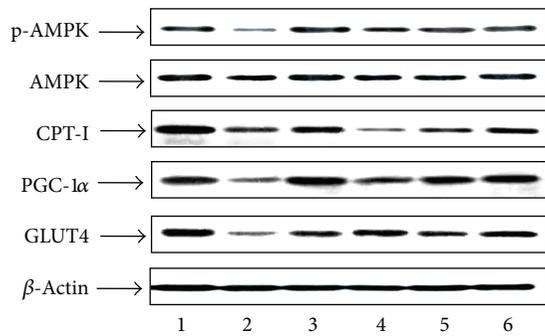


FIGURE 6: The p-AMPK, AMPK, PGC-1 $\alpha$ , CPT-1 and GLUT4 protein expressions in myocardial tissue of different groups. 1: Sham group; 2: TAC group; 3: Captopril group; 4: QL-L group; 5: QL-M group; 6: QL-H group.

was related to the increase of myocardial high-energy phosphate content as well as the improvement of energy reserves and the metabolic state. We argue that QL may regulate the glycolipid substrate metabolism by activating AMPK/PGC-1 $\alpha$  axis and reduce the accumulation of free fatty acids and lactic acid, to protect cardiac myocytes and mitochondrial function.

## Acknowledgments

This research was funded by the National Basic Research Program of China (973 Program) (Grant no. 2012 CB518606). The authors are thankful to China Shijiazhuang Yiling Pharmaceutical Co., LTD. for providing the study drug (Qili Qiangxin capsule powder) and are thankful to Key Laboratory of Network Disease of Hebei Province and Key Laboratory of State Administration of Traditional Chinese Medicine (Collateral Disease of Cardiovascular) (Shijiazhuang) for their great help.

## References

- [1] E. Agabiti-Rosei, M. L. Muiesan, and M. Salvetti, "Evaluation of sub clinical target organ damage for risk assessment and treatment in the hypertensive patients: left ventricular hypertrophy," *Journal of the American Society of Nephrology*, vol. 17, supplement 2, pp. S104–S108, 2006.
- [2] S. Maule, A. Milan, T. Grosso, and F. Veglio, "Left ventricular hypertrophy in patients with autonomic failure," *American Journal of Hypertension*, vol. 19, no. 10, pp. 1049–1054, 2006.
- [3] Y. L. Wu, *Context Theory*, China Science and Technology Press, 2010.
- [4] C. Wei, Z. H. Jia, Y. L. Wu et al., "Protective effects of Qiliqiangxin capsule in rabbit heart ventricle reconstitution of chronic heart failure," *Chinese Journal of Difficult and Complicated Cases*, vol. 6, no. 3, pp. 144–147, 2007.
- [5] Z. L. Wu, D. L. Xu, S. Lin et al., "The effects of Qiliqiangxin capsule on cardiac function and the plasma arginine vasopressin of rats with chronic heart failure," *Chinese Journal of Difficult and Complicated Cases*, vol. 10, no. 2, pp. 120–122, 2011.
- [6] J. Zhang and H. X. Wang, "Observed the effect of Astragaloside inhibition of myocardial hypertrophy and improve myocardial energy metabolism," *Chinese Traditional Patent Medicine*, vol. 34, no. 5, pp. 924–928, 2012.
- [7] J. Wang, L. Bai, J. Li et al., "Heart failure rat myocardial mitochondrial proteomics research," *Science in China C*, vol. 39, no. 11, pp. 1019–1027, 2009.
- [8] J. M. Li, Z. F. Yao, Y. Z. Zou et al., "The therapeutic potential of G-CSF in pressure overload induced ventricular reconstruction and heart failure in mice," *Molecular Biology Reports*, vol. 39, no. 1, pp. 5–12, 2012.
- [9] Y. L. Wu, C. H. Gu, G. C. Xu et al., "Randomly double-blind and multicenter clinical study of Qiliqiangxin capsule in the treatment of chronic heart failure," *Journal of Virology*, vol. 6, no. 5, pp. 263–266, 2007.
- [10] C. X. Liu, J. Y. Mao, X. L. Wang et al., "Systematic evaluation of Qiliqiangxin capsule in the treatment of chronic heart failure," *Chinese Medicines*, vol. 32, no. 4, pp. 539–544, 2010.
- [11] J. S. Ingwall, "Energy metabolism in heart failure and remodeling," *Cardiovascular Research*, vol. 81, no. 3, pp. 412–419, 2009.
- [12] M. G. Rosca and B. Tandler, "Hoppele CL. Mitochondria in cardiac hypertrophy and heart failure," *Journal of Molecular and Cellular Cardiology*, vol. 13, no. 12, pp. S0022–S2828, 2012.
- [13] W. C. Stanley, F. A. Recchia, and G. D. Lopaschuk, "Myocardial substrate metabolism in the normal and failing heart," *Physiological Reviews*, vol. 85, no. 3, pp. 1093–1129, 2005.
- [14] P. Jiao, J. Ma, B. Feng et al., "FFA-induced adipocyte inflammation and insulin resistance: involvement of ER stress and IKK $\beta$  pathways," *Obesity*, vol. 19, no. 3, pp. 483–491, 2011.
- [15] A. R. Aroor, C. H. Mandavia, and J. R. Sowers, "Insulin resistance and heart failure: molecular mechanisms," *Heart Failure Clinics*, vol. 8, no. 4, pp. 609–617, 2012.
- [16] M. F. Essop and L. H. Opie, "Metabolic therapy for heart failure," *European Heart Journal*, vol. 25, no. 20, pp. 1765–1768, 2004.
- [17] B. Tan, X. Li, Y. Yin et al., "Regulatory roles for L-arginine in reducing white adipose tissue," *Frontiers in Bioscience*, vol. 17, no. 6, pp. 2237–2246, 2012.
- [18] L. Deldicque, D. Theisen, and M. Francaux, "Regulation of mTOR by amino acids and resistance exercise in skeletal muscle," *European Journal of Applied Physiology*, vol. 94, no. 1–2, pp. 1–10, 2005.
- [19] M. López, C. J. Lelliott, and A. Vidal-Puig, "Hypothalamic fatty acid metabolism: a housekeeping pathway that regulates food intake," *BioEssays*, vol. 29, no. 3, pp. 248–261, 2007.
- [20] J. Li, X. Hu, P. Selvakumar et al., "Role of the nitric oxide pathway in AMPK-mediated glucose uptake and GLUT4 translocation in heart muscle," *American Journal of Physiology*, vol. 287, no. 5, pp. E834–E841, 2004.
- [21] E. H. Jeninga, K. Schoonjans, and J. Auwerx, "Reversible acetylation of PGC-1: connecting energy sensors and effectors to guarantee metabolic flexibility," *Oncogene*, vol. 29, no. 33, pp. 4617–4624, 2010.
- [22] G. Ugucconi and D. A. Hood, "The importance of PGC-1 $\alpha$  in contractile activity-induced mitochondrial adaptations," *American Journal of Physiology*, vol. 300, no. 2, pp. E361–E371, 2011.
- [23] V. A. Lira, D. L. Brown, A. K. Lira et al., "Nitric oxide and AMPK cooperatively regulate PGC-1 $\alpha$  in skeletal muscle cells," *Journal of Physiology*, vol. 588, no. 18, pp. 3551–3566, 2010.

## Research Article

# Tongguan Capsule Protects against Myocardial Ischemia and Reperfusion Injury in Mice

Jianyong Qi,<sup>1</sup> Juan Yu,<sup>2</sup> Lei Wang,<sup>1</sup> Liheng Guo,<sup>1</sup> Shiyu Ma,<sup>1</sup> Donghui Huang,<sup>1</sup> Miao Zhou,<sup>3</sup> Jiashin Wu,<sup>4</sup> and Minzhou Zhang<sup>1</sup>

<sup>1</sup> Intensive Care Laboratory, Guangdong Province Hospital of Chinese Medicine, 2nd Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510120, China

<sup>2</sup> Animal Laboratory, Guangdong Province Hospital of Chinese Medicine, 2nd Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510006, China

<sup>3</sup> Department of Oral and Maxillary Surgery, Stomatology Hospital of Guangzhou Medical University, Guangzhou 510140, China

<sup>4</sup> University of South Florida, Tampa, FL 33612, USA

Correspondence should be addressed to Minzhou Zhang; minzhouzhang@yahoo.com.cn

Received 15 March 2013; Revised 19 July 2013; Accepted 1 August 2013

Academic Editor: Keji Chen

Copyright © 2013 Jianyong Qi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Myocardial ischemia/reperfusion (I/R) can induce lethal ventricular arrhythmia and myocardial infarction. One of the clinical strategies for managing patients with high risk of myocardial I/R is to prevent the occurrence of arrhythmias and limit the size of infarction following a coronary episode. Tongguan Capsule (TGC) is one of the popular herbal remedies in treating coronary artery disease in the clinics of Chinese medicine. However, the potential roles and mechanisms of TGC in reducing I/R injury are still unclear. The present study statistically assessed the effectiveness of TGC in reducing I/R injury by comparing the infarct size (IS), risk region (RR), and arrhythmia (in electrocardiogram) among four groups of surgically created mice models of myocardial I/R: SHAM, I/R, VER (I/R with verapamil 20 mg/kg pretreatment), and TGC (I/R with TGC 5 g/kg/d pretreatment). We found that IS was significantly smaller in the TGC and VER groups than I/R group, and the incidence of arrhythmias was reduced in the TGC group compared with I/R group, although there were no differences in RR among the four groups. We conclude that TGC is effective in reducing I/R injury in mice. These results provided an experimental basis for clinical application of TGC in reducing I/R injury.

## 1. Introduction

Acute myocardial infarction is the most common cause of cardiac death. Early reperfusion after coronary obstruction represents the most effective means of therapy. However, myocardial ischemia reperfusion (I/R) can induce lethal ventricular arrhythmia and myocardial infarction [1–3]. Nearly 50% of myocardial infarction occur following I/R [4]. In the United States alone, approximately 1 million people suffer from myocardial infarction every year. Additionally, 700 patients undergo cardioplegic arrest for various cardiac surgeries [5]. Despite the different etiologies that lead to partial or complete arrest of cardiac circulation, both patient groups share myocardial ischemia and reperfusion injury as a common pathophysiological feature [6]. Myocardial ischemia

and reperfusion injury was first described by Jennings et al. in 1960 [7]. They observed that reperfusion accelerated the development of necrosis in a canine coronary ligation model with histological changes after 30 to 60 minutes of I/R comparable to the degree of necrosis normally seen after 24 hours of permanent coronary occlusion.

A traditional Chinese medicine, Tongguan Capsule (TGC), formulated mainly with the following herbs: *Astragalus mongholicus* and *salvia miltiorrhiza*, etc. [8, 9], has been widely used in China to treat patients with angina pectoris and acute coronary syndrome [10, 11]. A recent clinical trial [12] confirmed its effectiveness to improve symptoms and reduce angina pectoris and complications along with few adverse effects. Clinical trials demonstrated that TGC is effective and safe for treating angina pectoris [13]. TGC was shown

to activate eNOS signaling pathway in a pharmacodynamic study [14].

Although effective for treating angina pectoris, the effects of TGC on myocardial I/R injury remain unclear. Our previous studies found that pretreatment with TGC at high doses for 3 days before pituitrin intraperitoneal injection (ip) reduced myocardial ischemia in rats [14]. The current study evaluated a hypothesis that TGC can protect heart against I/R injury. To evaluate this hypothesis, we examined the myocardial injury after ischemia and reperfusion in mouse hearts pretreated with TGC and compared the effects of TGC with control groups. We observed that TGC could significantly reduce the infarct size (IS) of I/R. Therefore, this study experimentally demonstrated that TGC is an effective Chinese herbal medicine to control and reduce I/R injury.

## 2. Materials and Methods

**2.1. Animals and Reagents.** This study was performed in accordance with the guidelines and with approval from the Institutional Animal Care and Use Committee of Guangdong Province Hospital of Chinese Medicine, Guangzhou University of Traditional Chinese Medicine, and with the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (8th edition, Washington DC, 2011).

Ten to twelve weeks old male wild-type C57BL/6J mice ( $25 \pm 5$  g body weight) were obtained from the Experimental Animal Center of Guangdong Province. Triphenyl-tetrazolium chloride (TTC) and Evans blue were purchased from DingGuo Biotechnology Corp (Beijing, China); 10% Neutral Buffered Formalin were purchased from WEX Corp (Guangzhou, China); Pentobarbital sodium were purchased from Sigma-Aldrich Corp. (Guangzhou, China); TGC was produced by Guangdong Province Hospital of Chinese Medicine (Guangzhou, China), batch number 100519.

**2.2. I/R Model In Vivo.** The murine model of I/R has been previously described in detail [15, 16]. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), intubated, and ventilated with room air at a rate of 110 strokes/min and with a tidal volume of 0.25 mL using a mouse ventilator (Inspira, Harvard Apparatus, Holliston, MS, USA). The chest was opened through a left thoracotomy with the aid of a dissecting microscope. An 8-0 nylon suture was passed under the mid-left anterior descending coronary artery (LAD, 2-3 mm from the tip of the left auricle), and a nontraumatic occluder was applied on the artery. Ischemia was elicited by a 30 min coronary occlusion followed by 24 hours reperfusion (Figure 1). Significant changes, including widening of the QRS complex and elevation of ST segment in electrocardiography, were indicators of successful coronary occlusion. The chest was closed in layers, and animals were weaned from the ventilator when they resumed spontaneous breathing [17, 18].

**2.3. In Vitro Tissue Staining.** At the end of 24-hour reperfusion, the heart was perfused with 1X phosphate buffer

solution (1X PBS, pH 7.4) through an aortic cannula. The ligature around the LAD was retied. Two mL of 1% Evans blue dye was injected into the left coronary artery by reversing perfusion through the aorta, and the dye was circulated and uniformly distributed, except in the portion of the heart previously perfused by the occluded coronary artery (risk region, RR). The heart was quickly excised, and both atria and the right ventricle were removed. The left ventricle was weighed and sliced horizontally to yield six slices. After being weighted individually, the slices were incubated in 1% TTC prepared with 1X PBS for 8–15 minutes at 37°C, fixed in 10% neutral buffered formaldehyde for 24–48 h, and then photographed under a microscope with a digital camera [19].

**2.4. Infarct Size (IS) Measurement.** The areas stained with Evans blue (blue area, Normal Zone, NZ), TTC (red staining, Risk Region, RR), and TTC-negative area (white area, Infarct Size, IS) were measured digitally using Image Pro-plus (Version 6.0). The myocardial infarct size was measured and expressed as a percentage of infarct size over the total RR. We identified infarct, at-risk, and nonischemic areas based on tissue staining and measured infarct sizes by computerized video planimetry [20].

**2.5. Heart Rhythm Analysis.** Continuous electrocardiographic monitoring (RM6240; ChengDu Instruments) was performed during in vivo myocardial I/R with LAD ligation. Heart rate and rhythm were analyzed throughout the experiment. The incidence and type of arrhythmias, including atrial premature beats, heart block, and ventricular tachycardia, were evaluated during I/R based on limb lead recordings.

**2.6. Statistical Analysis.** Data are reported as mean  $\pm$  SEM. Bonferroni's post hoc method was used to assess the significance of differences using GraphPad Prism version 4.0. Incidence of arrhythmia was evaluated by  $\chi^2$  test. A *P* value of  $<0.05$  was considered statistically significant [21, 22].

## 3. Results

**3.1. Protocol.** To evaluate the cardioprotective effects of TGC on I/R injury, we firstly created a murine ischemia model as described earlier. Our previous study showed that the best protective effects to myocardial ischemia occurred when TGC was administrated 3 days before pituitrin intraperitoneal injection (ip) in rats [14]. Based on our previous study, literature, and clinical usage in patients (with dose conversion between humans oral usage and animals), TGC powder (Guangdong Province Hospital, Guangzhou, batch number 100519) at a dose of 5 g/kg body weight mixed with 0.5 mL saline was administered daily via direct gastric gavage, for 3 days prior to surgery. Four groups of mice were studied: SHAM group, I/R group, VER group and TGC group (Figure 1). Mice in I/R were subjected to 30 min of coronary occlusion followed by 24 h of reperfusion. VER group (verapamil group, 20 mg/kg, i.p) served as a positive control of the protective effects of drug ischemia preconditioning. The I/R group and SHAM group received saline (0.5 mL/day) for

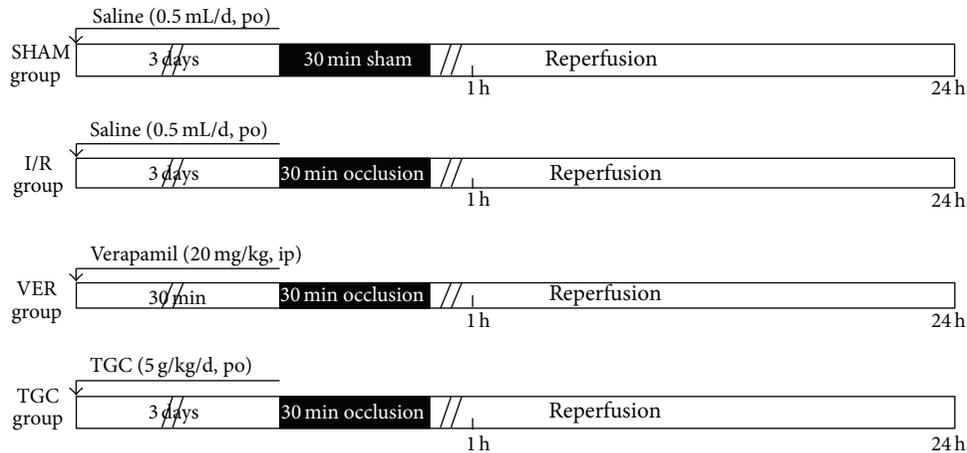


FIGURE 1: Experimental protocols. Four groups (SHAM, I/R, VER, and TGC groups) of mice were studied for infarct size analysis. Days 1–3: mice were subjected to either saline (SHAM and I/R groups) or TGC via direct gastric gavage daily. Day 4: mice in SHAM, I/R, TGC, and VER groups (verapamil 20 mg/kg i.p. pretreatment) were subjected to a 30 min LAD occlusion. Day 5: after 24 h of reperfusion following LAD occlusion, all animals were sacrificed for subsequent measurement of infarct size.

the same duration (3 days). To evaluate whether the high dose was safe for mice, we carried out a preliminary experiment by gavaging 2 mice for 3 days. Both mice survived and were healthy, so we proceeded with the current experiment.

**3.2. Exclusions.** A total of 47 mice were used in the current experiments. Three mice died (Table 1, 6% total mortality). This mortality was quite low for open-chest LAD ligation surgeries in mice. In all, twelve mice (26%) were excluded because of death (3 mice), severe bleeding during surgery (3 mice), technical problems (1 mouse, malfunction of the ventilation system, damage to the coronary vessels), or inadequate postmortem staining (4 mice). Thirty-five mice (74%) successfully completed the entire protocol and were included in the results (Table 1).

**3.3. Risk Region and Infarct Size.** We used infarct size, which is the gold standard for I/R injury evaluation, to investigate the cardioprotective effects of TGC on I/R injury. As shown in Figure 2, we can clearly differentiate infarct size (IS, white area) from risk region (RR, red area) and RR from normal zone (NZ, dark blue). As illustrated in Figure 3(a), there were no significant differences among the four groups in their LV weights and in their weights of the region at risk (Table 2). In the I/R group, the 30 min of coronary occlusion followed by 24 h of reperfusion resulted in an infarct size of  $30.2 \pm 2.7\%$  of the region at risk (Figure 3(c)). In contrast, the TGC group had an infarct size of  $19.3 \pm 2.0\%$  of the region at risk ( $7.8 \pm 1.0\%$  of LV) (Figure 3(b), Table 2). The VER group, which was the positive control group of protective effect of I/R injury, had an infarct size of  $17.1 \pm 2.4\%$  of the RR (Figure 3(c)). Large, confluent areas of infarction spanned most of the thickness of the LV wall. Assessment of cell death at 24 h represented the final extent of myocardial infarction in this model (see the example of TGC group in Figure 2). The room temperature

TABLE 1: Reasons for excluding mice from study.

Group	SHAM	I/R	VER	TGC	Total
Bleeding	1	1	1	0	3
Death	0	1	2	1	4
Technical problems	0	1	0	0	1
Poor postmortem staining	0	2	1	1	4
Mice instrumented	6	13	7	9	35
Mice excluded	1	5	4	2	12
Mortality rate (%)	0	6	18	9	11
Mice included in study	6	13	7	9	35
Mice included in study (%)	86	72	64	82	74

SHAM group, I/R group, I/R + saline; VER group, I/R + verapamil, TGC group: I/R + TGC.

I/R surgery possibly helped in limiting the size of infarct. The small SEM (Figure 3(c)) and similar sizes of infarct in both groups indicated stability of this I/R model.

**3.4. Arrhythmia.** Since I/R can induce both arrhythmia and myocardial infarction, we also analyzed the roles of TGC on occurrences of arrhythmia by examining the electrocardiogram (ECG) in the in vivo murine model of I/R injury. Figures 4(a)–4(c) illustrate three representative ECG complexes on the baseline, ischemia, and reperfusion stages. Ischemia dramatically altered transmural ECG. The most notable features were a gradual increasing inversion of the T-wave and a decrease in excitability as illustrated by the amplitude of R-wave (Figure 3(b)), subsequently followed by ST-segment elevation with continuous ischemia. Excitability recovered completely after 15 min of reperfusion. It is well recognized

TABLE 2: Size of left ventricle, risk region and infarction in study.

	SHAM	I/R	VER	TGC
LV Wt, mg	99.0 ± 4.6	100.3 ± 4.9	90.7 ± 2.6	95.4 ± 2.9
Risk region Wt, mg	41.4 ± 4.6	44.8 ± 3.2	58.5 ± 6.4	59.2 ± 3.5
Infarct Wt, mg	0.9 ± 0.5	13.6 ± 1.1	9.8 ± 1.3	11.5 ± 1.0
Risk region, % of LV	51.9 ± 6.4	51.6 ± 3.6	65.5 ± 5.5	62.1 ± 3.2
Infarct, % of risk region	2.7 ± 1.2	30.2 ± 2.7 <sup>#</sup>	17.1 ± 2.4 <sup>**</sup>	19.3 ± 2.0 <sup>**</sup>
Infarct, % of LV	1.1 ± 0.5	11.5 ± 1.1 <sup>#</sup>	7.8 ± 1.0 <sup>*</sup>	8.0 ± 0.8 <sup>*</sup>

Data are mean ± SEM. LV Wt: left ventricular weight; RR: region at risk. There were no significant differences in the age, sex, and LV weight of the mice among the four groups. Also, there were no significant differences in the region at risk among the four groups. <sup>#</sup> $P < 0.001$  I/R group compared with SHAM, <sup>\*</sup> $P < 0.05$ , <sup>\*</sup> $P < 0.01$  IS (percentage of LV and RR) in TGC and VER groups versus I/R group, respectively.

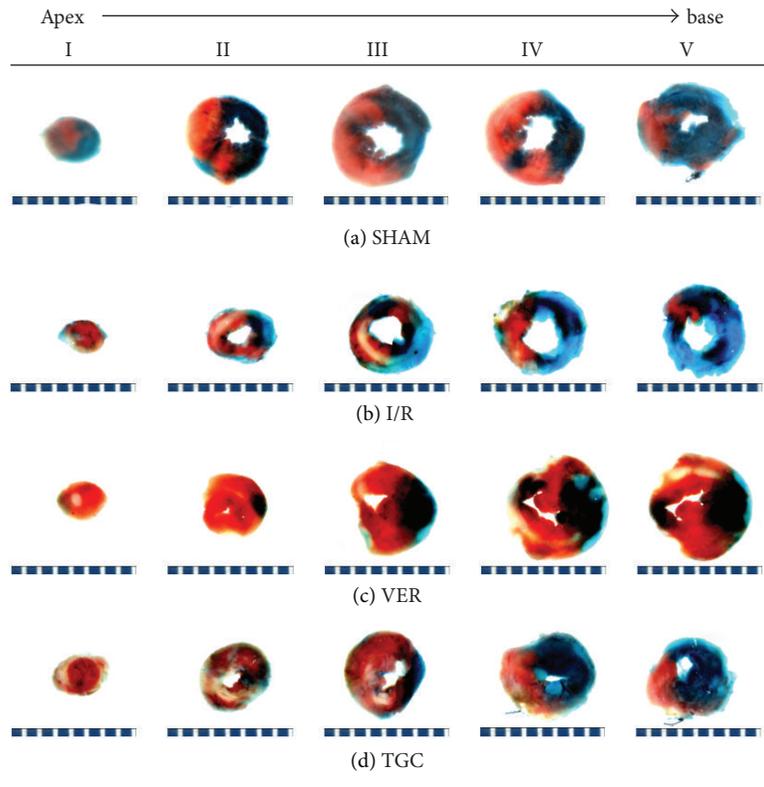


FIGURE 2: Example dye staining of the normal, risk, and infarcted regions. Photomicrographs ( $\times 10$ ) of heart sections obtained from mice subjected to myocardial ischemia/reperfusion (30 minutes/24 hours) treated with SHAM ((a), blank control), I/R ((b), negative control), VER ((c), positive control), and TGC (d). Blue-stained portion: nonischemic, normal region; red-stained portion: ischemic/reperfused, risk but not infarcted region; unstained portion (white area): ischemic/reperfused, infarcted region. Scale at bottom is in mm.

that reperfusion after ischemia is a powerful trigger of cardiac arrhythmias (with peak arrhythmia occurrences during reperfusion after 20–30 min ischemia) [2]. We observed that premature ventricular contractions occurred frequently at the onset of reperfusion in the I/R model (Figure 3(c)) in consistency with a previous report [23]. The results (Figure 4(d)) showed that I/R injury induced arrhythmias frequently in untreated hearts (incidence: 45.5%) but much less frequently in hearts pretreated with TGC (incidence: 27.2%). Thus, our results demonstrated that TGC was effective in reducing I/R-induced arrhythmias.

**3.5. Correlations.** To evaluate the relations between the risk region and infarct size, we performed linear analysis of their correlation. It is widely believed that bigger RR produces bigger IS [24]. However, our results showed that the size of the infarction was not linearly related to the size of the region at risk ( $r = 0.23, 0.33, 0.29,$  and  $0.38$  in the SHAM, I/R, VER, and TGC groups, resp.) (Figure 5). There was only a slight tendency of IS increase with RR. Considering similar RR in all I/R hearts, which was  $\sim 50\%$  and independent from other variables, our data suggest that IS is a property of mice type and surgical interventions.

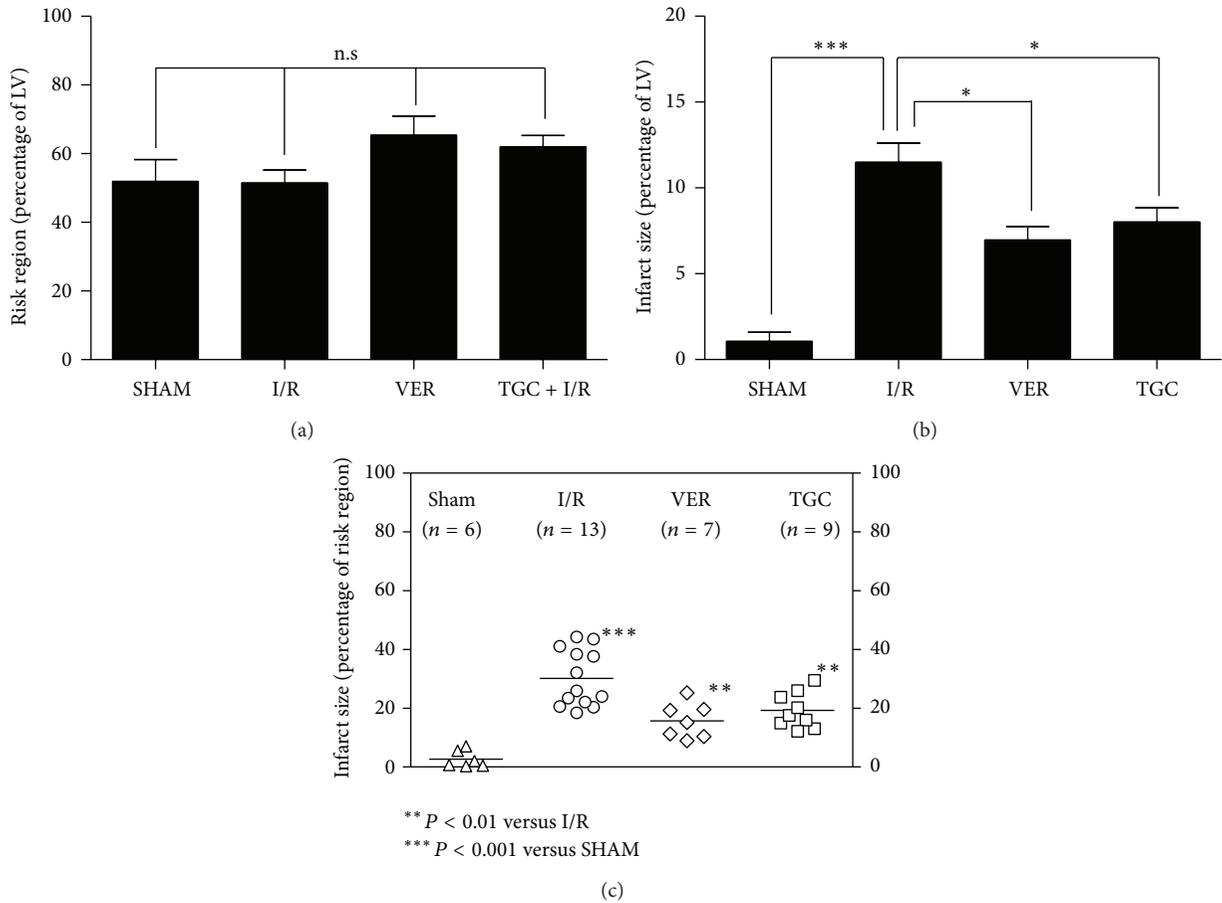


FIGURE 3: The risk regions and infarct sizes. (a) Myocardial risk region (RR) expressed as percent of left ventricle. (b, c) myocardial infarct sizes (IS) expressed as percent of total LV (b) and ischemic reperfusion area ((c), risk region, RR). Data are presented as mean  $\pm$  SEM, \*\*\*  $P < 0.001$  I/R group compared with SHAM, \*  $P < 0.05$ , \*\*  $P < 0.01$  IS (percentage of LV and RR) in TGC and VER groups versus I/R group, respectively.

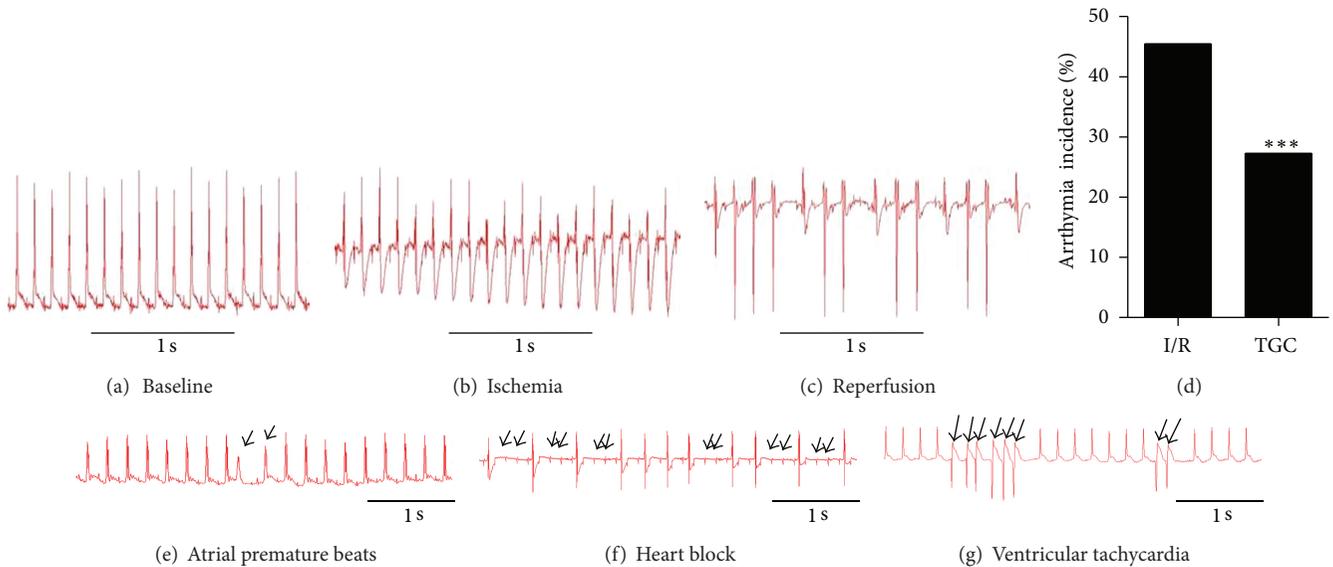


FIGURE 4: Reduced incidence of arrhythmia in TGC group. Representative ECG complexes in baseline (a), ischemia stage (b) and reperfusion stage (c). Ischemia produced dramatic alterations in the transmural ECG with a gradual increasing inversion of the T-wave and a decrease in the amplitude of R-wave (b). Premature ventricular contractions occurred frequently at the onset of reperfusion (c). Incidence of arrhythmia were reduced in TGC group (d), \*\*\*  $P < 0.01$  versus I/R group ( $\chi^2$  test). Various types of arrhythmia, including atrial premature beats (e), heart block (f), and ventricular tachycardia (g) were observed during I/R injury.

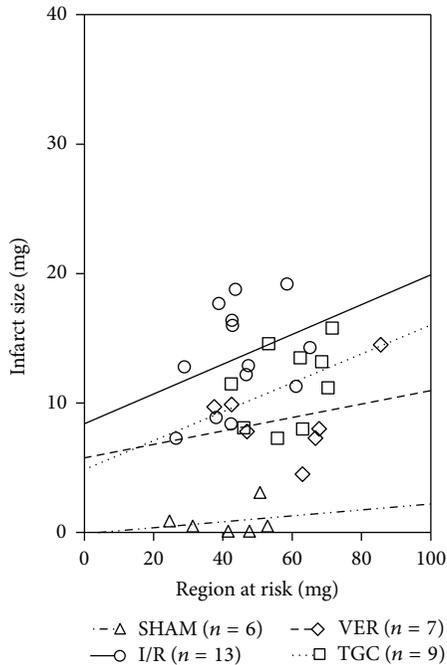


FIGURE 5: Relationships between the size of region at risk and the size of myocardial infarction. Linear regression analysis showed that the infarct size was unrelated to the size of the risk region in all groups. Linear regression equations: SHAM group,  $y = 0.02301x - 0.08$ ,  $r = 0.23$ ,  $P > 0.05$ ; I/R group,  $y = 0.1151x + 8.4$ ,  $r = 0.33$ ,  $P > 0.05$ ; VER group,  $y = 0.05201x + 5.767$ ,  $r = 0.29$ ,  $P > 0.05$ ; TGC group,  $y = 0.1115x + 4.86$ ,  $r = 0.38$ ,  $P > 0.05$ .

#### 4. Discussion

This is the first study to investigate the cardioprotective effects of TGC against I/R injury in mice. We determined the effects of TGC on ischemic and reperfusion injury in an anaesthetized opened-chest murine model of acute myocardial ischemia. The results demonstrate that TGC markedly reduced the infarct size of reperfusion injury and the mortality of acute ischemia/reperfusion in mice.

The effects of against reperfusion injury by several cardioprotective drugs, including reactive oxygen species scavengers, calcium channel blockers, adenosine, and nicorandil, have recently been evaluated in clinical trials and found to be inadequate [25]. These unsatisfactory results could be due to the presence of multiple mechanisms of reperfusion injury and a lack of therapies targeting these mechanisms simultaneously. The complex profile of active ingredients in TGC may possibly overcome the deficiencies of these single-target drugs in protecting against I/R injury. One of our previous studies [14] reported that eNOS activity was increased in myocardium and serum after TGC administration. Astragaloside IV, one of the major components of TGC, was reported to protect against myocardial infarction by increasing the ATP-sensitive potassium current and by improving intracellular calcium handling [26–28]. *Salvia miltiorrhiza* (Danshen), another major component of TGC, has scavenging effects on free radicals and protects myocardial mitochondrial membrane from ischemia reperfusion

injury [29]. Danshensu, which is an active component of *Salvia miltiorrhiza*, is considered effective in against myocardial ischemia/reperfusion injury and inhibits apoptosis of H9c2 cardiomyocytes via Akt and ERK1/2 phosphorylation [30]. Therefore, the protective effects of TGC against I/R injury can possibly be mediated through multiple signaling pathways.

We performed considerable amount of preliminary works before investigating the effects of TGC on MI/R injury. Because temperature can be a major determinant of infarct size [31, 32], this variable was evaluated in a preliminary study comparing mice with and without body temperature controlling (by using heating pads and heat lamps while continuously monitoring rectal temperature) throughout the experiment. We found 2 technical challenges that severely limited the use of body temperature controlling. The first one was time consuming to keep the mice at 36.4–37.6°C. The body temperature of mice usually decreased 2–4°C after anesthetizing and would fluctuate during the surgery. Secondly, using a heating pad and/or lamp to maintain a normal body temperature increased the rate of breathe and lowered the degree of anesthesia during the open-chest surgery and was associated with excessively high rate of postsurgery death. In our preliminary experiments, the death rate reached 80% when a heating device was used to keep normal body temperature during surgery. Because of this, we performed the surgery without a heating device, which reduced the death rate to 6% (see Table 1). Because all the four groups had the same protocol, thus, the effects of unheated body temperature during surgery would not affect the comparison among the groups.

The reliability of the measurements of infarct size was of paramount importance in the outcome of the present investigation. We implemented several modifications of the postmortem perfusion technique during the development of this protocol and achieved major improvements in the quality of tissue staining. The final protocol described in Figure 2 resulted in excellent staining and clear delineation of both region at risk and infarction, as demonstrated in Figure 2. Because of the quality of staining, the measurements of infarct size in this study were accurate and reproducible. The average infarct size in the I/R mice (negative control group) was  $30.2 \pm 2.7\%$  of the region at risk in the current study, similar to the average infarct size of  $34.4 \pm 9.2\%$  in I/R mice after the same I/R protocol (30 min coronary occlusion, 24 h reperfusion) reported by Michael et al. [33] and to the infarct size of  $33.4 \pm 4.5\%$  in six I/R mice subjected to 30 min of occlusion and 2 h of reperfusion reported by Hutter et al. [34], although the infarct size is slightly smaller than observed by Guo et al. in mice [24] [ $50.9 \pm 2.6\%$  of the region at risk]. Differences in body temperature during surgery might contribute to the differences in the infarction sizes.

There were no statistical differences among our groups of mice in their heart-to-body weight ratios, age, body weight, and risk region (Table 2). A major concern in the design of these experiments was to ensure the results would be physiologically relevant. The minuscule size of the murine heart necessitates miniaturization of the procedures used in larger species and therefore poses a unique challenge in

terms of maintaining general experimental conditions within normal values and avoiding artifacts.

## 5. Conclusion

The present study showed that a high loading dose of TGC 3 days before LAD ligation reduced the size of infarction by ischemia reperfusion injury. This result experimentally proved the effectiveness of TGC as a clinical therapy to protect against ischemia reperfusion injury at an early stage. Traditional Chinese medicine has been of great benefit to Asian people for centuries. However, evidence-based experimental verifications of their effectiveness and mechanistic studies have been insufficient. Experimental evidences of the TGC-induced cardioprotection can help to explain the improved outcomes after the immediate administration of TGC to patients with acute coronary syndrome and may lead to the development of better drugs and/or new therapeutic applications of TGC.

## Conflict of Interests

No conflict of interests, financial or otherwise, is declared by the author(s).

## Authors' Contribution

Jianyong Qi and Juan Yu contributed to the work equally.

## Acknowledgments

The authors thank Eric S. Bennett (University of South Florida) for general support. This study was supported by National Scientific Funding of Guangdong S20120410008010 (to Jianyong Qi), Guangdong Province Medical Research Foundation A2013235 (to Jianyong Qi), and National Scientific Funding of China 81173439 (to Minzhou Zhang) and 81202782 (to Lei Wang).

## References

- [1] R. Papp, M. Gönczi, M. Kovács, G. Seprényi, and A. Végh, "Gap junctional uncoupling plays a trigger role in the antiarrhythmic effect of ischaemic preconditioning," *Cardiovascular Research*, vol. 74, no. 3, pp. 396–405, 2007.
- [2] L. Nilsson, J. Hallén, D. Atar, L. Jonasson, and E. Swahn, "Early measurements of plasma matrix metalloproteinase-2 predict infarct size and ventricular dysfunction in ST-elevation myocardial infarction," *Heart*, vol. 98, no. 1, pp. 31–36, 2012.
- [3] N. Ueda, D. P. Zipes, and J. Wu, "Coronary occlusion and reperfusion promote early afterdepolarizations and ventricular tachycardia in a canine tissue model of type 3 long QT syndrome," *American Journal of Physiology*, vol. 290, no. 2, pp. H607–H612, 2006.
- [4] D. M. Yellon and D. J. Hausenloy, "Myocardial reperfusion injury," *New England Journal of Medicine*, vol. 357, no. 11, pp. 1074–1135, 2007.
- [5] A. S. Go, D. Mozaffarian, V. L. Roger et al., "Heart disease and stroke statistics—2013 update: a report from the American Heart Association," *Circulation*, vol. 127, no. 1, pp. e6–e245, 2013.
- [6] A. T. Turer and J. A. Hill, "Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy," *American Journal of Cardiology*, vol. 106, no. 3, pp. 360–368, 2010.
- [7] R. B. Jennings, H. M. Sommers, G. A. Smyth, H. A. Flack, and H. Linn, "Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog," *Archives of Pathology*, vol. 70, no. 7, pp. 68–78, 1960.
- [8] Q. F. Chen, M. Z. Zhang, and C. Yang, "Experimental study of the myocardial protection on septic rats by tongguan capsule," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 32, no. 9, pp. 1253–1258, 2012.
- [9] B. J. Chen, J. X. Feng, X. X. Su et al., "Effects of tongguan capsule on post-myocardial infarction ventricular remodeling and cardiac function in rats," *Chinese Journal of Integrative Medicine*, vol. 16, no. 2, pp. 157–161, 2010.
- [10] Z. Yong, M. Z. Zhang, and L. Wang, "The influence of Tongguan Capsule effect on cardiac function and serum SDF-1 of post-intervention patients of coronary artery diseases," *Journal of New Chinese Medicine*, vol. 43, no. 8, pp. 5–8, 2011.
- [11] J. Li, M. Z. Zhang, B. J. Chen, and E. A. et al., "Effect of Tongguan capsule on post-intervention patients of coronary heart disease with qi-deficiency and blood stasis syndrome," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 1, pp. 32–35, 2008.
- [12] Z. Q. Qiao, M. Z. Zhang, H. Liu, K. L. Chen, and S. Li, "A randomized double-blinded placebo-controlled clinical study of Tongguan capsule improving cardiac functional parameter of patients after PCI," *Chinese Archives of Traditional Chinese Medicine*, vol. 21, no. 7, pp. 882–883, 2003.
- [13] J. Y. Qi, M. Z. Zhang, J. Li et al., "The influence of Tongguan Capsule on coronary re-stenosis and hemodynamic," *Journal of Traditional Chinese Medicine*, vol. 21, no. 6, pp. 882–883, 2003.
- [14] J. L. Chen, W. K. Wu, Y. L. Han et al., "The dose-response relationship of Tongguan Capsule reducing myocardial ischemia and the effect on nitric oxide," *Journal of Guangzhou University of Traditional Chinese Medicine*, vol. 34, no. 4, pp. 301–306, 2007.
- [15] C. Depre, L. Wang, X. Sui et al., "H11 kinase prevents myocardial infarction by preemptive preconditioning of the heart," *Circulation Research*, vol. 98, no. 2, pp. 280–288, 2006.
- [16] Y. Guo, D. N. Tukaye, W. J. Wu et al., "The COX-2/PGI2 receptor axis plays an obligatory role in mediating the cardioprotection conferred by the late phase of ischemic preconditioning," *PLoS One*, vol. 7, no. 7, Article ID e41178, 2012.
- [17] Y. Guo, Q. Li, W. J. Wu et al., "Endothelial nitric oxide synthase is not necessary for the early phase of ischemic preconditioning in the mouse," *Journal of Molecular and Cellular Cardiology*, vol. 44, no. 3, pp. 496–501, 2008.
- [18] Q. Li, Y. Guo, Q. Ou et al., "Gene transfer as a strategy to achieve permanent cardioprotection II: rAAV-mediated gene therapy with heme oxygenase-1 limits infarct size 1 year later without adverse functional consequences," *Basic Research in Cardiology*, vol. 106, no. 6, pp. 1367–1377, 2011.
- [19] P. Ping, C. Song, J. Zhang et al., "Formation of protein kinase C $\epsilon$ -Lck signaling modules confers cardioprotection," *Journal of Clinical Investigation*, vol. 109, no. 4, pp. 499–507, 2002.
- [20] Y. T. Xuan, Y. Guo, Y. Zhu, O. L. Wang, G. Rokosh, and R. Bolli, "Endothelial nitric oxide synthase plays an obligatory role in the late phase of ischemic preconditioning by activating the protein

- kinase Cε-p44/42 mitogen-activated protein kinase-pSer-signal transducers and activators of transcription1/3 pathway," *Circulation*, vol. 116, no. 5, pp. 535–544, 2007.
- [21] J. Y. Qi, M. Xu, Z. Z. Lu, and Y. Y. Zhang, "14-3-3 inhibits insulin-like growth factor-I-induced proliferation of cardiac fibroblasts via a phosphatidylinositol 3-kinase-dependent pathway," *Clinical and Experimental Pharmacology and Physiology*, vol. 37, no. 3, pp. 296–302, 2010.
- [22] J. Qi, M. Xu, Z. Lu, and Y. Zhang, "Differential expression of 14-3-3ε during physiological, pathological cardiac hypertrophy and chronic heart failure in mice," *Gene Therapy and Molecular Biology*, vol. 13, no. 1, pp. 71–81, 2009.
- [23] T. B. Garcia and G. T. Miller, *Arrhythmia Recognition: The Art of Interpretation*, Jones and Bartlett Publishers, Sudbury, Mass, USA, 2004.
- [24] Y. Guo, W. J. Wu, Y. Qiu, X. L. Tang, Z. Yang, and R. Bolli, "Demonstration of an early and a late phase of ischemic preconditioning in mice," *American Journal of Physiology*, vol. 275, no. 4, pp. H1375–H1387, 1998.
- [25] J. P. Monassier, "Reperfusion injury in acute myocardial infarction: from bench to cath lab. Part II: clinical issues and therapeutic options," *Archives of Cardiovascular Diseases*, vol. 101, no. 9, pp. 565–575, 2008.
- [26] X. H. Han, P. Liu, Y. Y. Zhang, N. Zhang, F. R. Chen, and J. F. Cai, "Astragaloside IV regulates expression of ATP-sensitive potassium channel subunits after ischemia-reperfusion in rat ventricular cardiomyocytes," *Journal of Traditional Chinese Medicine*, vol. 31, no. 4, pp. 321–326, 2011.
- [27] Z. P. Li and Q. Cao, "Effects of astragaloside IV on myocardial calcium transport and cardiac function in ischemic rats," *Acta Pharmacologica Sinica*, vol. 23, no. 10, pp. 898–904, 2002.
- [28] X. L. Xu, X. J. Chen, H. Ji et al., "Astragaloside IV improved intracellular calcium handling in hypoxia-reoxygenated cardiomyocytes via the sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase," *Pharmacology*, vol. 81, no. 4, pp. 325–332, 2008.
- [29] B. L. Zhao, W. Jiang, Y. Zhao, J. W. Hou, and W. J. Xin, "Scavenging effects of *Salvia miltiorrhiza* on free radicals and its protection for myocardial mitochondrial membranes from ischemia-reperfusion injury," *Biochemistry and Molecular Biology International*, vol. 38, no. 6, pp. 1171–1182, 1996.
- [30] Y. Yin, Y. Guan, J. Duan et al., "Cardioprotective effect of Danshensu against myocardial ischemia/reperfusion injury and inhibits apoptosis of H9c2 cardiomyocytes via Akt and ERK1/2 phosphorylation," *European Journal of Pharmacology*, vol. 699, no. 3, pp. 219–226, 2013.
- [31] S. L. Hale and R. A. Kloner, "Myocardial temperature in acute myocardial infarction: protection with mild regional hypothermia," *American Journal of Physiology*, vol. 273, no. 1, pp. H220–H227, 1997.
- [32] L. M. Schwartz, S. G. Verbinski, R. S. Vander Heide, and K. A. Reimer, "Epicardial temperature is a major predictor of myocardial infarct size in dogs," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 6, pp. 1577–1583, 1997.
- [33] L. H. Michael, M. L. Entman, C. J. Hartley et al., "Myocardial ischemia and reperfusion: a murine model," *American Journal of Physiology*, vol. 269, no. 6, pp. H2147–H2154, 1995.
- [34] J. J. Hutter, R. Mestril, E. K. W. Tam, R. E. Sievers, W. H. Dillmann, and C. L. Wolfe, "Overexpression of heat shock protein 72 in transgenic mice decreases infarct size in vivo," *Circulation*, vol. 94, no. 6, pp. 1408–1411, 1996.

## Research Article

# Inhibitory Effects of Glycyrrhetic Acid on the Delayed Rectifier Potassium Current in Guinea Pig Ventricular Myocytes and HERG Channel

Delin Wu,<sup>1</sup> Linqing Jiang,<sup>1</sup> Hongjin Wu,<sup>1</sup> Shengqi Wang,<sup>2</sup> Sidao Zheng,<sup>1</sup> Jiyuan Yang,<sup>1</sup> Yuna Liu,<sup>1</sup> Jianxun Ren,<sup>1</sup> and Xianbing Chen<sup>1</sup>

<sup>1</sup> Beijing Hospital of Integrated Traditional Chinese and Western Medicine, Beijing 100039, China

<sup>2</sup> Beijing Institute of Radiation Medicine, Beijing 100850, China

Correspondence should be addressed to Hongjin Wu; whjyuanzhang@yahoo.com.cn

Received 19 March 2013; Revised 7 July 2013; Accepted 11 July 2013

Academic Editor: Hao Xu

Copyright © 2013 Delin Wu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Background.** Licorice has long been used to treat many ailments including cardiovascular disorders in China. Recent studies have shown that the cardiac actions of licorice can be attributed to its active component, glycyrrhetic acid (GA). However, the mechanism of action remains poorly understood. **Aim.** The effects of GA on the delayed rectifier potassium current ( $I_K$ ), the rapidly activating ( $I_{Kr}$ ) and slowly activating ( $I_{Ks}$ ) components of  $I_K$ , and the HERG K<sup>+</sup> channel expressed in HEK-293 cells were investigated. **Materials and Methods.** Single ventricular myocytes were isolated from guinea pig myocardium using enzymolysis. The wild type HERG gene was stably expressed in HEK293 cells. Whole-cell patch clamping was used to record  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ) and the HERG K<sup>+</sup> current. **Results.** GA (1, 5, and 10  $\mu$ M) inhibited  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ) and the HERG K<sup>+</sup> current in a concentration-dependent manner. **Conclusion.** GA significantly inhibited the potassium currents in a dose- and voltage-dependent manner, suggesting that it exerts its antiarrhythmic action through the prolongation of APD and ERP owing to the inhibition of  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ) and HERG K<sup>+</sup> channel.

## 1. Introduction

Cardiac arrhythmias are associated with significant morbidity and mortality in developed and developing countries. Although nonpharmacologic approaches, ablative therapy, and implantable defibrillators, for example, are being used more and more commonly as complementary and alternative interventions for the treatment of cardiac arrhythmias, drug therapy was traditionally the mainstay of arrhythmia treatment. Improved understanding of the cardiomyocyte ion channels holds the promise of identifying novel targets for the treatment of cardiac arrhythmias. Furthermore, the limited efficacy and the potency of provoking life-threatening arrhythmias of present drugs have generated interest in finding new antiarrhythmic agents [1].

Potassium channels constitute the most abundant family of ion channels involved in cardiac physiological or pathophysiological processes, disruptions of which could give

rise to prolongation of the action potential and potentiate occurrence of lethal arrhythmias subsequently [2, 3]. In the hearts of many mammalian species including humans, delayed rectifier K<sup>+</sup> current ( $I_K$ ), the major outward potassium current responsible for ventricular repolarization, can be divided into at least two different components, rapidly activating ( $I_{Kr}$ ) and slowly activating ( $I_{Ks}$ ). The human ether-à-go-go-related gene (HERG) encodes the  $\alpha$ -subunit for the rapid delayed rectifier potassium channel ( $I_{Kr}$ ) in cardiac myocytes.  $I_{Kr}$  and  $I_{Ks}$  are pivotal in cardiac repolarization, especially in the later phases of the action potential [4, 5]. Although the structural and functional aspects of potassium channels have been widely examined, only a few K<sup>+</sup> channel modulators are being used clinically at present for the unwanted adverse effects [2, 6]. As an alternative to find new antiarrhythmic agents, there has been increased interest in investing natural compounds that are effective against cardiac arrhythmia.

*Glycyrrhiza radix* is a commonly prescribed herb to prevent palpitations in Chinese traditional medicine for about 2000 years, derived from the dried roots and rhizomes of *Glycyrrhiza uralensis*, *G. glabra*, and *G. inflata*. Glycyrrhizin, the major constituent of *G. glabra*, is a glycoside, which occurs as an admixture of sodium, potassium and calcium salts [7]. Orally administered, glycyrrhizin is poorly absorbed by the intestinal tract and is hydrolyzed by  $\beta$ -D-glucuronidase-containing intestinal bacteria to yield two molecules of D-glucuronic acid and the aglycone glycyrrhetic acid (GA), a pentacyclic triterpene [8]. If intravenously administered, GA is metabolized in the liver by lysosomal  $\beta$ -D-glucuronidase to the 3-monoglucuronide of glycyrrhetic acid. This metabolite is excreted via the bile into the intestine, where it is transformed by bacteria into GA, which can be reabsorbed, causing a pronounced delay in terminal plasma clearance [9].

The use of *Glycyrrhiza uralensis* as a pharmacological remedy dates back far into the past [10]. Various pharmacological properties of licorice have been proved including cardioprotective [11, 12], antiulcer, anti-inflammatory, spasmolytic, antioxidative, contravariant, antiviral, anticancer, and hepatoprotective effects, as well as eliminating phlegm and reinforcing memory [7, 13–15]. Many components have been isolated from licorice including triterpene saponins, flavonoids, isoflavonoids, and chalcones. Triterpene saponins are the main components of *Glycyrrhiza radix* and its pharmacological activities are comparatively well understood and clear.

Recently, wide-ranging studies have provided evidence that GA is cardioprotective; this action involves different pathways. In rat cardiac mitochondria [16], GA was shown to increase permeability and concomitant release of proapoptotic factors. In particular, GA, acting as a gap junction inhibitor, influences connexin 43, the major gap junction-forming protein in adult cardiac ventricles and a regulator of mitochondrial function [17]. Studies have demonstrated that GA and its derivatives affect the inotropic, lusitropic, chronotropic, and coronary performances of the mammalian heart and the signal transduction pathways that could be involved [9]. Furthermore, research has demonstrated that GA reduces cardiac sodium currents [18], particularly the late  $I_{Na}$ . These findings might help to elucidate the traditional use of licorice in therapy for cardiovascular disorders [19]. It has also been reported that 18 $\beta$ -GA has significant potential for development as a novel antiarrhythmic agent and for treating myocardial ischemia by preferentially blocking the  $I_{Na,L}$  [20]. However, the effects of GA on the potassium channel, especially the rapid and slow components of the delayed rectifier potassium current, are not clearly defined.

In this study, we investigated the effects of GA on the  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ) in guinea pig ventricular myocytes. We also extended our study to investigate the effects of GA on the human ether-à-go-go-related gene (HERG)  $K^+$  channel current expressed in HEK-293 cells. Our study could provide theoretical support for developing the significant potential of GA as a novel antiarrhythmic agent.

## 2. Materials and Methods

**2.1. Materials.** Glycyrrhetic acid ((GA) molecular weight 470.6, purity > 99%) was obtained from the Chinese Biological Product Assay Institute (Beijing, China). The white powder was dissolved in dimethylsulfoxide (DMSO) as stock. The percentage of DMSO in the final solution was less than 0.1%. Collagenase II was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Protease XIV,  $Na_2ATP$ ,  $MgATP$ ,  $CdCl_2$ , L-Glutamic acid, Taurine, and EGTA were purchased from Sigma Co. Other reagents were of analytical reagent grade. Male guinea pigs weighing 300–350 g were provided by the Vital river Company, Certificate no.: SCXK (Jing) 2006-0009.

**2.2. Solutions.**  $Ca^{2+}$ -free Tyrode's solution (mM) contained NaCl 137, KCl 5.4,  $NaH_2PO_4$  1.2,  $MgCl_2$  1.2, HEPES 10, glucose 10, and Taurine 10 (adjusted to pH 7.36–7.38 with NaOH).

Kraft-Brühe (KB) solution (mM) contained L-glutamic acid 50, KCl 40,  $KH_2PO_4$  20, Taurine 20,  $MgCl_2$  3.0, KOH 70, EGTA 0.5, HEPES 10, and glucose 10 (adjusted to pH 7.25 with KOH).

For  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ) recordings from ventricular myocytes, the control bath solution contained (mM) NaCl 135, KCl 5.4,  $CaCl_2$  1.0,  $CdCl_2$  0.2,  $NaH_2PO_4$  0.33,  $MgCl_2$  1.0, HEPES 5, and glucose 5 (adjusted to pH 7.36–7.38 with NaOH). Drugs were added to the bath solution. Pipettes had tip resistances of 1.5–2 M $\Omega$  when filled with a solution containing (mM) KCl 140,  $MgCl_2$  1, HEPES 5, EGTA 10, and  $Na_2ATP$  2 (adjusted to pH 7.25 with KOH).

To record HERG activity in the HEK 293 cells, the internal solution contained (mM) KCl 130,  $MgCl_2$  1, HEPES 10, EGTA 10, and  $MgATP$  5 (adjusted to pH 7.25 with KOH). The external solution contained (mM) NaCl 136, KCl 5.4,  $CaCl_2$  1,  $MgCl_2$  1, HEPES 10, and glucose 10 (adjusted to pH 7.36–7.38 with NaOH).

**2.3. Isolation of Single Ventricular Myocytes.** Adult guinea pigs were fully anesthetized by intraperitoneal injection of urethane (40 mg/kg). The hearts were quickly removed and mounted on a Langendorff column, and cardiac myocytes were isolated as follows. The heart was dissected and rinsed in cold oxygenated  $Ca^{2+}$ -free Tyrode's solution and then perfused in the Langendorff apparatus at 37°C. Perfusion with  $Ca^{2+}$ -free Tyrode's solution for 5 min was followed first by 25  $\pm$  5 min perfusion with low  $Ca^{2+}$  (0.1 mmol/L) Tyrode's solution containing 0.04–0.06 g/L collagenase and 0.5–0.8 g/L BSA and then 5 min perfusion with collagenase-free Tyrode's solution containing 0.5–0.8 g/L BSA. The heart was then minced and the cells were filtered through 200  $\mu$ m nylon mesh, resuspended in Kraft-Brühe (KB) solution, and stored at room temperature (22–25°C) until use.

**2.4. Cell Culture.** The HEK293 cells that stably expressed the wild type HERG gene were kindly provided by Professor Xiaoyan Liu (Academy of Military Medical Sciences, Beijing, China). Dulbecco's Modified Eagle's Medium

(DMEM, Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1 mg/mL geneticin (G-418, Gibco), the cultures were passed every 3–5 days by use of a brief trypsin treatment. The cells were maintained at 37°C in 5% CO<sub>2</sub> and plated on a glass culture dish 2–3 days before electrophysiological experiments.

**2.5. Electrophysiological Recordings.** All currents were recorded using the conventional whole-cell patch-clamp technique. Borosilicate glass electrodes had tip resistances of 1–3 MΩ when filled with the pipette solution. All experiments were performed at room temperature (22–23°C) using an Axopatch 200B amplifier (Axon Instrument, USA). Adequate series resistances (less than five times the pipette resistances) were usually attained within 10 min after the gigaohm seal was formed. Measurements were taken using an Axopatch 200B amplifier (Axon Instruments). The current signals were filtered via a 4 kHz, 4-pole low-pass filter and digitized with an AD-DA converter (Digidata 1440, Axon Instruments) for subsequent analysis using pCLAMP 10.0 software.

**2.6. Statistical Analysis.** The data were analyzed with the use of Data Processing System (Version 7.05). All data are expressed as means ± SEM. Paired Student's *t*-tests were used for statistical comparisons when appropriate, and differences were considered significant at  $P < 0.05$ .

### 3. Results

**3.1. Effect of GA on the  $I_K$  of Guinea Pig Ventricular Myocytes.** We first tested the effect of GA on  $I_K$  using guinea pig ventricular myocytes.  $I_K$  were recorded by applying voltage pulses ranging from –10 to +80 mV for 5 s from the holding potential of –40 mV, and the repolarization potential was maintained at a constant –30 mV for the  $I_{K,tail}$  analysis. Figure 1(a) shows an example of a voltage-clamp recording from a single ventricular myocyte, with representative current traces given under control conditions and after exposure to 10 μM GA. Under control conditions, the depolarizing steps activated time-dependent outward currents. The amplitude of the outward currents measured at the end of the pulse ( $I_K$ ) increased with increasingly positive voltage steps. The current-voltage relationships for  $I_{K,step}$  and  $I_{K,tail}$  obtained at various concentrations of GA are shown in Figures 1(b) and 1(c). As the concentration of GA increased, the amplitude of  $I_{K,step}$  and  $I_{K,tail}$  decreased dose dependently (Figures 1(d) and 1(e)). The  $I_{K,step}$  measured at +80 mV was  $6.97 \pm 0.34$  pA/pF under control conditions and decreased to  $4.93 \pm 0.51$  pA/pF,  $3.35 \pm 0.55$  pA/pF, and  $2.12 \pm 0.29$  pA/pF after application of 1, 5, and 10 μM GA, respectively ( $n = 5$ ,  $P < 0.05$ ). Meanwhile, the  $I_{K,tail}$  evoked after repolarization to –30 mV was  $1.57 \pm 0.11$  pA/pF under control conditions and decreased to  $1.24 \pm 0.12$  pA/pF,  $0.67 \pm 0.15$  pA/pF and  $0.48 \pm 0.06$  pA/pF in the presence of 1, 5 and 10 μM GA, respectively ( $n = 5$ ,  $P < 0.05$ ). These results show that GA dose-dependently blocked  $I_{K,step}$  and  $I_{K,tail}$ .

**3.2. Effect of GA on  $I_{Kr}$  and  $I_{Ks}$  in Guinea Pig Ventricular Myocytes.** To investigate the effects of GA on the rapid and slow components of the delayed rectifier currents in guinea-pig ventricular myocytes, we used a voltage clamp protocol designed to separate the currents electrophysiologically. The holding potential was maintained at –40 mV. Our results revealed that depolarization to +60 mV activated both  $I_{Kr}$  and  $I_{Ks}$ . Repolarization to 0 mV revealed  $I_{Ks}$  as a deactivating  $I_{Ks,tail}$ , and subsequent repolarization to –40 mV resulted in deactivation of  $I_{Kr}$ . We confirmed that 2 μM E-4031, a selective blocker of  $I_{Kr}$ , blocked the rapid component of the delayed rectifier K<sup>+</sup> current but had no effect on  $I_{Ks}$  (Figure 2). As shown in Figures 2(b) and 2(c), 1, 5, and 10 μM GA dose dependently inhibited  $I_{Ks,tail}$  and  $I_{Kr,tail}$ . In the cells examined,  $I_{Ks,tail}$  measured at 0 mV was  $0.59 \pm 0.03$  pA/pF under control conditions and decreased to  $0.53 \pm 0.06$  pA/pF,  $0.33 \pm 0.06$  pA/pF, and  $0.16 \pm 0.03$  pA/pF after application of 1, 5, and 10 μM GA, respectively ( $n = 5$ ,  $P < 0.05$ ). The  $I_{Kr,tail}$  evoked after repolarization to –40 mV was  $1.57 \pm 0.11$  pA/pF under control conditions and decreased to  $0.57 \pm 0.06$  pA/pF,  $0.29 \pm 0.02$  pA/pF and  $0.21 \pm 0.05$  pA/pF in the presence of 1, 5 and 10 μM GA, respectively ( $n = 5$ ,  $P < 0.05$ ). This result shows that GA blocks the rapid and slow components of delayed rectifier K<sup>+</sup> current.

**3.3. Inhibition of HERG K<sup>+</sup> Currents and Concentration-Dependent Block by GA.** The HERG current was elicited from the holding potential of –80 mV by test pulses ranging from –60 to +30 mV in 10 mV steps. Each test pulse was followed by a repolarization step to –50 mV, which evoked large, slowly decaying outward tail currents. Currents were recorded first under control conditions, and then GA (5 μM) was washed into the bath for 10 min, with the cell kept at the holding potential, before current recording commenced in the presence of the drug (Figure 3(a)). In the absence of drug, the *I-V* relationship exhibited the characteristic bell-shaped curve increasing from –40 to 0 mV, and owing to the fast C-type inactivation of HERG channels, it decreased with further depolarization. GA reduced both the HERG current ( $I_{step}$ ) during the test potentials and the tail current ( $I_{tail}$ ) after a test pulse to 0 mV (Figures 3(c) and 3(d)). GA inhibited the tail current at all potentials, although voltage dependence was evident with a significantly weaker block at more negative potentials (Figure 3(d)).

GA blocked the HERG currents and the tail current in a concentration-dependent manner. The stimulus protocol used is illustrated in Figure 3(a). At GA concentrations of 1, 5, and 10 μM, the HERG current amplitude at 0 mV and the peak tail current amplitude were measured, normalized for each cell to the control value and then averaged ( $n = 5$ ). The fractional block of  $I_{step}$  was  $20.46 \pm 2.7\%$  in the 1 mM group ( $P < 0.05$ ),  $29.93 \pm 3.1\%$  in the 5 mM group ( $P < 0.05$ ), and  $56.38 \pm 4.2\%$  in the 10 mM group ( $P < 0.05$ ); the corresponding fractional blocks of  $I_{tail}$  were  $19.26 \pm 2.5\%$  ( $P < 0.05$ ),  $36.57 \pm 3.0\%$  ( $P < 0.05$ ), and  $55.29 \pm 3.6\%$  ( $P < 0.05$ ), respectively (Figure 4).

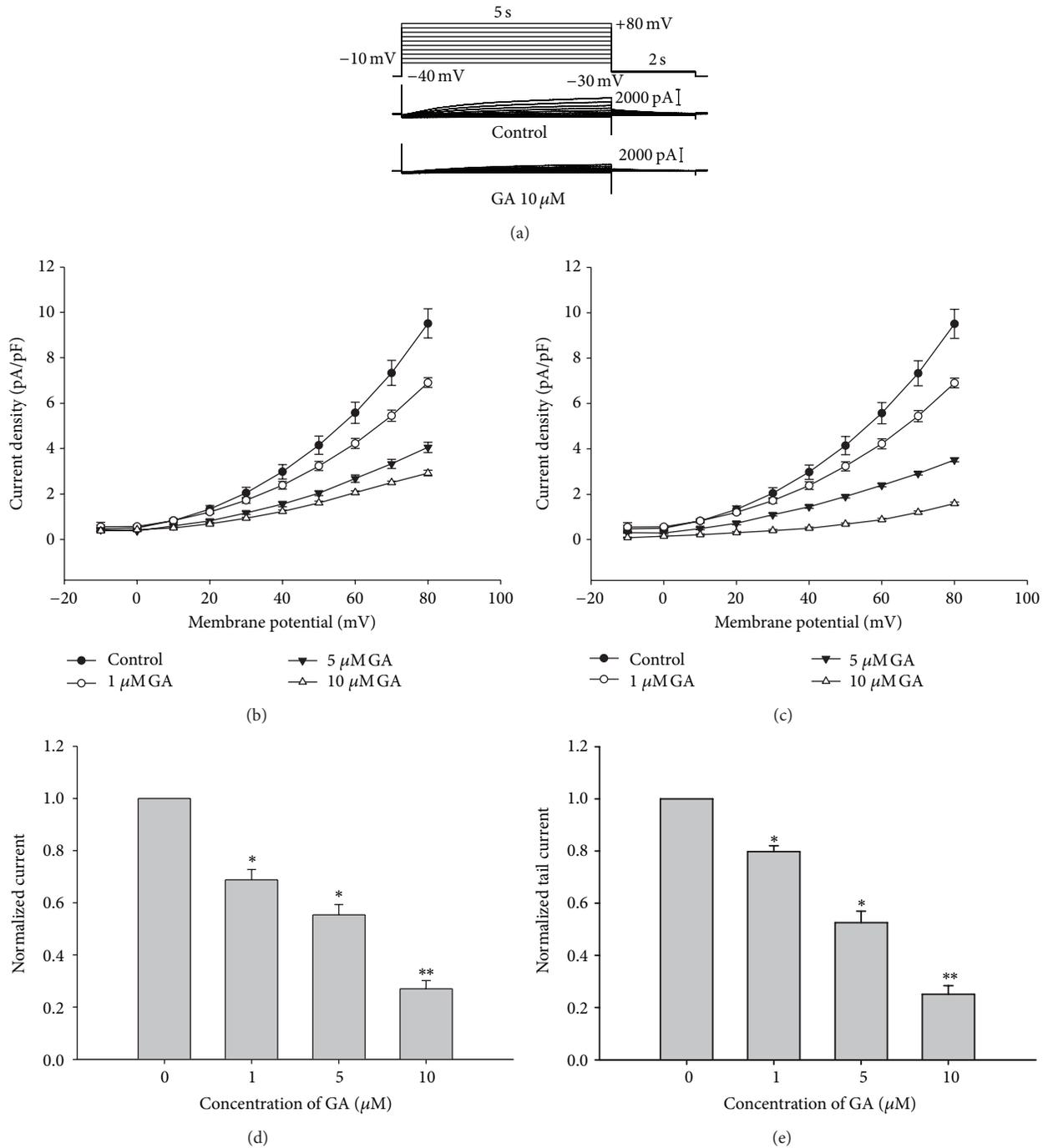


FIGURE 1: Effect of GA on the  $I_K$  of guinea pig ventricular myocytes. (a) Current traces elicited by 5 s test pulses from -10 to +80 mV with 10 mV increments, and tail currents recorded at -30 mV for 2 s. Representative traces of  $I_K$  recorded from the same cell under control condition and after applying GA. Current-voltage relationship of  $I_{K,step}$  (b) and  $I_{K,tail}$  (c) observed in five cells tested in the absence and presence of GA (1, 5, and 10  $\mu\text{M}$ ). Summary of the effects of GA (1, 5, and 10  $\mu\text{M}$ ) on  $I_{K,step}$  (d) and  $I_{K,tail}$  (e), normalized relative to the control current ( $n = 5$ , \* $P < 0.05$ , \*\* $P < 0.01$ ).

**3.4. Effects of GA on HERG Channel Kinetics.** Drugs that block ion channels often alter the voltage dependence or kinetics of channel gating. Therefore, we examined the effects of GA on the voltage dependence of activation and rectification and on the kinetics of inactivation and deactivation. The

activation curves were constructed by normalizing the tail currents recorded with the protocol used in Figure 3(a). The activation curve showed that the threshold voltage for HERG current activation was close to -50 mV and that it was fully activated with voltage steps to -10 mV. The rate of activation

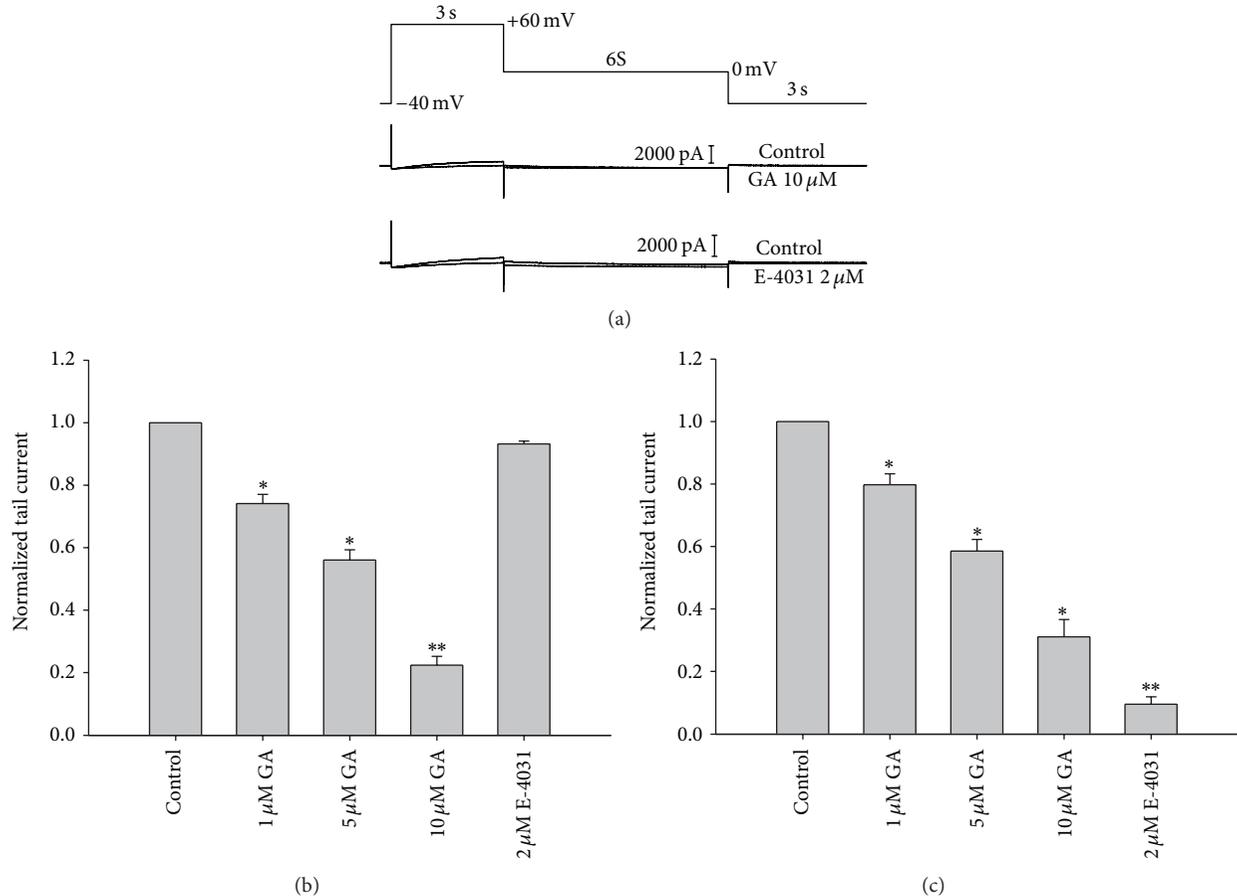


FIGURE 2: Effect of GA on the slow and rapid components of the delayed rectifier  $K^+$  current in guinea pig ventricular myocytes. (a) Representative traces of the rapid ( $I_{Kr}$ ) and slow ( $I_{Ks}$ ) components of the delayed rectifier  $K^+$  channel tail currents before and after treatment with  $10 \mu\text{M}$  GA and  $2 \mu\text{M}$  E-4031. The effects of  $1$ ,  $5$ , and  $10 \mu\text{M}$  GA and  $2 \mu\text{M}$  E-4031 on  $I_{Ks,tail}$  (b) and  $I_{Kr,tail}$  (c), normalized relative to the control current ( $n = 5$ ,  $P < 0.05$ ). The tail current amplitudes were differences between peak outward current and the steady state current at the end of the repolarizing voltage pulses. (\* $P < 0.05$ , \*\* $P < 0.01$ ).

was similar before and after exposure to  $5 \mu\text{M}$  GA.  $V_{1/2}$  values were  $-19.83 \pm 2.36$  mV in the control and  $-22.56 \pm 1.84$  mV in GA ( $P > 0.05$ ,  $n = 5$ ). Thus,  $5 \mu\text{M}$  GA had little effect on the voltage dependence of activation (Figure 3(b)).

To measure inactivation, a special protocol was used that inactivated the channel at a holding potential of  $40$  mV, recovered the channel from inactivation at various potentials from  $-120$  to  $20$  mV in  $10$  mV steps, and measured the resulting peak outward current at constant  $20$  mV as a measure of steady-state inactivation (Figure 5(a)). Mean data from five cells were fitted to a Boltzmann function, yielding inactivation  $V_{1/2}$  values of  $-51.27 \pm 3.21$  mV in the control and  $-62.68 \pm 2.85$  mV in  $5 \mu\text{M}$  GA ( $P < 0.05$ ,  $n = 5$ ) (Figure 5(c)). The time course of the development of inactivation was also assessed. Monoexponential curve fitting of the inactivation time-course yielded time constant values (Figure 5(b);  $n = 5$ ). From  $-120$  to  $-20$  mV, the time constants of inactivation were significantly lower in the presence of  $5 \mu\text{M}$  GA ( $P < 0.05$ ,  $n = 5$ ) than in the absence of the drug.

The effect of GA on the onset of inactivation of the HERG current was investigated using a three-pulse protocol. The channels were first inactivated by clamping the membrane

at  $40$  mV followed by a prepulse to  $-100$  mV. This prepulse was sufficiently long to allow rapid recovery of channels from inactivation but short enough to prevent significant channel deactivation. Following recovery from the prepulse, a series of test pulses were delivered to potentials ranging from  $-120$  to  $20$  mV, resulting in outward inactivating currents (Figure 6(a)). The time constants for the onset of inactivation were obtained by fitting exponential functions to the decaying current traces during the third pulse of the protocol and were significantly decreased following perfusion with  $5 \mu\text{M}$  GA ( $P < 0.05$ ,  $n = 5$ ) (Figure 6(c)).

To determine recovery from inactivation, the fully activated  $I$ - $V$  protocol shown in Figure 6(b) was used: a depolarizing pulse to  $40$  mV to inactivate the HERG channels, followed by different repolarizing pulses to test potentials between  $-120$  and  $20$  mV in  $10$  mV steps. The prepulse potential at  $40$  mV was positive enough to induce full conductance of the HERG channels but also inactivated many of the channels. The rate of recovery from inactivation was obtained by fitting a single exponential to the initial increase in tail-current amplitude, whereas the time constant of deactivation was ascertained by fitting a single exponential to the decay

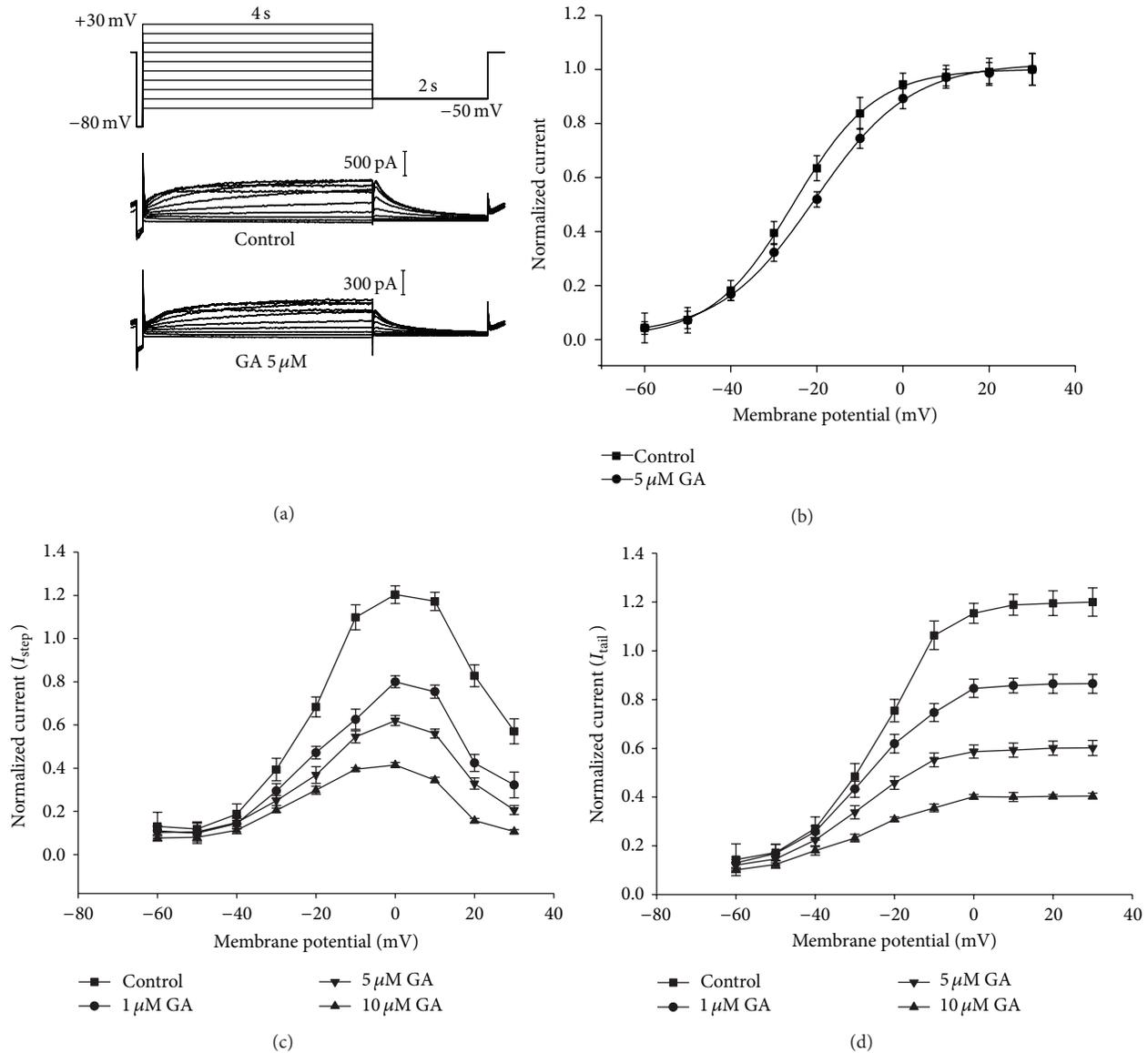


FIGURE 3: Current-voltage relationship for HERG channels and blockade by GA. (a) HERG currents are illustrated under control conditions and in the presence of 5  $\mu$ M GA recorded using the pulse protocol. (b) Voltage-dependent activation curves for the control and following GA exposure. Tail currents under control conditions and in the presence of 5  $\mu$ M GA were normalized and fitted to the Boltzmann sigmoidal function. There were no significant differences in half-activation voltage ( $V_{1/2}$ ) of 5  $\mu$ M compared with the control ( $P > 0.05$ ,  $n = 6$ ). Data were expressed as mean  $\pm$  SEM. Statistical comparisons were made using a two-tailed Student's  $t$ -test. Normalized (to respective control values)  $I$ - $V$  relationships for current measured at the end of depolarizing steps (c) and tail currents (d) in the control and the presence of 1  $\mu$ M, 5  $\mu$ M, and 10  $\mu$ M GA. Data are expressed as mean  $\pm$  SEM;  $n = 6$ . Statistical comparisons were made using a two-tailed Student's  $t$ -test ( $P < 0.05$ ,  $n = 6$ ).

of the tail current. However, 5  $\mu$ M GA did not change the deactivation rate significantly ( $P > 0.05$ ,  $n = 5$ ) (Figure 6(d)).

#### 4. Discussion

Cardiac  $K^+$  channels play a pivotal role in maintaining normal cardiac electrical activity. They regulate the resting membrane potential and excitability, participate in repolarization, and determine the shape and duration of cardiac action potential. A malfunction of the  $K^+$  channels due to either gene

mutations or drug blockade alters not only cardiomyocyte excitability but also the electrical balance of depolarization and repolarization, which causes a long or short QT interval in the electrocardiogram (ECG) and underlies different types of cardiac arrhythmia [21, 22]. Therefore, cardiac  $K^+$  channels are important targets for antiarrhythmic drugs.

In the present study, we have firstly provided the evidence that the antiarrhythmic ionic mechanism of GA is related to the inhibition of potassium currents ( $I_{Kr}$ ,  $I_{Ks}$ ) in guinea pig ventricular myocytes. The cardiac ion channel gene products

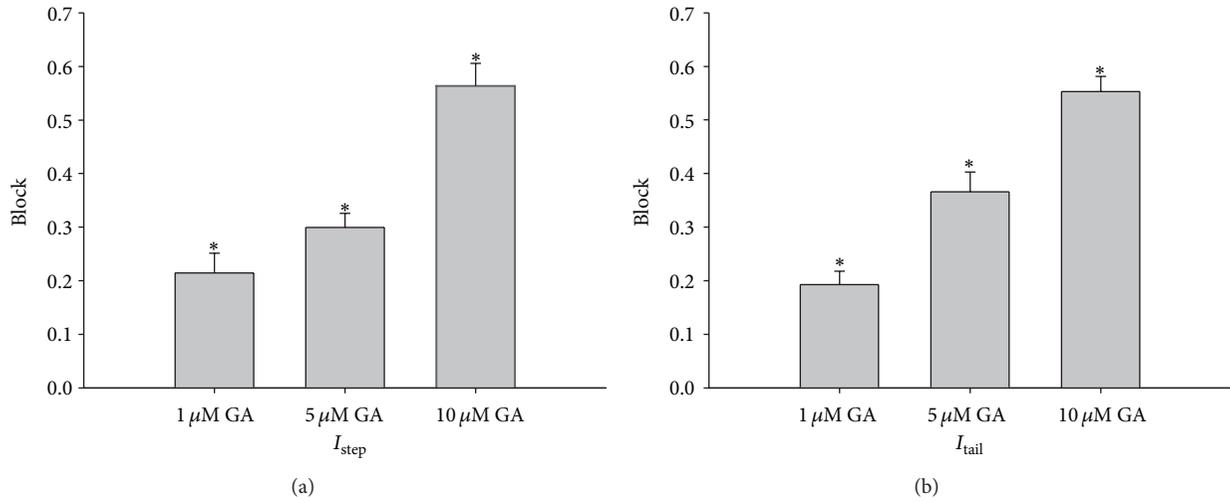


FIGURE 4: Inhibitory effect of GA on HERG current. Mean fractional block of HERG at a test potential of 0 mV at GA concentrations of 1, 5, and 10  $\mu$ M was determined in HEK293 cells that stably expressed HERG channels.

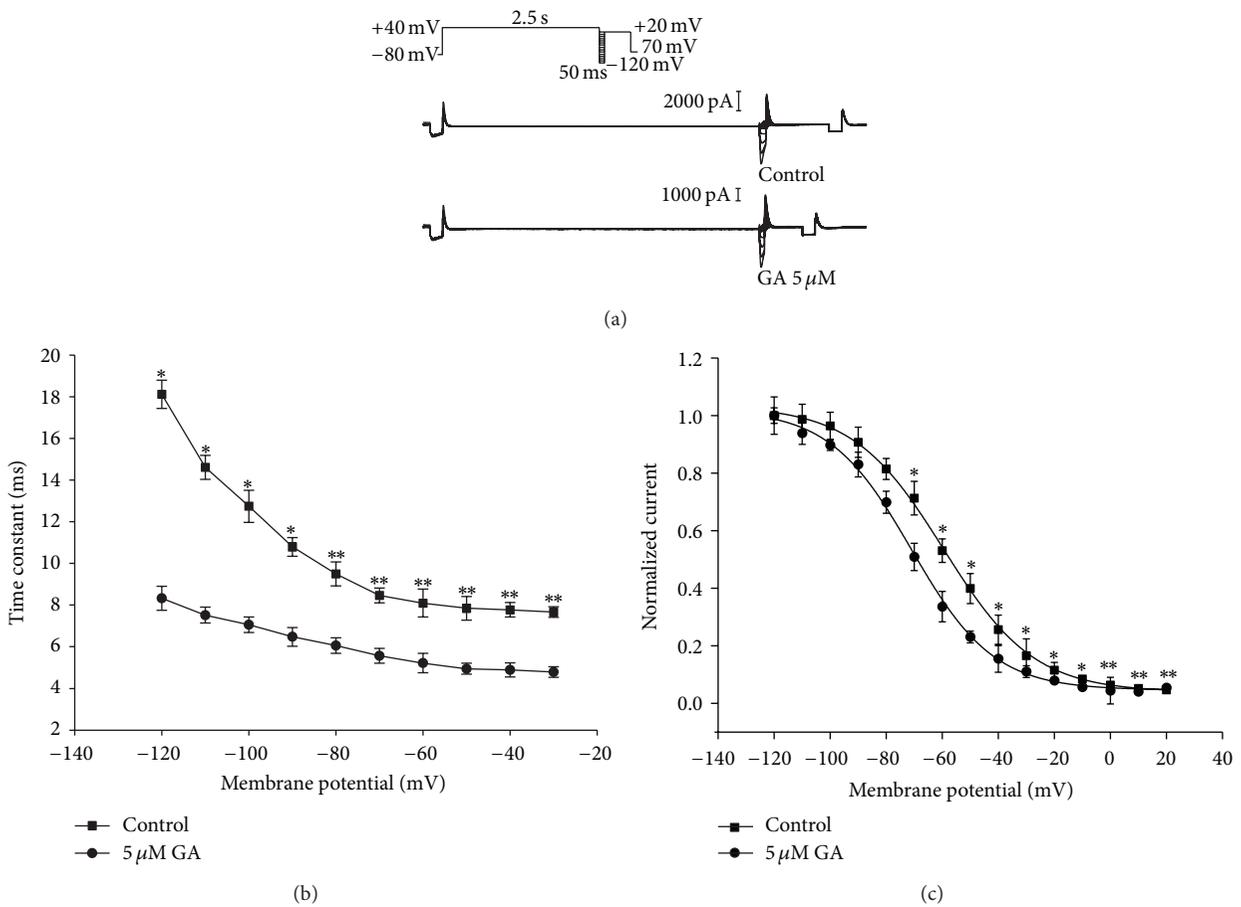


FIGURE 5: Effect of GA on normalized steady-state inactivation curves. (a) Representative active current traces for steady-state inactivation using a three-pulse protocol with various interpulse repolarization levels (from -120 mV to 20 mV). (b) The time constant of inactivation was significantly reduced at all membrane potential values ( $P < 0.05$ ,  $n = 5$ ). (c) Normalized steady-state inactivation curves before and after 5  $\mu$ M GA application ( $P < 0.05$ ,  $n = 5$ ). Data were expressed as mean  $\pm$  SEM. Solid lines represent fits to the Boltzmann sigmoidal function. Statistical comparisons were made using a two-tailed Student's  $t$ -test. (\*  $P < 0.05$ , \*\*  $P < 0.01$ ).

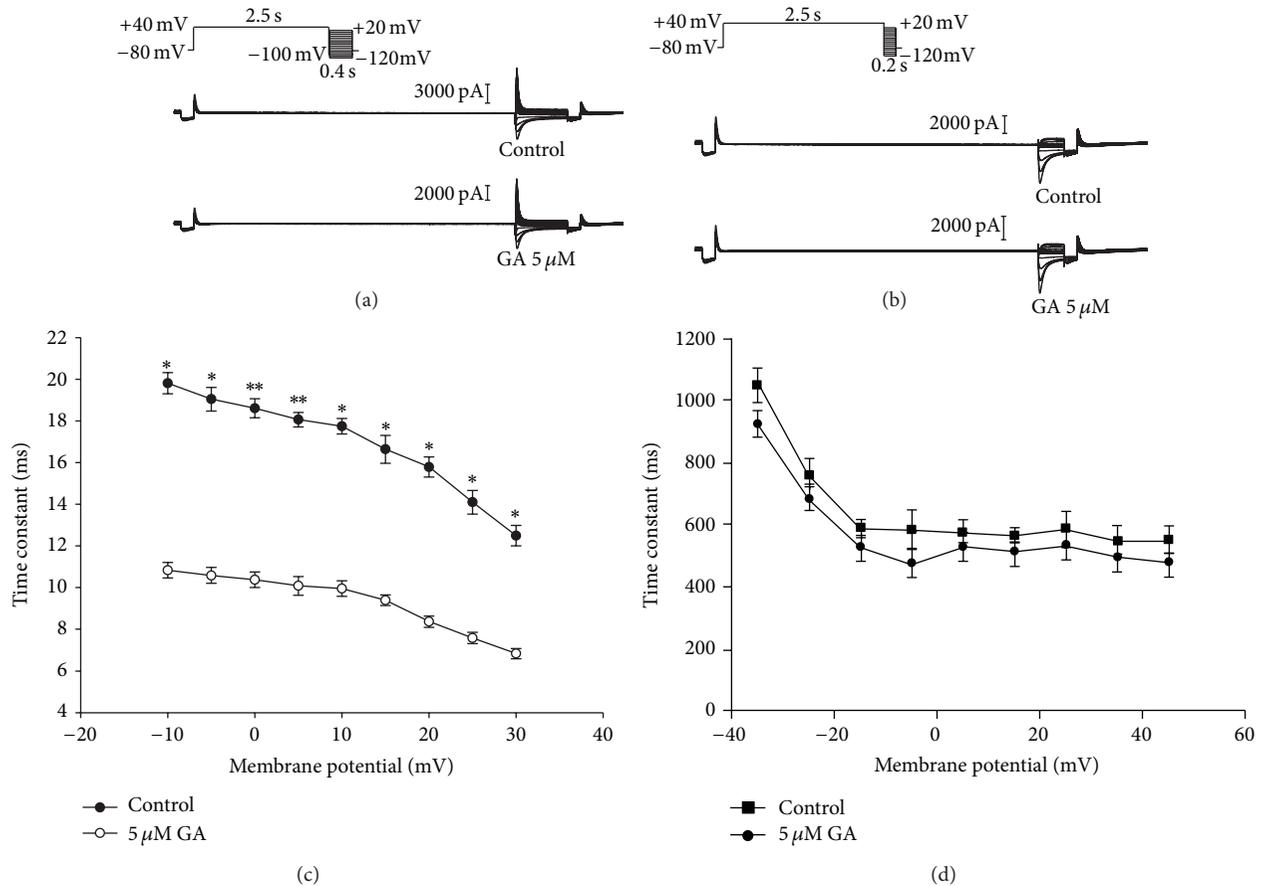


FIGURE 6: Effect of GA on kinetic time constants. (a) Representative current traces for the onset of inactivation of the HERG current using the protocol are shown on the figure. The time constants for the onset of inactivation were obtained by fitting the exponential function to the decaying current traces during the third pulse of the protocol. (b) Representative current traces for HERG current recovery from inactivation elicited by the protocol are shown on the figure. (c) Time constants for onset of inactivation were plotted against membrane potential. Data were expressed as mean  $\pm$  SEM ( $P < 0.05$ ,  $n = 5$ ). (d) Time constants for deactivation in the absence or presence of 5  $\mu$ M GA ( $P > 0.05$ ,  $n = 5$ ). Solid lines represent fits of a single exponential function to the descending phase of the tail current. Data were expressed as mean  $\pm$  SEM, and statistical comparisons were made using a two-tailed Student's  $t$ -test. (\* $P < 0.05$ , \*\* $P < 0.01$ ).

that are targets for GA are unknown. However, previous studies have demonstrated that the peak and late  $I_{Na}$ , studied in the *Xenopus* oocytes expressing either human  $Na_v1.5$  or mutant  $Na_v1.5$ - $\Delta$ KPQ of the  $\alpha$ -subunit channel, were strongly reduced by GA [19], resulting in a prolongation of the action potential. We therefore tested GA for a possible block of HERG expressed in HEK293 cells. The results confirmed that GA blocks HERG.

One of the main objectives of studying ionic channels is to provide a theoretical basis for the clinical treatment of tachyarrhythmia. Many active ingredients of Chinese medicine could block the HERG channel, decrease  $I_{Kr}$ , and lead to the acquired long QT syndrome, which makes the channel the therapeutic target for anti-arrhythmia [23]. It is thought that HERG channel inactivation is important in channel blocking by some (but not all) drugs, either by increasing drug-binding affinity or by facilitating the optimal orientation of the S6 aromatic residues to which drugs bind. In this study, the extent of channel inactivation was significantly

altered (Figure 5(c)), and the time course of inactivation seemed to be accelerated (Figure 5(b)). Furthermore, the time constants for the onset of inactivation were significantly smaller following perfusion with GA (Figure 6(c)). These effects of GA on channel kinetics were consistent with affinity for the inactivated state, suggesting that GA blocks the HERG channel by affecting its inactivation but not its activation. Our results demonstrated that GA has inhibitory effects on  $I_{Kr}$ ,  $I_{Ks}$  in guinea pig ventricular myocytes, and the HERG potassium channel, and the inhibition was in a concentration-dependent manner. The increase in action potential duration induced by GA is mainly due to its blocking effects on  $I_{Kr}$ ,  $I_{Ks}$ , and HERG and are its major mechanisms of antiarrhythmic action.

$I_{Kr}$  and  $I_{Ks}$  are important in cardiac repolarization [24].  $I_{Kr}$  channels open rapidly upon depolarization of the action potential but are quickly inactivated. The channel inactivation is released following repolarization with a slow deactivation [25]. Owing to this inward rectification property,  $I_{Kr}$  contributes a little during the plateau of the cardiac action

potential and progressively increases at phase 3 repolarization of the action potential [26]. Therefore,  $I_{Kr}$  is pivotal in cardiac repolarization, especially in the later phases of the action potential, due to its unique kinetics.  $I_{Ks}$  activates slowly with almost no inactivation after activation [25, 27] and contributes to the phase 2 slow repolarization of cardiac action potential.  $I_{Ks}$  has been demonstrated in cardiac tissues/myocytes from various species including human [24, 26, 27]. The physiological contribution of  $I_{Ks}$  to the human ventricular action potential is limited; however, during tonic sympathetic stimulation or when the cardiac repolarization reserve is attenuated,  $I_{Ks}$  becomes important in limiting APD prolongation owing to its slow deactivation [26].  $I_{Ks}$  is expressed heterogeneously in different regions of the heart. In the canine ventricle,  $I_{Ks}$  density is greater in epicardial and endocardial cells than in the M cells [28, 29]. This lower  $I_{Ks}$  density in the M cells is considered to be related to the steeper APD rate relationship and their greater tendency to display longer APD and to develop EADs at slow heart rates or in response to QT-prolonging drugs [29].

Any abnormality in channel density or function (up- or downregulation) may result in changes of currents and APD, even inducing arrhythmias. The inhibition of  $I_{Kr}$ ,  $I_{Ks}$ , and HERG by GA would induce a prolongation of APD, which could contribute to its antiarrhythmic actions because prolongation of APD could prevent or terminate the reentrant excitation and prolong the refractory period. Class III antiarrhythmic agents, blockers of  $I_{Kr}$  such as d-sotalol, exert a proarrhythmic effect with reverse frequency-dependent manner, which is largely attenuated at fast rates and enhanced at lower stimulation frequencies. This reverse frequency-dependent effect [30] could lead to an increase in the dispersion of repolarization and favor the occurrence of cardiac arrhythmias. Jurkiewicz and Sanguinetti [31] proposed that the reverse frequency-dependent effect on APD of typical class III agents is a consequence of selective blockade of  $I_{Kr}$ .  $I_{Kr}$  blockers prolong atrial and ventricular APD and the QT interval and can cause TdP, which can degenerate into ventricular fibrillation and sudden cardiac death [2]. Proarrhythmia induced by  $I_{Kr}$  blockers is related to [32–35] (a) excessive prolongation of APD near plateau voltages, which favor the development of early after-depolarizations; (b) a more marked prolongation of the APD in M cells than in subepicardial or subendocardial ventricular muscle, possibly because of the relative scarcity of  $I_{Ks}$  in M cells [29]. Thus, triggered focal activity and ventricular reentry associated with increased inhomogeneity of repolarization across the ventricular wall would lead to the development of TdP [3, 4]. It has been suggested that  $I_{Ks}$  accumulation at increased frequencies decreases the relative importance of  $I_{Kr}$ , reducing the impact of  $I_{Kr}$  blockade on APD prolongation. The authors suggested that the compounds which inhibit  $I_{Ks}$  might be devoid of reverse use-dependence. Actually, the agent that blocks both components of  $I_K$  might have a more consistent effect on action potentials at different frequencies and a better safety profile than a specific  $I_{Kr}$  blocker [36].

Control of cardiac electrical activity is well organized by an array of ion channels activated with a delicate balance between inward and outward ion currents [37]. Upon receiving an incoming impulse, cardiac cells are excited with rapid membrane depolarization followed by a relatively slow repolarization. Repolarization disorders, either excessive slowing or acceleration of the rate, can cause electrical perturbations resulting in cardiac arrhythmias, while excessive blockade of the HERG channel might increase the risk of arrhythmogenic activities. A good antiarrhythmic drug should affect multiple channels and keep invalids free from further episodes of arrhythmia [23]. Taking this into consideration, the drugs currently available in clinics are not satisfactory. Previous studies have revealed that GA can block peak and late  $I_{Na}$  [19, 20]. In our task group, the results suggested that GA not only blocks  $I_{Kr}$  (HERG) and  $I_{Ks}$  but also inhibits  $I_{Ca-L}$ . The effect of GA on multiple channels might make it a promising antiarrhythmic that can lead the cardiac cell to restore normal sinus rhythm and prevent further arrhythmia. Further basic and clinical studies will be needed to explore whether GA has proarrhythmic actions.

## 5. Conclusion

In conclusion, our study demonstrated that GA has an inhibitory effect on  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ) and the HERG potassium channel expressed in HEK293 cells. The results indicate that the antiarrhythmic activity and prolonged action potential of GA could be due to the blocking of  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ) and the HERG channel. It is thought that a good antiarrhythmic drug should affect multiple channels and be able to restore normal sinus rhythm and keep patients free from further episodes of arrhythmia. GA not only blocks the HERG channel,  $I_K$  ( $I_{Kr}$ ,  $I_{Ks}$ ), but also inhibits  $I_{Na}$ , so the results reveal that it has significant potential for development as a novel antiarrhythmic agent, particularly targeting the genesis of arrhythmias. Therefore, our findings might help to elucidate the traditional use of licorice in the treatment of cardiovascular disorders. Nonetheless, further evaluation of the therapeutic potential of GA is warranted.

## Authors' Contribution

Delin Wu and Linqing Jiang contributed equally to the paper.

## Acknowledgments

This work was supported by a Grant from the Beijing Key Disciplines Project of Traditional Chinese Medicine (no. JZZ-312) and National Natural Science Foundation of China (no. 81273890) to Dr Hongjin Wu. The authors thank Drs. Jianquan Zheng and Xiaoyan Liu (Beijing Institute of Pharmacology and Toxicology, Beijing, China) for generously providing the HEK293 cells that stably expressed the wild type HERG gene.

## References

- [1] D. Darbar and D. M. Roden, "Future of antiarrhythmic drugs," *Current Opinion in Cardiology*, vol. 21, no. 4, pp. 361–367, 2006.
- [2] C. E. Hill, H. Hickey, and S. L. Sandow, "Role of gap junctions in acetylcholine-induced vasodilation of proximal and distal arteries of the rat mesentery," *Journal of the Autonomic Nervous System*, vol. 81, no. 1–3, pp. 122–127, 2000.
- [3] T. Murai, K. Muraki, Y. Imaizumi, and M. Watanabe, "Levcromakalim causes indirect endothelial hyperpolarization via a myo-endothelial pathway," *The British Journal of Pharmacology*, vol. 128, no. 7, pp. 1491–1496, 1999.
- [4] H. Fukuta, M. Koshita, Y. Yamamoto, and H. Suzuki, "Inhibition of the endothelium-dependent relaxation by  $18\beta$ -glycyrrhetic acid in the guinea-pig aorta," *Japanese Journal of Physiology*, vol. 49, no. 3, pp. 267–274, 1999.
- [5] H. J. Taylor, A. T. Chaytor, W. H. Evans, and T. M. Griffith, "Inhibition of the gap junctional component of endothelium-dependent relaxations in rabbit iliac artery by  $18\alpha$ -glycyrrhetic acid," *The British Journal of Pharmacology*, vol. 125, no. 1, pp. 1–3, 1998.
- [6] S. C. Kwon, W. B. Pyun, G. Y. Park, H. Choi, K. Paik, and B. Kang, "The involvement of  $K^+$  channels and the possible pathway of EDHF in the rabbit femoral artery," *Yonsei Medical Journal*, vol. 40, no. 4, pp. 331–338, 1999.
- [7] C. Fiore, M. Eisenhut, R. Krausse et al., "Antiviral effects of Glycyrrhiza species," *Phytotherapy Research*, vol. 22, no. 2, pp. 141–148, 2008.
- [8] B. Ploeger, T. Mensinga, A. Sips, W. Seinen, J. Meulenbelt, and J. DeJongh, "The pharmacokinetics of glycyrrhizic acid evaluated by physiologically based pharmacokinetic modeling," *Drug Metabolism Reviews*, vol. 33, no. 2, pp. 125–147, 2001.
- [9] M. L. Parisella, T. Angelone, A. Gattuso, M. C. Cerra, and D. Pellegrino, "Glycyrrhizin and glycyrrhetic acid directly modulate rat cardiac performance," *Journal of Nutritional Biochemistry*, vol. 23, no. 1, pp. 69–75, 2012.
- [10] D. Armanini, C. Fiore, M. J. Mattarello, J. Bielenberg, and M. Palermo, "History of the endocrine effects of licorice," *Experimental and Clinical Endocrinology and Diabetes*, vol. 110, no. 6, pp. 257–261, 2002.
- [11] V. Battaglia, A. M. Brunati, C. Fiore et al., "Glycyrrhetic acid as inhibitor or amplifier of permeability transition in rat heart mitochondria," *Biochimica et Biophysica Acta*, vol. 1778, no. 1, pp. 313–323, 2008.
- [12] K. S. Kilgore, E. J. Tanhehco, J. L. Park, K. B. Naylor, M. B. Anderson, and B. R. Lucchesi, "Reduction of myocardial infarct size in vivo by carbohydrate-based glycomimetics," *Journal of Pharmacology and Experimental Therapeutics*, vol. 284, no. 1, pp. 427–435, 1998.
- [13] A. M. Aly, L. Al-Alousi, and H. A. Salem, "Licorice: a possible anti-inflammatory and anti-ulcer drug," *AAPS PharmSciTech*, vol. 6, no. 1, pp. E74–E82, 2005.
- [14] J. R. Lee, S. J. Park, H. S. Lee et al., "Hepatoprotective activity of licorice water extract against Cadmium-induced toxicity in rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 6, no. 2, pp. 195–201, 2009.
- [15] H. Hibasami, H. Iwase, K. Yoshioka, and H. Takahashi, "Glycyrrhizin induces apoptosis in human stomach cancer KATO III and human promyelotic leukemia HL-60 cells," *International Journal of Molecular Medicine*, vol. 16, no. 2, pp. 233–236, 2005.
- [16] A. Rodríguez-Sinovas, D. García-Dorado, M. Ruiz-Meana, and J. Soler-Soler, "Protective effect of gap junction uncouplers given during hypoxia against reoxygenation injury in isolated rat hearts," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 290, no. 2, pp. H648–H656, 2006.
- [17] F. Goubaeva, M. Mikami, S. Giardina, B. Ding, J. Abe, and J. Yang, "Cardiac mitochondrial connexin 43 regulates apoptosis," *Biochemical and Biophysical Research Communications*, vol. 352, no. 1, pp. 97–103, 2007.
- [18] J. Y. Yang, H. J. Wu, and D. L. Wu, "Effects of glycyrrhetic acid on sodium ion channel currents of rats' ventricular myocardial cells," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 32, no. 7, pp. 944–947, 2012.
- [19] Y. Du, S. Zhang, H. Wu et al., "Glycyrrhetic acid blocks cardiac sodium channels expressed in *Xenopus oocytes*," *Journal of Ethnopharmacology*, vol. 125, no. 2, pp. 318–323, 2009.
- [20] Y. M. Du, C. K. Xia, N. Zhao, Q. Dong, M. Lei, and J. H. Xia, " $18\beta$ -glycyrrhetic acid preferentially blocks late Na current generated by  $\delta$ KPQ Nav1.5 channels," *Acta Pharmacologica Sinica*, vol. 33, no. 6, pp. 752–760, 2012.
- [21] P. J. Kannankeril and D. M. Roden, "Drug-induced long QT and torsade de pointes: recent advances," *Current Opinion in Cardiology*, vol. 22, no. 1, pp. 39–43, 2007.
- [22] W. Zareba and I. Cygankiewicz, "Long QT syndrome and short QT syndrome," *Progress in Cardiovascular Diseases*, vol. 51, no. 3, pp. 264–278, 2008.
- [23] H. J. Wu, A. R. Zou, F. Xie et al., "Effect of matrine on human ether a-go-go related gene (HERG) channels expressed in Chinese hamster ovary cells," *Chinese Journal of Integrative Medicine*, vol. 16, no. 5, pp. 430–434, 2010.
- [24] G. R. Li, J. Feng, L. Yue, M. Carrier, and S. Nattel, "Evidence for two components of delayed rectifier  $K^+$  current in human ventricular myocytes," *Circulation Research*, vol. 78, no. 4, pp. 689–696, 1996.
- [25] M. C. Sanguinetti and N. K. Jurkiewicz, "Two components of cardiac delayed rectifier  $K^+$  current. Differential sensitivity to block by class III antiarrhythmic agent," *Journal of General Physiology*, vol. 96, no. 1, pp. 195–215, 1990.
- [26] N. Jost, L. Virág, M. Bitay et al., "Restricting excessive cardiac action potential and QT prolongation: a vital role for IKs in human ventricular muscle," *Circulation*, vol. 112, no. 10, pp. 1392–1399, 2005.
- [27] F. Charpentier, J. Mérot, G. Loussouarn, and I. Baró, "Delayed rectifier  $K^+$  currents and cardiac repolarization," *Journal of Molecular and Cellular Cardiology*, vol. 48, no. 1, pp. 37–44, 2010.
- [28] G. R. Li, C. P. Lau, A. Ducharme, J. C. Tardif, and S. Nattel, "Transmural action potential and ionic current remodeling in ventricles of failing canine hearts," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 283, no. 3, pp. H1031–H1041, 2002.
- [29] D. W. Liu and C. Antzelevitch, "Characteristics of the delayed rectifier current ( $I(Kr)$  and  $I(Ks)$ ) in canine ventricular epicardial, midmyocardial, and endocardial myocytes: a weaker  $I(Ks)$  contributes to the longer action potential of the M cell," *Circulation Research*, vol. 76, no. 3, pp. 351–365, 1995.
- [30] L. M. Hondeghem and D. J. Snyders, "Class III antiarrhythmic agents have a lot of potential but a long way to go. Reduced effectiveness and dangers of reverse use dependence," *Circulation*, vol. 81, no. 2, pp. 686–690, 1990.
- [31] N. K. Jurkiewicz and M. C. Sanguinetti, "Rate-dependent prolongation of cardiac action potentials by a methanesulfonanilide class III antiarrhythmic agent: specific block of rapidly activating delayed rectifier  $K^+$  current by dofetilide," *Circulation Research*, vol. 72, no. 1, pp. 75–83, 1993.

- [32] D. M. Roden, J. R. Balsler, A. L. George Jr., and M. E. Anderson, "Cardiac ion channels," *Annual Review of Physiology*, vol. 64, pp. 431–475, 2002.
- [33] J. Tamargo, "Drug-induced torsade de pointes: from molecular biology to bedside," *Japanese Journal of Pharmacology*, vol. 83, no. 1, pp. 1–19, 2000.
- [34] W. Haverkamp, G. Breithardt, A. J. Camm et al., "The potential for QT prolongation and pro-arrhythmia by non-antiarrhythmic drugs: clinical and regulatory implications: report on a policy conference of the european society of cardiology," *Cardiovascular Research*, vol. 47, no. 2, pp. 219–233, 2000.
- [35] W. S. Redfern, L. Carlsson, A. S. Davis et al., "Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development," *Cardiovascular Research*, vol. 58, no. 1, pp. 32–45, 2003.
- [36] P. T. Sager, P. Uppal, C. Follmer, M. Antimisiaris, C. Pruitt, and B. N. Singh, "Frequency-dependent electrophysiologic effects of amiodarone in humans," *Circulation*, vol. 88, no. 3, pp. 1063–1071, 1993.
- [37] J. M. Nerbonne and R. S. Kass, "Molecular physiology of cardiac repolarization," *Physiological Reviews*, vol. 85, no. 4, pp. 1205–1253, 2005.

## Review Article

# The Roles of Traditional Chinese Medicine: Shen-Fu Injection on the Postresuscitation Care Bundle

**Qian Zhang and Chunsheng Li**

*Department of Emergency Medicine, Beijing Chaoyang Hospital, Capital Medical University,  
8 Workers' Stadium South Road, Chaoyang District, Beijing 100020, China*

Correspondence should be addressed to Chunsheng Li; [lcscyyy@163.com](mailto:lcscyyy@163.com)

Received 3 December 2012; Revised 20 May 2013; Accepted 31 July 2013

Academic Editor: Ka Kit Hui

Copyright © 2013 Q. Zhang and C. Li. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Survival rates following in-hospital and out-of-hospital cardiac arrests remain disappointingly low. Organ injury caused by ischemia and hypoxia during prolonged cardiac arrest is compounded by reperfusion injury that occurs when a spontaneous circulation is restored. A bundle of procedures, which may need to be administered simultaneously, is required. The procedures include prompt identification and treatment of the cause of cardiac arrest, as well as a definitive airway and ventilation together. Additional benefit is possible with appropriate forms of early goal-directed therapy and achieving therapeutic hypothermia within the first few hours, followed by gradual rewarming and ensuring glycaemic control to be within a range of 6 to 10 mmol/L. All these would be important and need to be continued for at least 24 hours. Previous studies have showed that the effects of Shen-Fu injection (SFI) are based on aconitine properties, supplemented by ginsenoside, which can scavenge free radicals, improve energy metabolism, inhibit inflammatory mediators, suppress cell apoptosis, and alleviate mitochondrial damage. SFI, like many other complex prescriptions of traditional Chinese medicine, was also found to be more effective than any of its ingredient used separately *in vivo*. As the postresuscitation care bundle is known to be, the present paper focuses on the role of SFI played on the postresuscitation care bundle.

## 1. Introduction

Cardiac arrest (CA) results in whole-body ischemia/reperfusion and represents the most severe shock state, during which the delivery of oxygen and metabolic substrates is abruptly halted, and metabolites are no longer removed. Cardiopulmonary resuscitation (CPR) only partially reverses this process [1]. Furthermore, the myocardium is a main target tissue during this form of ischemia/reperfusion, and the destruction of tissue progresses even after circulation has been successfully restored.

In patients who initially achieve return of spontaneous circulation (ROSC) after CA, the significant subsequent morbidity and mortality are largely due to the cerebral and cardiac dysfunction that accompanies prolonged whole-body ischemia. CA contributes to hemodynamic disorders that cause the systemic release of massive oxygen free radical, lactic acid, and metabolites of arachidonic acid, which could reach the different tissues by blood circulation and cause

ischemia/reperfusion injury [2, 3]. Inadequate tissue oxygen delivery can persist even after ROSC; accumulated oxygen debt leads to activation of immunologic pathways and systemic inflammation, which increases the risk of multiple organ failure. This condition had many features in common with sepsis [4]. The pathophysiological changes cause multiple organ dysfunctions and have recently been termed the "postcardiac arrest syndrome," which comprises anoxic brain injury, postcardiac arrest myocardial dysfunction, systemic ischemia/reperfusion response, and persistent precipitating pathology [5].

Shen-Fu injection (SFI), which has been commonly used in China for nearly 800 years, is a well-known traditional Chinese medical formulation containing ginseng (*Panax*; family: Araliaceae) and fuzi (*Radix aconiti lateralis preparata*, *Aconitum carmichaeli* Debx.; family: Ranunculaceae), which is produced using multistage countercurrent extraction and macroporous resin adsorption technology (Ya'an Sanjiu Pharmaceutical Co., Ltd., China). Its quality is controlled strictly

according to the standard of China Ministry of Public Health and fingerprint technology was adopted in the process of production to ensure its quality [6]. Their chemical structures are as shown in Figure 1.

Lots of clinical and epidemiological studies have demonstrated that SFI has significant protective effects on ischemia/reperfusion injury of the brain, spinal cord, kidney, intestine, liver, and especially the heart [7–11]. However, relatively little is known about the effects of SFI on postresuscitation care bundle. The present paper focuses on the association between postresuscitation care bundle and SFI and explains the roles of SFI on the postresuscitation care bundle treatment.

## 2. Postresuscitation Care Bundles

Bundles are a group of “therapies” built around the best evidence-based guidelines, which, when implemented together, produce greater benefit in terms of outcome than the individual therapeutic interventions [12]. The conception of care bundles has been proposed based on the holistic principle that the whole is greater than the sum of its parts. Bundles play a useful role to help remove the constraints imposed by these deviations and variations by means of constructing the elements into packages that must be implemented in strict compliance for every patient, at each and every single time to ensure uniformity as well as universality [13].

In the year of 2005, the final ring of the chain of survival was updated to reflect the importance of postresuscitation care in determining the ultimate outcome following CA [14]. Organ injury caused by ischemia and hypoxia during prolonged CA is compounded by reperfusion injury that occurs when a spontaneous circulation is restored. These insults trigger a systemic inflammatory response, similar to that associated with sepsis [15]. Here, we propose a “postresuscitation care bundle” which comprises (1) early coronary reperfusion and hemodynamic optimization, (2) airway and ventilation management, (3) therapeutic hypothermia, (4) neurological enhancement measures and monitoring, and (5) glycaemic control [16].

**2.1. Early Coronary Reperfusion and Hemodynamic Optimization.** Patients resuscitated from CA who have electrocardiographic criteria for ST-elevation myocardial infarction should undergo immediate coronary angiography, with subsequent percutaneous coronary intervention (PCI) if indicated. If PCI is not available, thrombolytic therapy is an appropriate alternative for postcardiac arrest management of ST-elevation myocardial infarction [17]. This may also be helpful for monitoring blood pressures during therapeutic hypothermia and for accurate measurement of hemodynamic parameters so as to determine the most appropriate combination of medications for maintenance of perfusion. Central venous pressure monitoring would also be useful.

**2.2. Airway and Ventilation Management.** Airway control is crucial in the initial stages of postresuscitation management.

Insertion of a definitive airway (if not done yet) guided by capnography is followed by a chest radiograph for confirmation of tube position. While mechanical ventilation would be required so as to reduce the work of breathing, the rate of ventilation and tidal volume would need to be adjusted in order to maintain arterial oxyhemoglobin saturation at  $\geq 94\%$  [18, 19]. The concerns that hyperoxaemia during the reperfusion phase after ROSC with 100% oxygen may lead to increased brain lipidperoxidation, increased metabolic dysfunction, and neurological degeneration, as well as concerns about its impact on short-term functional outcome, have resulted in calls of ventilation with room air or an inspired oxygen fraction titrated to maintain a pulse oximetry reading of 94% to 98% [20]. Weaning the patients from 100% oxygen to the  $FiO_2$  required to maintain  $SpO_2$  at the above stated levels should begin once ROSC is achieved [21].

**2.3. Therapeutic Hypothermia.** It has been well demonstrated that brain temperatures during the first 24 hours after ROSC have a significant effect on survival and neurological recovery in patients who remain comatose soon after ROSC [22]. Preclinical and clinical evidence strongly supports mild therapeutic hypothermia as an effective therapy for the postcardiac arrest syndrome [23]. Unconscious adult patients with spontaneous circulation after out-of-hospital VF cardiac arrest should be cooled to 32–34°C for at least 12 to 24 hours. Induced hypothermia might also benefit unconscious adult patients with spontaneous circulation after out-of-hospital cardiac arrest from a nonshockable rhythm or in-hospital cardiac arrest [24].

**2.4. Neurological Enhancement Measures and Monitoring.** Postcardiac arrest brain injury is a result of initial ischemic injury followed by reperfusion injury occurring within the hours and days after ROSC [25]. Features indicating occurrence of brain injury in post-ROSC patients include coma, seizures, myoclonus, and various degrees of neurocognitive dysfunction, ranging from memory deficits to a persistent vegetative state and finally brain death. The neurological prognosis in the majority of comatose CA survivors cannot be reliably predicted until at least 72 hours after CPR. Currently, the principal neuroprotective measures recommended include normoventilation with controlled oxygenation to avoid hyperoxaemia and minimize the likelihood of lowering cerebral perfusion or aggravating cerebral ischemia, achieving normoglycaemia to optimize neuronal recovery and therapeutic hypothermia to minimize the cerebral and multisystem metabolic functions until biochemical and cellular parameters are better optimized [26].

**2.5. Glycaemic Control.** Hyperglycaemia occurring post-ROSC has been associated with increased mortality and worse neurological outcomes [27]. Similarly, hypoglycaemia is also associated with poor outcomes in critically ill patients [28]. The strategy is to maintain blood sugar levels at 6–10 mmol/L. Blood glucose concentrations must be monitored frequently in these patients, and hyperglycemia should be treated with an insulin infusion. A target glucose range with

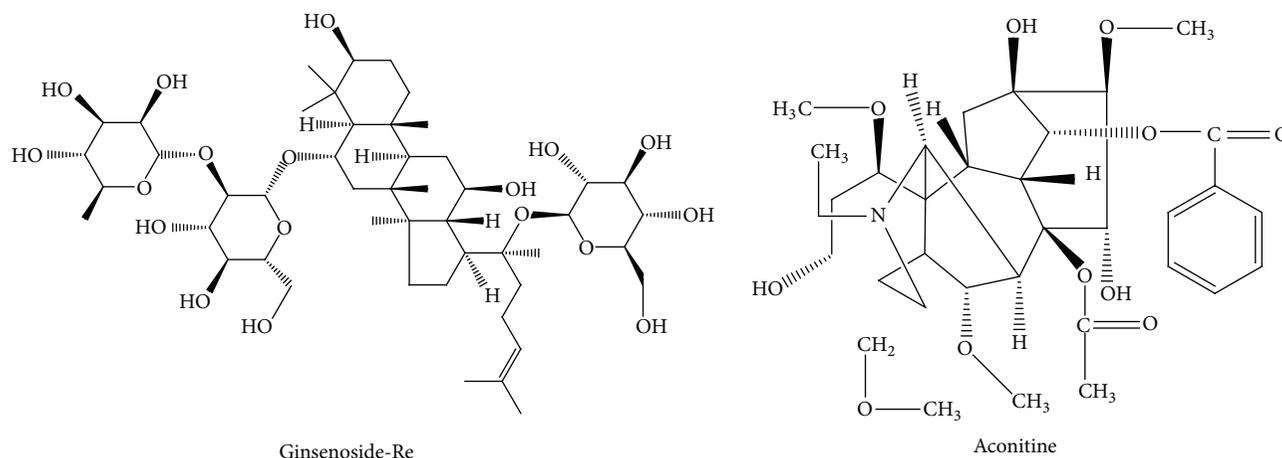


FIGURE 1: The chemical structures of ginsenoside-Re and Aconitine.

an upper value of 8.0 mmol/L (144 mg/dL) has been suggested by others [29–31]. The lower value of 6.1 mmol/L (110 mg/dL) may not reduce mortality any further but instead may expose patients to the potentially harmful effects of hypoglycemia [29]. The incidence of hypoglycemia in another recent study of intensive insulin therapy exceeded 18%, and some have cautioned against its routine use in the critically ill patients [32–34]. Regardless of the chosen glucose target range, blood glucose must be measured frequently, especially when insulin is started and during cooling and rewarming periods.

### 3. The Roles of SFI on the Postresuscitation Care Bundle Treatment

**3.1. SFI Mitigates the Postresuscitation Myocardial Dysfunction.** Postresuscitation myocardial dysfunction has been implicated as one of the major causes of fatal outcomes in patients who fail to survive hospitalization after CPR [35]. Results from Ji et al. showed that SFI significantly increased mean arterial pressure and improved the cardiac output and ejection fraction after ROSC. SFI also can attenuate postresuscitation myocardial dysfunction through beneficial effects on energy metabolism and remarkable antioxidant capacity. Meanwhile, it is also demonstrated that SFI can prevent and cure different kinds of arrhythmia (tachycardia and bradycardia), while such two-way regulating effect has rarely been found in western medicine [36]. Furthermore, in an in vitro study, Gu et al. reported the cardioprotective effects of SFI by decreasing myocardial injury, improving myocardial ultrastructure, inhibiting Bcl-2, Bax, and caspase 3 expressions, and reducing myocardial apoptosis [37].

**3.2. SFI Extenuates Postresuscitation Lung Injury.** Since post-ROSC patients are at risk of acute lung injury or acute respiratory distress syndrome, the standard recommendation for ventilation would be normocapnia. Excessive tidal volumes would not also be recommended owing to the risks of increased intrathoracic pressures with attendant reduced

venous return and cardiac output. Zhang et al. have reported that SFI resulted in improving oxygenation index, respiratory index, oxygen extraction ratio, dynamic lung compliance, airway resistance, external vascular lung water index, and pulmonary vascular permeability index, which indicates that SFI can effectively protect pulmonary gas exchange function, increase oxygen consumption and extraction, decrease structural lung damage, and alleviate pulmonary edema after ROSC [38]. A recent study has also demonstrated that SFI could reduce postresuscitation lung injury and improve lung immune function by regulating lung imbalance of Th1/Th2 [39].

**3.3. SFI Alleviates Postresuscitation Cerebral Injury.** In an in vitro study, Yang et al. showed that cerebral injury is aggravated progressively at the early phase following CPR. SFI could alleviate cerebral injury after CPR, and this protective effect might be of dosage effect relationship. Large dosage of SFI could promote the expression of neuron-specific enolase in neurons, which might alleviate the cerebral injury after CPR [40].

**3.4. SFI and Glycaemic Control.** Hyperglycemia is common after CA due to the upregulated stress response. Tight blood glucose control remains controversial in the critical care setting. It has been shown to improve survival in a surgical intensive care setting, but this outcome did not convey to the medical intensive care setting [41]. The blood glucose concentration should be monitored frequently in the postcardiac arrest syndrome patient, especially when instituting the therapeutics hypothermia. In an in vitro study, Shan-shan et al. reported that SFI could increase insulin secretion by increasing PI3K expression, which can improve hyperglycemia during CPR. The effects were likely caused by increased PI3K contents in islets. The mechanism may be partly related to SFI reversing the disequilibrium of oxidization and antioxidation [42].

## 4. The Holistic Approach with SFI

**4.1. The Effect of SFI on  $\text{Na}^+$ - $\text{K}^+$ -ATPase/ $\text{Ca}^{2+}$ -ATPase Activity.**  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity is an electrogenic process in which two  $\text{Na}^+$  ions extrude out of the cell, whereas one  $\text{K}^+$  ion enters the cell, thereby maintaining an appropriate transmembrane  $\text{Na}^+$  gradient.  $\text{Ca}^{2+}$ -ATPase enzyme is another sarcolemmal enzyme. Intracellular calcium loading is considered to represent the common denominator of ischemia/reperfusion-induced cell dysfunction and death. Various studies showed that  $\text{Na}^+$ - $\text{K}^+$ -ATPase and  $\text{Ca}^{2+}$ -ATPase enzymes may play a key role in the prevention of ischemia/reperfusion [43–45]. Luo et al. have found that SFI could restore the ability of  $\text{Na}^+$ - $\text{K}^+$ -ATPase and  $\text{Ca}^{2+}$ -ATPase enzyme activities, and it may be one of the mechanisms by which SFI could attenuate the postresuscitation myocardial dysfunction [46]. Additionally, the effect of SFI's blockage on the sodium channels in cardiac myocytes may be one of the important molecular mechanisms for its cardiac active effectiveness.

**4.2. Effects of SFI on Oxygen Free Radicals.** CPR can be viewed as a process of whole-body ischemia/reperfusion [47]. A large amount of oxygen free radicals (OFRs) produced during the ischemia/reperfusion play a major role in myocardial damage. Malondialdehyde (MDA) is an end product of lipid peroxidation that causes cellular damage and disruption of cell membranes when tissue antioxidants are exhausted. MDA has been found to increase in myocardial tissue after myocardial ischemia/reperfusion [48]. Superoxide dismutase (SOD) is a metalloenzyme that catalyzes the dismutation of  $\text{O}_2^-$  into  $\text{O}_2$  and  $\text{H}_2\text{O}_2$  and affords protection against OFRs damage [49]. The activity of SOD could reflect the in vivo scavenging capability of OFRs. Reducing of MDA content and attenuating the decrease of SOD activities in myocardial tissue have cardioprotective effects [50]. A recent study has shown that, comparing with saline group, A recent study has shown that the MDA content was significantly decreased in SFI-treated myocardium subjected to ischemia/reperfusion injury. At the same time, the activities of serum SOD in the SFI group were significantly higher than in the saline group [51]. These results indicate that SFI can reduce MDA content, attenuate the decrease of SOD activities in myocardial tissue, and thus alleviate the degree of myocardial injury.

**4.3. SFI Mitigates the Impact of Calcium Overload.**  $\text{Ca}^{2+}$  is a ubiquitous signal for regulating cellular function, including survival and death [52]. While a small amount of  $\text{Ca}^{2+}$  is necessary for the optimal physiological function of the heart, growing evidence suggests that an increased cytosolic free  $\text{Ca}^{2+}$  overload is one of the major contributors of myocardial injury induced by ischemia/reperfusion [53–55]. Therefore,  $\text{Ca}^{2+}$  handling in the postischemic myocardium has become a prime target. A recent study showed that the protective effects of SFI on myocardial ischemia/reperfusion injury were realized by repressing the opening of  $\text{Ca}^{2+}$  channel myocardial cell membrane, reducing inflow of  $\text{Ca}^{2+}$ , inhibiting calcium

overload, decreasing OFRs, and suppressing inflammation [56]. These results indicate that SFI can mitigate the impact of calcium overload.

**4.4. The Relationship between SFI and PI3K/Akt Signaling Pathway.** Some researches have showed that PI3K/Akt signaling pathway plays a crucial role in protecting the myocardium from ischemia/reperfusion injury [57, 58]. Strong evidence shows that endothelial nitric oxide synthase (eNOS) is an important target of Akt and eNOS plays the role of an important cardiovascular protective molecule [59]. Recent studies show that increased expression of Akt and eNOS alleviates the ischemia/reperfusion injury [60, 61]. Wu et al. showed that SFI treatment resulted in an enhanced level eNOS which was significantly higher than that of saline-treated ischemia/reperfusion group [62]. These results suggested that SFI-induced cardioprotective effects are mediated through Akt-induced eNOS phosphorylation.

**4.5. SFI Prevents Cardiomyocytes Apoptosis.** In a present study, after 24 hours post-ROSC, significant myocardial damage and apoptosis emerged, accompanied by increased protein expression of Bcl-2, Bax, and active caspase-3 [63]. The most principal pathways for apoptosis initiation are termed the Bcl-2/Bax-controlled pathway. Furthermore, downregulation of Bcl-2/Bax expression after postresuscitation might result in the activation of the caspase family of proteases, such as caspase 3, which is responsible for the induction of apoptotic cell death, leading to internucleosomal DNA fragmentation [64]. In an in vitro study, Wang et al. reported that treatment with different doses of SFI protected cardiac myocyte cultures from hypoxia/reoxygenation-induced apoptosis. Caspase-3 activation was decreased in hypoxic/reoxygenated cardiomyocytes cotreated with SFI when compared to hypoxia/reoxygenation alone treated cultures. Expression of the Bcl-2 proteins was increased in SFI-treated cardiomyocytes subjected to hypoxia/reoxygenation [65]. These results showed that SFI could significantly attenuate postresuscitation myocardial dysfunction by modulating apoptosis.

## 5. Conclusion

In summary, postcardiac arrest syndrome is associated with complex pathophysiological changes and a high mortality rate, and a multidisciplinary approach to treatment is optimal. SFI might be useful in the treatment of postcardiac arrest syndrome, incorporating the multilayer and multi-target advantages of TCM. Above all, SFI plays a key role during postresuscitation care bundle. Furthermore, with the initiation of large-scale, multicenter studies and further research into the pharmacological actions of SFI, we believe that SFI will eventually be widely used for postresuscitation care bundle treatment.

## Conflict of Interests

The authors declare that they have no commercial associations or sources of support that might pose a conflict of interests.

## Acknowledgment

The Shen-Fu injection was supplied by the Ya'an Sanjiu Pharmaceutical Co., Ltd., China.

## References

- [1] J. Herlitz, J. Engdahl, L. Svensson, K.-A. Ångquist, J. Silfverstolpe, and S. Holmberg, "Major differences in 1-month survival between hospitals in Sweden among initial survivors of out-of-hospital cardiac arrest," *Resuscitation*, vol. 70, no. 3, pp. 404–409, 2006.
- [2] I. Jacobs, V. Nadkarni, J. Bahr et al., "Cardiac arrest and cardiopulmonary resuscitation outcome reports: update and simplification of the Utstein templates for resuscitation registries. A statement for healthcare professionals from a task force of the International Liaison Committee on Resuscitation (American Heart Association, European Resuscitation Council, Australian Resuscitation Council, New Zealand Resuscitation Council)," *Circulation*, vol. 110, no. 21, pp. 3385–3397, 2004.
- [3] I. G. Stiell, G. A. Wells, B. Field et al., "Advanced cardiac life support in out-of-hospital cardiac arrest," *The New England Journal of Medicine*, vol. 351, no. 7, pp. 647–656, 2004.
- [4] C. Adrie, M. Adib-Conquy, I. Laurent et al., "Successful cardiopulmonary resuscitation after cardiac arrest as a "sepsis-like" syndrome," *Circulation*, vol. 106, no. 5, pp. 562–568, 2002.
- [5] D. F. Gaieski, B. S. Abella, and M. Goyal, "CPR and postarrest care: overview, documentation, and databases," *Chest*, vol. 141, no. 4, pp. 1082–1089, 2012.
- [6] Z. H. Zhu, L. Z. Xiong, H. L. Dong, W. N. Hu, X. L. Zeng, and L. C. Hou, "Dose response effects of Shenfu injection on ischemic reperfusion injury of spinal cord in rabbit," *Chinese Journal of Anesthesiology*, vol. 20, pp. 664–668, 2000.
- [7] Z.-Y. Xia, L.-Y. Zhan, Y.-H. He, and X.-Y. Liu, "The effect of Shen-Fu on gastrointestinal tract injury and its potential mechanism during cardio-pulmonary bypass in patients undergoing cardiac surgery," *Chinese Journal of Traumatology*, vol. 6, no. 4, pp. 245–248, 2003.
- [8] X. Y. Liu, H. D. Zou, J. F. Yu, H. B. Huang, and G. X. Xiong, "Protective effect of Shen-fu injection on multiple organ damage in rabbit during ischemia reperfusion," *Chinese Journal of Anesthesiology*, vol. 17, pp. 430–432, 1997.
- [9] B.-J. Zhang, Y.-L. Wang, C.-Y. Wang, and J.-J. Ke, "Effect of Shenfu injection on nuclear factor- $\kappa$ B during myocardial ischemia/reperfusion injury in rats," *Chinese Journal of Traumatology*, vol. 8, no. 4, pp. 200–204, 2005.
- [10] T. P. Zhang and M. Zhao, "Protective effect of Shen-Fu injection on heart injury induced by ischemia/reperfusion," *Journal of Molecular and Cellular Cardiology*, vol. 42, pp. S209–S210, 2007.
- [11] Z. R. Tang, "Effect of Shen-fu on hemodynamics of hypovolemic shock and oxygen delivery," *Chinese Journal of Pathophysiology*, vol. 21, no. 10, pp. 1954–1957, 2005.
- [12] R. G. Masterton, "Sepsis care bundles and clinicians," *Intensive Care Medicine*, vol. 35, no. 7, pp. 1149–1151, 2009.
- [13] M. M. Levy, P. J. Pronovost, R. P. Dellinger et al., "Sepsis change bundles: converting guidelines into meaningful change in behavior and clinical outcome," *Critical Care Medicine*, vol. 32, no. 11, pp. S595–S597, 2004.
- [14] S. J. Fletcher and A. C. Quinn, "The surviving sepsis campaign and sepsis care bundles: substance or sophistry?" *Anaesthesia*, vol. 61, no. 4, pp. 313–315, 2006.
- [15] C. Adrie, I. Laurent, M. Monchi, A. Cariou, J. F. Dhainaut, and C. Spaulding, "Postresuscitation disease after cardiac arrest: a sepsis-like syndrome?" *Current Opinion in Critical Care*, vol. 10, no. 3, pp. 208–212, 2004.
- [16] J. P. Nolan and J. Soar, "Post resuscitation care—time for a care bundle?" *Resuscitation*, vol. 76, no. 2, pp. 161–162, 2008.
- [17] I. Laurent, M. Monchi, J.-D. Chiche et al., "Reversible myocardial dysfunction in survivors of out-of-hospital cardiac arrest," *Journal of the American College of Cardiology*, vol. 40, no. 12, pp. 2110–2116, 2002.
- [18] L. N. Tremblay and A. S. Slutsky, "Ventilator-induced lung injury: from the bench to the bedside," *Intensive Care Medicine*, vol. 32, no. 1, pp. 24–33, 2006.
- [19] L. B. Ware and M. A. Matthay, "The acute respiratory distress syndrome," *The New England Journal of Medicine*, vol. 342, no. 18, pp. 1334–1349, 2000.
- [20] J. P. Muizelaar, A. Marmarou, J. D. Ward et al., "Adverse effects of prolonged hyperventilation in patients with severe head injury: a randomized clinical trial," *Journal of Neurosurgery*, vol. 75, no. 5, pp. 731–739, 1991.
- [21] R. Knafelj, P. Radsel, T. Ploj, and M. Noc, "Primary percutaneous coronary intervention and mild induced hypothermia in comatose survivors of ventricular fibrillation with ST-elevation acute myocardial infarction," *Resuscitation*, vol. 74, no. 2, pp. 227–234, 2007.
- [22] A. Zeiner, M. Holzer, F. Sterz et al., "Hyperthermia after cardiac arrest is associated with an unfavorable neurologic outcome," *Archives of Internal Medicine*, vol. 161, no. 21, pp. 2007–2012, 2001.
- [23] D. F. Gaieski, R. A. Band, B. S. Abella et al., "Early goal-directed hemodynamic optimization combined with therapeutic hypothermia in comatose survivors of out-of-hospital cardiac arrest," *Resuscitation*, vol. 80, no. 4, pp. 418–424, 2009.
- [24] J. P. Nolan, P. T. Morley, T. L. Vanden Hoek et al., "Therapeutic hypothermia after cardiac arrest. An advisory statement by the Advanced Life Support Task Force of the International Liaison Committee on Resuscitation," *Resuscitation*, vol. 57, no. 3, pp. 231–235, 2003.
- [25] R. R. Noppens, R. F. Kelm, R. Lindemann, K. Engelhard, C. Werner, and O. Kempfski, "Effects of a single-dose hypertonic saline hydroxyethyl starch on cerebral blood flow, long-term outcome, neurogenesis, and neuronal survival after cardiac arrest and cardiopulmonary resuscitation in rats," *Critical Care Medicine*, vol. 40, no. 7, pp. 2149–2156, 2012.
- [26] J. Hovdenes, J. H. Laake, L. Aaberge, H. Haugaa, and J. F. Bugge, "Therapeutic hypothermia after out-of-hospital cardiac arrest: experiences with patients treated with percutaneous coronary intervention and cardiogenic shock," *Acta Anaesthesiologica Scandinavica*, vol. 51, no. 2, pp. 137–142, 2007.
- [27] A. Kennedy and J. Soar, "Management of Glucose post cardiac arrest," <http://www.bestbets.org/bets/bet.php?id=1043>.
- [28] Y. M. Arabi, H. M. Tamim, and A. H. Rishu, "Hypoglycemia with intensive insulin therapy in critically ill patients: predisposing factors and association with mortality," *Critical Care Medicine*, vol. 37, no. 9, pp. 2536–2544, 2009.

- [29] G. van den Berghe, A. Wilmer, G. Hermans et al., "Intensive insulin therapy in the medical ICU," *The New England Journal of Medicine*, vol. 354, no. 5, pp. 449–461, 2006.
- [30] T. Oksanen, M. B. Skrifvars, T. Varpula et al., "Strict versus moderate glucose control after resuscitation from ventricular fibrillation," *Intensive Care Medicine*, vol. 33, no. 12, pp. 2093–2100, 2007.
- [31] H. Losert, F. Sterz, R. O. Roine et al., "Strict normoglycaemic blood glucose levels in the therapeutic management of patients within 12 h after cardiac arrest might not be necessary," *Resuscitation*, vol. 76, no. 2, pp. 214–220, 2008.
- [32] S. J. Finney, C. Zekveld, A. Elia, and T. W. Evans, "Glucose control and mortality in critically ill patients," *Journal of the American Medical Association*, vol. 290, no. 15, pp. 2041–2047, 2003.
- [33] F. M. Brunkhorst, C. Engel, F. Bloos et al., "Intensive insulin therapy and pentastarch resuscitation in severe sepsis," *The New England Journal of Medicine*, vol. 358, no. 2, pp. 125–139, 2008.
- [34] P. E. Marik and J. Varon, "Intensive insulin therapy in the ICU: is it now time to jump off the bandwagon?" *Resuscitation*, vol. 74, no. 1, pp. 191–193, 2007.
- [35] C.-Y. Hsu, C.-H. Huang, W.-T. Chang et al., "Cardioprotective effect of therapeutic hypothermia for postresuscitation myocardial dysfunction," *Shock*, vol. 32, no. 2, pp. 210–216, 2009.
- [36] X.-F. Ji, L. Yang, M.-Y. Zhang, C.-S. Li, S. Wang, and L.-H. Cong, "Shen-Fu injection attenuates postresuscitation myocardial dysfunction in a porcine model of cardiac arrest," *Shock*, vol. 35, no. 5, pp. 530–536, 2011.
- [37] W. Gu, C. Li, W. Yin, Z. Guo, X. Hou, and D. Zhang, "Shen-fu injection reduces postresuscitation myocardial dysfunction in a porcine model of cardiac arrest by modulating apoptosis," *Shock*, vol. 38, no. 3, pp. 301–306, 2012.
- [38] M.-Y. Zhang, X.-F. Ji, S. Wang, and C.-S. Li, "Shen-Fu injection attenuates postresuscitation lung injury in a porcine model of cardiac arrest," *Resuscitation*, vol. 83, no. 9, pp. 1152–1158, 2012.
- [39] W. Gu, C. Li, W. Yin, X. Hou, and D. Zhang, "Effects of shen-fu injection on the expression of t-cell-specific transcription factors t-bet/gata-3 in porcine postresuscitation lung injury," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 464650, 8 pages, 2013.
- [40] F. J. Yang, Y. S. Zheng, D. X. Li, and W. L. Deng, "Effect of Shenfu injection on micro-circulation," *Journal of Biomedical Engineering*, vol. 20, pp. 91–94, 2003 (Chinese).
- [41] G. van den Berghe, P. Wouters, F. Weekers et al., "Intensive insulin therapy in critically ill patients," *The New England Journal of Medicine*, vol. 345, no. 19, pp. 1359–1367, 2001.
- [42] T. Shan-shan, S. Min, and K. Wei, "Effect of insular phosphatidylinositol 3kinase on shenfu injection improving hyperglycemia in rabbits undergoing cardiopulmonary bypass," *Journal of Clinical Anaesthesiology*, vol. 26, no. 4, 2010.
- [43] S.-Y. Zheng, J. Sun, X. Zhao, and J.-G. Xu, "Protective effect of Shen-Fu on myocardial ischemia-reperfusion injury in rats," *American Journal of Chinese Medicine*, vol. 32, no. 2, pp. 209–220, 2004.
- [44] S. M. Krause, W. E. Jacobus, and L. C. Becker, "Alterations in cardiac sarcoplasmic reticulum calcium transport in the postischemic 'stunned' myocardium," *Circulation Research*, vol. 65, no. 2, pp. 526–530, 1989.
- [45] M. S. Kim and T. Akera, "O<sub>2</sub> free radicals: cause of ischemia-reperfusion injury to cardiac Na<sup>+</sup>-K<sup>+</sup>-ATPase," *The American journal of physiology*, vol. 252, no. 2, part 2, pp. H252–H257, 1987.
- [46] J. Luo, S. Min, K. Wei, and J. Cao, "Ion channel mechanism and ingredient bases of Shenfu Decoction's cardiac electrophysiological effects," *Journal of Ethnopharmacology*, vol. 117, no. 3, pp. 439–445, 2008.
- [47] L. Wiklund, H. S. Sharma, and S. Basu, "Circulatory arrest as a model for studies of global ischemic injury and neuroprotection," *Annals of the New York Academy of Sciences*, vol. 1053, pp. 205–219, 2005.
- [48] M. V. Cohen, "Free radicals in ischemic and reperfusion myocardial injury: is this the time for clinical trials?" *Annals of Internal Medicine*, vol. 111, no. 11, pp. 918–931, 1989.
- [49] K. H. Haider and W. H. Stimson, "Cardiac myofibrillar proteins: biochemical markers to estimate myocardial injury," *Molecular and Cellular Biochemistry*, vol. 194, no. 1-2, pp. 31–39, 1999.
- [50] H. Esterbauer, R. J. Schaur, and H. Zollner, "Chemistry and Biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes," *Free Radical Biology and Medicine*, vol. 11, no. 1, pp. 81–128, 1991.
- [51] S.-Y. Zhen, J.-G. Xu, and Z.-Z. Zhao, "The protective effect of shenfu injection on myocardium against ischemia reperfusion injury in rats," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 24, no. 6, pp. 541–544, 2004.
- [52] L. Chen, X.-Y. Lu, J. Li, J.-D. Fu, Z.-N. Zhou, and H.-T. Yang, "Intermittent hypoxia protects cardiomyocytes against ischemia-reperfusion injury-induced alterations in Ca<sup>2+</sup> homeostasis and contraction via the sarcoplasmic reticulum and Na<sup>+</sup>/Ca<sup>2+</sup> exchange mechanisms," *American Journal of Physiology. Cell Physiology*, vol. 291, no. 5, p. C1099, 2006.
- [53] R. B. Singh and N. S. Dhalla, "Ischemia-reperfusion-induced changes in sarcolemmal Na<sup>+</sup>/K<sup>+</sup>-ATPase are due to the activation of calpain in the heart," *Canadian Journal of Physiology and Pharmacology*, vol. 88, no. 3, pp. 388–397, 2010.
- [54] H. K. Saini-Chohan and N. S. Dhalla, "Attenuation of ischemia-reperfusion-induced alterations in intracellular Ca<sup>2+</sup> in cardiomyocytes from hearts treated with N-acetylcysteine and N-mercaptopyropionylglycine1," *Canadian Journal of Physiology and Pharmacology*, vol. 87, no. 12, pp. 1110–1119, 2009.
- [55] H. Gao, L. Chen, and H.-T. Yang, "Activation of  $\alpha$ 1B-adrenoceptors alleviates ischemia/reperfusion injury by limitation of mitochondrial Ca<sup>2+</sup> overload in cardiomyocytes," *Cardiovascular Research*, vol. 75, no. 3, pp. 584–595, 2007.
- [56] W.-H. Zhang, S.-B. Fu, F.-H. Lu et al., "Involvement of calcium-sensing receptor in ischemia/reperfusion-induced apoptosis in rat cardiomyocytes," *Biochemical and Biophysical Research Communications*, vol. 347, no. 4, pp. 872–881, 2006.
- [57] P. H. Sugden, "Ras, akt, and mechanotransduction in the cardiac myocyte," *Circulation Research*, vol. 93, no. 12, pp. 1179–1192, 2003.
- [58] B. D. Manning and L. C. Cantley, "AKT/PKB signaling: navigating downstream," *Cell*, vol. 129, no. 7, pp. 1261–1274, 2007.
- [59] S. V. Penumathsa, M. Thirunavukkarasu, S. M. Samuel et al., "Niacin bound chromium treatment induces myocardial Glut-4 translocation and caveolar interaction via Akt, AMPK and eNOS phosphorylation in streptozotocin induced diabetic rats after ischemia-reperfusion injury," *Biochimica et Biophysica Acta*, vol. 1792, no. 1, pp. 39–48, 2009.
- [60] F. Gao, E. Gao, T.-L. Yue et al., "Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation," *Circulation*, vol. 105, no. 12, pp. 1497–1502, 2002.

- [61] R. Schulz, M. Kelm, and G. Heusch, "Nitric oxide in myocardial ischemia/reperfusion injury," *Cardiovascular Research*, vol. 61, no. 3, pp. 402–413, 2004.
- [62] Y. Wu, Z.-Y. Xia, Q.-T. Meng et al., "Shen-fu injection preconditioning inhibits myocardial ischemia-reperfusion injury in diabetic rats: activation of eNOS via the PI3K/Akt pathway," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 384627, 9 pages, 2011.
- [63] E. Palojoki, A. Saraste, A. Eriksson et al., "Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 280, no. 6, pp. H2726–H2731, 2001.
- [64] L. Scorrano and S. J. Korsmeyer, "Mechanisms of cytochrome c release by proapoptotic BCL-2 family members," *Biochemical and Biophysical Research Communications*, vol. 304, no. 3, pp. 437–444, 2003.
- [65] Y.-L. Wang, C.-Y. Wang, B.-J. Zhang, and Z.-Z. Zhang, "Shenfu injection suppresses apoptosis by regulation of Bcl-2 and caspase-3 during hypoxia/reoxygenation in neonatal rat cardiomyocytes in vitro," *Molecular Biology Reports*, vol. 36, no. 2, pp. 365–370, 2009.

## Research Article

# Doinseunggitang Ameliorates Endothelial Dysfunction in Diabetic Atherosclerosis

Jung Joo Yoon,<sup>1,2</sup> Yun Jung Lee,<sup>1,2</sup> Ok Ju Park,<sup>3</sup> So Min Lee,<sup>1,2</sup> Yong Pyo Lee,<sup>1,2</sup>  
Nam Geun Cho,<sup>3</sup> Dae Gill Kang,<sup>1,2</sup> and Ho Sub Lee<sup>1,2</sup>

<sup>1</sup> College of Oriental Medicine and Professional Graduate School of Oriental Medicine, Wonkwang University, Shinyong-dong, Iksan 570-749, Jeonbuk, Republic of Korea

<sup>2</sup> Hanbang Body-fluid Research Center, Wonkwang University, Shinyong-dong, Iksan 570-749, Jeonbuk, Republic of Korea

<sup>3</sup> Department of Acupuncture & Moxibustion Medicine, Oriental Medicine hospital, Wonkwang University, Republic of Korea

Correspondence should be addressed to Dae Gill Kang; dgkang@wku.ac.kr and Ho Sub Lee; host@wku.ac.kr

Received 23 March 2013; Revised 23 May 2013; Accepted 23 May 2013

Academic Editor: Myeong S. Lee

Copyright © 2013 Jung Joo Yoon et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Atherosclerosis, a chronic and progressive disease characterized by vascular inflammation, is a leading cause of death in diabetes patients. Doinseunggitang (DYSGT), traditional prescription, has been used for promoting blood circulation to remove blood stasis. The aim of this study was to investigate the beneficial effects of DYSGT on endothelial dysfunction in diabetic atherosclerosis animal model. Apolipoprotein E knockout (ApoE KO) mice fed on a Western diet were treated with DYSGT (200 mg/kg/day). DYSGT significantly lowered blood glucose level and glucose tolerance as well as systolic blood pressure. Metabolic parameter showed that DYSGT markedly decreased triglyceride and LDL-cholesterol levels. In the thoracic aorta, the impairment of vasorelaxation response to acetylcholine and atherosclerotic lesion was attenuated by DYSGT. Furthermore, DYSGT restored the reduction of endothelial nitric oxide synthase (eNOS) expression, leading to the inhibition of intracellular adhesion molecule-1 (ICAM-1) and endothelin-1 (ET-1) expression. In conclusion, DYSGT improved the development of diabetic atherosclerosis via attenuation of the endothelial dysfunction, possibly by inhibiting ET-1, cell adhesion molecules, and lesion formation. Therefore, these results suggest that Korean traditional prescription Doinseunggitang may be useful in the treatment and prevention of diabetic vascular complications.

## 1. Introduction

Atherosclerosis and the associated cardiovascular disease (e.g., myocardial infarction, stroke, and peripheral vascular disease) are the principal cause of morbidity and mortality in diabetes patients. Atherosclerosis is defined as a chronic and progressive disease characterized by an inflammatory response of the arterial wall [1–3]. Vascular tone is an important factor in regulation of arterial blood pressure. Changes in vascular smooth muscle tone and the internal diameter of vessels can profoundly alter tissue perfusion and can impair the ability of arteries to respond to vasodilators and vasoconstrictors [4, 5]. The endothelium-dependent vasorelaxation that is induced by acetylcholine (ACh) is mediated by nitric oxide (NO), which acts through soluble guanylyl cyclase and cyclic GMP. Thus, this phenotypic change appears to result

from a decline in NO bioavailability due to impaired NO biosynthesis and inactivation of NO by superoxide, which leads to hypertension. These impaired vascular responses are also shown in hypercholesterolemia and obesity [6, 7].

Endothelin (ET-) 1 expression is significantly higher in aortic and mesenteric arteries of hypertensive animal models. Hypertensive patients with high plasma ET-1 levels often exhibit elevated cell adhesion molecule levels and increased risks for developing hypertension-induced organ damage [8]. One early phase of atherosclerosis involves the recruitment of inflammatory cells from the circulation and their endothelial migration [9]. This process is predominantly mediated by cellular adhesion molecules, which are expressed on the vascular endothelium and on circulating leukocytes in response to several inflammatory stimuli. Selectins and their ligands are involved in the rolling and tethering of leukocytes on

the vascular wall. Intracellular adhesion molecule-1 (ICAM-1) induces firm adhesion of inflammatory cells at the vascular surface [10].

Peroxisome proliferator activated receptors (PPARs) are a family of nuclear transcription factors, of which there are four members:  $\alpha$ ,  $\beta$ ,  $\gamma$ -1, and  $\gamma$ -2. PPAR- $\gamma$  plays an important role in regulating inflammatory, and PPAR- $\gamma$  agonist has been shown to reduce atherosclerosis in hypercholesterolemia or hyperplasia [11, 12]. It is highly expressed in all major cell types participating in atherosclerotic process to regulate transcription of a variety of genes encoding proteins involved in glucose and lipid metabolism.

Although atherosclerosis is not a distinguishing feature described in ApoE-deficient humans [13], ApoE-deficiency alone proved to be sufficient for aortic atherosclerotic plaques to develop in mice. In addition, high fat and cholesterol diet markedly accelerate plaque development in these mice [14]. The lesion development and plaque composition in ApoE KO mice are also similar to those in humans, establishing them as an excellent animal model for studying the pathogenesis of atherosclerosis.

Doinseunggitang (Taohe Chengqi Tang; Chinese) which is a traditional medicinal prescription has been used orally for promoting blood circulation to remove blood stasis. Doinseunggitang is one of the herbal mixtures documented in Shang Han Lun and this prescription is composed of *Glycyrrhizae uralensis* Fischer, *Rheum undulatum* Linne, *Prunus persica* L., and *Cinnamomum cassia* Presl. In clinic, Doinseunggitang has been documented to treat chronic hepatitis, amenorrhea, diabetes mellitus, acute necrotic enteritis, and chronic pyelonephritis [15]. However, the action mechanisms for this effectiveness of Doinseunggitang remained obscure. Here, we investigated the beneficial effects of Doinseunggitang (DYSGT) on vascular dysfunction in Western-diet-fed ApoE KO mice.

## 2. Methods

**2.1. Preparation of DYSGT.** The formula of DYSGT consists of five herbs including *Glycyrrhizae uralensis* Fischer (15 g), *Rheum undulatum* Linne (75 g), *Prunus persica* L. (37.5 g), and *Cinnamon cassia* Presl. (7.5 g) mixed and ground into a crude powder. The DYSGT (135 g) was boiled with 1 L of distilled water at 100°C for 2 h. The extract was centrifuged at 990  $\times$ g for 30 min at 4°C and resulting supernatant was lyophilized to produce a powder (12.45 g), which was then kept at -70°C until using this experiment.

**2.2. Experimental Animals.** Six-week-old male ApoE gene deficient C57BL6J mice (ApoE KO) and normal C57BL6J mice were obtained from Central Lab. Animal Inc. (Seoul, Republic of Korea) and housed in metabolic cages with an automatic temperature and relative humidity (22  $\pm$  2°C, 50~60%), and lighting (12 h light/dark cycle) condition. They were given free access to food and DW, and the consumptions were measured biweekly, respectively. They were fed a pelletized commercial chow diet for acclimatization for 2 weeks on arrival. After acclimatization, animals were randomly

TABLE 1: Anhydrous milk fat typically contains approximately 0.3% cholesterol. On this basis, D12079B contains approximately 0.21% cholesterol.

	gm %	kcal %
Protein	20	17
Carbohydrate	50	43
Fat	21	41
	Total kcal/gm	4.7 100
Ingredient	gm	kcal
Casein, 80 Mesh	195	780
DL-Methionine	3	12
Corn starch	50	200
Maltodextrin 10	100	400
Sucrose	341	1364
Cellulose	50	0
Milk fat, anhydrous*	200	1800
Corn oil	10	90
Mineral Mix S10001	35	0
Calcium carbonate	4	0
Vitamin Mix V10001	10	40
Choline bitartrate	2	0
Cholesterol, USP*	1.5	0
Ethoxyquin	0.04	0
Total	1001.54	4686

divided into four groups ( $n = 12$ ), namely, (1) the control group (C57BL6J mice + regular diet + DW), (2) ApoE KO control group (ApoE KO + Western diet + DW), (3) positive control group (ApoE KO + Western diet + rosiglitazone 10 mg/kg/day), (4) DYSGT group (ApoE KO + Western diet + DYSGT 200 mg/kg/day). The peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) agonist, rosiglitazone, was chosen as a positive control, which is an antidiabetic agent for the treatment of type 2 diabetes. The control and ApoE KO control groups received regular diet and Western diet, respectively, for 12 weeks. The Western diet (WTD) was purchased from Research Diets, Inc. (Table 1).

**2.3. Measurement of Blood Pressure (SBP).** SBP was determined using a noninvasive tail-cuff monitor (MK2000; Muromachi Kikai, Tokyo, Japan). At least eight determinations were made in every session, and the mean of the lowest five values within 5 mmHg was taken as the SBP level.

**2.4. Plasma Biochemical Analysis.** The concentration of glucose in blood was measured with whole blood sample obtained from vein of tail using a One Touch Ultra Blood Glucose Meter and Test Strip (Life Scan Inc., CA) at biweekly, respectively. Blood samples were taken by periorbital vein for biochemical analysis. Plasma insulin levels were measured based on ELISA method using commercial mice insulin ELISA kit (Shibayagi Co., Gunma, Japan). LDL cholesterol, total protein, triglyceride (TG), blood urea nitrogen (BUN)

levels in plasma were enzymatically measured using a commercially available kits (ARKRAY, Inc., Minami-ku, Kyoto, Japan).

**2.5. Quantitative Histopathology.** Aortae isolated from all groups were fixed in 10% (v/v) formalin in 50 mM potassium phosphate buffer (pH 7.0) for 48 h at 4°C. The tissues were subsequently embedded in paraffin and cross-sections (6  $\mu$ m) of the aortic arch in each group were stained by use of hematoxylin and eosin (H&E). For quantitative histopathologic comparisons, the mean of 10 sections was taken and intima-to-media ratio was determined by Axiovision 4 Imaging/Archiving software (Axiovision 4, Carl Zeiss, Germany).

**2.6. Measurement of Atherosclerotic Lesions by Oil Red O Staining.** Mice were euthanized, and thoracic and abdominal aorta were used for en face staining with Oil Red O to visualize neutral lipid (cholesteryl ester and triglycerides) accumulation. In brief, the aorta was removed, cleaned, and cut open with the luminal surface facing up and then immersion-fixed in 10% formalin in 10 mM phosphate-buffered saline. After rinsing with phosphate-buffered saline, the aorta was thoroughly cleaned of adventitial fat using microforceps and spring iris scissors under a stereoscopic microscope. The inner aortic surface was stained with Oil Red O for 25 min at room temperature after rinsing with 60% isopropyl alcohol and distilled water. Images of Oil red O stained aortas were taken with a Axiovision 4 Imaging/Archiving software (Axiovision 4, Carl Zeiss, Germany).

**2.7. Recording of Isometric Vascular Tone.** Vascular tone was determined as previously described by Kang et al. [16]. At the end of the experiment, mice were sacrificed by decapitation. The thoracic aorta was rapidly and carefully dissected and placed into ice-cold Krebs solution (118 mM NaCl, 4.7 mM KCl, 1.1 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.5 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, and 10 mM glucose, pH 7.4). The aortae were removed free of connective tissue and fat and cut into rings with a width of approximately 3 mm. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. The aortic rings were suspended by means of two L-shaped stainless steel wires inserted into the lumen in a tissue bath containing Krebs solution at 37°C. A gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> was continuously bubbled through the bath. The base line load placed on the aortic rings was 1.0 g. Changes in isometric tension were recorded using a Grass model FT 03 force displacement transducer (Grass Technologies, Quincy, MA) connected to a model 7E polygraph recording system (Grass Technologies). The aortic relaxation by the cumulative addition of ACh was performed in the presence of endothelium.

**2.8. Immunohistochemical Staining of ET-1, ICAM-1, and eNOS in Aortic Tissue.** Slides were immunostained by Invitrogen's Histostain-SP kits using the Labeled-Strept-avidin-Biotin (LAB-SA) method. Slides were immersed in 3% hydrogen peroxide for 1 min at room temperature to block endogenous peroxidase activity and rinsed with PBS. And

then, slides were incubated with 10% nonimmune goat serum for 20 min at room temperature to block nonspecific staining and incubated with a primary antibodies of ET-1, ICAM-1, and eNOS (Santa Cruz Biotechnology, Santa Cruz, CA) at a final dilution of 1:1000, in humidified chambers for overnight at 4°C. All slides were incubated with biotinylated secondary antibody for 20 min at room temperature and then incubated with horseradish-peroxidase-conjugated streptavidin for 20 min at room temperature, followed by detection with 3-amino-9-ethylcarbazole (AEC) as chromogen and counterstaining with hematoxylin (Zymed, CA). For the quantitative analysis, the average score of 10–20 randomly selected area was calculated using NIH Image analysis software, Image J (NIH, Bethesda, MD).

**2.9. Immunofluorescence Staining of ICAM-1 and ET-1 in Aortic Tissue.** Slides of the pancreatic frozen section were incubated with 10% nonimmune goat serum for 1 h at room temperature to block nonspecific staining, and incubated with primary antibodies of ICAM-1, and ET-1 (1:100, Santa Cruz Biotechnology) for overnight at 4°C. After washing, fluorescein-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated donkey anti-goat IgG (1:200, Molecular Probes, Carlsbad, CA) were incubated for 1 h at room temperature. After washing 3 times, the sections were mounted and observed by Olympus fluorescence microscopy. The expressions of insulin in aortic tissue were observed by Olympus microscopy equipped with an Olympus DP 70 camera. For the quantitative analysis, the average score of 10–20 randomly selected area was calculated using NIH Image analysis software, Image J (NIH, Bethesda, MD).

**2.10. Protein Preparation and Western Blot Analysis.** Thoracic aortae and muscle were homogenized in a buffer consisting of 250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM potassium phosphate buffer (pH 7.6). Large tissue debris and nuclear fragments were removed by two successive low-speed spins (3,500 rpm, 5 min; 8,000 rpm, 10 min, 4°C). The recovered protein (40  $\mu$ g) was separated by 10% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes using a Mini-Protean II apparatus (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat milk powder in 0.05% Tween 20-phosphate buffered saline (PBS-T) for 1 h prior to incubation in the presence of primary antibodies to ET-1, ICAM-1, PPAR- $\gamma$ , and  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA) at a final dilution of 1:1000 overnight at 4°C. The blot was washed several times with PBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. After the membrane was washed several times with PBST, the bound secondary antibody was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membrane using a ChemiDoc image analyzer (Bio-Rad).

**2.11. Statistical Analysis.** Values are shown as mean  $\pm$  S.E. Statistical analyses were performed using analysis of variance

TABLE 2: Effect of DYSGT on body weight, blood pressure, and plasma biomarker levels in ApoE KO mice.

	Body weight (g)		Systolic blood pressure (mmHg)		BUN (mg/dL)	LDL cholesterol ( $\mu\text{g}/\mu\text{L}$ )	TG (mg/dL)
	Start	Final	Start	Final			
	Parameter						
Cont.	23.09 $\pm$ 0.90	28.58 $\pm$ 0.98	103.92 $\pm$ 2.00	105.42 $\pm$ 1.80	0.67 $\pm$ 0.05	0.19 $\pm$ 0.02	44.92 $\pm$ 4.81
ApoE KO	22.76 $\pm$ 0.83	37.18 $\pm$ 0.88**	103.08 $\pm$ 2.69	118.92 $\pm$ 2.26**	1.32 $\pm$ 0.05*	1.02 $\pm$ 0.06**	102.42 $\pm$ 6.67**
Ros.	21.96 $\pm$ 0.44	35.3 $\pm$ 0.87	100.92 $\pm$ 2.04	105.58 $\pm$ 1.69##	0.60 $\pm$ 0.03#	0.84 $\pm$ 0.04#	51.75 $\pm$ 5.38##
DYSGT	21.92 $\pm$ 0.66	35.17 $\pm$ 1.32	100.33 $\pm$ 3.35	106.25 $\pm$ 2.52##	0.70 $\pm$ 0.04#	0.86 $\pm$ 0.03#	47.83 $\pm$ 5.55##

Data are mean  $\pm$  SE values ( $n = 12$ ); \* $P < 0.05$ , \*\* $P < 0.01$  versus control; # $P < 0.05$ , ## $P < 0.01$  versus ApoE KO. Cont.: control; ApoE KO: apolipoprotein E knockout; Ros: rosiglitazone; DYSGT: Doinseunggitang.

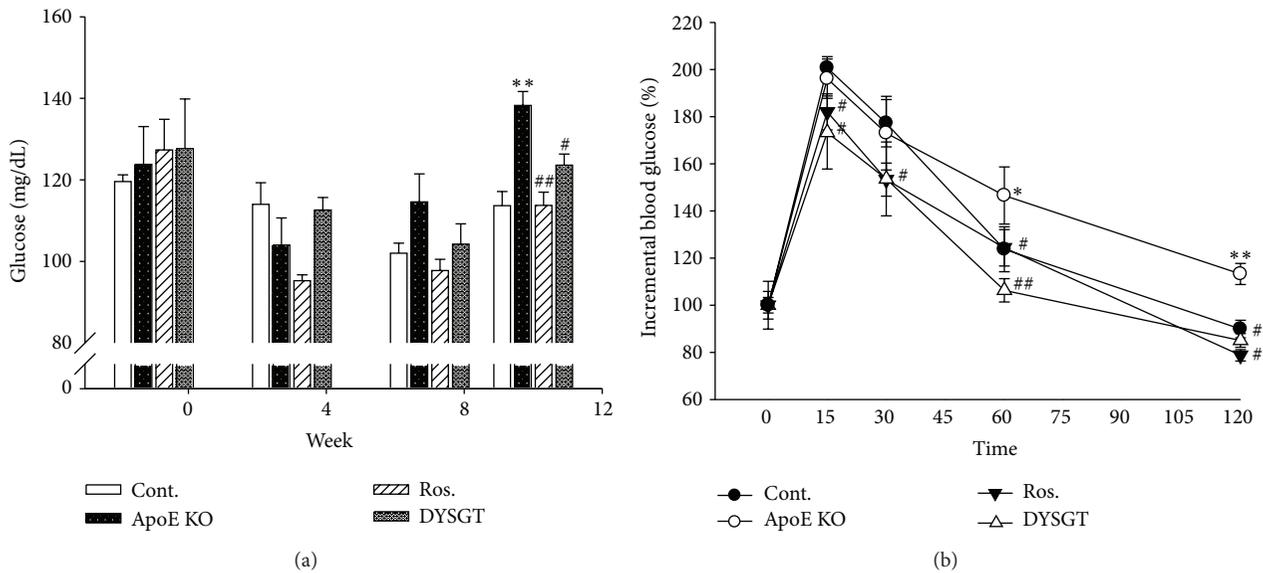


FIGURE 1: Effect of DYSGT on blood glucose levels (a) and glucose tolerance test (b) in ApoE KO mice. Values are expressed as mean  $\pm$  SE values ( $n = 12$ ); \* $P < 0.05$ , \*\* $P < 0.01$  versus cont.; # $P < 0.05$ , ## $P < 0.01$  versus ApoE KO.

followed by Student's  $t$ -test and one-way ANOVA. Differences with a value of  $P < 0.05$  were considered statistically significant.

### 3. Results

**3.1. Effect of DYSGT on Changes in Body Weight, Systolic Blood Pressure, and Plasma Biomarker Levels.** At time of sacrifice, mean body weight was as shown in Table 2, WTD-fed ApoE KO mice showed significantly increased body weight compared with RD-fed control mice. However, there were no differences of body weight among the WTD-fed ApoE KO mice groups. The systolic blood pressure (SBP) level after 12 weeks was significantly decreased in DYSGT-treated ApoE KO mice compared with the untreated WTD-fed ApoE KO mice ( $P < 0.01$ ). As similar in level, rosiglitazone-treated ApoE KO mice indicated a remarkable decrease in that level.

The BUN, LDL cholesterol, and TG levels of blood plasma were significantly increased in WTD-fed ApoE KO mice compared with RD-fed control mice. However, biochemical analysis of blood samples of ApoE KO mice showed that administration of DYSGT at a dose of 200 mg/kg/day resulted

in a significant decrease of BUN ( $1.32 \pm 0.05$  versus  $0.7 \pm 0.04$  mg/dL,  $P < 0.05$ ) in comparison with ApoE KO (nontreated) mice. Moreover the administration of DYSGT resulted in a significant decrease of LDL-cholesterol ( $1.02 \pm 0.06$  versus  $0.86 \pm 0.03$   $\mu\text{g}/\mu\text{L}$ ,  $P < 0.05$ ), and TG levels ( $102.42 \pm 6.67$  versus  $47.83 \pm 5.55$  mg/dL,  $P < 0.01$ ) when compared with ApoE KO mice. However, HDL-cholesterol level was not increased in DYSGT. Similarly, in Ros-treated ApoE KO mice, BUN, LDL cholesterol, and TG levels were significantly lower than those levels of ApoE KO mice.

**3.2. Effect of DYSGT on Blood Glucose and Glucose Tolerance Test Levels.** Fasted blood glucose level of untreated WTD-fed ApoE mice was significantly higher than that of the RD-control mice at the 12 weeks. Interestingly, the blood glucose levels were markedly reduced in DYSGT-treated ApoE KO mice ( $P < 0.05$ ) (Figure 1(a)). Glucose tolerance was significantly better in DYSGT-treated ApoE KO mice than in untreated WTD-fed ApoE mice, as shown by the much smaller rise in blood glucose in DYSGT-treated ApoE KO mice over the investigated 15 min time period following administration of 1 g/kg glucose (Figure 1(b)). Similarly,

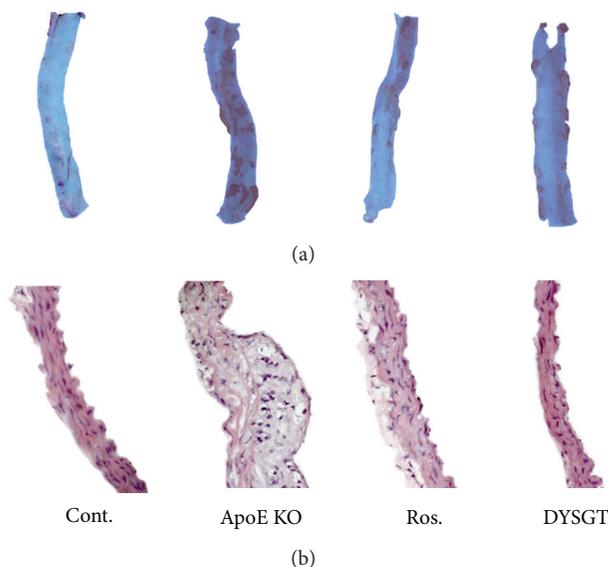


FIGURE 2: Atherosclerotic lesions in aortic root and aorta of ApoE KO mice. The aorta was obtained from mice fed a regular diet or western diet with or without oral administration of DYSGT (200 mg/kg/day) for 12 weeks ( $n = 6-8$ ). Representative photomicrographs of oil red O (pink color) staining (a) and H&E staining (b) in cross-sections of descending aorta (100x magnification).

these levels of Ros-treated ApoE KO mice were significantly decreased compared with untreated WTD-fed ApoE mice.

**3.3. Reduction in Lipid Accumulation in the Aorta and Aortic Valve.** To investigate whether DYSGT treatment could inhibit lipid accumulation in the aorta of ApoE KO mice fed a western diet, we assessed Oil Red O staining. Consistent with the change in lipid profile, western diet in ApoE KO mice induced lipid-rich plaque, whereas treatment with DYSGT significantly inhibited the development of atherosclerosis (Figure 2(a)). Microscopic examination of H&E staining revealed that atherosclerotic lesions such as roughened endothelial layers and fibrous cap formation were shown in WTD-fed ApoE KO mice. However, chronic treatment with DYSGT maintained the smooth and soft character of the tunica intima and decreased the intima-media thickness in aortic sections (Figure 2(b)).

**3.4. Effect of DYSGT on Changes in Vascular Tone of WTD-Fed ApoE KO Mice.** Vasorelaxation responses to ACh were measured in the thoracic aorta of WTD-fed ApoE KO mice (Figure 3(a)). Significant impairment of vasorelaxation was evident in thoracic aorta of WTD-fed ApoE KO mice compared with RD-fed control mice. DYSGT treatment restored the vasorelaxation response. On the other hand, the vasorelaxant response to sodium nitroprusside (SNP), a NO donor, was unchanged, and DYSGT and rosiglitazone did not affect this response (Figure 3(b)).

**3.5. Effect of DYSGT on Vascular Inflammation in Aortic Tissue.** The protein expression of ET-1, ICAM-1, and PPAR- $\gamma$  in the descending aortas of all groups of mice was examined by Western blot analysis. Expression of ET-1, ICAM-1 in WTD-fed ApoE KO mice was significantly increased compared with control mice fed RD. The group treated with DYSGT at 200 mg/kg/day showed a lower band intensity of ET-1 and ICAM-1 compared with untreated WTD-fed ApoE KO mice. Densitometric analysis indicated that DYSGT significantly decreased ET-1 (82.09%) and ICAM-1 (69.02%) protein expression compared with non-treated ApoE KO mice (100%) (Figure 4(a)). Conversely, the PPAR- $\gamma$  protein expression was suppressed in the aorta of WTD-fed compared with RD-fed control mice. Rosiglitazone and DYSGT treatment markedly restored PPAR- $\gamma$  expression levels by 1.0-fold and 0.73-fold (WTD-fed ApoE KO = 0.4-fold), respectively ( $P < 0.01$ ) (Figure 4(b)).

Figure 5 shows representative micrographs of ET-1, ICAM-1, and eNOS expression using immunohistochemistry. As a result, in the control group, expression of ET-1 was observed in blood vessel intima (atherosclerotic lesion) and in endothelium covering atherosclerotic lesion as well as in endothelium outside the lesion. DYSGT treatment markedly decreased the levels of ET-1 by 57%, respectively (Figures 5(a) and 5(d)). Similarly, ICAM-1 was significantly increased in WTD-fed ApoE KO mice. Treatment with rosiglitazone and DYSGT significantly decreased ICAM-1 expression by 48% and 67%. (Figures 5(b) and 5(d)). The eNOS expression was suppressed in the aorta of WTD-fed ApoE KO mice compared with RD-fed control mice (Figure 5(c)). DYSGT treatment markedly restored eNOS expression levels by 1.9-fold, respectively ( $P < 0.05$ ) (Figure 5(d)). Similarly, in WTD-fed ApoE KO mice, immunofluorescence of the aorta tissues showed that ICAM-1 and ET-1 expression were decreased in rosiglitazone and DYSGT groups compared with ApoE KO group (Figure 6).

## 4. Discussion

In the present study, we investigated the protective role of DYSGT in diabetic atherosclerosis using western diet-ApoE KO mice. In addition to the prevention of atherosclerosis, we clearly demonstrated improvement of endothelial dysfunction. Atherosclerosis is a consequence of chronic inflammation of the vessel wall [3]. One of the key events is the recruitment of leukocytes and the adhesion of platelets to the endothelium overlying the plaque [17-19]. The present study demonstrated that DYSGT significantly reduced blood glucose levels in WTD-fed ApoE KO mice. Glucose tolerance also was significantly better in DYSGT, suggesting a beneficial effect of insulin resistance. Thus, further study is required to clarify the activation of insulin signaling induced by DYSGT. Following administration of DYSGT for 12 weeks, there was no difference of body weight. However, it is clear that DYSGT markedly reduced LDL cholesterol, triglyceride levels. Consistent with increasing LDL cholesterol and triglyceride, lipid accumulation in the thoracic aorta was shown in WTD-fed ApoE mice. We suspected that western diet/hyperlipidemia-induced obesity is dependent of various administration

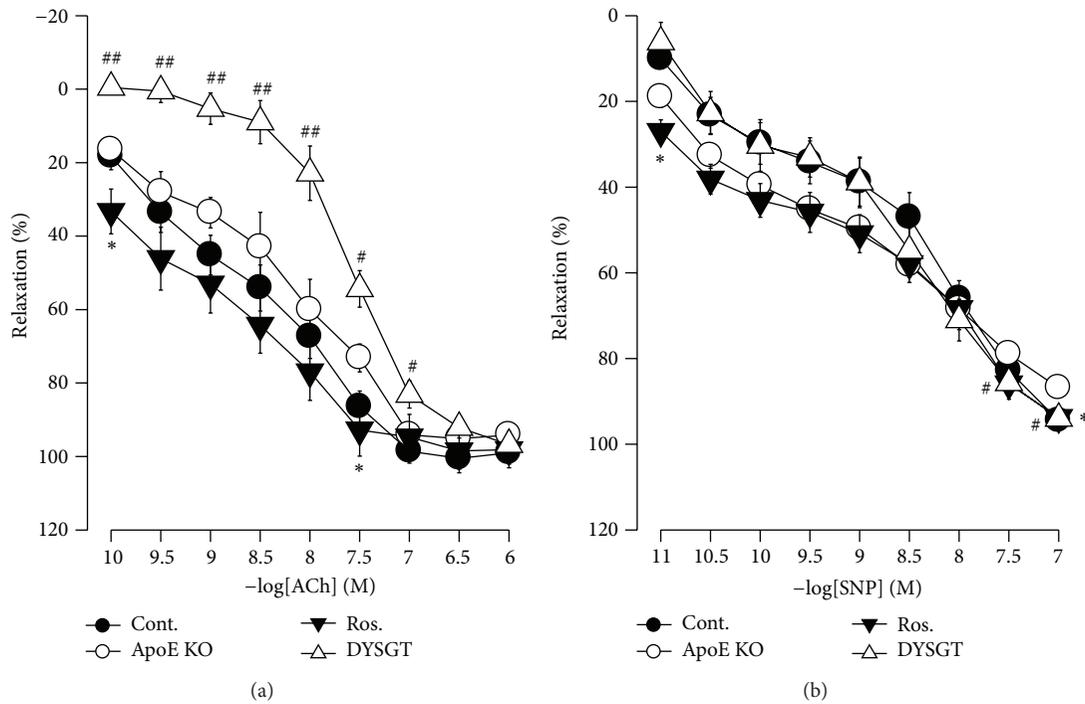


FIGURE 3: Effects of DYSGT on relaxation of thoracic aorta induced by (a) acetylcholine or (b) sodium nitroprusside (SNP) in WTD-fed ApoE KO mice. Data are mean  $\pm$  S.E. values ( $n = 5$ ). \*  $P < 0.05$ , versus cont.; #  $P < 0.05$ , versus ApoE KO.

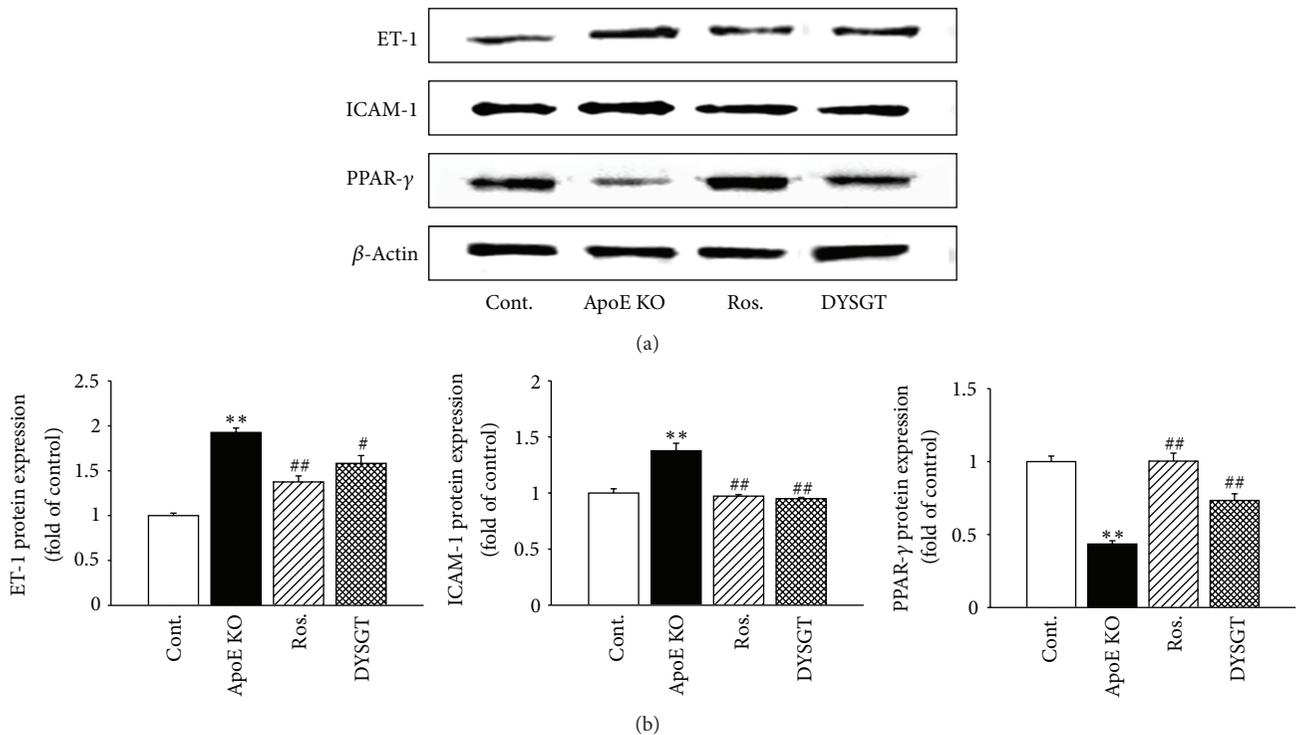


FIGURE 4: Effect of DYSGT on ET-1, ICAM-1, and PPAR- $\gamma$  protein expression in the aorta of ApoE KO mice. Western blots and corresponding densitometric analyses of ET-1, ICAM-1, and PPAR- $\gamma$  in aortic tissue. Values are expressed as mean  $\pm$  S.E. ( $n = 4$ ); \*\*  $P < 0.01$  versus cont.; #  $P < 0.05$ , ##  $P < 0.01$  versus ApoE KO.

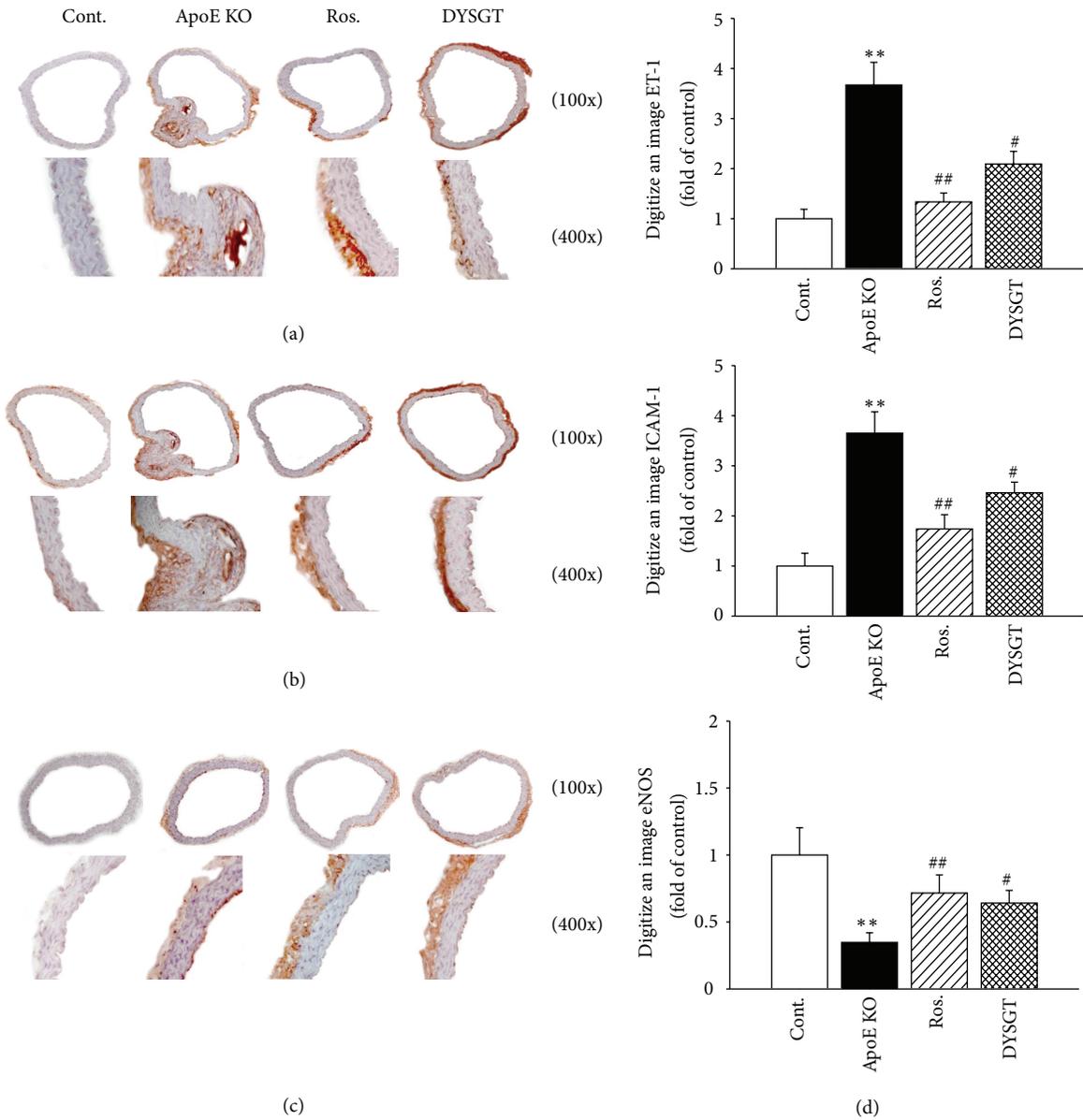


FIGURE 5: Effect of DYSGT on (a) ET-1, (b) ICMA-1, and (c) eNOS immunoreactivity in aorta of ApoE KO mice. Immunohistochemical staining of ET-1, ICMA-1, and eNOS in aorta from cont., ApoE KO mice, ApoE mice treated with rosiglitazone, and ApoE mice treated with DYSGT. (d) Quantitative analysis of ET-1, ICMA-1, and eNOS positive area, respectively. The average score of 5–8 randomly selected sites per section of aorta was calculated. Data expressed as mean  $\pm$  S.E.; \*\* $P < 0.01$  versus cont.; \* $P < 0.05$ , ## $P < 0.01$  versus ApoE KO. Original magnification, 100x and 400x.

periods (e.g., >12 weeks). At least, it is clear that DYSGT attenuated lipid accumulation and atherosclerotic lesions in the thoracic aorta. These results suggest that DYSGT is specific to vessel leading to antiatherosclerosis property.

The vascular endothelium, which lies between circulating blood and vascular smooth muscle and senses changes or abnormalities in blood flow and blood pressure, plays an important role in modulation of vascular tone [20–22]. In our results, blood pressure was determined using the tail-cuff technique. The mean SBP with 12 weeks of WTD-fed ApoE KO mice was significantly increased; however,

DYSGT significantly decreased this trend. In addition, WTD-fed ApoE KO mice also caused endothelial dysfunction as evidenced by decreased ACh-induced vascular tone and increased ET-1 expression. DYSGT exerted endothelium-dependent vasodilation in thoracic aortic smooth muscle. There were no significant differences of sodium SNP-induced dilation between DYSGT treated groups and the control group. These findings suggest that the hypotensive effects of DYSGT are mediated by ACh and further via the endothelium-dependent NO/cGMP pathway. In fact, other studies have also reported defective acetylcholine response

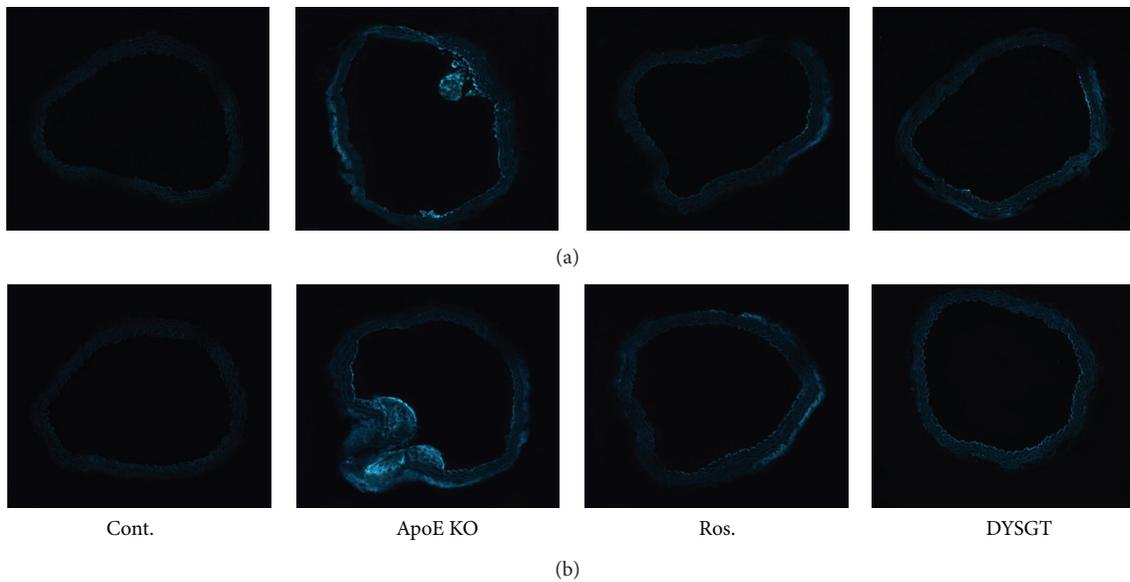


FIGURE 6: Immunofluorescence staining of (a) ICAM-1 and (b) ET-1 in the aorta. Representative histological sections are thoracic aorta of Cont., ApoE KO mice, ApoE mice treated with rosiglitazone, and ApoE mice treated with DYSGT incubated with anti-ICAM-1, anti-ET-1 antibodies, respectively. Original magnification: 100x.

without a corresponding change in SNP response in aortas of obese rats fed a high fat diet, and impaired relaxation of the aorta induced by acetylcholine but not SNP has been seen in obese Zucker rats as a consequence of endothelial dysfunction [23, 24]. It has been well documented that endothelium-dependent vascular relaxation is abnormal in both hypercholesterolemia and atherosclerosis because the ability of NO to maintain vascular tone is impaired [25, 26]. In addition, DYSGT reduced blood pressure via the inhibition of ET-1 expression. Thus, we suggest a protective role of DYSGT on vasoconstriction-mediated hypertension, and further progression to vascular dysfunction in diabetic atherosclerosis model.

Endothelial dysfunction will include not only reduced vasodilation but also vascular inflammation and atherosclerotic lesions [27, 28]. Blocking of inflammatory mediators can decrease the size of the atherosclerotic lesion. Adhesion molecules such as VCAM-1 and ICAM-1 play a significant role in the process of atherosclerosis as they ensure the recruitment of inflammatory cells. The study of ICAM-1 expression was reported by several authors in various models of atherosclerosis [29–31]. It has been shown that ICAM-1 is detected in the regions predisposed to atherosclerotic lesion formation in normocholesterolemic rabbits, and the expression of both molecules is upregulated by a high-cholesterol diet in rabbits and mice [29]. To examine the effect of DYSGT on vascular inflammation, adhesion molecule ICAM-1 expression was measured in the thoracic aorta. The WTD-fed ApoE KO mice had significantly increased levels of aortic expression of ICAM-1. However, this increase was significantly reduced by DYSGT. These data suggested that vascular inflammation is related to vasoconstriction; that is, eNOS-mediated NO production is required to defend

against diabetic atherosclerosis, especially vascular dysfunction. We previously reported that *Prunella vulgaris* exerts anti-inflammatory effect by inducing eNOS expression in vascular endothelial cell [32]. Furthermore, *Prunella vulgaris*-induced eNOS/NO expression is involved in repairment of vasodilation and vascular inflammation in db/db mice [33]. In fact, NO, an important physiological regulator of vascular homeostasis, is implicated in the pathophysiology of atherosclerosis [6, 7]. Recent study shows that both eNOS and nNOS significantly inhibit atherosclerosis in ApoE KO mice [34, 35]. Thus, we could not rule out the involvement of nNOS in the improvement of present animal model. Next study will be clarify the possible role of DYSGT in the activation of specific NOS for the involvement of vascular protection against diabetic atherosclerosis. Another defense system is PPAR- $\gamma$ , potent antidiabetic drug, since both eNOS and PPAR- $\gamma$  expression were recovered by DYSGT. Effect of DYSGT in type II diabetes is similar to rosiglitazone which is ameliorated in insulin sensitivity. However, recently reported side effect, weight gain or heart attack, is different although the present study did not show those effects [11, 12]. Thus, these finding suggested that DYSGT is a safe and potent traditional drug for the treatment of vascular dysfunction in diabetic atherosclerosis.

Doinseunggitang (DYSGT), traditional prescription, has been used for promoting blood circulation to remove blood stasis. In our study, treatment with DYSGT in WTD-fed ApoE KO mice reduced hypertension as well as insulin resistance. DYSGT also improved LDL cholesterol and triglyceride levels and reduced vascular inflammation. Thus, to our knowledge, this study provides the first evidence that Doinseunggitang apparent antihypertensive, hyperlipidemic, and antivascular inflammatory effects are in agreement with

traditional medicinal effect. Therefore, these findings, at least in part, indicate that Doinseunggitang protects vascular function against initiation and development of atherosclerosis.

## Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) (no. 2010-0029465).

## References

- [1] R. Ross, "Atherosclerosis—an inflammatory disease," *The New England Journal of Medicine*, vol. 340, no. 2, pp. 115–126, 1999.
- [2] P. Libby, P. M. Ridker, and G. K. Hansson, "Progress and challenges in translating the biology of atherosclerosis," *Nature*, vol. 473, no. 7347, pp. 317–325, 2011.
- [3] A. J. Lusis, "Atherosclerosis," *Nature*, vol. 407, no. 6801, pp. 233–241, 2000.
- [4] K. M. Ito, M. Okayasu, C. Koshimoto et al., "Impairment of endothelium-dependent relaxation of aortas and pulmonary arteries from spontaneously hyperlipidemic mice (*Apodemus sylvaticus*)," *Vascular Pharmacology*, vol. 47, no. 2-3, pp. 166–173, 2007.
- [5] T. Namikoshi, N. Tomita, M. Satoh et al., "Olmesartan ameliorates renovascular injury and oxidative stress in Zucker obese rats enhanced by dietary protein," *American Journal of Hypertension*, vol. 20, no. 10, pp. 1085–1091, 2007.
- [6] T. Kubozono, A. Koike, O. Nagayama et al., "High diastolic blood pressure during exercise is associated with hypercholesterolemia in patients with coronary artery disease," *International Heart Journal*, vol. 46, no. 1, pp. 79–87, 2005.
- [7] H. Nishimatsu, E. Suzuki, H. Satonaka et al., "Endothelial dysfunction and hypercontractility of vascular myocytes are ameliorated by fluvastatin in obese Zucker rats," *American Journal of Physiology*, vol. 288, no. 4, pp. H1770–H1776, 2005.
- [8] L. Li, Y. Chu, G. D. Fink, J. F. Engelhardt, D. D. Heistad, and A. F. Chen, "Endothelin-1 stimulates arterial VCAM-1 expression via NADPH oxidase-derived superoxide in mineralocorticoid hypertension," *Hypertension*, vol. 42, no. 5, pp. 997–1003, 2003.
- [9] F. Andreotti, I. Porto, F. Crea, and A. Maseri, "Inflammatory gene polymorphisms and ischaemic heart disease: review of population association studies," *Heart*, vol. 87, no. 2, pp. 107–112, 2002.
- [10] S. Blankenberg, S. Barbaux, and L. Tiret, "Adhesion molecules and atherosclerosis," *Atherosclerosis*, vol. 170, no. 2, pp. 191–203, 2003.
- [11] R. E. Law, W. P. Meehan, X. Xi et al., "Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia," *Journal of Clinical Investigation*, vol. 98, no. 8, pp. 1897–1905, 1996.
- [12] S. Theocharis, A. Margeli, P. Vielh, and G. Kouraklis, "Peroxisome proliferator-activated receptor- $\gamma$  ligands as cell-cycle modulators," *Cancer Treatment Reviews*, vol. 30, no. 6, pp. 545–554, 2004.
- [13] E. J. Schaefer, R. E. Gregg, and G. Ghiselli, "Familial apolipoprotein E deficiency," *Journal of Clinical Investigation*, vol. 78, no. 5, pp. 1206–1219, 1986.
- [14] S. Ishibashi, M. S. Brown, J. L. Goldstein, R. D. Gerard, R. E. Hammer, and J. Herz, "Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery," *Journal of Clinical Investigation*, vol. 92, no. 2, pp. 883–893, 1993.
- [15] Q. Chen, *Pharmacology and Application of Chinese Herbs*, SMC, Taipei, Taiwan, 1989.
- [16] D. G. Kang, J. K. Lee, D. H. Choi, E. J. Sohn, M. K. Moon, and H. S. Lee, "Vascular relaxation by the methanol extract of sorbus cortex via NO-cGMP pathway," *Biological and Pharmaceutical Bulletin*, vol. 28, no. 5, pp. 860–864, 2005.
- [17] R. Ross, "The pathogenesis of atherosclerosis: a perspective for the 1990s," *Nature*, vol. 362, no. 6423, pp. 801–809, 1993.
- [18] M. Gawaz, H. Langer, and A. E. May, "Platelets in inflammation and atherogenesis," *Journal of Clinical Investigation*, vol. 115, no. 12, pp. 3378–3384, 2005.
- [19] S. Zadelaar, R. Kleemann, L. Verschuren et al., "Mouse models for atherosclerosis and pharmaceutical modifiers," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 8, pp. 1706–1721, 2007.
- [20] N. F. Voelkel and R. M. Tuder, "Hypoxia-induced pulmonary vascular remodeling: a model for what human disease?" *Journal of Clinical Investigation*, vol. 106, no. 6, pp. 733–738, 2000.
- [21] H. G. Zecchin, F. B. M. Priviero, C. T. Souza et al., "Defective insulin and acetylcholine induction of endothelial cell-nitric oxide synthase through insulin receptor substrate/Akt signaling pathway in aorta of obese rats," *Diabetes*, vol. 56, no. 4, pp. 1014–1024, 2007.
- [22] A. H. Siddiqui and T. Hussain, "Enhanced AT1 receptor-mediated vasocontractile response to ANG II in endothelium-denuded aorta of obese Zucker rats," *American Journal of Physiology*, vol. 292, no. 4, pp. H1722–H1727, 2007.
- [23] H. G. Zecchin, F. B. M. Priviero, C. T. Souza et al., "Defective insulin and acetylcholine induction of endothelial cell-nitric oxide synthase through insulin receptor substrate/Akt signaling pathway in aorta of obese rats," *Diabetes*, vol. 56, no. 4, pp. 1014–1024, 2007.
- [24] A. H. Siddiqui and T. Hussain, "Enhanced AT1 receptor-mediated vasocontractile response to ANG II in endothelium-denuded aorta of obese Zucker rats," *American Journal of Physiology*, vol. 292, no. 4, pp. H1722–H1727, 2007.
- [25] H. Takase, P. Morcau, C. F. Küng, E. Nava, and T. F. Lüscher, "Antihypertensive therapy prevents endothelial dysfunction in chronic nitric oxide deficiency: effect of verapamil and trandolapril," *Hypertension*, vol. 27, no. 1, pp. 25–31, 1996.
- [26] M. Gervais, S. Pons, A. Nicoletti, C. Cosson, J. Giudicelli, and C. Richer, "Fluvastatin prevents renal dysfunction and vascular NO deficit in apolipoprotein E-deficient mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 2, pp. 183–189, 2003.
- [27] Q. N. Diep, F. Amiri, R. M. Touyz et al., "PPAR $\alpha$  activator effects on Ang II-induced vascular oxidative stress and inflammation," *Hypertension*, vol. 40, no. 6, pp. 866–871, 2002.
- [28] D. H. Endemann and E. L. Schiffrin, "Nitric oxide, oxidative excess, and vascular complications of diabetes mellitus," *Current Hypertension Reports*, vol. 6, no. 2, pp. 85–89, 2004.
- [29] K. Iiyama, L. Hajra, M. Iiyama et al., "Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation," *Circulation Research*, vol. 85, no. 2, pp. 199–207, 1999.

- [30] H. Li, M. I. Cybulsky, M. A. Gimbrone Jr., and P. Libby, "An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium," *Arteriosclerosis and Thrombosis*, vol. 13, no. 2, pp. 197–204, 1993.
- [31] Y. Nakashima, E. W. Raines, A. S. Plump, J. L. Breslow, and R. Ross, "Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the apoE-deficient mouse," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 18, no. 5, pp. 842–851, 1998.
- [32] S. M. Hwang, Y. J. Lee, J. J. Yoon et al., "Prunella vulgaris suppresses HG-induced vascular inflammation via Nrf2/HO-1/eNOS activation," *International Journal of Molecular Sciences*, vol. 13, no. 1, pp. 1258–1268, 2012.
- [33] S. M. Hwang, J. S. Kim, Y. J. Lee et al., "Anti-diabetic atherosclerosis effect of Prunella vulgaris in db/db mice with type 2 diabetes," *The American Journal of Chinese Medicine*, vol. 40, pp. 937–951, 2012.
- [34] P. J. Kuhlencordt, R. Gyrko, F. Han et al., "Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice," *Circulation*, vol. 104, no. 4, pp. 448–454, 2001.
- [35] P. J. Kuhlencordt, S. Hötten, J. Schödel et al., "Atheroprotective effects of neuronal nitric oxide synthase in apolipoprotein E knockout mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 7, pp. 1539–1544, 2006.

## Review Article

# Oral *Panax notoginseng* Preparation for Coronary Heart Disease: A Systematic Review of Randomized Controlled Trials

Qinghua Shang,<sup>1,2</sup> Hao Xu,<sup>2</sup> Zhaolan Liu,<sup>3</sup> Keji Chen,<sup>2</sup> and Jianping Liu<sup>3</sup>

<sup>1</sup> Beijing University of Chinese Medicine, Beijing 100029, China

<sup>2</sup> Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>3</sup> Centre for Evidence-Based Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China

Correspondence should be addressed to Keji Chen; keji\_chen@yahoo.com and Jianping Liu; jianping\_l@hotmail.com

Received 22 April 2013; Revised 13 July 2013; Accepted 13 July 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Qinghua Shang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

This systematic review aims to evaluate current evidence for the benefit and side effect of oral *Panax notoginseng* preparation for coronary heart disease (CHD). We included 17 randomized clinical trials (17 papers and 1747 participants). Comparing with no intervention on the basis of conventional therapy, oral *Panax notoginseng* did not show significant effect on reducing cardiovascular events, but it could alleviate angina pectoris (including improving the symptoms of angina pectoris [RR 1.20; 95% CI 1.12 to 1.28; 7 trials,  $n = 791$ ], improving electrocardiogram [RR 1.35; 95% CI 1.19 to 1.53; 8 trials,  $n = 727$ ], decreasing the recurrence of angina pectoris [RR 0.38; 95% CI 0.16 to 0.94; 1 trials,  $n = 60$ ], duration of angina pectoris [RR -1.88; 95% CI -2.08 to -1.69; 2 trials,  $n = 292$ ], and dosage of nitroglycerin [MD -1.13; 95% CI -1.70 to -0.56; 2 trials,  $n = 212$ ]); oral *Panax notoginseng* had no significant difference compared with isosorbide dinitrate on immediate effect for angina pectoris [RR 0.96; 95% CI 0.81 to 1.15; 1 trial,  $n = 80$ ]. In conclusion, oral *Panax notoginseng* preparation could relieve angina pectoris related symptoms. However, the small sample size and potential bias of most trials influence the convincingness of this conclusion. More rigorous trials with high quality are needed to give high level of evidence, especially for the potential benefit of cardiovascular events.

## 1. Introduction

Coronary heart disease (CHD) is one of the most leading causes of morbidity and mortality in many countries with large economic and human burdens, and it accounts for 20% of overall mortality in the United State [1]. It is reported that Ischaemic heart disease is the second leading cause for males and the third leading cause of global burden of disease for females, accounting for 6.8% and 5.3% respectively [2]. Although the benefit of some conventional drugs, such as aspirin and statin, have been demonstrated in reducing CHD mortality, annually 17.3 million people die from cardiovascular disease (CVD) worldwide (WHO 2008), and over 80% of CVD deaths take place in low and middle income countries, it is reported that by 2030 more than 23 million people will die annually from CVDs [3].

In recent years, traditional medicines have been playing more and more important roles in the maintenance of health, the prevention and treatment of diseases, and

plant-based drug discovery [4–8]. Chinese herbal medicine or its products have been administered widely for treating CHD in China. There are more than one hundred kinds of patent herbal medicine for CHD available at present. Puerarin injection [9], Danshen preparations [10], Tongxinluo [11], compound salvia pellet [12], Suxiao jiuxin wan [13] or traditional Chinese herbal products [14] have been shown as potential benefits recently by systematic reviews. Sanqi is one of the most widely used herbal medicines in China, with function of invigorating the blood circulation according to TCM theory. *Panax notoginseng* was the active and effective component purified from sanqi. Oral *Panax notoginseng* products included xuesaitong capsule, xuesaitong dripping pills, xuesaitong pill, xuesaitong effervescent tablet, xuesaitong granule, xuesaitong dispersible tablet, sanqishutong capsule, *Panax notoginseng* saponins (PNS) tablet and PNS capsule. The content of *Panax notoginseng* varies in different agents. All of the agents have been used in clinic for patients with CHD for decades of years. Recent researches found

its antioxidative [15], antiatherogenic, lipid-lowering, and anti-inflammatory [16] effects and angiogenic effect [17]. A Cochrane systematic review indicated that *Panax notoginseng* was effective in preventing stroke [18]. Some recent clinical trials also proved that it could benefit CHD patients [19, 20]. Therefore, this systematic review aims to evaluate the safety and effectiveness of oral *Panax notoginseng* preparations for CHD patients.

## 2. Method

**2.1. Inclusion Criteria.** We included randomized controlled trials (RCTs) or cross-over trials in English and Chinese regardless of publication type in this review. Quasirandomized trials were excluded and the first stage of data was used if it was cross-over trial. Any adult participant with CHD meeting with at least one of the current or past definitions or guidelines of CHD (including acute coronary syndrome (ACS) and X syndrome) was considered. Those who did not introduce diagnostic criteria in the text but stated patients with definite CHD were also included. The trial was included if oral *Panax notoginseng* preparation was in intervention group regardless of dosage, treatment course, and agents; trials should be excluded if there were other Chinese herbal medicines in intervention group; trials also should be excluded if there was a combination of *Panax notoginseng* preparation and a kind of western medicine on the basis of control group. Chinese herbal injection should be excluded in this review. Placebo, no intervention, or nitrate was considered in control group, Chinese herbal medicine in control group should be excluded. Oral *Panax notoginseng* preparations versus conventional therapy (except for nitroglycerin) were excluded for limited extension.

Outcome measures include primary outcomes: all cause mortality, cardiovascular events (e.g., CHD mortality, incidence of myocardial infarction (MI), revascularization, and rehospitalization for unstable angina); secondary outcomes: quality of life, attack of angina pectoris (measuring by recurrence of angina pectoris, frequency of angina pectoris, duration of angina pectoris, dosage of nitroglycerin, decrement of nitroglycerin, efficacy of angina pectoris, and others), electrocardiogram (ECG), and adverse events. We defined the efficacy of angina pectoris as improvement was more than 50%; the efficacy of ECG as elevation of ST segment was more than 0.05 mv.

**2.2. Search Strategy.** Two review authors (Qinghua Shang, Hao Xu) searched the following databases up to January 2013 independently for the identifications of trials (publication or nonpublication): the Cochrane Library, Pubmed, Chinese Biomedical database (CBM), China National Knowledge Infrastructure (CNKI), Chinese VIP Information (VIP), and Wanfang databases. We used the terms as follows: coronary heart disease, CHD, coronary artery disease, angina pectoris, myocardial infarction, acute coronary syndrome, cardi\*, sanqi, sanchi, jinbuhuan, tiansanqi, tianqi, panlongqi, tongpitiegu, xueshancao, liuyuelin, xuesaitong, xueshuantong,

notoginseng, pseudoginseng, *Panax notoginseng*, ginsenosides *Panax*, sanchinoside, and so forth. Because of different characteristics of various databases, MeSH terms and free text terms were used regardless of the report types in full text, title, keyword, subject terms, or abstract.

**2.3. Data Extraction and Quality Assessment.** Two review authors (Qinghua Shang, Hao Xu) independently extracted data according to a data extraction form made by the authors. Disagreements were resolved by consensus or consultation from a third reviewer (Jianping Liu or Zhaolan Liu). The methodological quality of trials was assessed independently using criteria from the Cochrane Handbook for Systematic Review of Interventions, Version 5.0.1 (Qinghua Shang, Hao Xu) [15]. The items included random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other biases. We judged each item from three levels ("Yes" for a low of bias, "No" for a high risk of bias, and "Unclear" otherwise), and then we assessed the trials and categorized them into three levels: low risk of bias (all the items were in low risk of bias), high risk of bias (at least one item was in high risk of bias), and unclear risk of bias (otherwise).

**2.4. Data Synthesis.** We used Revman 5.1 software provided by the Cochrane Collaboration for data analyses. Studies were stratified by the types of comparisons. We will express dichotomous data as risk ratio (RR) and its 95% confidence intervals (CI). Continuous outcome will be presented as mean difference (MD) and its 95% CI. Heterogeneity was recognized significant when  $I^2 \geq 50\%$ . Fixed effects model was used if there is no significant heterogeneity of the data; random effects model was used if significant heterogeneity existed ( $50\% < I^2 < 85\%$ ). Sensitive analysis would be used if there was any heterogeneity (including differences of clinical characteristics among trials and the statistical heterogeneity); subgroup analysis would be used in patients prescribed Xuesaitong softy capsule. Publication bias was explored using a funnel plot.

## 3. Results

**3.1. Description of Included Trials.** 17 RCTs (17 papers) [19, 21–36] were included. All of the papers were published in Chinese and 2 were in postgraduate dissertations (unpublished study) [23, 24]. The whole process of trials selection was demonstrated in Figure 1. The characteristics of included trials were listed in Table 1.

1747 Participants were included (864 in the intervention group and 883 in the control group). 906 males and 581 females were included in 17 trials (two of the trials did not report the number in each gender group). A total of 7 criteria of CHD (including ACS) were involved. 5 trials [21, 27, 30, 33, 36] did not introduce criteria of CHD but mentioned that "patients with CHD were eligible to be included." One

TABLE 1: Characteristics of trials.

Study ID	Type of CHD and syndrome	Members (I/C)	Age	Gender (M/F)	Interventions group	Control group	Product	Outcome evaluation
Du and Chen 2009 [21]	UA	56/56	58.8 ± 9.2	62/50	C + Xuesaitong softy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (aspirin, β blocker agent, nitroglycerin, CCB, low molecular heparin 5–7 days, antihypertensive drugs and medicine used to treat 2 diabetes)	Xuesaitong softy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China, Z19990022, containing PNS 60 mg/capsule)	Angina pectoris (extension, frequency, duration), dosage of nitroglycerin, Ads.
Ge and Zhao 2010 [22]	UA	48/48	I: 56; C: 54 (in average)	I: 22/26 V: 25/23	C + Xuesaitong softy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (aspirin, β blocker agent, nitroglycerin, CCB, low molecular heparin 5–7 days, antihypertensive drugs and medicine used to treat 2 diabetes)	Xuesaitong softy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming China, Z19990022, containing PNS 60 mg/capsule)	Angina pectoris reliefement, ECG, Ads.
Han and Chen 2008 [23]	PCI patients	30/30	I: (64.1 ± 10.8); C: (63.7 ± 11.7)	I: 23/7; C: 21/9	C + Xuesaitong softy capsule, 2 capsules, BID in the first 2 weeks, then 1 capsule, TID, 12 weeks	Conventional therapy (anticoagulant agent, antiplatelet agent, medicine for modifying blood lipid, antihypertensive drug and medicine used to treat 2 diabetes)	Xuesaitong softy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China, Z19990022, containing PNS 60 mg/capsule)	Angina pectoris, rehospitalization
Ji and Zhang 2003 [24]	UA	30/90	II: (69.0 ± 7.5); I2: (69.2 ± 6.0); I3: (68.5 ± 5.4); C: (68.7 ± 7.3)	II: 20/10; I2: 18/12; I3: 21/9; C: 17/13	II: C + coarse power 1 g, TID; I2: C + semi-micron power 1 g, TID I3: C + micron power 1 g, TID	Isosorbide Mononitrate 20 mg BID; Aspirin 75 mg QD; Metoprolol 25 mg BID; D/TZ 30 mg, TID or QID; Plendil 5 mg QD or BID or Acertil 4 mg, QD for hypertension; Nitroglycerol 0.5 mg sublingual administration or nitroglycerol injection 10 mg, iv.	<i>Panax notoginseng</i> coarse power: WF-2000 pulverizer; <i>Panax notoginseng</i> micron power: BFM-6 pulverizer; <i>Panax notoginseng</i> semi-micron power: BFM-6 pulverizer and starch.	Efficacy of Angina pectoris, ECG, symptoms, Ads
Liu et al. 2008 [19]	UA and BSS	30/30	I: (64.6 ± 5.4); C: (63.6 ± 4.5)	Unclear	C + Xuesaitong softy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (no detail)	Xuesaitong softy capsule (Yunnan weihe Pharmaceutical company, containing PNS 60 mg/capsule)	Syndrome, pulse, heart rate, heart rhythm, blood pressure, angina pectoris, ECG

TABLE 1: Continued.

Study ID	Type of CHD and syndrome	Members (I/C)	Age	Gender (M/F)	Interventions group	Control group	Product	Outcome evaluation
Meng 2003 [25]	UA and SA	60/20	I: (61–78); C: (61–78)	I: 44/16 C: 16/4	PNS pill, 2 tablets, sublingual when angina pectoris attacked	Isorbide dinitrate when angina pectoris attacked (5 mg/tables)	PNS pill, 2 tablet*, Sublingual	Duration of angina pectoris reliefment, blood pressure, heart rate, and ECG after 2 hours of prescription.
Song et al. 2005 [26]	Unclear	50/50	I: (36–77), (61.21 ± 5.73); C: (38–74), (60.77 ± 5.61) in average	I: 31/19 C: 33/17	C + Xuesaitong sofy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (aspirin, β blocker agent, nitroglycerin, CCB, low molecular heparin 5–7 days, antihypertensive drugs and medicine used to treat diabetes)	Xuesaitong sofy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China)	Efficacy of angina pectoris, ECG, dosage of nitroglycerin
Wan 2011 [27]	UA	26/26	I: 65.7 in average; C: No report	I: 15/11 C: 13/13	C + Xuesaitong sofy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (aspirin, β blocker agent and et al.)	Xuesaitong sofy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China)	Efficacy of angina pectoris, ECG
Wang et al. 2009 [28]	UA	100/100	36–75	Unclear	T1: C1 + Xuesaitong sofy capsule, 2 capsules, BID, 30 days; T2: C2 + trimetazidine + Xuesaitong sofy capsule, 2 capsules, BID, 30 days;	C1: conventional therapy (ant platelet, Nitrates, CCB, β blocker agent, statin, trimetazidine); C2: Conventional therapy (ant platelet, Nitrates, CCB, β blocker agent, astatine)	Xuesaitong sofy capsule	Efficacy of angina pectoris and cardiovascular events in 30 d followup.
Wei 2010 [29]	Unclear	90/90	60.4 ± 3.5	113/67	C + Xuesaitong sofy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (Nitrate, β blocker agent, CCB, low molecular heparin)	Xuesaitong sofy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China)	Angina pectoris, Ads, ECG
Yan 2005 [30]	Unclear	24/24	I: (48–67), 60 in average; C: (47–69), 62 in average	I: 13/11 C: 14/10	Isorbide mononitrat 5 mg TID + <i>Panax notoginseng</i> power 6 g BID, 7 days	Isorbide Mononitrate, 10 mg, TID	<i>Panax notoginseng</i> power 6 g BID	Efficacy of angina pectoris, ECG, ADs
Yu 2010 [31]	UA	50/50	I: (64.18 ± 12.13); C: (62.8 ± 10.8)	I: 29/21 C: 28/22	C + Xuesaitong sofy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (aspirin, β blocker agent, nitroglycerin, CCB and et al.)	Xuesaitong sofy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China)	Efficacy of angina pectoris, ECG, ADs, cardiovascular events
Zhou and Bai 2009 [32]	Unclear	43/43	65 ± 6	I: 32/11 C: 34/9	C + Xuesaitong sofy capsule, 2 capsules, TID, 4 weeks	Conventional therapy (nitrate, Metoprolol, aspirin, Nitroglycerin if necessary)	Xuesaitong sofy capsule <sup>A</sup> (Luotai, Kunming Pharmaceutical incorporated corporation)	Efficacy of angina pectoris, ECG

TABLE 1: Continued.

Study ID	Type of CHD and syndrome	Members (I/C)	Age	Gender (M/F)	Interventions group	Control group	Product	Outcome evaluation
Kuang et al. 2011 [33]	UA	90/90	I: (56.3 ± 6.9); C: (57.1 ± 7.2)	I: 47/43 C: 46/44	C + Xuesaitong softy capsule, 2 capsules, BID, 4 weeks	Conventional therapy (aspirin, β blocker agent, nitroglycerin, CCB, low molecular dextran, and others)	Xuesaitong softy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China, containing PNS 60 mg/capsule)	Efficacy of angina pectoris, ECG, ADs
Bao 2011 [34]	SA, BSS	63/64	I: (52.3 in average); C: (51.6 in average)	I: 35/28 C: 37/27	C + Sanqi guanxinning tablets (Z53020028), 2–4 tables, TID, 6 weeks	Conventional therapy (nitroglycerin, β blocker agent, and others)	Sanqi guanxinning tablets <sup>ΔΔ</sup> (Z53020028)	Efficacy of angina pectoris
Zhao and Li 2012 [35]	SA	60/58	I: (57.4 ± 9.9, 42–70); C: (59.6 ± 9.7, 41–68)	I: 38/22 C: 40/18	C + Xuesaitong softy capsule, 2 capsules, TID, 4 weeks	Conventional therapy (aspirin, J20080078, 100 mg) Qd, isosorbide mononitrate (H20030418, 60 mg Qd), β blocker (H32025391)	Xuesaitong softy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China, ZI9990022)	Efficacy of ECG
Yang 2012 [36]	Unclear	14/14	(67.3 ± 1.1), 51–78	19/9	C + Xuesaitong softy capsule, 2 capsules BID in the first two weeks, 1 capsule BID in the later weeks	Conventional therapy (aspirin, β blocker agent, nitroglycerin, CCB, and others)	Xuesaitong softy capsule* (Shenghuo Pharmaceutical Holdings Yunnan kunming, China, containing PNS 60 mg/capsule)	Frequency of angina pectoris, dosage of nitroglycerin, frequency of premature ventricular contraction

BSS: blood stasis syndrome; PNS: *panax notoginseng* saponins; I: intervention group; C: control group; DTZ: diltiazem; ECG: electrocardiogram; Ads: adverse event.

\*Xuesaitong softy capsule produced by Shenghuo Pharmaceutical Holdings, Yunnan kunming, China (ZI9990022) contains PNS 60 mg/capsule.

\*\*There was no purity of PNS pill in this trial. According to the internet, PNS pill produced by Yunnan Weihe Pharmaceutical company contains PNS 50 mg/pill.

<sup>Δ</sup>Xuesaitong softy capsule<sup>Δ</sup> (Luotai, Kunming Pharmaceutical incorporated corporation, China) contains PNS 100 mg/capsule.

<sup>ΔΔ</sup>Sanqi guanxinning tablets (Z53020028), there is no introduction in the paper about the composition and the purity. According to the internet, Sanqi guanxinning produced by Yunnan JinBuFuan (group) Co., ltd. pharmaceutical branch, containing 100 mg PNS/tablet.

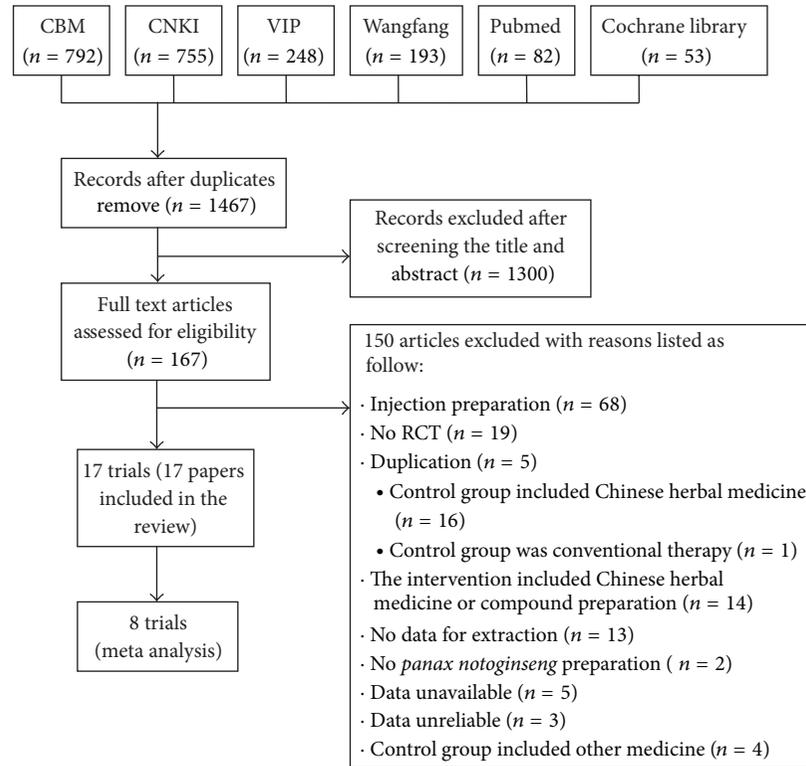


FIGURE 1: The process of included and excluded studies.

trial [23] included patients who need to take percutaneous coronary intervention (PCI) the next day; 8 trials [19, 21, 22, 24, 27, 28, 31, 33] included patients with unstable angina; 2 trials [34, 35] included patients with stable angina pectoris; 1 trial [25] included patients with either stable angina or unstable angina; the other 5 trials [26, 29, 30, 32, 36] did not introduce the types of CHD, but two of them recruited hospitalized patients [29, 32].

Patients in 11 trials [19, 21–23, 26–29, 31, 33, 36] were prescribed Xuesaitong softy capsule 2 tablet (120 mg, 60 mg *Panax notoginseng* Saponins [PNS] in each capsule) BID (regulation was conducted for the course); patients in 2 trials [32, 35] were prescribed Xuesaitong softy capsule 2 tablet (120 mg, 60 mg PNS in each capsule) TID; patients in 1 trial [34] were prescribed Sanqi guanxinning pills 2–4 pills (100 mg PNS in each pill) TID; 1 trial [25] prescribed PNS tablets 2–4 pill (50 mg PNS in each pill) TID, oral administration or sublingual administration, 2 trials [26, 30] used sanqi power (the purity is unclear) in the treatment group. The treatment course of treatment ranged from 7 days to 6 months.

There were 2 comparisons in the review according to various control groups: (1) *Panax notoginseng* preparations and conventional therapy versus conventional therapy (15 trials) [19, 21–24, 26–29, 31–36]; (2) *Panax notoginseng* preparations and conventional therapy versus nitrates and conventional therapy (2 trials) [25, 30]. Two trials [24, 28] were designed as three groups and four groups, respectively. Wang et al. [28] designed three groups with 2 comparisons: *Panax notoginseng* preparations and conventional therapy versus

conventional therapy; *Panax notoginseng* preparations and trimethazine and conventional therapy versus conventional therapy; however, we extracted the data of first comparison for inclusion criteria. Ji and Zhang [24] designed four groups with 3 comparisons: *Panax notoginseng* coarse power and conventional therapy versus conventional therapy; *Panax notoginseng* semi-micron power and conventional therapy versus conventional therapy; *Panax notoginseng* micron power and conventional therapy versus conventional therapy; however, we summed up the three groups included *Panax notoginseng* as intervention group and conventional therapy as control group for data analysis.

**3.2. Methodological Quality of Included Trials.** According to the criteria introduced above, no trial was evaluated as low risk of bias. Only one trial of the 17 trials reported the method to generate the allocation sequence (random number table) [23]. Two trials were assessed as having adequate concealment (concealed letter cover) [19, 23]. No trial reported blinding method. One trial [31] reported the result of followup. No trial reported information on withdrawal/dropout. All of trials provided baseline data for the comparability among groups. The results of the assessment of risk of bias are presented in a “risk of bias summary” figure produced by Revman 5.1 automatically (Figure 2).

### 3.3. Effect Estimates of Outcomes (Tables 2 and 3)

**3.3.1. Cardiovascular Mortality.** There was only 1 trial [31] that reported the cardiovascular mortality in the comparisons of

TABLE 2: Analysis of cardiovascular events and angina pectoris.

Outcomes (comparisons)	Treatment group (n/N)	Control group (n/N)	RR	95% CI
(1) Cardiovascular mortality				
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Yu 2010 [31]	1/50	2/50	0.50	[0.05, 5.34]
(2) Myocardial infarction incidence				
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Wang et al. 2009 [28]	0/100	3/100	0.14	[0.01, 2.73]
Yu 2010 [31]	0/50	2/50	0.20	[0.01, 4.06]
	Overall all (FEM, $I^2 = 0\%$ )		0.17	[0.02, 1.37]
(3) Incidence of intractable angina pectoris				
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Wang et al. 2009 [28]	6/100	11/100	0.55	[0.21, 1.42]
(4) Rehospitalization incidence for unstable angina				
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Han and Chen 2008 [23]	1/30	3/30	0.33	[0.04, 3.03]
(5) Recurrence of angina pectoris				
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Han and Chen 2008 [23]	5/30	13/30	0.38	[0.16, 0.94]
(6) Nitroglycerol decrease				
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Ji and Zhang 2003 [24]	19/30	65/120	1.17	[0.85, 1.61]
Song et al. 2005 [26]	26/50	14/50	1.86	[1.11, 3.12]
	Overall all (REM, $I^2 = 59\%$ )		1.41	[0.89, 2.24]
(7) Angina pectoris relief				
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Ge and Zhao 2010 [22]	44/48	36/48	1.22	[1.02, 1.47]
Ji and Zhang 2003 [24]	24/30	77/120	1.25	[1.00, 1.56]
Wan 2011 [27]	24/26	19/26	1.26	[0.98, 1.64]
Wei 2010 [29]	84/90	75/90	1.12	[1.01, 1.25]
Yu 2010 [31]	48/50	43/50	1.12	[0.98, 1.27]
Zhou and Bai 2009 [32]	37/43	30/43	1.23	[0.98, 1.55]
Bao 2011 [34]	57/63	45/64	1.29	[1.08, 1.54]
	Overall all (FEM, $I^2 = 0\%$ , $N = 791$ )		1.20	[1.12, 1.28]
Subgroup analysis (excluded Ji and Zhang [24])	Overall (FEM, $I^2 = 0\%$ , $N = 641$ )		1.19	[1.11, 1.27]
(8) Electrocardiogram improvement				
15.1 <i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy				
Ge and Zhao 2010 [22]	42/48	36/48	1.17	[0.96, 1.42]
Ji and Zhang 2003 [24]	67/86	19/29	1.19	[0.89, 1.58]
Liu et al. 2008 [19]	12/30	8/30	1.50	[0.72, 3.14]
Song et al. 2005 [26]	36/50	27/50	1.33	[0.98, 1.82]
Wan 2011 [27]	19/26	12/26	1.58	[0.98, 2.55]
Yu 2010 [31]	28/50	19/50	1.47	[0.96, 2.27]
Zhou and Bai 2009 [32]	35/43	27/43	1.30	[0.99, 1.70]
Zhao and Li 2012 [35]	24/60	12/58	1.93	[1.07, 3.49]
	Overall all (FEM, $I^2 = 0\%$ , $N = 727$ )		1.35	[1.19, 1.53]
Subgroup analysis (excluded Ji and Zhang [24])	Overall (FEM, $I^2 = 0\%$ , $N = 612$ )		1.39	[1.21, 1.59]
15.2 <i>Panax notoginseng</i> preparation and conventional therapy versus isosorbide dinitrate and conventional therapy				
Meng 2003 [25]	19/60	8/20	0.79	[0.41, 1.52]
(9) Angina pectoris immediate effect				
<i>Panax notoginseng</i> preparation and conventional therapy versus isosorbide dinitrate and conventional therapy				
Meng 2003 [25]	52/60	18/20	0.96	[0.81, 1.15]

FEM: fixed effects model; REM: random effects model; RR: relative risk; CI: credibility interval.

TABLE 3: Analysis of efficacy of angina pectoris.

Angina pectoris (comparison)	Intervention group		Control group		Weight (%)	MD	95% CI
	Mean	SD	Mean	SD			
(1) Angina pectoris frequency							
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy (times/week)							
Du and Chen 2009 [21]	3.24	0.61	5.63	0.92	33.6	-2.39	[-2.68, -2.10]
Kuang et al. 2011 [33]	3.53	0.61	6.83	1.92	14.1	-3.30	[-3.72, -2.88]
Song et al. 2005 [26]	0.75	0.79	1.36	1.31	32.4	-0.61	[-1.03, -0.19]
Wei 2010 [29]	4.27	0.87	6.58	0.75	34.0	-2.31	[-2.55, -2.07]
	Overall (REM, $I^2 = 96%$ , $N = 572$ )				<b>100</b>	<b>-2.16</b>	<b>[-3.02, -1.30]</b>
Sensitive analysis (excluded Song et al. 2005 [26] Kuang et al. [33])	Overall (FEM, $I^2 = 0%$ , $N = 292$ )					<b>-2.34</b>	<b>[-2.53, -2.16]</b>
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy (times/day)							
Yang 2012 [36]	1.22	0.97	3.98	1.89	28	-2.76	[-3.87, -1.65]
(2) Angina pectoris duration (minute/time)							
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy							
Du and Chen 2009 [21]	2.86	0.72	4.82	0.63	60.7	-1.96	[-2.21, -1.71]
Kuang et al. 2011 [33]	2.23	0.62	4.78	0.83	45.4	-2.55	[-2.76, -2.34]
Wei 2010 [29]	4.56	1.08	6.32	1.05	39.3	-1.76	[-2.07, -1.45]
	Overall (REM, $I^2 = 91%$ , $N = 472$ )				100	-2.10	[-2.58, -1.62]
Sensitive analysis (excluded Kuang et al., [33])	Overall (FEM, $I^2 = 0%$ , $N = 292$ )					<b>-1.88</b>	<b>[-2.08, -1.69]</b>
(3) Dosage of nitroglycerol							
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy (mg/week)							
Du and Chen 2009 [21]	2.94	2.26	4.26	1.94	53.0	-1.32	[-2.10, -0.54]
Song et al. 2005 [26]	2.95	2.25	3.87	1.97	47.0	-0.92	[-1.75, -0.09]
	Overall (FEM, $I^2 = 0%$ , $N = 212$ )				100	-1.13	[-1.70, -0.56]
<i>Panax notoginseng</i> preparation and conventional therapy versus conventional therapy (mg/day)							
Yang 2012 [36]	1.3	0.4	5.4	2.8	100	-4.10	[-5.58, -2.62]

FEM: fixed effects model; REM: random effects model; MD: mean difference; CI: credibility interval.

*Panax notoginseng* preparations (Xuesaitong softy capsule) and conventional therapy versus conventional therapy with no significant difference between the two groups [RR 0.50; 95% CI 0.05 to 5.34; 1 trial,  $n = 100$ ]. In the followup of 4 months, 2 patients died of heart failure in the conventional therapy group and 1 patient died of arrhythmia in the combined therapy group.

**3.3.2. Incidence of Myocardial Infarction (MI).** There were 2 studies [28, 31] reporting MI incidence in one comparison. Compared with no intervention on the basis of conventional therapy, *Panax notoginseng* preparations (Xuesaitong softy capsule) showed no significant reduction of incidence of MI (RR 0.17; 95% CI 0.02 to 1.37; 2 trials,  $n = 300$ ) [28, 31].

**3.3.3. Incidence of Intractable Angina Pectoris.** One trial [28] reported the intractable angina pectoris in 2 different comparisons. In the comparisons of *Panax notoginseng* preparation (Xuesaitong softy capsule) and conventional therapy versus conventional therapy, *Panax notoginseng* preparation (Xuesaitong softy capsule) showed no significant difference (RR 0.55; 95% CI 0.21 to 1.42; 1 trial,  $n = 200$ ) in controlling intractable angina pectoris.

**3.3.4. Rehospitalization for Unstable Angina.** There was 1 trial [23] reporting rehospitalization. Compared with no treatment on the basis of conventional therapy, *Panax notoginseng* preparation (Xuesaitong softy capsule) showed no significant difference in the number of rehospitalization (RR 0.33; 95% CI 0.04 to 3.03; 1 trial,  $n = 60$ ).

**3.3.5. Recurrence of Angina Pectoris.** One trial [23] reported recurrence of angina pectoris. Compared with no treatment on the basis of conventional therapy, *Panax notoginseng* preparation (Xuesaitong softy capsule) showed significant difference in reducing recurrence of angina pectoris (RR 0.38; 95% CI 0.16 to 0.94; 1 trial,  $n = 60$ ).

**3.3.6. Reduction of Nitroglycerin.** The definition of successful nitroglycerin reduction was that the patients in the trial stopped using nitroglycerin or the dosage of nitroglycerin was cut off more than 50% after the trial. Two trials [24, 30] reported the condition of nitroglycerin. The results showed no significant improvement of *Panax notoginseng* preparation comparing with no treatment on the basis of conventional therapy (RR 1.41; 95% CI 0.89 to 2.24; 2 trials,  $n = 250$ ).

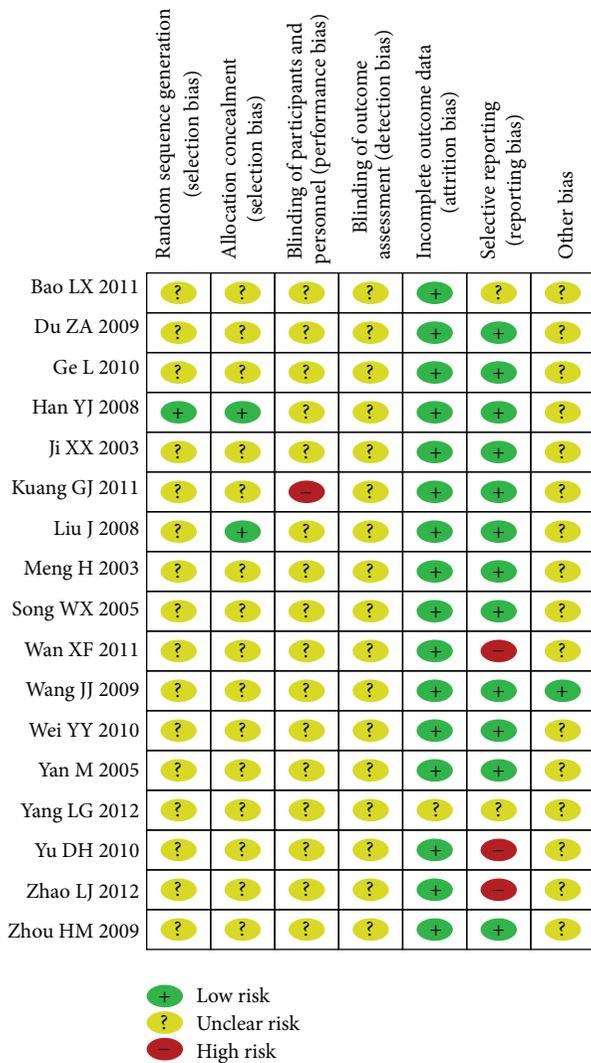


FIGURE 2: Risk of bias summary.

3.3.7. *Angina Pectoris Alleviation.* We defined the efficacy of angina pectoris as alleviation of more than 50%. There were 7 studies [22, 24, 27, 29, 31–33] reporting angina pectoris alleviation. The results showed significant improvement of *Panax notoginseng* preparations as compared with no treatment on the basis of conventional therapy (RR 1.20; 95% CI 1.12 to 1.28; 7 trials,  $n = 791$ ). Subgroup analysis showed that Xuesaitong softy capsule in 6 trials [17, 23, 25, 27–29] was more effective than no treatment in the basis of conventional therapy (RR 1.19; 95% CI 1.11 to 1.27; 6 trials,  $n = 641$ ).

3.3.8. *Electrocardiogram Improvement.* We defined the efficacy of ECG as elevation of depressed ST segment of more than 0.05 mv. There were 9 trials [19, 22, 24–27, 31, 32, 35] reporting the electrocardiogram improvement. The results in 8 trials [19, 22, 24, 26, 27, 31, 32, 35] showed significant improvement of *Panax notoginseng* preparation comparing with no treatment on the basis of conventional therapy (RR 1.35; 95% CI 1.19 to 1.53; 8 trials,  $n = 727$ ). 1 trial [25]

showed that notoginsenoside pill had no immediate effect on improving ECG compared with isosorbide dinitrate (RR 0.79; 95% CI 0.41 to 1.52; 1 trial,  $n = 80$ ). Subgroup analysis showed that Xuesaitong softy capsule [19, 22, 26, 27, 31, 32, 35] was superior to no treatment on the basis of conventional treatment in improving ECG (RR 1.39; 95% CI 1.21 to 1.59; 7 trials,  $n = 612$ ).

3.3.9. *Angina Pectoris Immediate Effect.* There was only one trial [25] which reported the angina pectoris immediate effect. 2 notoginsenoside pills were prescribed in this trial when angina pectoris happened. The criterion was defined as remarkably effective (angina was alleviated in 3 minutes); effective (angina was alleviated in 3–5 minutes); no effect (angina was alleviated in more than 5 minutes or need to add other medicines). The result indicated that notoginsenoside pill had similar effect compared with isosorbide dinitrate (RR 0.96; 95% CI 0.81 to 1.15; 1 trial,  $n = 80$ ).

3.3.10. *Angina Pectoris Frequency.* There were 4 studies [21, 26, 29, 33] reporting frequency of angina pectoris in the unit of times/week. Compared with no intervention on the basis of conventional therapy, *Panax notoginseng* preparation (Xuesaitong softy capsule) showed a reduction in angina pectoris frequency (MD -2.16; 95% CI -3.02 to -1.30; 4 trials,  $n = 572$ ). Sensitivity analysis also indicated that *Panax notoginseng* preparation was effective in reducing angina pectoris frequency (MD -2.34; 95% CI -2.53 to -2.16; 2 trials,  $n = 292$ ) [21, 29]. There was 1 trial [33] which reported the frequency of angina pectoris in the unit of times/day. The result indicated that *Panax notoginseng* (Xuesaitong softy capsule) could reduce angina pectoris frequency compared with no treatment on the basis of conventional therapy (MD -2.76; 95% CI -3.87 to -1.65; 1 trial,  $n = 28$ ).

3.3.11. *Angina Pectoris Duration.* There was 3 trials [21, 29, 33] reporting the duration of angina pectoris. The result showed that *Panax notoginseng* preparation (Xuesaitong softy capsule) significantly reduced angina pectoris duration comparing with no treatment on the basis of conventional therapy (MD -2.10; 95% CI -2.58 to -1.62; 3 trials,  $n = 472$ ). However, there was significant statistical heterogeneity among these three trials ( $I^2 = 91%$ ). Further sensitivity analysis also indicated the benefit of *Panax notoginseng* preparation (Xuesaitong softy capsule) in reducing angina pectoris frequency in hospitalized patients (MD -1.88; 95% CI -2.08 to -1.69; 2 trials,  $n = 292$ ) [21, 29].

3.3.12. *Dosage of Nitroglycerol.* There were 2 studies [21, 26] reporting dosage of nitroglycerol in the unit of mg/week. Compared with no intervention on the basis of conventional therapy, oral *Panax notoginseng* preparation (Xuesaitong softy capsule) showed a reduction of nitroglycerol dosage (MD -1.13; 95% CI -1.70 to -0.56; 2 trials,  $n = 212$ ). There was 1 study [36] reporting dosage of nitroglycerol in the unit of mg/day, which showed *Panax notoginseng* preparation (Xuesaitong softy capsule) also reduced the nitroglycerol

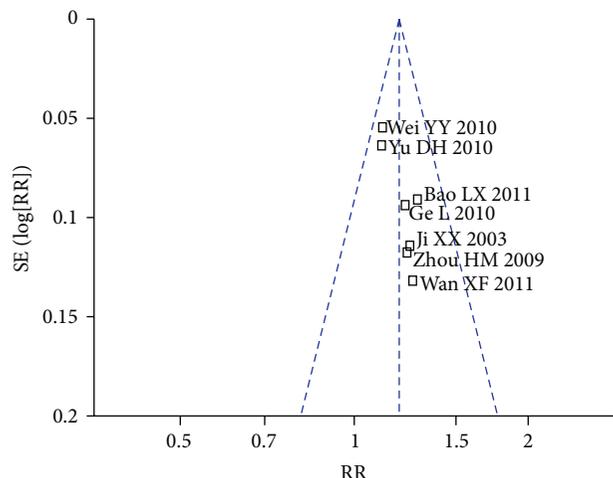


FIGURE 3: Funnel plot of comparison: conventional therapy and *Panax notoginseng* preparation versus conventional therapy, outcome: 3.3.7 Angina Pectoris Alleviation.

dosage significantly (MD  $-4.10$ ; 95% CI  $-5.58$  to  $-2.62$ ; 1 trial,  $n = 28$ ).

**3.4. Publication Bias.** A funnel plot analysis of the 7 trials in comparison of *Panax notoginseng* preparation and conventional therapy versus conventional therapy on angina pectoris improvement was conducted and shown in Figure 3; there might be a publication bias in this review for small sample, negative report, and low quality of the included trials.

**3.5. Adverse Events.** There were 9 trials [21–25, 29–31, 33] reporting adverse events (Ads) (Table 4). 6 trials [21–24, 29, 33] indicated no Ads in the duration of treatment. 1 trial [25] reported reduction of blood pressure and increase of heart rate (RR 0.03; 95% CI 0.00 to 0.543; 1 trial,  $n = 80$ ); 1 trial [30] reported nausea (RR 3.0; 95% CI 0.13 to 70.16; 1 trial,  $n = 48$ ); 1 trial [30] reported dizziness (RR 0.33; 95% CI 0.01 to 7.80; 1 trial,  $n = 48$ ); 1 trial [30] reported vomit (RR 0.33; 95% CI 0.01 to 7.80; 1 trial,  $n = 48$ ); 1 trial [31] reported erythra (RR 3.00; 95% CI 0.13 to 71.92; 1 trial,  $n = 100$ ). All Ads were not significantly different between the intervention group and the control group (Table 4).

## 4. Discussion

This systematic review included 17 RCTs and a total of 1747 participants. The review showed that, (1) comparing with no intervention on the basis of conventional therapy, oral *Panax notoginseng* showed no significant improvement for reducing the cardiovascular events, but it could relieve angina pectoris and related symptoms (including reducing the recurrence of angina pectoris, duration and frequency of angina pectoris, and dosage of nitroglycerol, as well as ECG changes); (2) oral *Panax notoginseng* showed similar immediate effect on angina pectoris compared with nitrate, but we could not make a significant conclusion from this equivalence due to small sample and low methodological quality trial; (3) The results

also showed that oral *Panax notoginseng* was safe for CHD patients according to the information in hand, but it was too limited to make a conclusion for high risk bias and small sample in these trials.

Oral *Panax notoginseng* preparations have been used widely for treating CHD in China. Most of the researchers paid more attention to their pharmacological mechanism. Yang et al. comprehensively collected the pharmacological action of *Panax notoginseng* and concluded that it could provide protective effects against cardiovascular diseases through many pharmacological mechanisms including improving myocardial microcirculation, reducing arrhythmia, regulating blood lipid, preventing atherosclerosis, lowering blood pressure, and antishock [37]. Du et al. summarized the experiments on *Panax notoginseng* for MI and concluded that *Panax notoginseng* could inhibit the inflammatory reaction and improve ischemia reperfusion injury in patients with MI [38]. Chan et al. concluded that Trilinolein purified from *Panax notoginseng* could provide protective effects against cardiovascular disease including reducing thrombogenicity and arrhythmia and increase erythrocyte deformability. It was also an antioxidant which could counteract free radical damage associated with atherogenesis and myocardial damage [39]. All these experiments provided us laboratory evidence on protective effect of *Panax notoginseng* for CHD. Although many clinical trials were conducted on effect of oral *Panax notoginseng* preparations for CHD, there was no critical appraisal for these up to now. There was still not enough evidence for clinicians to prescribe oral *Panax notoginseng* preparations in CHD patients.

The impact of this review was to take a light on oral *Panax notoginseng* for CHD. Although it failed to prove the protective effect of *Panax notoginseng* on major cardiovascular events (cardiovascular mortality, MI incidence, and rehospitalization), it demonstrated that *Panax notoginseng* preparation might be recommended for improving symptoms of angina pectoris.

However, before translating the conclusion of this review to clinical practitioners, we have to consider the following weaknesses in this review. (1) Firstly, the “randomization” was not clear in most of the trials for insufficient reporting of generation methods of the allocation sequence, allocation concealment. Most trials stated only that patients were randomly assigned. (2) Secondly, no trial used placebo in control group, most of trials did not introduce double blind in this review, and one trial introduced blinding of outcome assessment. Therefore, in nonplacebo-controlled and nondouble blind trials, placebo effects may add to the complexity of interpreting the conclusion. (3) Thirdly, most of the trials did not introduce the study plan, and attrition bias and selective reporting bias might exist in this conclusion. (4) Fourthly, funnel plot indicated that publication bias would exist in this review. The reasons we considered were as follows: we only selected trials published in Chinese and English trials published in other languages or originated from other countries might be omitted; we only identified unpublished studies from conference paper or academic thesis, and negative trials might not be reported and induce publication bias.

TABLE 4: Adverse Events.

Study ID	ADs
Du and Chen 2009 [21]	No abnormal changes appeared and no Ads was reported in the trial.
Ge and Zhao 2010 [22]	Blood regular test, urine regular test, and blood biochemistry test had no changes compared with the previous.
Han and Chen 2008 [23]	No serious Ads were reported in the trial; blood, urine, and stool routine tests, blood biochemistry test had no changes comparing with the previous.
Ji and Zhang 2003 [24]	Blood, urine, and stool routine tests, and blood biochemistry test had no changes compared with the previous. No Ads was reported in the trial.
Meng 2003 [25]	Reduction of blood pressure and increase of heart rate: intervention group: 0/60; control group: 5/20. RR: 0.03. 95% CI: [0.00, 0.54].
Wei 2010 [29]	Blood, urine, and stool routine tests, and blood biochemistry test had no changes compared with the previous. No Ads was reported in the trial.
Yan 2005 [30]	Nausea: intervention group (1/24), control group (0/24), RR: 3.0, 95% CI: [0.13, 70.16]. Dizziness: intervention group (0/24), control group (1/24), RR: 0.33, 95% CI: [0.01, 7.80]. Vomit: intervention group (0/24), control group (1/24), RR: 0.33, 95% CI: [0.01, 7.80].
Yu 2010 [31]	Erythra: intervention group (1/50), control group: (0/50). RR: 3.00; 95% CI: [0.13, 71.92].
Kuang et al. 2011 [33]	No abnormal changes appeared and no Ads was reported in the trial.

Note: ADs: Adverse Events.

Although this review suggested some benefit of *Panax notoginseng* preparation for CHD, the recommendation should be discreet due to poor quality and high risk bias of these trials, further rigorously designed, and well reported RCTs are still needed to prove the effectiveness and safety of *Panax notoginseng* preparation for CHD.

## 5. Conclusion

In this systematic review, oral *Panax notoginseng* preparation did not show benefit on reducing major cardiovascular events and relapse (including cardiovascular death, MI incidence, incidence of intractable angina pectoris, and rehospitalization), although it was effective in alleviating angina pectoris (including the recurrence, frequency, and duration of angina pectoris, ECG presentation, and dosage of nitroglycerin) with low adverse reaction. However, the small sample size and potential bias of most trials influence the convincingness of this conclusion. Before recommending oral *Panax notoginseng* preparation as an alternative herbal medicine in CHD patients, more rigorous trials with high quality are needed to prove the benefit of oral *Panax notoginseng* preparation and provide high level of evidence.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Authors' Contribution

Jianping Liu and Keji Chen conceived and designed the review and performed interpretation of the review; Qinghua Shang and Hao Xu developed the search strategy, did the literature search, study selection, data extraction, data analyses, and interpretation; Zhaolan Liu gave many suggestions

in designing and performing the review. All of authors contributed to the writing of the review.

## Acknowledgments

The current work was partially supported by the National Key Basic Research Program of China (no. 2006CB504803) and the Twelfth Five-year Plan of China (no. 2013BAI02B01 and 2013BAI13B01). Jian-Ping Liu and Zhao-Lan Liu was supported by grant number 2011-CXTD-09 from Beijing University of Chinese Medicine, and were partially funded by the grant number 2011ZX09302-006-01-03(5) by the Ministry of Science and Technology of China.

## References

- [1] American Heart Association, "Heart disease and stroke statistics—2007 update," 2007, <http://www.americanheart.org/statistics>.
- [2] World Health Organization, "The World Health Report 2003—Shaping the Future," 2003, <http://www.who.int/whr/2003/en/>.
- [3] [http://www.who.int/cardiovascular\\_diseases/en/](http://www.who.int/cardiovascular_diseases/en/), [http://www.who.int/cardiovascular\\_diseases/resources/atlas/en/](http://www.who.int/cardiovascular_diseases/resources/atlas/en/).
- [4] H. Xu and K. Chen, "Integrative medicine: the experience from China," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 3–7, 2008.
- [5] N. Robinson, "Integrative medicine—traditional Chinese medicine, a model?" *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 21–25, 2011.
- [6] H. Xu and K.-J. Chen, "Integrating traditional medicine with biomedicine towards a patient-centered healthcare system," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 83–84, 2011.
- [7] G. Dobos and I. Tao, "The model of western integrative medicine the role of chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 11–20, 2011.

- [8] S. P. Balasubramani, P. Venkatasubramanian, S. K. Kukkupuni, and B. Patwardhan, "Plant-based Rasayana drugs from Ayurveda," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 88–94, 2011.
- [9] Q. Wang, T. X. Wu, X. Y. Chen et al., "Puerarin injection for unstable angina pectoris," *Cochrane Library*, 2009.
- [10] T. X. Wu, J. Ni, and J. F. Wei, "Danshen (Chinese medicinal herb) preparations for acute myocardial infarction," *Cochrane Library*, 2009.
- [11] T. X. Wu, R. A. Harrison, X. Y. Chen et al., "Tongxinluo (tong xin luo or tong-xin-luo) capsule for unstable angina pectoris," *Cochrane Library*, 2009.
- [12] G. Wang, L. Wang, Z.-Y. Xiong, B. Mao, and T.-Q. Li, "Compound salvia pellet, a traditional Chinese medicine, for the treatment of chronic stable angina pectoris compared with nitrates: a meta-analysis," *Medical Science Monitor*, vol. 12, no. 1, pp. SR1–SR7, 2006.
- [13] X. Duan, L. Zhou, T. Wu et al., "Chinese herbal medicine suxiao jixun wan for angina pectoris," *Cochrane Database of Systematic Reviews*, no. 1, Article ID CD004473, 2008.
- [14] Q. Zhuo, Z. Yuan, H. Chen, and T. Wu, "Traditional Chinese herbal products for stable angina," *Cochrane Database of Systematic Reviews*, no. 5, Article ID CD004468, 2010.
- [15] J.-W. Guo, L.-M. Li, G.-Q. Qiu et al., "Effects of Panax notoginseng saponins on ACE2 and TNF- $\alpha$  in rats with post-myocardial infarction-ventricular remodeling," *Zhong Yao Cai*, vol. 33, no. 1, pp. 89–92, 2010.
- [16] J.-B. Wan, S. M.-Y. Lee, J.-D. Wang et al., "Panax notoginseng reduces atherosclerotic lesions in ApoE-deficient mice and inhibits TNF- $\alpha$ -induced endothelial adhesion molecule expression and monocyte adhesion," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 15, pp. 6692–6697, 2009.
- [17] Z. R. Zhang, X. Li, Y. H. Wang et al., "Angiogenic effect of total saponins extracted from root and flower of panax notoginseng in zebrafish model," *Universitatis Traditionis Medicalis Sinensis Pharmacologiaeque Shanghai*, vol. 27, no. 1, pp. 45–49, 2013.
- [18] X. Y. Chen, M. K. Zhou, Q. F. Li et al., "Sanchi for acute ischaemic stroke," *Cochrane Library*, 2008.
- [19] X. Liu, J. Li, G. Yang, and J. Wang, "Study on effect of promoting blood circulation drugs components in treating unstable angina in patients with blood stasis inflammatory levels," *Zhongguo Zhongyao Zazhi*, vol. 33, no. 24, pp. 2950–2953, 2008.
- [20] J. P. T. Higgins and S. Green, "Cochrane handbook for systematic reviews of interventions, version 5. 0. 2 [updated september 2009]," The Cochrane Collaboration, 2011, <http://www.cochrane-handbook.org/>.
- [21] Z. A. Du and G. L. Chen, "Effect of Xuesaitong softy capsule for angina pectoris on endothelin and C reaction protein," *Chinese Journal of Modern Drug Application*, vol. 3, no. 4, pp. 140–141, 2009.
- [22] L. Ge and S. Z. Zhao, "Effect of Xuesaitong softy capsule for unstable angina pectoris," *Hebei Journal of Traditional Chinese Medicine*, vol. 32, no. 8, pp. 1223–1224, 2010.
- [23] Y. J. Han and Q. X. Chen, *Clinical study on intervention of Xuesaitong soft capsule on coronary heart disease patients after PCI [M.S. thesis]*, Guangzhou University of Chinese Medicine, 2008.
- [24] X. X. Ji and W. G. Zhang, *Clinical and experimental study of micro-powder of panax notoginseng on both treating unstable angina and protecting in isoproterenol induced myocardial necrosis in rats [M.S. thesis]*, Shandong University of Chinese Medicine, 2003.
- [25] H. Meng, "Analysis of Panax Notoginseng Saponins tablets for 60 patients with angina pectoris," *Academic Journal of Traditional Chinese Medicine*, vol. 21, no. 6, p. 1007, 2003.
- [26] W. X. Song, Z. T. Wang, and S. Y. Zeng, "Clinical observation of Xuesaitong soft capsule for angina pectoris," *Journal of Emergency in Traditional Chinese Medicine*, vol. 14, no. 8, pp. 707–708, 2005.
- [27] X. F. Wan, "Effect observation of Xuesaitong soft capsule for unstable angina pectoris," *Practical Journal of Cardiac Cerebral Pneumal and Vascular Disease*, vol. 19, no. 10, p. 1768, 2011.
- [28] J. J. Wang, S. L. Zhang, Q. Y. Liu, Q. F. Yang, and H. J. Wan, "Clinical effect of Xuesaitong soft capsule and trimetazidine for unstable angina pectoris," *Shandong Medical Journal*, vol. 49, no. 37, pp. 92–93, 2009.
- [29] Y. Y. Wei, "Effect of Xuesaitong soft capsule for 90 patients with angina pectoris," *Chinese Journal of Modern Drug Application*, vol. 4, no. 23, pp. 20–21, 2010.
- [30] M. Yan, "Report of isosorbide 5-mononitrate and panax notoginseng for angina pectoris," *Gansu Journal of Traditional Chinese Medicine*, vol. 18, no. 1, p. 28, 2005.
- [31] D. H. Yu, "Clinical effect analysis of Xuezhikang soft capsule for unstable angina pectoris," *Proceeding of Clinical Medicine*, vol. 19, no. 1, pp. 38–39, 2010.
- [32] H. M. Zhou and J. Bai, "Effect of Xuesaitong soft capsule for angina pectoris and influence on serum endothelin and nitric oxide," *Chinese Journal of Practical Meicine*, vol. 36, no. 14, p. 82, 2009.
- [33] G. J. Kuang, Z. J. Huang, C. W. Bai, Y. Q. Hu, and T. Dai, "Effect of Xuesaitong soft capsule for unstable angina pectoris and influence on Electrocardiogram QT dispersion degree," *Lingnan Journal of Emergency Medicine*, vol. 16, no. 6, pp. 455–456, 2011.
- [34] L. X. Bao, "Clinical trial of Sanqi Guanxinning for stable angina pectoris," *Journal of Chinese Medicine*, vol. 26, no. 162, pp. 1371–1372, 2011.
- [35] L. J. Zhao and F. E. Li, "Clinical observation of Xuesaitong softy capsule for 60 patients with stable angina pectoris," *Hebei Journal of Traditional Chinese Medicine*, vol. 34, no. 7, pp. 1049–1050, 2012.
- [36] L. G. Yang, "Clinical effect of xuesaitong for coronary heart disease," *Contemporary Medicine*, vol. 18, no. 35, pp. 79–80, 2012.
- [37] Z. G. Yang, A. Q. Chen, and S. D. Yu, "Research Progress of pharmacological actions of Panax notoginseng," *Shanghai Journal of Traditional Chinese Medicine*, vol. 39, no. 4, pp. 59–62, 2005.
- [38] W. S. Du, X. F. Xiao, M. D. Zhu et al., "Inflammatory response, damage, repairing in necrotic area and effect of Panax notoginseng for patient with myocardial infarction," *Lishizhen Medicine and Materia Medica Research*, vol. 21, no. 10, pp. 2560–2562, 2010.
- [39] P. Chan, G. N. Thomas, and B. Tomlinson, "Protective effects of trilinolein extracted from Panax notoginseng against cardiovascular disease," *Acta Pharmacologica Sinica*, vol. 23, no. 12, pp. 1157–1162, 2002.

## Review Article

# Integrative Western and Chinese Medicine on Coronary Heart Disease: Where Is the Orientation?

Siming Li<sup>1,2</sup> and Hao Xu<sup>2</sup>

<sup>1</sup> Graduate School, Beijing University of Chinese Medicine, Beijing 100029, China

<sup>2</sup> Cardiovascular Diseases Center, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

Correspondence should be addressed to Hao Xu; xuhaotcm@hotmail.com

Received 22 April 2013; Accepted 20 July 2013

Academic Editor: Keji Chen

Copyright © 2013 S. Li and H. Xu. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Coronary heart disease (CHD) is the leading cause of death. As the main treatment of CHD, modern medicine has improved dramatically in recent years. Although researches of TCM and integrative medicine on CHD are witnessed encouraging progress in many respects, the role TCM playing in the prevention and treatment of CHD has been unprecedentedly challenged under such circumstance of the very fast development of modern medicine. In order to share mutual complementary advantages of TCM and western medicine, this review summarizes the relatively prominent researches of TCM and integrative medicine on CHD in recent years, and illuminates the issue of the orientation of the further research of integrative medicine on CHD, including (1) original innovation of TCM etiology and pathogenesis, (2) combination of disease and TCM syndrome, (3) biological basis of TCM syndrome of CHD, (4) clinical design and quality control of integrative medicine research, (5) herb-drug interaction, (6) difficulties and hot issues of modern medicine.

## 1. Strengthening Original Innovation on the TCM Etiology and Pathogenesis of CHD

So far, there are so many researches of TCM and integrative medicine on CHD, however, with insufficient enhancement of clinical effectiveness. If the work on blood-stasis syndrome (BSS) is a milestone of TCM and integrative medical research on CHD, activating blood circulation by removing blood-stasis and its derived therapies have significantly improved the clinical effectiveness of TCM therapy on CHD [1], when comparing with the traditional therapeutic method of eliminating stagnation to activate yang mainly with formulae on the basis of *Trichosanthes* and *Allium*. After the research of BSS, it is urgent to strengthen the original innovation in the TCM and integrative medicine research. Focusing on the clinical problems or phenomenon, we should innovate TCM etiology and pathogenesis of CHD, which will bring about innovation on the treatment of CHD and then improve the clinical effectiveness. In this respect, the etiology and pathogenesis innovation research on “blood-stasis and toxin” conducted by the research team headed by academician Chen

Ke-ji can be used for reference. Directing at the clinical problem/phenomenon that “some patients with CHD have a stable condition for a long time while some develop acute cardiovascular events (ACEs),” the research team innovatively proposed the hypothesis of “blood-stasis and toxin causing catastrophe” on etiology and pathogenesis of CHD [2, 3], according to the modern medicine progress in the relevance of the vulnerable plaque rupture, the inflammatory response of atherosclerosis and acute coronary syndrome (ACS), and on the basis of the previous experimental research [4, 5], long-period clinical practice, as well as the TCM cognition of “toxin”. On that basis, they conducted a series of research combined literature reviews with experimental and clinical researches [6–8], which provides a model for TCM etiological innovation research. Especially, the prospective cohort study [9] enrolling 1503 stable patient with CHD designed by reference to the classical method of clinical epidemiological etiological research analyzed correlation factors of ACEs in the follow-up, proved the hypothesis of “blood-stasis and toxin causing catastrophe,” and established the criterion of syndrome differentiation of “toxin” for stable

CHD. It is of great significance for exerting TCM features and advantages of “preventive treatment” to early recognize high-risk patients and give intervention as soon as possible, and further reducing the incidence of ACEs. In addition, some scholars proposed the hypothesis of “endogenous collateral wind” in accordance with the characteristic of the acute onset and rapid progression of ACS, which is also a beneficial exploration [10]. All in all, in view of the different group of patients with CHD, to conduct further research on their pathological physiology proceeding from the similarities and differences in the theory of TCM and Western medicine and then to analyze combining with their characteristics of TCM etiology, pathogenesis, and syndrome, we may bring about the original innovation and the development of TCM therapy to further improve the clinical effectiveness.

## **2. Combination of Disease and TCM Syndrome: One of the Breakthrough Points of Integrative Medicine Research on CHD**

Combining disease with TCM syndrome is an important treatment model in TCM clinical practice. The theory of combining disease with syndrome reflects the principle of inheritance and innovation, which has been widely accepted by TCM and integrative medical practitioners for the advantages of scientificity and operability. Thus, it is the important breakthrough point of integrative medicine research on CHD. Previously, most of the TCM syndrome researches on CHD are cross-sectional studies focusing on syndrome distribution characteristics of CHD and different subgroups, which have made certain progress. Many results have shown that patients with CHD have the syndrome of asthenia in origin and asthenia in superficiality, and blood-stasis is the main syndrome element of CHD, followed by qi deficiency [11, 12]. There are also different characteristics in each subtypes: for instance, the TCM syndrome of exertional angina is primarily qi deficiency [13]; the syndrome distribution of AMI is mostly identical to which of CHD but more serious of both asthenia in origin and asthenia in superficiality [14, 15]. In recent years, characteristics of TCM syndrome distribution of CHD, such as increasing heat-accumulation [16], aggravating qi deficiency after coronary revascularization [17] are worthy of attention. The characteristics of syndrome distributions in patients with CHD combined with different diseases are shown in difference: for example, phlegm-dampness and blood-stasis syndrome may be the main characteristic of CHD combined with hyperlipidemia [18, 19]; when CHD combined with hypertension, BSS is manifested more obvious, while liver fire and hyperactivity of yang are also the common syndrome elements [20, 21]; when combined with diabetes mellitus (DM), BSS and turbid phlegm are common in excessive syndrome while deficiency of both qi and yin is most common in deficiency syndrome [20, 22]. A regional difference also exists in the TCM patterns of patients with CHD between the North and South China: the proportions of patients with qi-deficiency syndrome, turbid phlegm syndrome, or blood-stasis syndrome were generally higher in the South group, while the proportion of

patients with a congealing cold syndrome was identified to be obviously higher in the North group [23]. The research on diagnostic criteria of combining disease with syndrome is also going deeper, such as the establishment of the diagnostic criteria for CHD patients with BSS [24], which is the further research based on the diagnostic criteria of BSS and is of great significance in standardizing syndrome research and improving the level of TCM syndrome differentiation and treatment.

It is worth mentioning that in the process of the occurrence, development, and prognosis of CHD, TCM syndrome is always in a dynamic change, so the research on the evolution law of CHD syndromes may help us understand the pathogenesis and prognosis of CHD in each stage or population and then improve clinical syndrome differentiation and treatment. But research in this field is still less and need to be strengthened. Mei et al. [25] conducted a case-control study to explore the main factors and the influencing extent of the susceptibility of the Han population with CHD of BSS in Fuzhou area. The result showed that mental labors, hypertension, excessive consumption of oil and salt, depression, stress, and past relevant medical history are related to the predisposing factors for the Han population with CHD of BSS in Fuzhou area when compared with the population of non-BSS. It has provided clues for the cause of CHD of BSS. Through method of principal component and logistic regression analysis, we studied the distribution laws of TCM syndrome elements in 1072 CHD inpatients according to the 1-year follow-up of cardiovascular events [26]. The results showed that blood-stasis, qi deficiency, turbid phlegm, and yin deficiency were the main syndrome elements of CHD inpatients while qi deficiency and yin deficiency might be relevant TCM syndrome elements of the CHD in patients who suffered from follow-up ACEs. In addition, we applied multifactor dimensional reduction (MDR) and complex network method to analyze the evolution law of TCM syndrome in 1333 stable patients with CHD [27]. The result showed that with the time of follow-up, the TCM syndromes were kept in a dynamic evolution. Toxin resulting from blood-stasis, combination of toxin and blood-stasis, toxin consuming qi, and blood-stasis due to qi deficiency may be the key pathogenic mechanism and the law of syndrome development.

## **3. Research on Biological Basis of TCM Syndrome of CHD under the Guidance of Holistic Concept**

Treatment based on syndrome differentiation is one of the characteristics and advantages of TCM in preventing and treating disease, but how to conduct syndrome differentiation exactly is the key to improve the clinical efficacy. The rapid development of modern medicine has provided technical support and good opportunity for the expansion and extension of the four methods of diagnosis of TCM and the organic combination of traditional macroscopic and modern microcosmic syndrome differentiation. In recent years, Chinese scholars have studied relevant factors affecting TCM

syndrome differentiation of CHD and the biological basis of syndrome in the aspects of coronary artery lesion, cardiac function, changes in ECG, blood lipid, insulin resistance, homocysteine and inflammation factors, related gene, and so on [28]. They have made significant progresses and provided the basis for objectifying the TCM syndrome differentiation for CHD. In particular for some silent myocardial ischemia, adopting the method of microcosmic syndrome differentiation can make the treatment more targeted. However, indicators in most of these studies are single and just reflect one aspect of TCM syndrome's essential. The research on biological basis of TCM syndrome should be under the guidance of holistic view and system biology and by taking full advantage of modern omics technologies such as genomics, proteomics, and metabolomics. Researches on this respect have been launched and show favorable signs. For example, applying oligonucleotide microarray technology, through comparing the gene expression profiles among healthy control group, blood-stasis syndrome with CHD and without CHD group, Ma et al. [29] screened the differential genes correlated with CHD of BSS, analyzed gene ontology and pathway, and then confirmed the target gene by real-time reverse transcription polymerase chain reaction (RT-PCR). The results suggested that inflammation and immune response might cause the occurrence and development of blood-stasis syndrome to some extent. Yuan et al. [30] also found that the hereditary relevant differential genes of BSS of CHD were closely associated with inflammation, plaque formation, and endothelial injury by gene chip technique. Wu et al. [31] applied proteomic technology including two-dimensional electrophoresis (2DE), image analysis, and spectrometry detection to identify the change of plasma protein in healthy person and BSS patients with CHD and found that fibrinogen and granzyme might be considered as diagnostic biomarkers of BSS patients with CHD. Li et al. [32] screened out 13 differential proteins from platelet between BSS patients with CHD and healthy control group by proteomic technology, among which 7 were identified by spectrometry successfully. The results showed that CD41 and Actin $\gamma$  are the possible marker proteins that might play crucial roles in the occurrence and development of BSS patients with CHD. Zhao et al. [33] also applied proteomic technology and discovered that unstable angina-qi deficiency and blood-stasis syndrome (UA-QDBS) might belong to a kind of inflammatory reaction. There might be myocardial injury, abnormality of coagulation factor, lipid metabolic disorder, and oxygen transport obstacle in patients of UA-QDBS. Lu et al. [34] proposed a new approach of studying the biological basis of phlegm and blood-stasis syndrome in CHD based on metabolomics and correspondence of prescription and syndrome. Utilizing metabolomics, Jian et al. [35] found that the major plasmic metabolites in patients with CHD- blood-stasis syndrome (CHD-BSS) are arachidonic acid, octadecanoic acid, lactic acid, urea, citric acid,  $\beta$ -hydroxybutyric acid, oleic acid, glucose, and alanine. The results showed that CHD-BSS is related with lipid metabolism, glycometabolism, as well as the stress induced by hypoxia and agonism. Applying various omics technologies, we can conduct researches in the holistic level, and provide

strong technical support for the research on biological basis of TCM syndrome.

#### **4. Strengthening the Clinical Design and Quality Control of Integrative Medicine Research on CHD**

In recent years, with the concept of evidence-based medicine (EBM) widely accepted, a number of multicenter, randomized controlled trials (RCT) with large sample focusing on the prevention and treatment of CHD have been carried out successively both in the fields of Chinese medicine and integrative medicine, for example, the randomized, double-blind, placebo-controlled trial on the effect of Xuezhikang (XZK) for regulating blood lipids and secondary prevention of CHD [36]. The results showed that XZK-treated group decreased the recurrence of nonfatal myocardial infarction in patients with CHD by 62% compared with the placebo control group, and the occurrence of coronary death, coronary events, and total mortality was reduced by 45%, 30%, and 33.0%, respectively, which fills the blank of research on regulating blood lipids for secondary prevention of CHD in oriental populations. According to restenosis after percutaneous coronary intervention (PCI), a worldwide problem in the field of heart disease, the effectiveness and safety of XS0601 treatment based on conventional Western medicine have been demonstrated in a multicenter, randomized, double-blind, placebo-controlled trial [37, 38]. These trials provided objective evidence for integrative medicine in the prevention and treatment of CHD. Admittedly, RCT has a higher level of evidence than many other trials and is more persuasive. However, we should also avoid only-RCT-oriented clinical trials in clinical research.

When conducting clinical trials of integrative medicine on CHD, we should pay attention to the following three points.

- (1) Strengthen the top-level design of clinical trials and choose appropriate clinical research methods based on the clinical demands and research objectives. For most intervention studies, RCT including explanatory randomized controlled trial (ERCT) and pragmatic randomized controlled trial (PRCT) is usually the first choice. Recently, real-world study (RWS) [39], which is applicable to explore the effectiveness of complex intervention with integrative medicine [40] and the reevaluation of postmarketing drugs, has been drawn and is worthy of more attention, while researchers need to strengthen the control of confounding factors, to which modern statistical analysis methods involving instrumental variable and propensity scores can be introduced.
- (2) Outcome measures should be appropriate [41]. Avoid merely using subjective outcome measures like angina score and TCM syndrome score. On the contrary, objective outcome measures should be adopted, such as electrocardiogram treadmill exercise test on stable angina, and if possible, long-term follow-up of ACEs,

to improve persuasion of evidence. Meanwhile, we should pay attention to the quality of life, patient reported outcomes (PRO), and medical economics evaluation to highlight the characteristics of TCM and the advantage of integrative medicine.

- (3) Trials are designed and conducted according to good clinical practice (GCP) principle. Strengthen the implementation of process management and quality control of clinical trials, such as the international registration of clinical trials, ethical approval, the third-party evaluation of end point, and data management, and reported trials in accordance with the consolidated standards of reporting trials (CONSORT) statement 2010 [42], in order to improve the quality of original trials as source references of systematic reviews (SRs) and meta-analysis [43]. Combining the problems often existing in current clinical trial reports, the following details should be highlighted: estimation of sample size, description of random method and random allocation concealment, patient compliance and the completion status of follow-up, adverse drug reactions and its treatment, data processing of loss to follow-up (LFU) and intention to treat analysis (ITT) or not, and so forth.

## 5. Herb-Drug Interaction Is a Key Problem That Cannot Be Ignored in Integrative Medicine Research on CHD

If the combination of disease and syndrome is the integration of TCM and Western medicine in diagnostic level, then interaction of Chinese and Western medicine can be the practical problem we face in treating disease with integrative medicine. As the integrative medical model of patient-centered healthcare and combined application of botanical and chemical drugs evolving into a new trend of modern medicine in preventing and treating disease nowadays, the combined application of Chinese and Western medicine is increasing, and the potential interactions are drawing more and more attention [44].

Herb-drug interaction has two meanings. One is the advantageous function of effect-enhancing and toxicity-reducing. CHD and other chronic noncommunicable diseases are often related to multiple risk factors, complex interventions, and multitarget treatment are more appropriate. Combination therapy with a TCM formula has been the feature and advantage of TCM. Some international scholars also proposed the concept of “polypill” [45], which has caused widespread concern. Actually antihypertensive polypill has been widely accepted in the treatment of hypertension. Xuezhikang, a modern Chinese patent medicine, which has beneficial effects in lipid regulation and secondary prevention of CHD, could also be regarded as a natural polypill [46], and its multicomponent synergy may be the mechanism of pleiotropic effects. According to our clinical practice experience, the effectiveness of prescription for supplementing qi and activating blood circulation in combination with vasodilator agent and diuretics for patients with heart failure

(HF) after AMI is much better than Western medicine only, which suggests the potential synergistic effect of Chinese and Western medicine.

Another meaning of herb-drug interaction is the potential of increased risk of adverse reactions. International researches discovered that some botanical drug products including Chinese herbal medicine, such as *hypericum perforatum*, motherwort, ginseng, ginkgo biloba, salvia miltiorrhiza, garlic, and aconite, can possibly increase the risk of adverse events in patients with cardiovascular diseases by the interactions with other drugs, particularly in elderly patients who always consume multiple prescription medications in the same time for comorbidities [47]. In clinical practice, it was also found easily to cause digitalis toxicity when taken digitalis with semen lepidii, north acanthopanax bark, and so forth. In addition, patients after PCI are treated with dual antiplatelet therapy with aspirin and clopidogrel. Whether it increases the risk of bleeding or not when adding blood-invigorating and stasis-removing herbs needs to be further investigated.

So, herb-drug interaction is the issue that cannot be ignored in the research of integrative medicine on CHD. In clinical practice, doctors should notice the reference application of evidence-based medication [48, 49], pay great attention to adverse drug interaction that has been discovered, and avoid the off-label drug use, long-term drug overdosage and irrational drug combination, and so forth, so as to minimize the risk of adverse events. To the key population, more attention should be paid to strengthen monitoring and discovering the potential adverse effects of drug interactions timely.

Meanwhile, the firsthand material should be accumulated in routine clinical practice, as there is an important way to discover the meaningful clues of herb-drug interactions through the real-world data analysis. On this basis, design experiment scientifically, carry out the relevant studies involved in drug metabolism, pharmacokinetic, efficacy, toxicology, and the relevance of toxicity and efficacy, and conduct in-depth study of herb-drug interactions and their mechanism from drug absorption, distribution, transformation, metabolism, excretion, and so on.

## 6. Target Integrative Medicine Research on Difficulties and Hot Issues of Modern Medicine

The development of modern medicine has brought new hope for the prevention and treatment of CHD. However, some new problems have unavoidably been presented, such as aspirin resistance (AR), restenosis after PCI, late thrombosis of drug-eluting stent (DES), the no-reflow phenomenon after revascularization, vulnerable plaque, contradiction of therapeutic angiogenesis, the viability and differentiation ability of transplanted cells when operating stem cell transplantation, and residual cardiovascular risk. These are still challenges which modern medicine has to face positively. Focusing on these key issues that influence the curative effect, we should give full play to the characteristics of TCM, complement each

other's advantages of Chinese and Western medicine, and conduct researches scientifically.

For instance, to the clinical problem of AR, we may screen potential Chinese herbals which have antiplatelet effect, then explore its material basis and active ingredients, illustrate the target point of drug action, find the lead compound, and optimize its structure. An effective Chinese herb with high-efficacy and low-toxicity will be of great significance in the prevention and treatment of cardiovascular disease. Some domestic scholars [50] have recomposed Ferulic Acid and Ligustrazine to give full play to the advantages of Chinese compound prescription and observed its inhibitory effect on adenosine diphosphate glucose pyrophosphorylase (ADP) induced platelet aggregation in vivo. They found that the effect of compound prescription group was obviously superior to Ligustrazine group, which provided reference for the further study of antiplatelet with traditional Chinese preparation.

Stem cell transplantation is also a hot issue. It may be a new dawn for the treatment of myocardial infarction, and its preliminary clinical study outcome was inspiring. A study [51] indicated that bone marrow stem cell could transversely transform into myocardial cell and vascular endothelial cell in the environmental conditions of the heart, which finally remedy the damaged myocardium. Another study showed that ginsenoside Rg1 could induce bone marrow cells' migration to myocardium and differentiation to vascular endothelial cell by stimulating local myocardium to excrete granulocyte colony stimulating factor (G-CSF) [52]. When applied Chinese compound prescription based on ginseng and *Salvia miltiorrhiza* combined with bone marrow mononuclear cells autotransplantation through cardiac catheter to the model of myocardial infarction in swine, it promoted transplanted cell to survive, differentiate, and amplify, and many new myocardial cell and myocardial small vessel emerged. These facilitated the repair of damaged myocardial cells by synergies and complementing each other's advantages [53] and predicted a gratifying future. In addition, whether Chinese medicine could work on the inflammation reaction and local microcirculation after stem cell transplantation and immunological rejection after cell transplantation and whether it could improve transplanted cell viability and induce differentiation of transplanted cell need to be answered in the future investigations.

## 7. Conclusion

In conclusion, integrative medical research on CHD has made great development. Present studies shed light on the orientation of integrative medicine. From the progress of integrative medicine on CHD, in order to further complement each other's advantages of Chinese and Western medicine, researches should keep focusing research on original innovation of TCM etiology and pathogenesis, combination of disease and TCM syndrome, biological basis of TCM syndrome of CHD, difficulties and hot issues of modern medicine such as stem cell transplantation and strengthen clinical design and quality control of integrative medicine

research. In addition, herb-drug interaction should not be ignored.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Acknowledgments

The current work was partially supported by the National Key Basic Research Program of China (no. 2006CB504803) and the Twelve Five-year Plan of China (nos. 2013BAI02B01 and 2013BAI13B01).

## References

- [1] K. J. Chen, "Blood stasis syndrome and its treatment with activating blood circulation to remove blood stasis therapy," *Chinese Journal of Integrative Medicine*, vol. 18, no. 12, pp. 891–896, 2012.
- [2] H. Xu, D.-Z. Shi, and H.-J. Yin, "Blood-stasis and toxin causing catastrophe hypothesis and acute cardiovascular events: proposal of the hypothesis and its clinical significance," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 10, pp. 934–938, 2008.
- [3] D.-Z. Shi, H. Xu, H.-J. Yin, J.-C. Zhang, and K.-J. Chen, "Combination and transformation of toxin and blood stasis in etiopathogenesis of thrombotic cerebrocardiovascular diseases," *Journal of Chinese Integrative Medicine*, vol. 6, no. 11, pp. 1105–1108, 2008.
- [4] C. Wen, H. Xu, and Q.-F. Huang, "Effect of drugs for promoting blood circulation on blood lipids and inflammatory reaction of atherosclerotic plaques in ApoE gene deficiency mice," *Journal of Chinese Integrative Medicine*, vol. 25, no. 4, pp. 345–349, 2005.
- [5] M.-X. Zhou, H. Xu, K.-J. Chen, and E. A. et al, "Effects of some active ingredients of Chinese drugs for activating blood circulation and detoxicating on blood lipids and atherosclerotic plaque inflammatory reaction in ApoE-gene knockout mice," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 2, pp. 126–130, 2008.
- [6] H. Xu, D. Qu, and F. Zheng, "Clinical manifestations of "blood-stasis and toxin" in patients with stable coronary heart disease," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 30, no. 22, pp. 125–129, 2010.
- [7] Y. Feng, H. Xu, D. Qu, F. Zheng, D.-Z. Shi, and K.-J. Chen, "Study on the tongue manifestations for the blood-stasis and toxin syndrome in the stable patients of coronary heart disease," *Chinese Journal of Integrative Medicine*, vol. 17, no. 5, pp. 333–338, 2011.
- [8] M. Xue, H. J. Yin, and C. F. Wu, "Effect of Chinese drugs for activating blood circulation and detoxifying on indices of thrombosis, inflammatory reaction, and tissue damage in a rabbit model of toxin-heat and blood stasis syndrome," *Chinese Journal of Integrative Medicine*, vol. 19, no. 1, pp. 42–47, 2013.
- [9] K. J. Chen, D. Z. Shi, and H. Xu, "The criterion of syndrome differentiation and quantification for stable coronary heart disease caused by etiological toxin of Chinese medicine," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 31, no. 3, pp. 313–314, 2011.
- [10] X. Wang and D. Y. Hu, "Clinical study on the hypothesis of 'endogenous collateral wind'on acute coronary syndrome,"

- China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 23, no. 3, pp. 204–208, 2008.
- [11] J. B. Zhong, S. Y. Dong, J. Wang et al., “Literature analysis on syndrome elements of 2689 patients with angina pectoris,” *Chinese Journal of Information on TCM*, vol. 13, no. 5, pp. 100–101, 2006.
  - [12] J. X. Zhou, M. Tang, and J. Li, “Analysis of Chinese syndrome features and combination laws of 2029 patients with coronary heart disease angina,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 31, no. 6, pp. 753–755, 2011.
  - [13] H.-L. Wu, X.-M. Ruan, and X.-B. Yang, “Analysis on TCM syndrome distribution laws in 319 patients with coronary heart disease,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 27, no. 6, pp. 498–500, 2007.
  - [14] X. C. Wang, Y. B. Nong, Q. Lin et al., “Analysis on the combination characteristics of TCM syndromes of 138 cases with coronary heart disease,” *Journal of Traditional Chinese Medicine*, vol. 49, no. 1, pp. 62–63, 2008.
  - [15] L. Wang, H. X. Liu, Z. D. Zou et al., “Research on TCM syndrome characteristics of hospitalized patients with acute myocardial infarction in TCM hospitals of Beijing,” *Chinese Journal of Integrative Medicine on Cardio/Cerebrovascular Disease*, vol. 6, no. 4, pp. 379–380, 2008.
  - [16] J. Wang, Y. W. Xing, K. W. Yao et al., “Study on Chinese medicine syndrome elements of coronary heart disease angina pectoris and its clinical applications,” *Journal of Hubei College of Traditional Chinese Medicine*, vol. 11, no. 3, pp. 3–5, 2009.
  - [17] Y. Ren, Y. Wu, M. Z. Zhang et al., “Study on Chinese medicine syndromes characteristics and distribution rule around percutaneous coronary intervention perioperative period,” *Chinese Journal of Integrative Medicine on Cardio/Cerebrovascular Disease*, vol. 8, no. 6, pp. 639–641, 2010.
  - [18] G. R. Yu, Y. Q. He, Y. G. Guo et al., “Clinical studies on relationship of traditional Chinese medicine syndrome of Coronary heart disease with insulin resistance, lipids and erythrocyte membrane atpase,” *Journal of Traditional Chinese Medicine*, vol. 41, no. 2, pp. 111–112, 2002.
  - [19] D. X. Wei, M. Liu, Y. C. Pang et al., “Study on the correlation between Chinese medicine syndrome types and blood lipid levels in patients with coronary heart disease,” *Journal of Emergency in Traditional Chinese Medicine*, vol. 19, no. 3, pp. 441–442, 2010.
  - [20] G. Shi and T. Liu, “Clinical epidemiological investigation on Chinese medicine syndrome in patients with Coronary heart disease,” *Chinese Archives of Traditional Chinese Medicine*, vol. 25, no. 8, pp. 1675–1676, 2007.
  - [21] C.-J. Bai, Y. Zhou, L. Wang, D.-L. Zhang, and Y. Yang, “Delamination of cardiovascular risk factor, staging and grading of hypertension and the changing characteristics of blood lipids and hemorheological indexes in hypertensive patients with different syndromes of traditional Chinese medicine,” *Chinese Journal of Clinical Rehabilitation*, vol. 9, no. 23, pp. 145–147, 2005.
  - [22] Y. W. Xing, J. Wang, Y. H. Gao et al., “Study on characters of TCM syndrome and pathological changes of coronary artery in patients of coronary artery disease combined with diabetes,” *Chinese Journal of Information on TCM*, vol. 14, no. 9, pp. 20–21, 2007.
  - [23] Y. Ren, M. Z. Zhang, K. J. Chen et al., “Clinical and epidemiological investigation of TCM syndromes of patients with coronary heart disease in China,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 714517, 5 pages, 2012.
  - [24] C. G. Fu, Z. Y. Gao, and P. L. Wang, “Study on the diagnostic criteria for coronary heart disease patients of blood stasis syndrome,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 32, no. 9, pp. 1285–1286, 2012.
  - [25] L. J. Mei, S. Q. Xiong, T. Wang et al., “A case control study of influential factors for the Han population with coronary heart disease of blood stasis syndrome in Fuzhou area,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 32, no. 2, pp. 168–171, 2012.
  - [26] O. Li and H. Xu, “The occurrence of cardiovascular events of coronary heart disease inpatients and study on Chinese medicine syndrome distribution laws,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 32, no. 5, pp. 603–606, 2012.
  - [27] S. W. Li, *Study on evolution rule of Chinese medicine syndrome in stable phase of coronary heart disease [Ph.D. thesis]*, Beijing University of Traditional Chinese Medicine, Beijing, China, 2011.
  - [28] X. T. Yu, L. Zhang, and H. Xu, “Progress in research on relevant factors affecting TCM syndrome differentiation of CHD,” *Chinese Journal of Integrative Medicine on Cardio/Cerebrovascular Disease*, vol. 7, no. 5, pp. 581–584, 2009.
  - [29] X.-J. Ma, H.-J. Yin, and K.-J. Chen, “Investigation of gene expression profiles in patients with blood stasis syndrome,” *Journal of Chinese Integrative Medicine*, vol. 6, no. 4, pp. 355–360, 2008.
  - [30] Z. K. Yuan, L. P. Wang, and X. P. Huang, “The screening and the functional pathway analysis of differential genes correlated with coronary heart disease of blood stasis syndrome,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 32, no. 10, pp. 1314–1318, 2012.
  - [31] H. J. Wu, Z. C. Ma, Y. Gao et al., “Study on GAP in blood-stasis type of coronary heart disease by using proteomic technique,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 3, no. 3, pp. 189–191, 2005.
  - [32] X. F. Li, Y. R. Jiang, Z. Y. Gao et al., “Screening, identification and analysis of platelet differential functional proteins in patients with coronary heart disease of blood-stasis pattern,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 30, no. 5, pp. 467–473, 2010.
  - [33] H.-H. Zhao, N. Hou, and W. Wang, “Study on proteomic specificity of unstable angina with qi deficiency and blood stasis syndrome,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 29, no. 6, pp. 489–492, 2009.
  - [34] X. Y. Lu, H. Xu, G. Li, and T. Zhao, “Study on correspondence between prescription and syndrome and the essence of phlegm and blood stasis syndrome in coronary heart disease based on metabolomics,” *Chinese Journal of Integrative Medicine*, 2012.
  - [35] W. X. Jian, Z. K. Yuan, and X. P. Huang, “Detection and analysis on plasma metabolomics in patient with coronary heart disease of xin-blood stasis syndrome pattern,” *Chinese Journal of Integrative Medicine*, vol. 30, no. 6, pp. 579–584, 2010.
  - [36] Z. Lu, W. Kou, B. Du et al., “Effect of xuezhikang, an extract from red yeast Chinese rice, on coronary events in a Chinese population with previous myocardial infarction,” *American Journal of Cardiology*, vol. 101, no. 12, pp. 1689–1693, 2008.
  - [37] K.-J. Chen, D.-Z. Shi, H. Xu et al., “XS0601 reduces the incidence of restenosis: a prospective study of 335 patients undergoing percutaneous coronary intervention in China,” *Chinese Medical Journal*, vol. 119, no. 1, pp. 6–13, 2006.

- [38] Q.-H. Shang, H. Xu, X.-Y. Lu, C. Wen, D.-Z. Shi, and K.-J. Chen, "A multi-center randomized double-blind placebo-controlled trial of Xionghao Capsule in preventing restenosis after percutaneous coronary intervention: a subgroup analysis of senile patients," *Chinese Journal of Integrative Medicine*, vol. 17, no. 9, pp. 669–674, 2011.
- [39] F. Tian and Y.-M. Xie, "Real-world study: a potential new approach to effectiveness evaluation of traditional Chinese medicine interventions," *Journal of Chinese Integrative Medicine*, vol. 8, no. 4, pp. 301–306, 2010.
- [40] Z.-Y. Gao, H. Xu, D.-Z. Shi, C. Wen, and B.-Y. Liu, "Analysis on outcome of 5284 patients with coronary artery disease: the role of integrative medicine," *Journal of Ethnopharmacology*, vol. 141, no. 2, pp. 578–583, 2012.
- [41] J. Luo and H. Xu, "Outcome measures of Chinese herbal medicine for coronary heart disease: an overview of systematic reviews," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 927392, 9 pages, 2012.
- [42] K. F. Schulz, D. G. Altman, D. Moher, and CONSORT Group, "CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials," *Annals of Internal Medicine*, vol. 152, no. 11, pp. 726–732, 2010.
- [43] Y. Qiu, H. Xu, and D. Shi, "Traditional chinese herbal products for coronary heart disease: an overview of cochrane reviews," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 417387, 5 pages, 2012.
- [44] H. Xu and K.-J. Chen, "Herb-drug interaction: an emerging issue of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 3, pp. 195–196, 2010.
- [45] N. J. Wald and M. R. Law, "A strategy to reduce cardiovascular disease by more than 80%," *British Medical Journal*, vol. 326, no. 7404, pp. 1419–1423, 2003.
- [46] Y. Feng, H. Xu, and K. J. Chen, "Natural polypill xuezhikang: its clinical benefit and potential multicomponent synergistic mechanisms of action in cardiovascular disease and other chronic conditions," *Journal of Alternative Complementary Medicine*, vol. 18, no. 4, pp. 318–328, 2012.
- [47] A. Tachjian, V. Maria, and A. Jahangir, "Use of herbal products and potential interactions in patients with cardiovascular diseases," *Journal of the American College of Cardiology*, vol. 55, no. 6, pp. 515–525, 2010.
- [48] H. Xu and K.-J. Chen, "Making evidence-based decisions in the clinical practice of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 6, pp. 483–485, 2010.
- [49] X.-F. Yan, Q. Ni, J.-P. Wei, and H. Xu, "Evidence-based practice method of integrative Chinese and western medicine based on literature retrieval through PICO question and complementary and alternative medicine topics," *Chinese Journal of Integrative Medicine*, vol. 16, no. 6, pp. 542–548, 2010.
- [50] Z. Y. Tan, T. Jiang, C. P. Tang, J.-L. Luo, H.-T. Tan, and R.-S. Chen, "The inhibitory effect of tetramethylpyrazine and ferulate on platelet aggregation," *Chinese Journal of New Drugs*, vol. 12, no. 7, pp. 529–531, 2003.
- [51] F. Norol, P. Merlet, R. Isnard et al., "Influence of mobilized stem cells on myocardial infarct repair in a nonhuman primate model," *Blood*, vol. 102, no. 13, pp. 4361–4368, 2003.
- [52] N.-Y. Wang, C.-J. Lu, and X.-H. Chen, "Study on effect of ginsenoside Rg1 in promoting myocardial vascular endothelial cell regeneration through induction on bone marrow stem cell's migration and differentiation in rabbits of myocardial infarction," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 25, no. 10, pp. 916–919, 2005.
- [53] L. D. Li, R. L. Zhang, C. Y. Liu et al., "The effects of Shuanglongfang therapy plus bone marrow mononuclear cells autotransplantation on myocardial infarction in swines," *Chinese Journal of New Drugs*, vol. 12, no. 12, pp. 999–1004, 2003.

## Research Article

# ITIH4: A New Potential Biomarker of “Toxin Syndrome” in Coronary Heart Disease Patient Identified with Proteomic Method

Hao Xu,<sup>1</sup> Qinghua Shang,<sup>1,2</sup> Hao Chen,<sup>3</sup> Jianpeng Du,<sup>1</sup> Jianyan Wen,<sup>4</sup> Geng Li,<sup>4</sup> Dazhuo Shi,<sup>1</sup> and Keji Chen<sup>1</sup>

<sup>1</sup> Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>2</sup> Beijing University of Chinese Medicine, Beijing 100029, China

<sup>3</sup> Wuxi Hospital of Traditional Chinese Medicine, Wuxi 214001, China

<sup>4</sup> China-Japan Friendship Hospital, Beijing 100029, China

Correspondence should be addressed to Dazhuo Shi; heartmail@263.net and Keji Chen; keji\_chen@yahoo.com

Received 5 May 2013; Accepted 15 June 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Hao Xu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Objective.** This trial aims to look for the protein biomarker of “toxin syndrome” of CHD patients. **Methods.** We have performed two trials in this paper. The first trial was a randomized controlled trial (RCT) of the plasma proteome in unstable angina (UA) patients by Maldi-Tof Mass. The second trial was a nested case-control study in 1503 stable CHD patients with one-year followup for acute cardiovascular events (ACEs). **Results.** In the RCT study, 12 protein spots were found to be the differential protein for the significant differences between the difference of before and after treatment in group A and group B; 2 of them (3207.37 Da and 4279.95 Da) was considered to be unique to “toxin syndrome” for being differential proteins of group B but not group A. These 2 spots were identified as Isoform 1 of Fibrinogen alpha chain precursor (FGA, 3207.37 Da) and Isoform 2 of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4, 4279.95 Da), respectively. In the nested case-control study, the result of Western blot demonstrated that protein expression of ITIH4 in the group with followup ACEs was significantly lower than the matched group without followup ACEs ( $P = 0.027$ ). **Conclusion.** ITIH4 might be a new potential biomarker of CHD “toxin syndrome” in TCM, indicating the potential role in early identifying high-risk CHD patients in stable period.

## 1. Introduction

Syndrome differentiation is a unique diagnostic method of traditional Chinese medicine (TCM) [1, 2]. “Blood stasis syndrome” (BSS) is considered as a major and key syndrome in the process of coronary heart disease (CHD) in TCM [3, 4], and activating blood circulation and dissolving stasis has been a mainstream treatment for CHD. However, some stable CHD patients develop acute cardiovascular events (ACEs), while others do not, why? Based on this question, we proposed a hypothesis of “blood stasis and toxin” considering blood stasis was a constant pathogenesis in CHD, while “toxin” was the trigger in transforming to ACEs [5].

The original meaning of “toxin” is a kind of poisonous herb but it has been considered as a pathogenic factor in

a narrow sense and pathogenesis, medicine, and syndrome in a broad sense. It is often seen in the fields of epidemic febrile diseases and surgical diseases (such as carbuncle, abscess, hard furuncle, and sore). Zhang et al. [6] presented a theory of “artery carbuncle” according to previous studies that arteriosclerosis plaque has the characteristics such as redness, swelling, and being hot on the local scale, just like the traditional “toxin syndrome.” Heat-clearing and detoxifying treatment has been widely used in CHD, especially acute coronary syndrome patients [7–9]. Previous studies showed that Rhizoma Coptidis, Cyrtomium Rhizome, compound simiaoyongan decoction, and Huanglian Jiedu decoction could improve clinical symptoms by multiple mechanisms such as anti-inflammatory action, lipid regulation, and AS plaque reduction [10–19]. Furthermore, a lot of researches

indicated that drugs for activating blood circulation and detoxifying had a better effect on relieving angina than drugs for activating blood circulation only; it might be related to the effect of anti-inflammatory action [20–27].

Changes in macroscopic manifestation certainly have the corresponding microscopic biological basis. Inflammation has been proved to be a biomarker for CHD/ACS, and the proteome supported us with a new technology for studying it further. The proteome is a subject studying all the proteins in a cell, a kind of tissue, or an organism in specific conditions or at specific times and has been one of the most potential and effective approaches for decoding and revealing the biological foundation and essence of syndromes. Different syndromes sequentially have relevant differential protein expressions; meanwhile, one syndrome also has different protein expressions after treatment of different medicines. Therefore, the protein's characteristics of a specific syndrome can be reflected by the effectiveness of prescriptions corresponding to syndromes.

Berberine extracted from *Rhizoma Coptidis*, a representative herb of clearing heat and detoxifying, could inhibit the expressions of inflammatory factors such as thromboxane A2 and prostaglandin I2 after the injury of blood vessels [10]. Xiongshao capsule, consisting of active ingredients (Chuanxiongol and paeoniflorin), has shown beneficial effect in atherosclerosis or CHD in clinical and experimental studies [28–34]. Therefore, it was served as a representative Chinese medicine for activating blood circulation.

The aim of this study was to look for the protein biomarker of “toxin syndrome” of CHD patients, which is anticipated to help early identification of high-risk CHD patients in stable period.

## 2. Design and Ethics Statement

There are two parts in this paper (Figure 1). The first one was a randomized controlled trial (RCT) with 2 study groups conducted at 2 cooperating hospitals (Anzhen Hospital and Tongren Hospital) to look for biomarkers for “toxin syndrome” of TCM. The other was a nested case-control study with a follow-up for ACEs conducted at 5 cooperating hospitals (China Academy of Chinese Medical Sciences Xiyuan Hospital, China-Japan friendship Hospital, Anzhen Hospital, Tongren Hospital, and Fujian Integrative Medicine Clinic) to verify the biomarker found in RCT. The trials were carried out according to the Declaration of Helsinki, and the protocols were approved by the institutional review boards and ethics committees at each center. All the patients provided written informed consent.

## 3. Materials and Methods

### 3.1. Randomized Controlled Trial

**3.1.1. Patients.** Fasting serum samples were obtained from 64 patients with UA (ICD-10 : I20.0/20.1/20.9) [35, 36] aged between 40 and 75 years old. All of the patients who were admitted into the 2 cooperating hospitals were enrolled in

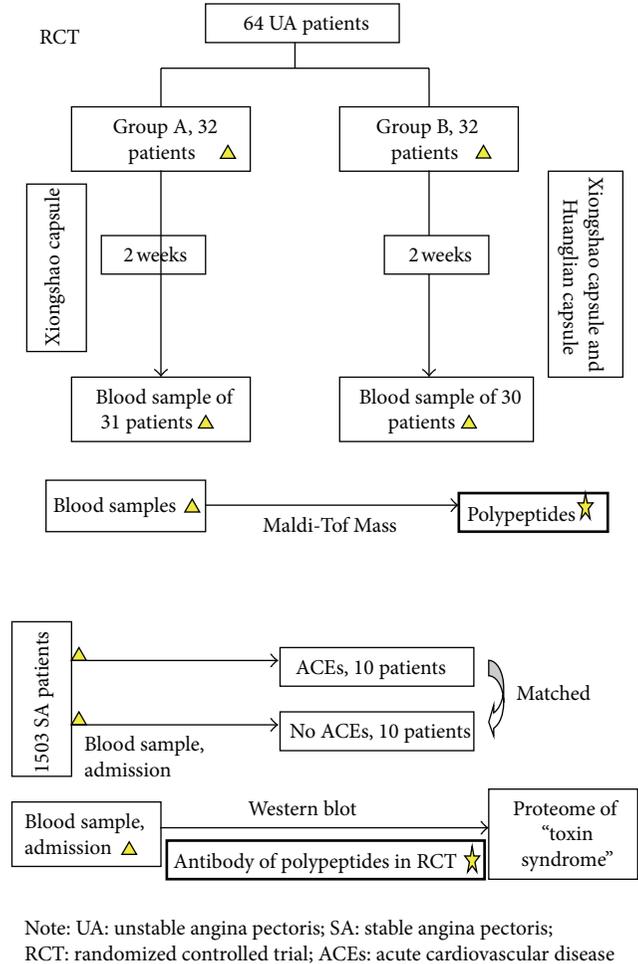


FIGURE 1: Flow chart of the study.

the study. The inclusion criteria were successful PCI in 48 hours after the first severe angina and BSS of TCM (including Qi-stagnation-blood-stasis syndrome and Qi-deficiency-blood-stasis syndrome) [37–39]. Patients were excluded if they met any of the following criteria: presence of (1) stable angina or acute myocardial infarction; (2) inflammation, fever, trauma, burn, or surgery in one recent month; (3) active tuberculosis or rheumatic autoimmune disease; (4) known renal insufficiency and serum creatinine >2.5 mg/dL in male and >2.0 mg/dL in female; (5) known hepatic insufficiency and alanine transaminase (ALT) > three times value of the normal level; (6) severe heart failure (EF < 35%); (7) complication by severe primary disease such as hematologic systems and psychological abnormalities; (8) malignancies; (9) organ transplantation; (10) participants of other clinical trials; (11) taking other Chinese patent drugs; (12) pregnancy or breast-feeding. Patients were removed from analysis if they could not estimate the efficacy for they did not take any medicine or did not participate reexamination as proposal.

**3.1.2. Groups and Drugs.** 64 eligible patients were randomly assigned in a 1:1 ratio to group of activating blood circulation

(Xiongshao capsule, Z20053499, Hospital preparation approved by Beijing drug administration, group A) or group of activating blood circulation and detoxification (Huanglian capsule, Z19983042, Hubei Xianglian Pharmaceutical Co., Ltd and Xiongshao capsule, group B). Randomization table was performed centrally with the use of SAS software and reserved by a specific person who did not participate this clinic research. Randomized number was obtained by telephone if any patient was eligible.

In the group of activating blood circulation, Xiongshao capsule was taken as 500 mg (2 capsules) 3 times per day for 2 weeks. In the group of activating blood circulation and detoxification, Huanglian capsule was taken as 500 mg (2 capsules) 3 times per day, and Xiongshao capsule was taken as 500 mg (2 capsules) 3 times per day for 2 weeks.

All patients received western standardized medication including antiplatelet drugs (aspirin and/or clopidogrel hydrogen sulfate), anticoagulant drugs (heparin or low molecular weight heparin), anti-ischemic drugs (nitrates,  $\beta$ -blocker, calcium channel blocker, and angiotensin-converting enzyme inhibitors), and statins.

**3.1.3. Data Collection.** At the beginning of the trial, all patients filled out a standardized questionnaire containing general information, past history, risk stratification of UA [40], angina score, primary symptom score of TCM [41], BSS score [42], medical treatment, and PCI surgery. In addition, to obtain the serum, at the beginning and the end of the trial, 2 ml of blood from each patient with an empty stomach was drawn into common coagulation-promoting tubes, centrifuged at 3000 r for 10 min at room temperature to remove insoluble materials, cells, and debris, and supernatants were kept at  $-80^{\circ}\text{C}$  until use.

**3.1.4. Reagents and Instruments.** The WCX magnetic bead kit (Bruker Daltonics Tech, Beijing, China), alpha-cyano-4-hydroxycinnamic acid (HCCA), MALDI-TOF MS (type: microflex, Bruker Daltonics Biosciences, Bremen, Germany), 100% ethanol (chromatographic grade), and 100% acetone (chromatographic grade) were freshly prepared (sigma).

**3.1.5. WCX Fractionation and MALDI-TOF MS Analysis.** The suspension in the WCX magnetic bead kit was mixed by shaking. After eluting and beating, the magnetic beads were separated from the protein, and the eluted peptide samples were transferred to a 0.5 mL clean sample tube for further MS analysis. Five microliters of HCCA substrate solution (0.4 g/L, dissolved in acetone and ethanol) and 0.8–1.2  $\mu\text{L}$  of elution were mixed. Then, 0.8–1.2  $\mu\text{L}$  of this mixture was applied to a metal target plate and dried at room temperature. Finally, the prepared sample was analyzed by MALDI-TOF MS. A range of 1000–10,000 Da peptide molecular weights was collected, and 400 shots of laser energy were used. Peptide mass fingerprints were obtained by accumulating 50 single MS signal scans.

**3.1.6. Peptide Sequence.** Experiment for 4280.13  $m/z$  peptide identification was performed using a nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC/ESI-mass spectrometry/mass spectrometry) system consisting of an Acquity UPLC system (Waters) and an LTQ Orbitrap XL mass spectrometer (Thermo Fisher) equipped with a nano-ESI source. The peptide solutions were loaded to a C18 trap column (nano-Acquity) (180  $\mu\text{m} \times 20 \text{ mm} \times 5 \mu\text{m}$  (symmetry)). The flow rate was 15  $\mu\text{L}/\text{min}$ . Then the desalted peptides were analyzed by C18 analytical column (nano-Acquity) (75  $\mu\text{m} \times 150 \text{ mm} \times 3.5 \mu\text{m}$  (symmetry)) at a flow rate of 400 nL/min. The mobile phases A (5% acetonitrile, 0.1% formic acid) and B (95% acetonitrile, 0.1% formic acid) were used for analytical columns. The gradient elution profile was as follows: 5%B–50%B–80%B–80%B–50%B–5%B in 100 min. The MS instrument was operated in a data-dependent model. The range of full scan was 400–2000  $m/z$  with a mass resolution of 100,000 ( $m/z$  400). The eight most intense monoisotope ions were the precursors for collision induced dissociation. Mass spectrometry was limited to two consecutive scans per precursor ion followed by 60 s of dynamic exclusion.

**3.1.7. Statistical Analysis.** ClinProTools (ClinProt software version 2.1, Bruker Daltonics) was used to subtract baseline, normalize spectra (using total ion current), and determine peak  $m/z$  values and intensities in the mass range of 1000 to 10,000 Da. The signal-to-noise (S/N) ratio should be higher than five. To align the spectra, a mass shift of no more than 0.1% was determined. The peak area was used as quantitative standardization. Student's  $t$ -test was used for analysis of normally distributed continuous data, while Wilcoxon test for nonnormally distributed continuous data. Chi-square test was used for categorical data analysis. A  $P$  value  $<0.05$  was considered significant.

### 3.2. Nested Case-Control Study

**3.2.1. Patients.** 1503 patients with stable CHD (old myocardial infarction or at least one significant ( $>50\%$ ) stenosis that was documented on a recent coronary angiogram and WHO [35]) younger than 80 years old were enrolled from 5 cooperating hospitals. Stable CHD was defined as no symptoms or stable exertional angina or patients in stable condition after ACS for at least 1 month. Patients were excluded if they met any of the following criteria: presence of (1) inflammation, fever, trauma, bure, or surgery in one recent month; (2) active tuberculosis or rheumatic autoimmune disease; (3) severe heart failure (EF  $< 35\%$ ); (4) complication by severe valvular heart disease, or cardiomyopathy; (5) complication by severe chronic obstructive pulmonary disease (COPD), pulmonary heart disease or respiratory failure; (6) known renal insufficiency and serum creatinine  $>2.5 \text{ mg/dL}$  in male and  $>2.0 \text{ mg/dL}$  in female; (7) known hepatic insufficiency and alanine transaminase (ALT)  $>$ three times value of the normal level; (8) complication by severe primary disease such as hematologic systems; (9) severe psychological abnormalities; (10) malignancies; (11) viscera transplantation; (11) life

expectancy less than 3 years. Patients were removed from analysis if a mistaken inclusion or lack of necessary record for analysis or failure to follow up for ACEs because of missing contact information took place.

**3.2.2. Data Collection.** In all patients, follow-up was scheduled at 0.5 and 1 year after inclusion of the trial. At every visit of the trial, information was obtained from each patient by use of a standardized questionnaire, the information regarding general information, past history, and the secondary cardiovascular events in follow-up. Physicians collecting information were unaware of the purpose of the study. Secondary cardiovascular events were defined as death from heart disease, nonfatal myocardial infarction (MI), or ischemic cerebrovascular events (stroke or transient ischemic attack). All the cardiovascular events were estimated by consulting medical records. In addition, the serum also was collected at every visit, and the method of blood collection, centrifugation, and storage was the same as that of RCT.

Twenty three patients were confirmed as ACEs during one-year follow-up, and 10 patients were selected for their well preserved serum sample. Another 10 patients with no follow-up ACEs were matched in a 1:1 ratio by sex, age ( $\pm 5$  years), hypertension history, diabetes history, and myocardial infarction history. All the sera at the admission of these 20 patients were adopted for verifying the differential protein of “toxin syndrome” obtained from RCT by Western blot method.

**3.2.3. Western Blot.** To detect the inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) obtained from RCT (see results section), blood serum stored in  $-80^{\circ}\text{C}$  refrigerator was assayed using Western blot as described before [43]. Additionally, ITIH4 antibody (1:2500, Sigma, USA) was used for detection of ITIH4. The horseradish peroxidase (HRP) conjugated anti-mouse IgG ( $0.1\text{ mL/cm}^2$ , Santa Cruz Biotechnology, UAS) was used as the secondary antibody, and signals were visualized using the enhanced chemiluminescence system (ECL, Pierce, USA).

**3.3. Statistical Analysis.** Statistical analysis was performed by a statistician in a blind fashion. Statistical analysis was performed with SPSS15.0 software. All tests were two tailed, and a statistical probability of  $<0.05$  was considered significant. Normality test and homogeneity test of variances were conducted. Frequency table, percentage or constituent ratio for describing enumeration data;  $\bar{X} \pm S$  for describing measurement data.  $\chi^2$  test or Fisher exact test if necessary was used for comparison of enumeration data,  $t$ -test was used for comparison of measurement data (corrected  $t$  test was used if variant heterogeneity), and Wilcoxon tests were used for abnormal distribution.

## 4. Results

**4.1. Patients' Characteristics in RCT.** 64 participants with UA were enrolled in 5 centers and were randomized into two

groups: 32 to receive Xiongshao capsule (group A) and 32 to receive Xiongshao capsule and Huanglian capsule (group B). During the course of the study, one patient was excluded in group A due to incomplete follow-up, while two patients were excluded in group B with 1 incomplete follow-up, and 1 noncompliance with medications. They were removed from statistics as the stated protocol. Thus finally, the population in analysis consisted of 61 patients, with 31 patients in group A and 30 patients in group B. The baseline characteristics of the UA patients were summarized in Table 1. The two groups were well matched with regard to baseline clinical and angiographic characteristics ( $P > 0.05$ ).

**4.2. Sample Processing in RCT.** During the course of the protein analysis, five blood samples were excluded from group A due to bad peptide mass spectrometry; thus, the population in differential protein analysis consisted of 56 patients, with 26 patients in group A and 30 patients in group B. Acquisition mass range 500–10000 Da (low-to-medium molecular mass range) would be studied in bioinformatics analysis.

**4.3. MALDI-TOF Mass Spectrometry Analysis of Peptides in Serum of RCT.** Statistical analysis of the data revealed that the expression of 24 spots was altered after treatment as compared with that at admission in group A (7 of them upregulated and 17 downregulated, Table 2, Figure 2). The expression of 15 spots was altered after treatment as compared with that at admission in group B (8 of them upregulated and 17 downregulated, Table 3, Figure 3), and 4 of the 15 spots were the same as group A. Twelve protein spots were found (Table 4) to be the differential protein for the significant differences between the difference of before-after treatment in group A and group B; 2 of them (3207.37 Da and 4279.95 Da) were considered to be unique to “toxin syndrome” for being differential proteins of group B but not group A. These 2 spots were identified by mass spectrometry (Figures 4 and 5).

**4.4. Identification of Protein Fragments by Proteome Analysis in RCT.** Isoform 2 of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and Isoform 1 of Fibrinogen alpha chain precursor (FGA) were identified in different spots by proteome analysis which can be served as biomarkers of “toxin syndrome” in CHD patients (Table 5).

**4.5. Western Blot.** A large multicenter nested casecontrol study was conducted for verifying the unique protein biomarker to “toxin syndrome” of TCM obtained from RCT. The admission blood samples of 20 patients were collected for Western blot (10 patients, resp. in the ACEs group and the matched group). We assay the serum protein concentrations and based on the readings load the same amount of protein. In the posttranslational process, the protein ITIH4 was modified and cleaved by plasma kallikrein to yield 100 kDa and 35 kDa fragments. Statistics indicated that protein expression

TABLE 1: Baseline information of two groups in RCT.

Groups	Group A	Group B
Age		
Minimum value (years)	48	42
Maximum value (years)	74	75
Mean value (years)	61.94 ± 8.41	61.24 ± 9.86
Sex		
Male (proportion)	22 (71%)	25 (86.2%)
Female (proportion)	9 (29%)	5 (13.8%)
Angina score	14.42 ± 4.86	14.89 ± 4.63
Primary symptom score of TCM	17.97 ± 6.74	18.94 ± 5.64
BSS score	10.89 ± 4.62	10.59 ± 3.38
Past history		
Hypertension ( <i>N</i> )	18	16
Diabetes ( <i>N</i> )	7	9
Dislipidemia ( <i>N</i> )	11	12
Stroke ( <i>N</i> )	2	4
Peripheral vascular atherosclerosis ( <i>N</i> )	5	3
Old myocardial infarction ( <i>N</i> )	4	1
Western medicine		
Aspirin ( <i>N</i> )	31	30
Clopidogrel hydrogen sulfate ( <i>N</i> )	31	30
Nitrates ( <i>N</i> )	21	16
β-blocker ( <i>N</i> )	28	30
ACEI/ARB ( <i>N</i> )	19	17
CCB ( <i>N</i> )	3	9
Low molecular weight heparin ( <i>N</i> )	16	19
Statins ( <i>N</i> )	30	29
UA risk stratification		
Low risk ( <i>N</i> )	0	0
Mediate risk ( <i>N</i> )	26	24
High risk ( <i>N</i> )	5	6
Number of stenosed coronary vessel		
1 vessel ( <i>N</i> )	8	12
2 vessels ( <i>N</i> )	12	7
3 vessels ( <i>N</i> )	11	11
Lesions nature		
De novo ( <i>N</i> )	28	26
Restenosis ( <i>N</i> )	3	4
Stent type		
Sirolimus-eluting stent ( <i>N</i> )	21	21
paclitaxel-eluting stent ( <i>N</i> )	8	6
Mixed drug-eluting stents ( <i>N</i> )	2	3
Total length of stents	22.94 ± 7.23	21.67 ± 9.69

Note: group A patients have taken the Xiongshao capsule; group B patients have taken the Xiongshao capsule and Huanglian capsule.

of ITIH4 in the ACEs group was significantly lower than that in the matched group ( $P = 0.027$ ) (Table 6, Figure 6). Therefore, the results of nested case-control study further demonstrated the biomarker identified in the RCT, which indicated that the reduced ITIH4 might be a unique protein biomarker/bioinformation of “toxin syndrome” in CHD patients.

## 5. Discussion

As the development of systems biology and the advancement of the human genome project increased, more and more attention has been paid on the importance of proteome. In this study, we identified 2 peptides (FGA and ITIH4) related to CHD “toxin syndrome” by “taking special drugs

TABLE 2: Comparison of before and after treatment in group A ( $\bar{X} \pm S$ ).

Mass (Da)	Ave $\pm$ StdDev (A-Q)	Ave $\pm$ StdDev (A-H)	<i>P</i>
1076.12	3.77 $\pm$ 2.35	2.54 $\pm$ 1.24	0.016099
1136.37	7.25 $\pm$ 3.12	5.42 $\pm$ 2.08	0.008065
1205.62	9 $\pm$ 3.32	6.92 $\pm$ 4.43	0.018784
1329.42	14.44 $\pm$ 7.31	10.26 $\pm$ 4.92	0.000268
1348.81	10.41 $\pm$ 4.08	7.09 $\pm$ 2.89	0.000806
1464.89	24.93 $\pm$ 13.64	13.52 $\pm$ 6.44	0.000293
1519.06	18.49 $\pm$ 8.17	11.39 $\pm$ 5.92	0.000111
1544.61	30.66 $\pm$ 17.33	19.41 $\pm$ 16.3	0.011662
1616.74	33.35 $\pm$ 22.17	18.7 $\pm$ 9.09	0.002143
2209.31	37.24 $\pm$ 18.84	25.45 $\pm$ 17.84	0.01935
2279.51	50.15 $\pm$ 17.26	40.99 $\pm$ 14.08	0.018232
2644.01	30.08 $\pm$ 20.39	22.42 $\pm$ 14.8	0.000347
2660.01	288.15 $\pm$ 231.67	206.03 $\pm$ 161.86	0.003587
2862.02	77.55 $\pm$ 78.74	45.15 $\pm$ 29.33	0.00951
3261.70	125.1 $\pm$ 63.45	159.89 $\pm$ 62.44	0.043161
3277.49	45.57 $\pm$ 23.64	59.7 $\pm$ 29.1	0.015557
4053.87	71.26 $\pm$ 38.16	95.63 $\pm$ 53.55	0.047052
4710.25	15.53 $\pm$ 5.5	12.84 $\pm$ 4.04	0.028146
4936.10	17.25 $\pm$ 15.28	9.77 $\pm$ 2.99	0.015955
4964.02	147.4 $\pm$ 184.34	59.95 $\pm$ 34.25	0.019728
5807.76	52.08 $\pm$ 23.63	70.92 $\pm$ 24.68	0.010841
5822.51	22.33 $\pm$ 13.95	31.77 $\pm$ 10.34	0.007368
5904.69	881.7 $\pm$ 598.83	1266.04 $\pm$ 415.59	0.00471
6049.22	27.36 $\pm$ 10.77	32.41 $\pm$ 10.1	0.023583

Note: paired sample *t* test was used, 2-tailed, and  $P < 0.05$  was considered significant; Ave: peak area/intensity average; StdDev: standard deviation of the peak area/intensity average; A-Q: before treatment in group A; A-H: after treatment in group A.

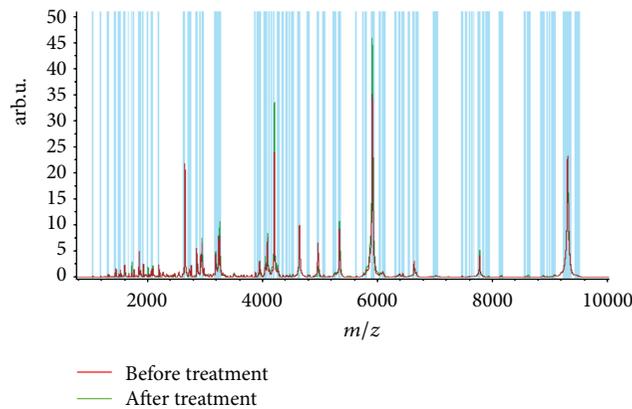


FIGURE 2: Peptide mass spectrometry before and after treatment in group A.

to ascertain syndromes” in RCT using MOLDI-TOF MS. Since fibrinogen has been proven to be a risk factor for ACEs in many previous studies [44–46], we only verified another differential protein, ITIH4, by Western blot in a subsequent nested case-control study. Finally, ITIH4 was ascertained to be a new biomarker for CHD “toxin syndrome,” which can also be served as a new risk predictor for ACEs in stable CHD patients.

BSS is one of the basic syndromes in CHD, and “toxin syndrome” is the key in pathogenesis of disease progression. BSS and “toxin syndrome” can coexist or transform to each other, which make up the whole pathological process of CHD [5]. From the macroscopic point of view, Xu et al. [47, 48] enrolled 254 stable CHD patients, collected the clinical information and ACEs in follow-up, and thus concluded a series of clinical manifestations for “toxin syndrome” in stable CHD patients

TABLE 3: Comparison of before and after treatment in group B ( $\bar{X} \pm S$ ).

Mass (Da)	Ave $\pm$ StdDev (B_Q)	Ave $\pm$ StdDev (B_H)	P
1616.74	32.83 $\pm$ 18.26	23.95 $\pm$ 12.74	0.033415
2209.31	29.04 $\pm$ 14.35	21.23 $\pm$ 10.27	0.022373
2881.04	49.8 $\pm$ 18.98	38.19 $\pm$ 10.15	0.010917
3207.37	56.15 $\pm$ 17.06	47.84 $\pm$ 12.01	0.023899
4053.87	60.23 $\pm$ 22.82	83.04 $\pm$ 42.14	0.010323
4266.31	35.94 $\pm$ 19.99	45.73 $\pm$ 19.4	0.020676
4279.95	18.9 $\pm$ 8.79	27.23 $\pm$ 23.79	0.025866
4817.85	20.34 $\pm$ 17.41	11.15 $\pm$ 7.93	0.012491
4936.10	21.91 $\pm$ 27.22	10.29 $\pm$ 3.94	0.02628
5066.25	25.87 $\pm$ 7.61	32.1 $\pm$ 11.2	0.019568
5248.63	21.01 $\pm$ 5.89	25.23 $\pm$ 8.04	0.025751
6378.01	47.98 $\pm$ 47.92	28.92 $\pm$ 10.34	0.045958
7833.86	10.85 $\pm$ 2.21	12.97 $\pm$ 4.82	0.040051
9064.40	30.36 $\pm$ 8.86	35.83 $\pm$ 10.12	0.012614
9290.26	907.98 $\pm$ 442.14	1126.48 $\pm$ 455.76	0.023601

Note: paired sample *t*-test was used, 2-tailed, and *P* < 0.05 was considered significant; Ave: peak area/intensity average; StdDev: standard deviation of the peak area/intensity average; B-Q: before treatment in group B; B-H: after treatment in group B; overstriking mass: unique to group B.

TABLE 4: Comparison of difference between before and after treatment in the two groups.

Mass (Da)	Ave $\pm$ StdDev (A_H - A_Q)	Ave $\pm$ StdDev (B_H - B_Q)	P
1329.42	-4.18 $\pm$ 5.02	0.4 $\pm$ 6.86	0.005918
1519.06	-7.1 $\pm$ 7.9	-2.47 $\pm$ 9.08	0.046435
2660.01	-82.12 $\pm$ 130.27	3.85 $\pm$ 184.48	0.047102
2881.04	4.86 $\pm$ 16.64	-11.62 $\pm$ 23.4	0.003442
<b>3207.37</b>	<b>6.56 <math>\pm</math> 21.34</b>	<b>-8.31 <math>\pm</math> 19.09</b>	<b>0.008696</b>
3277.49	14.13 $\pm$ 27.75	-9.87 $\pm$ 33.72	0.005087
3972.15	-8.69 $\pm$ 22.59	3.47 $\pm$ 15.5	0.02561
<b>4279.95</b>	<b>-2.49 <math>\pm</math> 14.27</b>	<b>8.32 <math>\pm</math> 19.41</b>	<b>0.020196</b>
5066.25	-0.43 $\pm$ 10.97	6.23 $\pm$ 13.8	0.049446
5807.76	18.84 $\pm$ 34.89	-2.2 $\pm$ 36.2	0.031283
5822.51	9.44 $\pm$ 16.49	-7.75 $\pm$ 32.03	0.013558
6088.68	8 $\pm$ 28.11	-8.21 $\pm$ 25.58	0.029233

Note: paired sample *t*-test was used, 2-tailed, and *P* < 0.05 was considered significant; Ave: peak area/intensity average; StdDev: standard deviation of the peak area/intensity average; "A\_H - A\_Q": subtraction between after and before treatment in group A; "B\_H - B\_Q": subtraction between after and before treatment in group B; overstriking mass: unique to "Toxin."

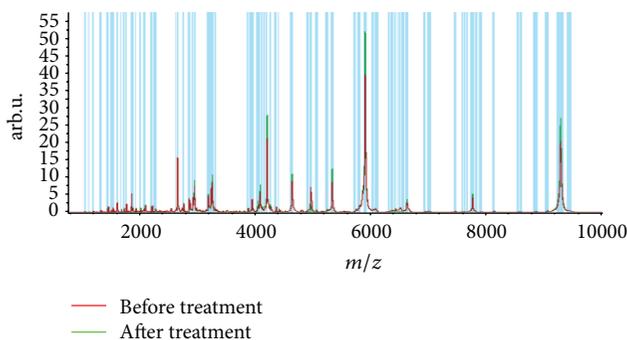


FIGURE 3: Peptide mass spectrometry before and after treatment in group B.

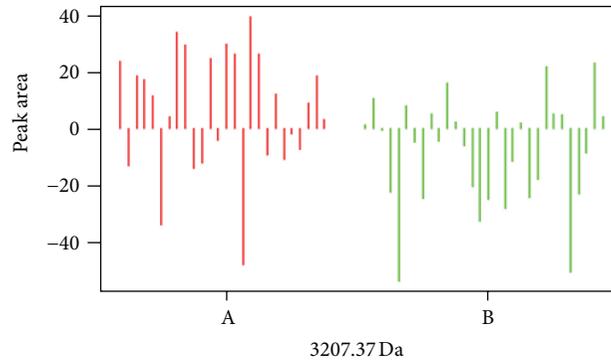


FIGURE 4: Difference of 3207 Da protein peak between group A and group B.

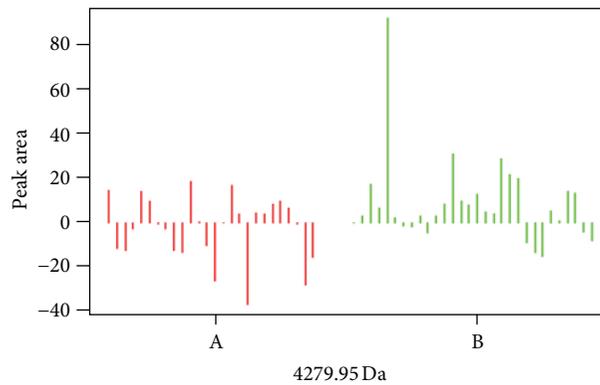


FIGURE 5: Difference of 4279.95 Da protein peak between group A and group B.

including pain in substernal, headache, uneven or irregular pulse, frequent pharyngalgia, and increased high-sensitivity C-reactive protein (hs-CRP). Other scholars [49] collected clinical information and then summarized a differentiation standard for “toxin-stasis syndrome” of ACS in Chinese medicine. From the microcosmic point of view, “inflammatory reaction” is always a research focus for CHD “toxin syndrome.” Wen et al. [23] investigated the effect on plaque stabilization among herbs regulating blood circulation (*Salvia Miltiorrhiza*, *Radix Paeoniae Rubra*), activating blood circulation (*Szechuan Lovage Rhizome*, *Panax Notoginseng*), and breaking blood stasis (*Peach Seed*, *Rhubarb Root Parched in Wine*) from inflammation, pathomorphology, cellular composition, and so on; indicated *Rhubarb root parched in wine* was the best for its effect of breaking blood stasis and detoxification. Zhou et al. [26] proposed the hypothesis of “activating blood circulation and detoxification— inflammatory reaction inhibition—plaque stabilization,” then compared the effect on plaque stabilization among herbs activating blood circulation (*Panax Notoginsenosides*), herbs for detoxification (*Goldthread Rhizome extract*) and herbs activating blood circulation and detoxification (*Rhubarb alcohol extract* and *Polygonum Cuspidatum extract*). The results indicated the superior effect of *Rhubarb alcohol extract* and *Polygonum Cuspidatum extract* on stabilizing vulnerable plaque, showing the “class effect” of herbs activating blood circulation

and detoxification in inhibiting inflammatory reaction and stabilizing plaque.

The function of ITIH4 in European Molecular Biology Laboratory-The European Bioinformatics Institute (EMBL-EBI) showed that ITIH4 is a type II acute-phase protein (APP) involved in inflammatory responses to trauma. And it may also play a role in liver development and regeneration [50]. Inter-alpha-trypsin inhibitor (ITI) family proteins are all composed by light chain (bikunin) and at least 6 heavy chains. Lots of researches have proved that bikunin could inhibit the activity of protease, but little studies have paid attention to heavy chains of ITI. In 2000, Japanese scholar Choi-Miura et al. found that inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP) could inhibit the aggregation and phagocytosis of actins in polymorphonuclear leukocyte, implying that IHRP might be a new APP involved in inflammatory responses [51]. In 2004, Fujita et al. showed that genetic locus mutation of ITIH4 might be one of possible factors for dyslipidemia [52]. Then, Piñeiro et al. proved that ITIH4 was a new APP isolated from cattle during experimental infection [53]. Recently, Kashyap et al. found that ITIH4 showed high expression in normal subjects but no expression or little expression in patients with acute ischemic stroke (AIS), and this protein could return to normal level in blood serum gradually as the patients were getting better. The scholars considered that ITIH4 was a novel biomarker in

TABLE 5: Peptides identification unique to “Toxin”.

Mass	IPI	Gene.Symbol	Amino acid sequence
4280.13 Da	IPI00218192.3	ITIH4 Isoform 2 of inter-alpha-trypsin inhibitor heavy chain H4	R.NVHSAGAAGSRMNFPGVLSSRQLGLPGPPDVPDHAAYHPFR
3206.42 Da	IPI00021885.1	FGA Isoform 1 of Fibrinogen alpha chain precursor	K.SSSYSKQFTSSTS YNRGDSTFESKSYKM*.A

TABLE 6: ITIH4 expression between ACEs group and matched group.

Groups	Patients	MOD	P
ACEs group	10	8.41 ± 4.04	0.027
Matched group	10	11.57 ± 5.34	

Note: paired sample *t*-test was used, 2-tailed, and  $P < 0.05$  was considered significant; MOD: mean optical density.

inflammatory responses for AIS due to its close relationship with S-100  $\beta$ , neuron specific enolase (NSE), interleukin-2 (IL-2), and interleukin-10 (IL-10) expression [54].

Results in this study showed that the group of activating blood circulation and detoxification could significantly increase ITIH4 expression and decrease FGA expression compared with the group of activating blood circulation and indicated that ITIH4 and FGA might be potential protein biomarkers for “toxin syndrome” of CHD. ITIH4 was further demonstrated in a nested case-control study, indicating its potential role as a new prewarning biomarker in stable CHD patients.

Before recommending the conclusion of this study to clinical practice, we have to consider the following weaknesses. We cannot ascertain whether the tendency of these two polypeptides is always the same in the process of “toxin syndrome” development. Therefore, a large prospective cohort study collecting information in more time points is necessary.

## 6. Conclusion

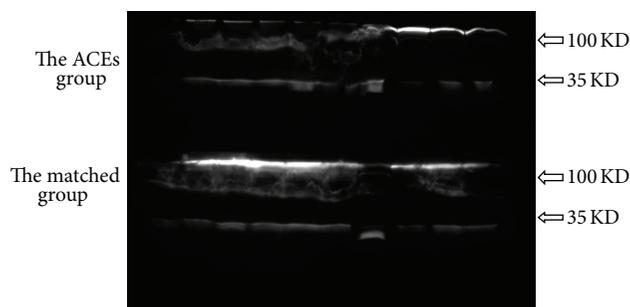
ITIH4 might be a new potential biomarker of CHD “toxin syndrome” in TCM, indicating the potential role as a pre-warning biomarker in stable CHD patients.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Authors' Contribution

Keji Chen and Dazhuo Shi conceived and designed the trials and performed interpretation of the results; Hao Xu and Qinghua Shang carried out the project and drafted the paper; Hao Chen and Jianpeng Du collected the information of all volunteers; and Geng Li and Jianyan Wen performed



Note: KD: unit of ITIH4 molecular weight

FIGURE 6: ITIH4 expression between the ACEs group and the Matched group.

the experiments in this trial. All authors read and discussed the paper, and all gave approval for the publication.

## Acknowledgments

The current work was partially supported by the National Key Basic Research Program of China (no. 2006CB504803) and the Twelve Five-Year Plan of China (nos. 2013BAI02B01 and 2013BAI13B01). Hao Xu and Qinghua Shang are cofirst authors.

## References

- [1] A. S. Ferreira and A. J. Lopes, “Chinese medicine pattern differentiation and its implications for clinical practice,” *Chinese Journal of Integrative Medicine*, vol. 17, no. 11, pp. 818–823, 2011.
- [2] M. F. Mei, “A systematic analysis of the theory and practice of syndrome differentiation,” *Chinese Journal of Integrative Medicine*, vol. 17, no. 11, pp. 803–810, 2011.
- [3] O. Li and H. Xu, “The occurrence of cardiovascular events of coronary heart disease inpatients and study on chinese medicine syndrome distribution laws,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 32, no. 5, pp. 603–606, 2012.
- [4] Z. Y. Gao, J. C. Zhang, H. Xu et al., “Analysis of relationships among syndrome, therapeutic treatment, and Chinese herbal medicine in patients with coronary artery disease based on complex networks,” *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 8, no. 3, pp. 238–243, 2010.
- [5] H. Xu, D. Z. Shi, H. J. Yin, J. C. Zhang, and K. J. Chen, “Blood-stasis and toxin causing catastrophe hypothesis and acute cardiovascular events: proposal of the hypothesis and its

- clinical significance," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 10, pp. 934–938, 2008.
- [6] Z. Zhang, G. L. Yang, H. Y. Zhang, M. Chen, Y. Chen, and Z. B. Luo, "A hypothesis of atherosclerosis plaque from the surgical carbuncle theory," *Liaoning Journal of Traditional Chinese Medicine*, vol. 35, no. 2, pp. 201–202, 2008.
  - [7] L. Wang, L. B. Wei, X. F. Liu, S. W. Ding, and M. Peng, "Toxin theory and acute coronary syndrome," *Chinese Journal of Integrative Medicine on Cardio-/Cerebrovascular Disease*, vol. 3, no. 12, pp. 1080–1081, 2005.
  - [8] W. X. Du, C. Y. Liu, H. X. Zhang, M. Zhang, and H. W. Song, "Acute myocardial infarction and TCM theory," *Chinese Journal of Integrative Medicine on Cardio-/Cerebrovascular Disease*, vol. 4, no. 5, pp. 434–436, 2006.
  - [9] W. Wu and R. Peng, "Progress of heat & toxin pathogenesis in coronary heart disease," *Journal of New Chinese Medicine*, vol. 39, no. 6, pp. 3–4, 2007.
  - [10] H. Duan, H. K. Zhang, J. J. Liu, W. J. Nie, Y. Ma, and Y. Q. Gao, "Effect of berberine on IL-6 of carotid artery after balloon injury in rabbits," *Journal of Medical Forum*, vol. 27, no. 10, pp. 6–9, 2006.
  - [11] M. Wu and J. Wang, "Advance on study in anti-atherosclerosis mechanism of berberine," *China Journal of Chinese Materia Medica*, vol. 33, no. 18, pp. 2013–2016, 2008.
  - [12] Z. Q. Lei, S. Y. Chen, and X. M. Gao, *Traditional Chinese Pharmacology*, Shanghai Science and Technology Press, 1st edition, 1995.
  - [13] S. W. He, R. H. Zhao, H. J. Wu, and A. H. Guo, "Effect of wild purslane on atherosclerosis formation in rabbit," *Chinese Journal of Preventive Medicine*, vol. 31, no. 2, p. 91, 1997.
  - [14] H. B. Tan, "Study of gynostemma pentaphylla on atherosclerosis in rabbit," *Chinese Journal of Gerontology*, vol. 27, no. 6, pp. 519–521, 2007.
  - [15] L. D. Xie, L. X. Chen, S. X. Wu, and B. Nie, "Overview of clinical and basic research of Simiao Yong'an decoction in the treatment of coronary heart disease," *Global Traditional Chinese Medicine*, vol. 5, no. 8, pp. 629–633, 2012.
  - [16] N. Zhu, X. M. Cao, Y. X. Cai, Y. Wu, and B. B. Wang, "Effect of huanglian jiedu decoction on CRP and TNF- $\alpha$  in coronary heart disease," *Journal of Emergency in Traditional Chinese Medicine*, vol. 21, no. 4, pp. 542–543, 2012.
  - [17] G. Q. Li, P. Huang, H. M. Cheng et al., "Effect of huanglian jiedu decoction on T cell expression regulated by CD4+ CD25+ in rats with atherosclerosis," *Guangdong Medical Journal*, vol. 31, no. 3, pp. 329–331, 2010.
  - [18] Z. Y. Guo, P. Huang, and G. Q. Li, "Effect of huanglian jiedu decoction on the expression of MCP-1/CCR2 mRNA in atherosclerosis rats," *Traditional Chinese Drug Research and Clinical Pharmacology*, vol. 21, no. 6, pp. 583–586, 2010.
  - [19] F. Zheng, M. X. Zhou, H. Xu, and K. J. Chen, "Effects of herbs with function of activating blood circulation and detoxication on serum inflammatory markers and blood lipids in stable patients with coronary heart disease," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 24, no. 9, pp. 1153–1157, 2009.
  - [20] X. H. Lu and S. W. Ding, "Clinical effect and mechanism on treating unstable angina pectoris by huanglian jiedu capsule," *Journal of Shandong University of Traditional Chinese Medicine*, vol. 29, no. 6, pp. 457–460, 2005.
  - [21] R. Yu, S. X. He, X. L. Ye, and X. C. Wang, "The effect of therapeutic method of blood-activating and detoxifying of TCM on serum level of sCD40L in the patients with acute coronary syndrome," *Journal of Emergency in Traditional Chinese Medicine*, vol. 17, no. 11, pp. 1500–1501, 2008.
  - [22] R. Yu, X. S. Hu, S. X. He, X. L. Ye, and X. X. Zhang, "The effect of huoxuejiedu decoction on serum level of MMP-1, MMP-9, TIMP-1 in the patients with acute coronary syndrome," *Journal of Emergency in Traditional Chinese Medicine*, vol. 17, no. 10, pp. 1337–1346, 2008.
  - [23] C. Wen, H. Xu, Q. F. Chen, P. Li, and X. Sheng, "Effects of herbs of activation blood on atherosclerotic plaque morphology in ApoE gene-deficient mice," *Chinese Journal of Pathophysiology*, vol. 21, no. 5, pp. 864–867, 2005.
  - [24] C. Wen, H. Xu, Q. F. Huang, and K. J. Chen, "Effect of drugs for promoting blood circulation on blood lipids and inflammatory reaction of atherosclerotic plaques in ApoE gene deficiency mice," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 25, no. 4, pp. 345–349, 2005.
  - [25] M. X. Zhou, H. Xu, K. J. Chen, L. Pan, C. Wen, and J. G. Liu, "Effects of some active ingredients of Chinese drugs for activating blood circulation and detoxicating on blood lipids and atherosclerotic plaque inflammatory reaction in ApoE-gene knockout mice," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 2, pp. 126–130, 2008.
  - [26] M. X. Zhou, H. Xu, K. J. Chen, L. Pan, C. Wen, and Y. R. Guo, "Effects of several herbal extractives with the effect of promoting blood flow and detoxication on atherosclerotic plaque stability in aorta of apoE-gene knockout mice," *Chinese Journal of Pathophysiology*, vol. 24, no. 11, pp. 2097–2102, 2008.
  - [27] J.-C. Zhang, K.-J. Chen, G.-J. Zheng et al., "Regulatory effect of Chinese herbal compound for detoxifying and activating blood circulation on expression of NF-kappaB and MMP-9 in aorta of apolipoprotein E gene knocked-out mice," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 21, no. 1, pp. 40–44, 2007.
  - [28] K.-J. Chen, D.-Z. Shi, H. Xu et al., "XS0601 reduces the incidence of restenosis: a prospective study of 335 patients undergoing percutaneous coronary intervention in China," *Chinese Medical Journal*, vol. 119, no. 1, pp. 6–13, 2006.
  - [29] H. Xu, K. J. Chen, D. Z. Shi, X. C. Ma, S. Z. Lv, and J. M. Mao, "Clinical study of Xiongshao capsule in preventing restenosis after coronary interventional treatment," *Chinese Journal of Integrative Medicine*, vol. 8, no. 3, pp. 162–166, 2002.
  - [30] Q.-H. Shang, H. Xu, X.-Y. Lu, C. Wen, D.-Z. Shi, and K.-J. Chen, "A multi-center randomized double-blind placebo-controlled trial of Xiongshao capsule in preventing restenosis after percutaneous coronary intervention: a subgroup analysis of senile patients," *Chinese Journal of Integrative Medicine*, vol. 17, no. 9, pp. 669–674, 2011.
  - [31] H. Xu, D. Z. Shi, K. J. Chen et al., "Effect of Xiongshao capsule on vascular remodeling in porcine coronary balloon injury model," *Chinese Journal of Integrative Medicine*, vol. 6, no. 4, pp. 278–282, 2000.
  - [32] L.-Z. Li, J.-G. Liu, L.-B. Ma et al., "Effect of Xiongshao capsule on lipid metabolism and platelet aggregation in experimental atherosclerosis rabbits," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 12, pp. 1100–1103, 2008.
  - [33] D. W. Zhang, L. Zhang, J. G. Liu, C. L. Wang, D. Z. Shi, and K. J. Chen, "Effects of Xiongshao capsule combined with ischemic postconditioning on monocyte chemoattractant protein-1 and tumor necrosis factor- $\alpha$  in rat myocardium with ischemic reperfusion injury," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 30, no. 12, pp. 1279–1283, 2010.

- [34] F. Q. Xu, H. Xu, J. G. Liu, and K. J. Chen, "Effects of Xiongshao capsule on the proliferation of vascular smooth muscle cells in rabbits with atherosclerosis," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 28, no. 10, pp. 912–916, 2008.
- [35] Report of the Joint International Society and Federation of Cardiology/World Health Organization Task Force on Standardization of Clinical Nomenclature, "Nomenclature and criteria for diagnosis of ischemic heart disease," *Circulation*, vol. 59, no. 3, pp. 607–609, 1979.
- [36] Chinese Society of Cardiology, "Guideline for diagnosis and treatment of patients with unstable angina and non-ST-segment elevation myocardial infarction," *Chinese Journal of Cardiology*, vol. 35, no. 4, pp. 295–304, 2007.
- [37] Subcommittee of Cardiovascular Diseases of China Society of Integrated Traditional Chinese and Western Medicine, "Criteria for TCM syndrome differentiation of patients with coronary heart disease," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 11, no. 5, pp. 257–258, 1991.
- [38] Professional Committee of Activating Blood Circulation in Chinese Association of the Integration of Traditional and Western Medicine, "Criteria for diagnosis of blood stasis syndrome," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 7, no. 3, p. 129, 1987.
- [39] C. G. Fu, Z. Y. Gao, P. L. Wang et al., "Study on the diagnostic criteria for coronary heart disease patients of blood stasis syndrome," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 32, no. 9, pp. 1285–1286, 2012.
- [40] Chinese Society of Cardiology, "Guideline on diagnosis and treatment of unstable angina pectoris and non-ST elevated myocardial infarction," *Chinese Journal of Cardiology*, vol. 35, no. 4, pp. 295–304, 2007.
- [41] Y. Y. Zheng, *Chinese Herbal Medicine Clinical Research Guiding Principles (for Trial Implementation)*, Chinese Medicine and Technology Publishing House, 2002.
- [42] K. J. Chen, *Study on Activating Blood Circulation and Clinical Application*, Peking Union Medical College Press, 1993.
- [43] Y. Gong, X. Wang, J. Liu et al., "NSPc1, a mainly nuclear localized protein of novel PcG family members, has a transcription repression activity related to its PKC phosphorylation site at S183," *FEBS Letters*, vol. 579, no. 1, pp. 115–121, 2005.
- [44] M. C. Tataru, H. Schulte, E. A. Von, J. Heinrich, G. Assmann, and E. Koehler, "Plasma fibrinogen in relation to the severity of arteriosclerosis in patients with stable angina pectoris after myocardial infarction," *Coronary Artery Disease*, vol. 12, no. 3, pp. 157–165, 2001.
- [45] M. Naito, "Effects of fibrinogen, fibrin and their degradation products on the behavior of vascular smooth muscle cells," *Nippon Ronen Igakkai Zasshi*, vol. 37, no. 6, pp. 458–463, 2000.
- [46] H. L. Ma, X. Lu, H. J. Yang et al., "Fibrinogen is the one of risk factors of coronary heart disease," *Chinese Journal of Thrombosis and Hemostasis*, vol. 14, no. 1, pp. 8–11, 2008.
- [47] H. Xu, D. Qu, F. Zheng, D. Z. Shi, and K. J. Chen, "Clinical manifestations of "Blood-stasis and Toxin" in patients with stable coronary heart disease," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 30, no. 2, pp. 125–129, 2010.
- [48] Y. Feng, H. Xu, D. Qu, F. Zheng, D. Z. Shi, and K. J. Chen, "Study on the tongue manifestations for the blood-stasis and toxin syndrome in the stable patients of coronary heart disease," *Chinese Journal of Integrative Medicine*, vol. 17, no. 5, pp. 333–338, 2011.
- [49] H. Chen, *The clinical research on differentiation standard for toxin-stasis syndrome of ACS in Chinese medicine [Doctoral dissertation]*, China Academy of Chinese Medical Sciences, 2009.
- [50] <http://www.uniprot.org/uniprot/Q14624>.
- [51] N. H. Choi-Miura, K. Takahashi, M. Yoda et al., "The novel acute phase protein, IHRP, inhibits actin polymerization and phagocytosis of polymorphonuclear cells," *Inflammation Research*, vol. 49, no. 6, pp. 305–310, 2000.
- [52] Y. Fujita, Y. Ezura, M. Emi et al., "Hypercholesterolemia associated with splice-junction variation of inter- $\alpha$ -trypsin inhibitor heavy chain 4 (ITIH4) gene," *Journal of Human Genetics*, vol. 49, no. 1, pp. 24–28, 2004.
- [53] M. Piñeiro, M. Andrés, M. Iturralde et al., "ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) is a new acute-phase protein isolated from cattle during experimental infection," *Infection and Immunity*, vol. 72, no. 7, pp. 3777–3782, 2004.
- [54] R. S. Kashyap, A. R. Nayak, P. S. Deshpande et al., "Inter- $\alpha$ -trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke," *Clinica Chimica Acta*, vol. 402, no. 1-2, pp. 160–163, 2009.

## Research Article

# Effects of Swedish Massage Therapy on Blood Pressure, Heart Rate, and Inflammatory Markers in Hypertensive Women

Izreen Supa'at,<sup>1</sup> Zaiton Zakaria,<sup>2</sup> Oteh Maskon,<sup>3</sup>  
Amilia Aminuddin,<sup>2</sup> and Nor Anita Megat Mohd Nordin<sup>2</sup>

<sup>1</sup> Faculty of Biomedical and Health Sciences, Universiti Selangor, Shah Alam, Malaysia

<sup>2</sup> Physiology Department, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia

<sup>3</sup> Medical Department, Cardiology Unit, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia

Correspondence should be addressed to Izreen Supa'at; izreens@gmail.com

Received 23 August 2012; Accepted 21 July 2013

Academic Editor: Ka Kit Hui

Copyright © 2013 Izreen Supa'at et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Swedish Massage Therapy (SMT) is known for its therapeutic relaxation effects. Hypertension is associated with stress and elevated endothelial inflammatory markers. This randomized control trial measured the effects of whole body SMT (massage group) or resting (control group) an hour weekly for four weeks on hypertensive women. Blood pressure (BP) and heart rate (HR) were measured before and after each intervention and endothelial inflammatory markers: vascular endothelial adhesion molecules 1 (VCAM-1) and intracellular adhesion molecules 1 (ICAM-1) were measured at baseline and after the last intervention. Massage group ( $n = 8$ ) showed significant systolic BP (SBP) reduction of 12 mmHg ( $P = 0.01$ ) and diastolic BP (DBP) reduction of 5 mmHg ( $P = 0.01$ ) after four sessions with no significant difference between groups. Reductions in HR were also seen in massage group after sessions 1, 3, and 4 with significant difference between groups. VCAM-1 showed significant reduction after four sessions: the massage group showed reduction of 998.05 ng/mL ( $P = 0.03$ ) and the control group of 375.70 ng/mL ( $P = 0.01$ ) with no significant differences between groups. There were no changes in ICAM-1. In conclusion, SMT or resting an hour weekly has effects on reducing BP, HR, and VCAM-1 in hypertensive women.

## 1. Introduction

The prevalence of hypertension in Malaysia is increasing from 32.9% in 1996 to 40.5% in 2004 for individuals above 30 years old [1] with a prevalence in women higher than men [2]. In 2004, 43% of women over 30 years old are hypertensive [3]. Hypertension is a major risk factor for cardiovascular disease (CVD). CVD is the primary cause of death in women in Malaysia as well as globally [4]. In addition, more women experienced the side effects of antihypertensive treatment such as calcium channel blockers and angiotensin converting enzyme inhibitors compared to men [4].

The development of primary hypertension has been closely associated with endothelial dysfunction. This was seen in the increased expression of interleukin 6 (IL-6), interleukin 1 (IL-1), tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), monocyte

chemoattractant protein 1 (MCP-1), VCAM-1, and ICAM-1 in hypertensive rats [5–7]. VCAM-1 and ICAM-1 are immunoglobulin superfamily (IGSF) molecules involved in cell to cell adhesion. VCAM-1 is expressed on endothelial, epithelial, macrophage, and dendritic cells. ICAM-1 is expressed on endothelial, epithelial, fibroblast, leucocytes, and tumour cells. VCAM-1 and ICAM-1 form attachments and assist transendothelial migration of leukocytes at sites of atherosclerosis [8]. These molecules are upregulated in response to inflammatory cytokines or oxidative low-density lipoprotein (ox-LDL) [9]. During an inflammation caused by injury, leukocytes will roll on the endothelium before firmly adhering to the vessel wall. Stable adhesion of leukocytes further upregulates adhesion molecules that will drive the transmigration of leukocytes across the endothelium to the site of injury. Leukocytes adhesion is opposed by tangential

tractive forces or shear stress induced by the blood flow velocity gradient near the vessel wall [10].

Shear stress is defined as the tangential drag force of blood passing along the surface of the endothelium [11]. Shear stress is directly proportional to blood viscosity and velocity and inversely proportional to blood vessel diameter (shear stress = blood viscosity  $\times$  blood velocity/vessel diameter). There are two types of shear stress: the atheroprotective laminar or pulsatile shear stress which occurs in straight blood vessels [12] and the atherogenic oscillatory shear stress which occurs at bends and bifurcation of arteries [13–15]. The physiological atheroprotective shear stress is more than 15 dyne/cm<sup>2</sup>. In the event of narrowing of the arteries, the shear stress here is low. Consequently, blood will rush out of the narrow opening at high velocity and create a higher shear stress on the arterial wall, therefore inducing endothelial-dependent nitric oxide-mediated vasodilation which brings the shear stress back to normal. However, this effect is blunted in hypertensive and hypercholesterolemic patients [16]. It was found that with increasing shear stress the expression of VCAM-1 is low and the expression of ICAM-1 is high. There was also no leukocytes adhesion to the vessel wall. In contrast, if there is a decrease in shear stress, the expression of VCAM-1 increases and the expression of ICAM-1 decreases with leukocytes adhesion to the vessel wall. This is indicative of the early stage of development of atherosclerosis [17, 18].

Swedish Massage Therapy (SMT) is a complementary treatment that is believed to provide relaxation and therefore able to reduce blood pressure caused by stress [19]. It is the most recognised and frequent used massage therapy [20]. It is characterised by long strokes applied according to the venous and lymphatic flow. It is a painless, gentle and nonforceful technique that is not associated with any serious adverse effects [21]. Massage therapy has been shown to decrease sympathetic activity and increase parasympathetic activity [22]. Therefore this therapy is able to decrease anxiety and stress [23, 24]. In addition, massage therapy is able to reduce blood pressure (BP) and heart rate (HR) in hypertensive individuals [25–27]. It also increases skin blood flow and suppleness and induces tissue relaxation [28]. The long strokes in massage compress the body tissues and when released increase blood flow to the local area [19].

The present study assessed the effects of SMT versus rest on hypertensive women. If SMT yields positive results in this study, it can be recommended as an adjunct or a complementary therapy to the conventional management of hypertension especially in women as the prevalence of hypertension in women is high [1, 2]. As far as the author's present knowledge extends, no studies have assessed the effects of whole body Swedish Massage Therapy on hypertensive women on BP, HR, and inflammatory markers. In view of the literature stated above, it was hypothesized that massage increases blood flow and thus increases shear stress on the blood vessel wall. The increase in shear stress reduces the expression of VCAM-1 and vWF and increases ICAM-1. In addition, through the activation of parasympathetic nervous system, massage is able to reduce BP and HR. Therefore Swedish Massage Therapy is expected to reduce BP, HR, and VCAM-1 and increase ICAM-1.

## 2. Methods

**2.1. Participants.** This study is an experimental randomized control trial that has been approved by the Ethics Committee of the Universiti Kebangsaan Malaysia Medical Centre (UKMMC) (Project Ethics Code: FF-280-2009). The participants were 35–60-year-old women recruited from the UKMMC records. These women must fulfil the following criteria:

- (a) body mass index (BMI) of less than 35 kg/m<sup>2</sup>;
- (b) SBP of 120–159 mmHg and DBP of 90–99 mmHg with or without treatment. If they are on antihypertensive or anticholesterol medications, they must be on only one medication of the same type and dose for at least six months;
- (c) normal liver, thyroid, and renal functions;
- (d) not taking any prescribed and/or traditional medications (apart from those stated in (b)) and supplements;
- (e) not smoking or drinking alcohol;
- (f) no other illnesses;
- (g) not pregnant;
- (h) have not experienced Swedish Massage Therapy.

Detailed explanation of the study was given to each woman. All women had signed the consent form before participating in this study.

The sample size is calculated based on paired samples that is, BP taken before and after intervention with continuous outcomes. The formula used in this study is taken from Chan 2003 [29]. The values for this formula are taken from another study [26] with a similar topic and significant results. The minimum number of subjects calculated is 16 with 8 subjects per group.

Twenty-three women fulfilled the above criteria and were screened for any health conditions that may influence their blood pressure. Blood pressure of these women was monitored for two weeks prior to the intervention. Blood samples were taken to ensure that the liver, thyroid, and renal function and the fasting blood glucose level were normal. Resting electrocardiogram (ECG) and stress tests were also carried out to ensure normal cardiac function. Fasting body composition was measured before and after the intervention to ensure that the body fluid distribution remains unchanged. Twenty women successfully passed their screening. These women were randomly assigned to two groups: the massage group and the control group using random numbers generated through the SPSS version 15 software. However, only 16 women (8 per group) successfully completed the intervention.

**2.2. Intervention.** In this study, the massage protocol is an hour of Swedish Massage Therapy to the whole body, once a week for four weeks. An hour of massage allows enough time to apply all the Swedish massage techniques to the whole body which was expected to produce positive effects on BP and

HR [30, 31]. Massage sessions once a week for four weeks are considered not too frequent as they prevent the subject from showing any signs of relaxation prior to the massage session as would be expected if the sessions are more frequent.

Eight women in the massage group underwent an hour of whole body Swedish Massage Therapy once a week for four weeks at the Clinical Trial Ward, UKMMC. A qualified massage therapist with a certificate in Holistic Therapy from the Institute of Bioproduct Development, Universiti Teknologi Malaysia, carried out the massage on each of the subjects. The massage techniques used are a combination of *petrissage* or kneading, *tapotement* or beating/hacking/cupping, and *effleurage* or long strokes. These techniques are applied at medium pressure. Olive oil was used as the lubricant. These massage sessions were carried out during working days between 8 and 10 a.m. The protocol used is described below.

- (1) The subject is requested to lie prone with only the right leg is exposed. Massage oil applied on the exposed leg.
- (2) Long strokes are applied on the posterior right leg.
- (3) The gastrocnemius muscle is kneaded using both thumbs.
- (4) Step (2) is repeated.
- (5) The medial and lateral parts of back of the thigh are
  - (a) kneaded using the palm of the hand;
  - (b) hacked or stricken with the medial side of the hand;
  - (c) pounded using the medial side of a clenched fist.
- (6) Lymphatic drainage is then carried out by applying long strokes along the venous or lymphatic vessels towards the nearest lymph nodes.
- (7) Step (2) is repeated.
- (8) The left leg is covered and steps 1–7 are repeated on the left leg.
- (9) Massage is then carried out on the back. Massage oil is applied on the whole back.
- (10) Long strokes are carried out using the palms of therapist hands from the lower back to the shoulders.
- (11) Kneading using fingers is applied parallel to the spine from the lower back to between the scapulae.
- (12) Step (10) is repeated.
- (13) Kneading using palms is applied on both loin areas and posterior to the lungs.
- (14) Lymphatic drainage was carried out from the lower back to the axillary and subclavicular lymph nodes.
- (15) Step (10) is repeated.
- (16) The subject is requested to turn over and lie supine with the right leg exposed. Massage oil is applied on the whole leg.
- (17) Long strokes are applied on the anterior right leg.
- (18) The anterior tibialis muscle is kneaded using both thumbs.
- (19) Step (17) is repeated.
- (20) The biceps femoris is kneaded using the palm of the hand.
- (21) Step (6) and Step (17) are repeated.
- (22) Massage is then carried out on the abdomen where massage oil is applied.
- (23) Long strokes are applied from the umbilicus to the xiphisternum, along the lower border of the ribs towards lateral of the abdomen and inferior towards the inguinal area.
- (24) Strokes are applied along the ascending, transverse, and descending colon.
- (25) Small, circular kneading using tips of fingers is applied clockwise around the umbilicus.
- (26) Lymphatic drainage is carried out through long strokes from the posterior loin area to the inguinal region.
- (27) Step (23) is repeated.
- (28) Massage is then carried out on the right arm where massage oil is applied.
- (29) Effleurage is applied using one hand to support the subject's arm and the other hand carrying out long strokes from the wrist to the scapula region.
- (30) The forearm is kneaded using the thumb.
- (31) The upper arm is kneaded using the palm of the hand.
- (32) Step (29) is repeated.
- (33) The subject is requested to sit up with her back to the massage therapist.
- (34) The massage therapy ends with massage to the scalp, neck, and shoulders.
- (35) The scalp is kneaded from the frontal area to the occipital area using fingers.
- (36) The temples are kneaded in circular motions using the heel of the hands.
- (37) The trapezius and deltoid muscles are kneaded according to the orientation of the muscle fibers using fingers.
- (38) BP and HR were taken. The subject is requested to dress.

Eight women in the control group underwent an hour of rest, once a week for four weeks at the same ward and at the same time. They were instructed to lie supine and rest either doing light reading or sleep. They are not allowed to listen to any music, watch television, exercise, or carry out any activities that may affect their BP.

**2.3. Measurements.** Blood pressure and HR were taken before and after each massage and rest session and 48 hours after the last session. Blood pressure was taken twice at five-minute interval using the mercury sphygmomanometer and HR through palpation.

10 mL of blood was collected in normal test tubes with no chemical preservatives at baseline, that is, before the first massage and rest session and after the last session ended. The blood was left to coagulate for two hours before being centrifuged at 3000 rpm for 10 minutes at 4°C. The serum is pipetted into 1 mL Eppendorf tubes and stored at -70°C before being analysed. The level of soluble VCAM-1 and ICAM-1 was analysed using kits through the enzyme-linked immunosorbent assay method (ELISA). The analysis protocols were carried out as instructed in the kits: VCAM-1 (BMS232TEN, Bender MedSystems, Austria) and ICAM-1 (BMS201INST, Bender MedSystems, Austria). The concentration of soluble VCAM-1 and ICAM-1 of each sample was calculated through the SOFTmaxPRO software through the measurements of the absorbance value of the study samples, the standard samples, and the control samples. The normal range of soluble VCAM-1 as stated in the kit is 400.6–1340.8 ng/mL and soluble ICAM-1 is 129.9–297.4 ng/mL.

**2.4. Statistical Analyses.** Data were analysed using SPSS software version 15. Due to the small number of samples (<20), nonparametric statistical tests were used. The Mann-Whitney *U* test was used to compare the readings between the two groups, and the Wilcoxon Signed Rank test was used to measure the changes in each group. For BP and HR, the difference between the preintervention readings and postintervention readings of each session is considered to be an acute change. The difference between the preintervention readings of BP and HR of session 1 and preintervention readings of subsequent sessions is considered to be chronic change. A four week chronic change is the difference between the preintervention reading of BP and HR of session 1 and reading 48 hours after session 4. All data is presented in median (interquartile range). The value of  $P < 0.05$  is accepted as the significant level.

### 3. Results

**3.1. Baseline Characteristics.** The baseline characteristics of the research participants are shown in Table 1. Women in the massage and control groups were comparable in all parameters except for a higher fasting blood glucose level in the latter. However, this was still within the normal range. In general, these women were overweight; resting BP showed Stage 1 hypertension, and lipid profile showed high cholesterol and high LDL while HDL levels were normal. More than 62% were nonmenopausal. In each group, three participants (37.5%) are on antihypertensive medications and only one (12.5%) is on anticholesterol medication.

**3.2. SBP Changes.** Figure 1 shows the trend of SBP changes for every session for both groups. During session 1, there was an acute significant reduction in the massage group from 143.00 (9.00) mmHg to 138.00 (16.75) mmHg ( $Z = -1.70$ ,

TABLE 1: Baseline characteristics of the research participants.

Parameters	Massage group ( <i>n</i> = 8)	Control group ( <i>n</i> = 8)	<i>P</i>
Age (years)	51.00 (10.00)	51.13 (11.00)	0.96
Employed (%)	50	37.5	0.61
Nonmenopausal (%)	62.5	75	0.59
On antihypertensives (%)	37.5	37.5	1.00
On anticholesterol (%)	12.5	12.5	1.00
Resting SBP (mmHg)	142.25 (18.38)	143.75 (7.50)	0.67
Resting DBP (mmHg)	81.25 (9.75)	89.50 (13.50)	0.53
Resting HR (bpm)	66.00 (15.50)	72.00 (3.00)	0.10
BMI (kg/m <sup>2</sup> )	29.02 (6.65)	28.15 (7.41)	0.83
Cholesterol (mmol/L)	5.80 (0.74)	6.42 (1.32)	0.13
HDL (mmol/L)	1.30 (0.42)	1.41 (0.20)	0.11
LDL (mmol/L)	3.95 (0.32)	4.44 (1.68)	0.49
Fasting blood glucose (mmol/L)*	4.95 (0.25)	5.50 (0.45)	0.01

Data in median (interquartile range). \* $P < 0.01$  is significant.

$P = 0.03$ ). Chronic significant reduction was seen after the first week in the control group from 140.00 (23.75) mmHg to 137.00 (11.75) mmHg with  $Z = -2.52$ ,  $P = 0.01$ , after the second and third weeks in both groups and after the fourth week in the massage group only at 12 mmHg ( $Z = -2.54$ ,  $P = 0.01$ ). There was no significant difference in SBP readings between massage and control groups.

**3.3. DBP Changes.** The trend in DBP changes is shown in Figure 2. During session 1, there was an acute significant DBP reduction of 7 mmHg in massage group ( $Z = -2.38$ ,  $P = 0.02$ ). During session 4, there was an acute significant reduction in the control group from 81.50 (8.75) mmHg to 80.00 (5.25) mmHg with  $Z = -2.03$ ,  $P = 0.04$ . In addition, chronic significant DBP reductions were seen after the second week (-2 mmHg, ( $Z = -2.21$ ,  $P = 0.03$ )), the third week (-8 mmHg, ( $Z = -2.54$ ,  $P = 0.01$ )), and the fourth week (-5 mmHg, ( $Z = -2.52$ ,  $P = 0.01$ )) in the massage group. There were no significant differences between the two groups in all DBP readings.

Figure 3 shows the trend of HR changes. There is an acute significant HR reduction in massage group after each session with significant differences between groups after session 1 ( $Z = -2.22$ ,  $P = 0.03$ ), session 3 ( $Z = -2.07$ ,  $P = 0.04$ ), and session 4 ( $Z = -2.03$ ,  $P = 0.04$ ). Chronic reduction was seen in the control group after the first week ( $Z = -2.26$ ,  $P = 0.02$ ) with no significant difference between groups.

**3.4. VCAM-1 Changes.** The changes in the level of VCAM-1 are displayed in Figure 4. Significant reduction in VCAM-1 was seen in massage group from 1988.30 (911.39) ng/mL to 990.25 (675.25) ng/mL ( $Z = -2.20$ ,  $P = 0.03$ ) and the control group from 1420.55 (861.07) ng/mL to 1044.85 (602.39) ng/mL ( $Z = -2.52$ ,  $P = 0.01$ ). No significant change was seen between groups.

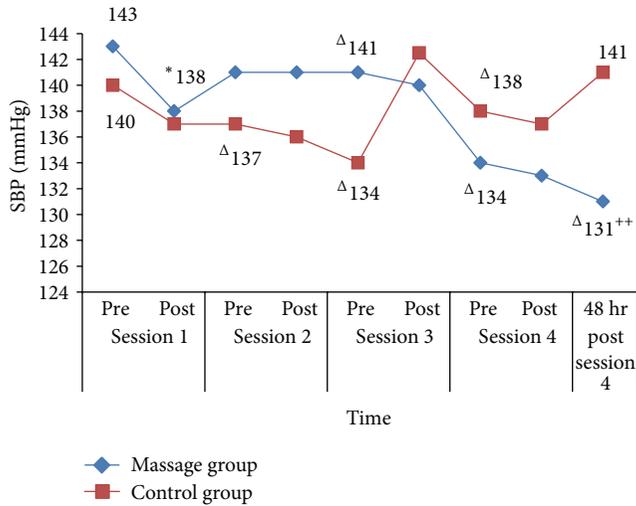


FIGURE 1: SBP changes for each session for message group and control group. \* $P < 0.05$  (acute changes within groups) and  $\Delta P < 0.05$  (chronic changes within groups). <sup>++</sup> $P < 0.05$  (baseline versus after session 4).

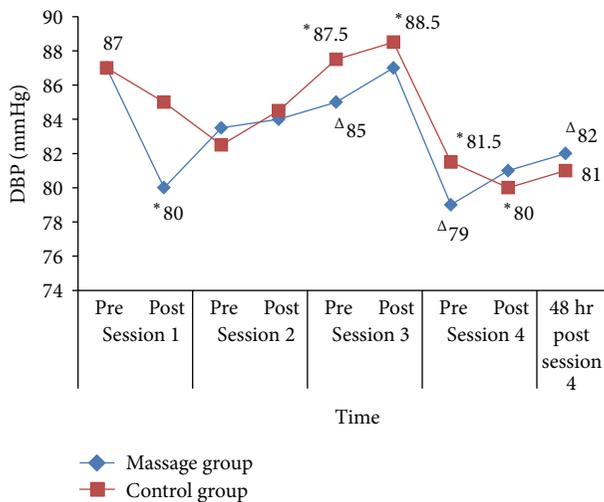


FIGURE 2: DBP changes for each session for message group and control group. \* $P < 0.05$  (acute changes within groups) and  $\Delta P < 0.05$  (chronic changes within groups).

3.5. ICAM-1 Changes. No significant changes were seen within the two groups and between groups as shown in Figure 5.

#### 4. Discussion

Massage group showed chronic significant reduction in SBP after two, three, and four weeks. These results are consistent with Olney (2005) [25], Hernandez-Reif et al. (2000) [26], and Moeini et al. (2011) [27] which showed that massage had long-term effects on the BP of hypertensive patients. However, the control group of this current study also showed chronic significant reduction in SBP after one, two, and three weeks. These results contradict the results of the authors

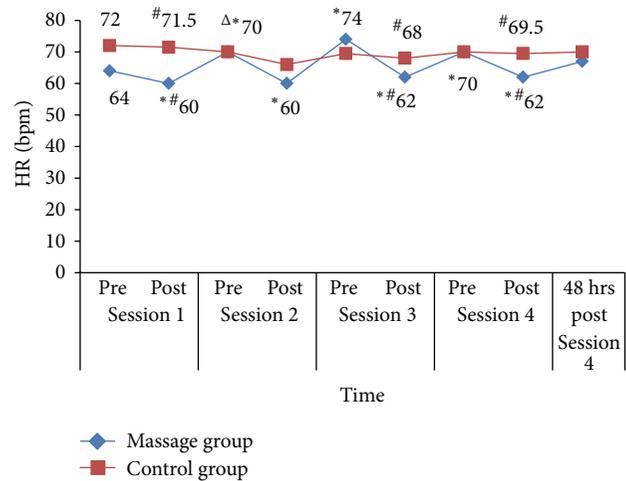


FIGURE 3: HR changes for each session for message group and control group. \* $P < 0.05$  (acute changes within groups),  $\Delta P < 0.05$  (chronic changes within groups), and # $P < 0.05$  (changes between groups).

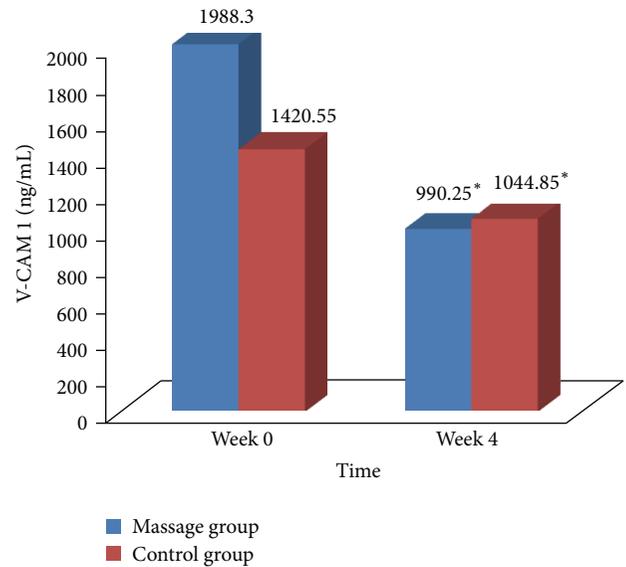


FIGURE 4: V-CAM-1 changes for message group and control group. \* $P < 0.05$  (reduction within groups).

mentioned. Olney (2005) [25], Hernandez-Reif et al. (2000) [26], and Moeini et al. (2011) [27] showed no significant changes in SBP of their control groups. Several factors may be able to explain these occurrences. Firstly, the rest session of this current study is of longer duration if compared to the studies of Olney [25] and Moeini et al. [27]. Secondly, during the resting period, the subject is free to use any relaxation methods, and the researcher is only present during the pre- and postintervention measurements. The combination of no supervision and longer duration of rest may have reduced the SBP of the control subjects. Acute reduction in SBP of 5 mmHg after session 1 in the message group was also seen,

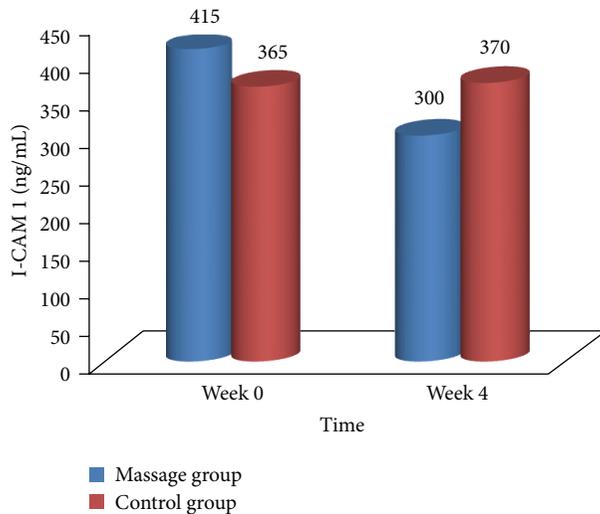


FIGURE 5: ICAM-1 changes for massage group and control group.

and this is consistent with the results of Hernandez-Reif et al. (2000) [26].

In parallel with the results of SBP, the massage group also showed significant chronic reduction in DBP after two, three, and four weeks of massage therapy and acute significant reduction of 7 mmHg after session 1. In summary, SMT is able to reduce both SBP and DBP, even though there is no significant difference between groups.

Heart rate of the massage group was reduced significantly after each session, and the changes were significant between groups after sessions one, three, and four. Even though the duration of the massage session is different from other studies, these results are consistent with studies that measure the effects of massage on normal individuals [22], breast cancer patients [32], hospice patients [33], migraine patients [34], and critical care patients [35].

The reduction in BP and HR could be explained through the comfortable feeling and relaxation, as well as the increase in parasympathetic activities induced by massage as shown by Ouchi et al. (2006) [22]. This is supported by Diego and Field (2009) [36] who showed that the massage applied at medium pressure for 15 minutes caused increase in the high-frequency component of HR variability which reflected an increase in vagal activities. In addition, there was a decrease in the ratio of low-frequency component to high-frequency component of HR variability which indicates a change from sympathetic activities to parasympathetic activities.

To date, there have been no studies that measure the effects of massage on endothelial inflammatory markers. In this current study, the massage group experienced a significant reduction of VCAM-1 from an abnormal level to a normal level with a greater magnitude difference compared to the control group which also showed significant reduction. Braun and Simonson 2008 [20] stated that SMT through *effleurage* and compression increases local blood flow. If blood viscosity remains unchanged, the increase in blood flow increases shear stress on the blood vessel wall. The increase in shear stress decreases the expression of VCAM-1 [18, 37]. This

is supported by the studies of Ando et al., (1994) [17], Korenaga et al. (1997)[38], and Helmlinger et al. (1995)[39] which showed decrease in the production of VCAM-1 at physiological shear stress of  $>15$  dyne/cm<sup>2</sup> and an increase in the production of VCAM-1 at shear stress of  $\pm 0-4$  dyne/cm<sup>2</sup>.

It was expected that the level of ICAM-1 of the massage group increases after the intervention. Walpolo et al. (1995) [18] showed that high shear stress (30.5 dyne/cm<sup>2</sup>) increases the expression of ICAM-1. Nagel et al. (1994) [40] who studied the effects of shear stress at 10 dyne/cm<sup>2</sup> on the expression of ICAM-1 on human umbilical vein endothelial cells (HUVEC) and Morigi et al. (1995) [10] who exposed HUVEC at shear stress of 8 dyne/cm<sup>2</sup> also reported an increase in the expression of ICAM-1. However, the current study showed no significant changes in both massage and control groups for ICAM-1. It may be that the shear stress created by massage is not large enough to have effects on ICAM-1. Further studies are warranted on the effects of massage on blood flow to confirm the effects discussed above.

## 5. Conclusion

This study has shown that Swedish Massage Therapy or resting an hour weekly significantly reduced BP, HR, and VCAM-1 through the effects that have been discussed. However, the effect of rest on BP does not extend to four weeks as compared to SMT. In addition, massage also reduces resting HR in hypertensive women.

## Acknowledgments

The authors would like to acknowledge all the staff at the Physiology Department, Cardiology Unit, Clinical Trial Ward, and the Centre for Research in Emergency Medicine, UKMMC, for all their support during this research. This research was funded by UKMMC Fundamental Research Grant (FF-280-2009).

## References

- [1] L. Rampal, S. Rampal, M. Z. Azhar, and A. R. Rahman, "Prevalence, awareness, treatment and control of hypertension in Malaysia: a national study of 16,440 subjects," *Public Health*, vol. 122, no. 1, pp. 11–18, 2008.
- [2] T. O. Lim, Z. Morad, R. H. Hussein et al., "Prevalence, awareness, treatment and control of hypertension in the Malaysian adult population: results from the National Health and Morbidity Survey 1996," *Singapore Medical Journal*, vol. 45, no. 1, pp. 20–27, 2004.
- [3] National Health & Morbidity Survey III (NHMS III) Conference Proceedings, Putrajaya, Malaysia, November 2007.
- [4] MOH/P/PAK/171.08(GU), *Clinical Practice Guidelines: Prevention of Cardiovascular Disease in Women*, Academy of Medicine of Malaysia, Kuala Lumpur, Malaysia, 1st edition, 2008.
- [5] D. Sanz-Rosa, M. P. Oubiña, E. Cediél et al., "Effect of AT1 receptor antagonism on vascular and circulating inflammatory mediators in SHR: role of NF- $\kappa$ B/I $\kappa$ B system," *American Journal of Physiology*, vol. 288, no. 1, pp. H111–H115, 2005.
- [6] Q. Capers IV, R. W. Alexander, P. Lou et al., "Monocyte chemoattractant protein-1 expression in aortic tissues of hypertensive rats," *Hypertension*, vol. 30, no. 6, pp. 1397–1402, 1997.

- [7] G. Luvarà, M. E. Pueyo, M. Philippe et al., "Chronic blockade of NO synthase activity induces a proinflammatory phenotype in the arterial wall: prevention by angiotensin II antagonism," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 18, no. 9, pp. 1408–1416, 1998.
- [8] A. J. H. Gearing and W. Newman, "Circulating adhesion molecules in disease," *Immunology Today*, vol. 14, no. 10, pp. 506–512, 1993.
- [9] J. Constans and C. Conri, "Circulating markers of endothelial function in cardiovascular disease," *Clinica Chimica Acta*, vol. 368, no. 1-2, pp. 33–47, 2006.
- [10] M. Morigi, C. Zoja, M. Figliuzzi et al., "Fluid shear stress modulates surface expression of adhesion molecules by endothelial cells," *Blood*, vol. 85, no. 7, pp. 1696–1703, 1995.
- [11] A. Gnasso, C. Irace, C. Carallo et al., "In vivo association between low wall shear stress and plaque in subjects with asymmetrical carotid atherosclerosis," *Stroke*, vol. 28, no. 5, pp. 993–998, 1997.
- [12] O. Traub and B. C. Berk, "Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 18, no. 5, pp. 677–685, 1998.
- [13] D. N. Ku, D. P. Giddens, C. K. Zarins, and S. Glagov, "Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low and oscillating shear stress," *Arteriosclerosis*, vol. 5, no. 3, pp. 293–302, 1985.
- [14] J. Ravensbergen, J. W. Ravensbergen, J. K. B. Krijger, B. Hillen, and H. W. Hoogstraten, "Localizing role of hemodynamics in atherosclerosis in several human vertebrobasilar junction geometries," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 18, no. 5, pp. 708–716, 1998.
- [15] C. K. Zarins, D. P. Giddens, and B. K. Bharadvaj, "Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress," *Circulation Research*, vol. 53, no. 4, pp. 502–514, 1983.
- [16] O. A. Paniagua, M. B. Bryant, and J. A. Panza, "Role of endothelial nitric oxide in shear stress-induced vasodilation of human microvasculature: diminished activity in hypertensive and hypercholesterolemic patients," *Circulation*, vol. 103, no. 13, pp. 1752–1758, 2001.
- [17] J. Ando, H. Tsuboi, R. Korenaga et al., "Shear stress inhibits adhesion of cultured mouse endothelial cells to lymphocytes by downregulating VCAM-1 expression," *American Journal of Physiology*, vol. 267, no. 3, pp. C679–C687, 1994.
- [18] P. L. Walpola, A. I. Gotlieb, M. I. Cybulsky, and B. L. Langille, "Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 15, no. 1, pp. 2–10, 1995.
- [19] S. Fritz, *Mosby's Fundamentals of Therapeutic Massage*, Mosby Elsevier, St. Louis, Mo, USA, 2009.
- [20] M. B. Braun and S. J. Simonson, *Introduction to Massage Therapy*, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 2nd edition, 2008.
- [21] E. Ernst, "The safety of massage therapy," *Rheumatology*, vol. 42, no. 9, pp. 1101–1106, 2003.
- [22] Y. Ouchi, T. Kanno, H. Okada et al., "Changes in cerebral blood flow under the prone condition with and without massage," *Neuroscience Letters*, vol. 407, no. 2, pp. 131–135, 2006.
- [23] K. C. Richards, R. Gibson, and A. L. Overton-McCoy, "Effects of massage in acute and critical care," *AACN Clinical Issues*, vol. 11, no. 1, pp. 77–96, 2000.
- [24] A. Moraska, R. A. Pollini, K. Boulanger, M. Z. Brooks, and L. Teitlebaum, "Physiological adjustments to stress measures following massage therapy: a review of the literature," *Evidence-Based Complementary and Alternative Medicine*, vol. 7, no. 4, pp. 409–418, 2010.
- [25] C. M. Olney, "The effect of therapeutic back massage in hypertensive persons: a preliminary study," *Biological Research for Nursing*, vol. 7, no. 2, pp. 98–105, 2005.
- [26] M. Hernandez-Reif, T. Field, J. Krasnegor, Z. Hossain, H. Theakston, and I. Burman, "High blood pressure and associated symptoms were reduced by massage therapy," *Journal of Bodywork and Movement Therapies*, vol. 4, no. 1, pp. 31–38, 2000.
- [27] M. Moeini, M. Givi, Z. Ghasempour, and M. Sadeghi, "The effect of massage therapy on blood pressure of women with prehypertension," *Iranian Journal of Nursing & Midwifery Research*, vol. 16, no. 1, pp. 61–70, 2011.
- [28] I. G. P. Duimel-Peeters, R. J. G. Halfens, M. P. F. Berger, and L. H. E. H. Snoeckx, "The effects of massage as a method to prevent pressure ulcers. A review of the literature," *Ostomy/Wound Management*, vol. 51, no. 4, pp. 70–80, 2005.
- [29] Y. H. Chan, "Randomised controlled trials (RCTS)—sample size: the magic number?" *Singapore Medical Journal*, vol. 44, no. 4, pp. 172–174, 2003.
- [30] J. M. Lovas, A. R. Craig, Y. D. Segal, R. L. Raison, K. M. Weston, and M. R. Markus, "The effects of massage therapy on the human immune response in healthy adults," *Journal of Bodywork and Movement Therapies*, vol. 6, no. 3, pp. 143–150, 2002.
- [31] M. Aourell, M. Skoog, and J. Carleson, "Effects of Swedish massage on blood pressure," *Complementary Therapies in Clinical Practice*, vol. 11, no. 4, pp. 242–246, 2005.
- [32] A. Billhult, C. Lindholm, R. Gunnarsson, and E. Stener-Victorin, "The effect of massage on immune function and stress in women with breast cancer—a randomized controlled trial," *Autonomic Neuroscience: Basic and Clinical*, vol. 150, no. 1-2, pp. 111–115, 2009.
- [33] S. S. Meek, "Effects of slow stroke back massage on relaxation in hospice clients," *Journal of Nursing Scholarship*, vol. 25, no. 1, pp. 17–21, 1993.
- [34] S. P. Lawler and L. D. Cameron, "A randomized, controlled trial of massage therapy as a treatment for migraine," *Annals of Behavioral Medicine*, vol. 32, no. 1, pp. 50–59, 2006.
- [35] J. A. Hayes and C. Cox, "Immediate effects of a five-minute foot massage on patients in critical care," *Complementary Therapies in Nursing & Midwifery*, vol. 6, no. 1, pp. 9–13, 2000.
- [36] M. A. Diego and T. Field, "Moderate pressure massage elicits a parasympathetic nervous system response," *International Journal of Neuroscience*, vol. 119, no. 5, pp. 630–638, 2009.
- [37] P. L. Walpola, A. I. Gotlieb, and B. L. Langille, "Monocyte adhesion and changes in endothelial cell number, morphology, and F-actin distribution elicited by low shear stress in vivo," *American Journal of Pathology*, vol. 142, no. 5, pp. 1392–1400, 1993.
- [38] E. Korenaga, J. Ando, K. Kosaki, M. Isshiki, Y. Takada, and A. Kamiya, "Negative transcriptional regulation of the VCAM-1 gene by fluid shear stress in murine endothelial cells," *American Journal of Physiology*, vol. 273, no. 5, pp. C1506–C1515, 1997.
- [39] G. Helmlinger, B. C. Berk, and R. M. Nerem, "Calcium responses of endothelial cell monolayers subjected to pulsatile and steady laminar flow differ," *American Journal of Physiology*, vol. 269, no. 2, pp. C367–C375, 1995.

- [40] T. Nagel, N. Resnick, W. J. Atkinson, C. F. Dewey Jr., and M. A. Gimbrone Jr., "Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells," *Journal of Clinical Investigation*, vol. 94, no. 2, pp. 885–889, 1994.

## Research Article

# Effect of Wenxin Granule on Ventricular Remodeling and Myocardial Apoptosis in Rats with Myocardial Infarction

Aiming Wu,<sup>1</sup> Jianying Zhai,<sup>2</sup> Dongmei Zhang,<sup>1</sup> Lixia Lou,<sup>1</sup> Haiyan Zhu,<sup>3</sup>  
Yonghong Gao,<sup>1</sup> Limin Chai,<sup>1</sup> Yanwei Xing,<sup>4</sup> Xiying Lv,<sup>1</sup> Lingqun Zhu,<sup>1</sup>  
Mingjing Zhao,<sup>1</sup> and Shuoren Wang<sup>1</sup>

<sup>1</sup> Key Laboratory of Chinese Internal Medicine of Ministry of Education and Beijing,

Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100700, China

<sup>2</sup> National Engineering Research Center for R&D of TCM Multi-Ingredient Drugs, Beijing 100079, China

<sup>3</sup> Beijing University of Chinese Medicine Institute for Cardiovascular Disease,

Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100700, China

<sup>4</sup> Guang'an Men Hospital, Chinese Academy of Chinese Medical Sciences, Beijing 100053, China

Correspondence should be addressed to Mingjing Zhao; [mjgx2004@163.com](mailto:mjgx2004@163.com) and Shuoren Wang; [doctor\\_wang@sohu.com](mailto:doctor_wang@sohu.com)

Received 1 April 2013; Revised 1 July 2013; Accepted 12 July 2013

Academic Editor: Keji Chen

Copyright © 2013 Aiming Wu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Aim.** To determine the effect of a Chinese herbal compound named Wenxin Granule on ventricular remodeling and myocardial apoptosis in rats with myocardial infarction (MI). **Methods.** Male Sprague-Dawley (SD) rats were randomly divided into four groups: the control group, the model group, the metoprolol group, and the Wenxin Granule group (WXKL group) with sample size ( $n$ ) of 7 rats in each group. An MI model was established in all rats by occlusion of the left anterior descending coronary artery (the control group was without occlusion). Wenxin Granule (1.35 g/kg/day), metoprolol (12 mg/kg/day), and distilled water (5 mL/kg/day for the control and model groups) were administered orally for 4 weeks. Ultrasonic echocardiography was used to examine cardiac structural and functional parameters. Myocardial histopathological changes were observed using haematoxylin and eosin (H&E) dyeing. Myocardial apoptosis was detected by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining. Serum angiotensin II (Ang II) concentration was measured using the enzyme-linked immunosorbent assay (ELISA). **Results.** It was found that Wenxin Granule could partially reverse ventricular remodeling, improve heart function, alleviate the histopathological damage, inhibit myocardial apoptosis, and reduce Ang II concentration in rats with MI. **Conclusions.** The results of the current study suggest that Wenxin Granule may be a potential alternative and complementary medicine for the treatment of MI.

## 1. Introduction

Myocardial infarction (MI) is an acute and critical disease of the cardiovascular system endangering human health [1]. The prevalence of MI continues to increase in a Japanese population [2]. With the impact of the aging Chinese population, the accelerated pace of modern life, changes in eating habits, and social, psychological, and other factors, the incidence of MI in China shows an increasing tendency [3]. The negative impact on family and society is serious due to the economic and death burdens resulting from MI. Prevention and control of this increased occurrence of MI in the Chinese population is currently unsatisfactory [4]. Currently, the reperfusion

therapy such as percutaneous coronary intervention (PCI) has been widely carried out [5], with the early mortality in patients with MI significantly reduced [6]. Whilst a large number of patients with MI survive into the recovery phase, patients are still confronted with the risks of recurrent acute cardiovascular events, readmission to hospital, and unfavorable quality of life [7, 8]. In addition, patients also need to manage difficulties such as severe left ventricular dysfunction and potentially the development of heart failure [9].

Ventricular remodeling and myocardial apoptosis are the primary causes of heart failure following MI and the major pathological factors affecting prognosis of heart failure following MI [10, 11]. Neither ventricular remodeling

nor myocardial apoptosis, however, is independent disease. Both diseases are the secondary pathophysiological response process following MI. Ventricular remodeling is the result of overall ventricular compensation represented by a series of changes in heart size, shape, wall thickness, cardiac tissue structure, and aggravation of heart function [12]. Apoptosis, also known as programmed cell death, is a physiological phenomenon. The increase in myocardial apoptosis following MI is one of many mechanisms involved in aggravated cardiac tissue injury [13]. The ventricular remodeling and myocardial apoptosis following MI are inextricably linked with each other [14, 15]. Excessive apoptosis may result in two different events. Whilst excessive apoptosis can accelerate the loss of myocardial cells, deteriorate heart function, and promote the development of ventricular remodeling [10], the ventricular remodeling can also aggravate myocardial ischemia and the excessive apoptosis result in hypoxic injury [16]. Regardless in both cases, ventricular remodeling and myocardial apoptosis are the primary reasons leading to heart failure following MI and ultimately death [17]. Therefore, both reverse ventricular remodeling and inhibition of myocardial apoptosis are beneficial to delay the incidence of heart failure after MI and reduce mortality in patients [18, 19].

Wenxin Granule is a Chinese herbal compound developed by the China Academy of Chinese Medical Sciences and funded by Chinese national “85” science and technology research project. It contains *Radix Codonopsis Pilosulae*, *Rhizoma Polygonati*, *Radix Notoginseng*, *Succinum* and *Radix et Rhizoma Nardostachyos*. In recent years, more and more clinicians have successfully applied Wenxin Granule in cardiovascular disease prevention and treatment, and have received a satisfactory clinical outcome [20–22]. Nevertheless, the role of Wenxin Granule in cardiovascular diseases requires further clinical evidence and definitive mechanisms of action. The current study focused on ventricular remodeling and myocardial apoptosis after MI, in an attempt to provide experimental evidence of the cardioprotective effect of Wenxin Granule in a rat model of MI.

## 2. Materials and Methods

**2.1. Animals.** Male Sprague-Dawley (SD) rats (190–210 g) were purchased from the animal laboratory of the Academy of Medical Sciences, Beijing, China (certificate number SCXK (Beijing) 2009-0007).

**2.2. Drugs and Reagents.** Wenxin Granule was produced by Shandong Buchang pharmaceutical Co., Ltd., Xi'an, China (Med-drug permit number Z10950026, China). According to the Chinese National Pharmacopoeia (National Pharmacopoeia Committee, 2010), the total amount of notoginseng saponin R1 (C47H80O18), ginseng saponin Rg1 (C42H72O15), and ginseng saponin Rb1 (C54H92O23) should not be less than 17.0 mg per bag (5 g). Metoprolol tartrate tablets were produced by AstraZeneca Pharmaceutical Co., Ltd., Jiangsu, China (Med-drug permit number H32025391, China). The terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) apoptosis

assay kit was purchased from Wuhan Boster Bio-Engineering limited company (product number MK1020, China). Rat angiotensin II (Ang II) enzyme-linked immunosorbent assay (ELISA) Kit (batch number 201211) was provided by Beijing UBIO Biotechnology Co., Ltd., China.

**2.3. Establishment of the Myocardial Infarction (MI) Rat Model [23].** Male SD rats were anaesthetised by intraperitoneal (i.p) injection of a 1% solution of sodium pentobarbital (50 mg/kg). The procedures performed consisted of endotracheal intubation; positive pressure ventilation; preoperative recording by twelve-lead electrocardiogram (ECG); one-lead monitoring; local skin disinfection; chest opening; thoracotomy device setup and opening of the pericardium; occlusion of the left anterior descending coronary artery at the location between the pulmonary cone and the left atrial appendage under its origin 2–3 mm. In the control group, the left anterior descending artery was not occluded. Additional twelve-lead ECG recordings were made postoperatively. Successful ligation was confirmed by ST segment elevation in postoperative ECG, compared with preoperative ones. After the coronary artery occlusion surgery, all animals were given penicillin by i.p injection for three days to prevent infection. One rat died due to surgical bleeding during the operation. Within 24 h after surgery, three rats died of ventricular fibrillation following acute MI. The rat mortality rate was 12.5%.

**2.4. Design and Allocation of Rats.** All rats used in this study received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were randomly divided into 4 groups: control, model, metoprolol, and Wenxin Granule (WXKL) groups with each group consisting of 7 rats. The rats in the WXKL group were administered oral doses of 1.35 g/kg of Wenxin Granule per day. Rats in the metoprolol group were treated with 12 mg/kg of metoprolol tartrate tablets per day. The Wenxin Granule and metoprolol tartrate tablets were grinded and then mixed with distilled water prior to administration. Rats in the control and the model groups were administered equivalent amounts of distilled water orally each day. The day after the coronary artery occlusion, all rats were administered treatment orally for 4 weeks. After 4 weeks of treatment, rats were injected with 1% solution of sodium pentobarbital (40 mg/kg), and an echocardiography was performed. Blood samples were taken from the abdominal aorta. After separation of serum, Ang II was determined using an ELISA assay. The heart was excised and weighed for calculation of the heart weight/body weight ratio. The heart samples were fixed in 4% paraformaldehyde for further pathological experiments.

**2.5. Echocardiography.** At 4 weeks after the coronary artery occlusion surgery, a noninvasive transthoracic echocardiography method was used to evaluate the structure and function of the left ventricle in each group. Under anesthesia by i.p injection of 40 mg/kg pentobarbital sodium, rats were fixed on their backs with their fur shaved and skin cleaned. The parasternal long axis view was selected by using a

high-frequency linear-array transducer. Then, the parameters of heart structure and function were checked in the two-dimensional ultrasound-guided M-curve. The parameters were automatically recorded and consisted of left ventricular posterior wall end-diastolic thickness (LVPWTD); left ventricular posterior wall end-systolic thickness (LVPWTs); interventricular septum end-diastolic thickness (IVSTd); interventricular septum end-systolic thickness (IVSTs); left ventricular end-diastolic inner diameter (LViDd); left ventricular end-systolic inner diameter (LViDs); end-diastolic volume (EDV); end-systolic volume (ESV); stroke volume (SV); ejection fraction (EF); and fractional shortening (FS). The instrument used was a Sino-Japanese joint AloCa5000 color ultrasound diagnostic apparatus. Echocardiography was operated by a technician who was blind to the grouping allocation.

**2.6. Myocardial Histopathology.** Rat heart samples were fixed in 4% paraformaldehyde and embedded in paraffin. The tissue slices (4  $\mu\text{m}$ ) of the heart underwent haematoxylin and eosin (H&E) staining. Histopathological changes were examined and photographed under a light microscope ( $\times 400$ ). The experiment of histopathology has been previously described [24].

**2.7. Apoptosis Detection.** Myocardial apoptosis was detected by the method of TUNEL. All procedures were performed as per the manufacturers' instructions. Diaminobenzidine (DAB) was used to label the nucleus. Samples were counterstained with hematoxylin. The nuclei were defined as apoptotic if the whole nuclear area of the cell was labeled positively. The apoptotic cells were counted manually in 5 high-power fields ( $\times 400$  magnification) by the Image Pro Plus 6.0 program. The apoptosis rate was calculated manually as the percentage of positively staining cells: apoptosis rate = number of apoptotic cells/total number of nucleated cells [25].

**2.8. Detection of Ang II.** Blood samples were taken from the abdominal aorta at 4 weeks after the coronary artery occlusion surgery. The serum was separated by centrifugation at 3,000 rpm for 10 min. The serum was kept at  $-70^{\circ}\text{C}$ . The serum was examined using the ELISA method to detect the levels of Ang II. The serum was further analyzed to quantify the concentration of Ang II in strict accordance with the manufacturers' protocols. The main assay procedures are as follows: (1) dilute standard; (2) inject samples and standard wells; (3) add both Ang II-antibody and Streptavidin-HRP, seal the sealing membrane, gently shake, and incubate for 60 min at  $37^{\circ}\text{C}$ ; (4) remove the membrane carefully, drain the liquid, and shake away the remaining water; (5) add chromogen solution A and then chromogen solution B to each well. Gently mixed, incubate for 10 min at  $37^{\circ}\text{C}$  in the dark; (6) add Stop Solution into each well to stop the reaction; (7) take blank well as zero, measure the optical density (OD) under the wavelength of 450 nm by using Thermo scientific Multiskan MK3 microplate reader.

**2.9. Statistical Analysis.** Data were analyzed using Statistical Package for Social Sciences (SPSS) for windows (version 13.0).

The measurement data were expressed as mean  $\pm$  standard deviation (SD). The data generated from multiple samples were statistically analyzed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test. The count data are expressed as frequency (%). The data from the multiple samples for grade materials were analyzed by the Kruskal-Wallis test. A value of  $P < 0.05$  was considered statistically significant.

### 3. Results

**3.1. The Heart Weight/Body Weight (HW/BW) Ratio and Ventricular Wall Thickness.** As shown in Figure 1(a), the HW/BW ratio in the model group was significantly increased compared with the control group ( $0.383 \pm 0.071$  versus  $0.312 \pm 0.008$  mg/g, resp.,  $P < 0.01$ ). The HW/BW ratio in the metoprolol group ( $0.325 \pm 0.024$  mg/g,  $P < 0.05$ ) and the WXKL group ( $0.333 \pm 0.017$  mg/g,  $P < 0.05$ ) was significantly decreased compared with the model group. As shown in Figure 1(b), there was no significant difference between LVPWTD, LVPWTs, and IVSTd among the four groups. The IVSTs in the control group was  $0.32 \pm 0.03$  cm, and those of the model ( $0.19 \pm 0.01$  cm,  $P < 0.01$ ), metoprolol ( $0.23 \pm 0.08$  cm,  $P < 0.01$ ), and the WXKL groups ( $0.25 \pm 0.05$  cm,  $P < 0.01$ ) were significantly lower in various degrees. Compared with the model group, the IVSTs in the WXKL group were significantly increased ( $P < 0.05$ ).

**3.2. Left Ventricular Contraction Movement, Internal Diameter, and Volume.** At 4 weeks after the coronary artery occlusion surgery, echocardiography was performed, and the typical echocardiography images were taken among different groups. As shown in Figure 2(a), the image of contraction movement in the control group is shaped like waves (red arrow). As shown in Figure 2(b), the waves in the model group weakened and even straightened (red arrow), indicating diminished and even disappearance of contraction movement. The left ventricle expanded significantly in the model group compared with the control group. As shown in Figures 2(c) and 2(d), the changes of left ventricular size and contraction weakness were alleviated in the metoprolol and the WXKL groups. Figures 2(e) and 2(f) show the quantitative analysis of the internal diameter and volume of the left ventricle. The LViDd, LViDs, and EDV in the other three groups were significantly increased to various extents compared with the control group ( $P < 0.01$ ,  $P < 0.05$ ). The ESV in the model and the metoprolol groups also increased significantly ( $P < 0.01$ ). The SV did not show any significant differences between the four groups. Compared with the model group, Wenxin Granule administration decreased LViDs ( $0.66 \pm 0.09$  versus  $0.49 \pm 0.12$  cm,  $P < 0.05$ ) and ESV ( $0.67 \pm 0.24$  versus  $0.30 \pm 0.23$  mL,  $P < 0.05$ ) but had no effects on LViDd ( $0.81 \pm 0.09$  versus  $0.72 \pm 0.08$  cm,  $P > 0.05$ ) and EDV ( $1.14 \pm 0.35$  versus  $0.84 \pm 0.26$  mL,  $P > 0.05$ ). Metoprolol had no effects on the left ventricular internal diameter and volume, neither in the systolic nor diastolic phase.

**3.3. Cardiac Function.** As shown in Figure 3, the EF and FS were significantly decreased to various extents in the other

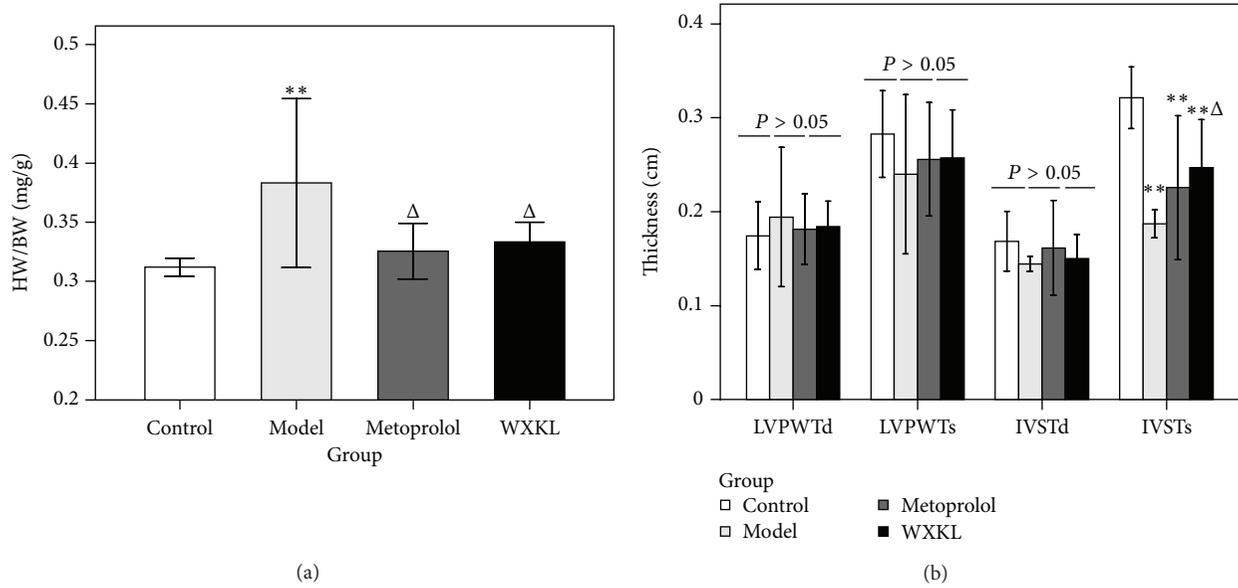


FIGURE 1: The HW/BW ratio and ventricular wall thickness. The heart weight, body weight, and ventricular wall thickness were detected at 4 weeks after the coronary artery occlusion surgery. (a) The HW/BW ratio. (b) The ventricular wall thickness. Values are expressed as mean  $\pm$  SD ( $n = 7$ ). \*\* $P < 0.01$ , versus control.  $\Delta P < 0.05$ , versus model.

TABLE 1: The myocardial histopathological changes among different groups (case (%),  $n = 7$ ).

Group	Necrosis				Inflammatory cells infiltrating			
	-	+	++	+++	-	+	++	+++
Control	7 (100%)	0	0	0	7 (100%)	0	0	0
Model	0	1 (14.3%)	4 (57.1%)	2 (28.6%)	0	1 (14.3%)	5 (71.4%)	1 (14.3%)
Metoprolol	0	5 (71.4%)	2 (28.6%)	0	0	1 (14.3%)	6 (85.7%)	0
WXXL	0	4 (57.1%)	3 (42.9%)	0	0	3 (42.9%)	4 (57.1%)	0
$\chi^2$		19.923				19.475		
$P$		0.000				0.000		

three groups compared with the control group ( $P < 0.01$ ). The EF in the model group was  $43.17 \pm 6.89\%$ , and those of the metoprolol group ( $57.51 \pm 18.31\%$ ,  $P < 0.05$ ) and the WXXL group ( $65.67 \pm 14.82\%$ ,  $P < 0.01$ ) significantly increased compared with the model group. The FS in the WXXL group was significantly increased compared with the model group ( $32.84 \pm 9.85$  versus  $18.57 \pm 3.59\%$ ,  $P < 0.01$ ).

**3.4. Myocardial Histopathological Findings.** Myocardial histopathological findings are shown in Figure 4. In the control group, myocardial fibers were arranged in an orderly fashion, cytoplasmic staining was uniform, and nucleus boundaries were clear. In the model group, myocardial fibers arrangement was discarded, numerous neutrophil granulocytes were seen to be infiltrating, and wide range of necrosis observed, while some cytoplasm showed intense staining. Compared with the model group, the previous histopathological changes were alleviated in both the metoprolol and the WXXL groups. According to the literature [24], the severity of necrosis and inflammatory cells infiltration were graded as follows according to staining intensity: -: absent; +: mild;

++: moderate; and +++: severe. As shown in Table 1, the severity of necrosis and inflammatory cells infiltrating in the metoprolol group and the WXXL group were significantly alleviated compared with the model group.

**3.5. Myocardial Apoptosis.** The technique of TUNEL staining was used to detect myocardial apoptosis at 4 weeks following coronary artery occlusion surgery. Normal nuclei were stained blue, whilst apoptotic nuclei were stained brownish yellow. As shown in Figure 5(a), in the control group, the majority of nuclei were stained blue (normal nuclei), with few nuclei staining brownish yellow (apoptotic nuclei). As shown in Figure 5(b), there was a large number of myocardial apoptotic nuclei in the model group stained brownish yellow. In Figures 5(c) and 5(d), myocardial nuclei apoptosis can be seen to be alleviated in the metoprolol and WXXL groups. The quantitative analysis data of positive staining is shown in Figure 5(e). Compared with the control group, the apoptosis rates were significantly increased at 4 weeks after the coronary artery occlusion surgery. The apoptosis rates in the model group were  $17.33 \pm 1.46\%$ . Those of the metoprolol group

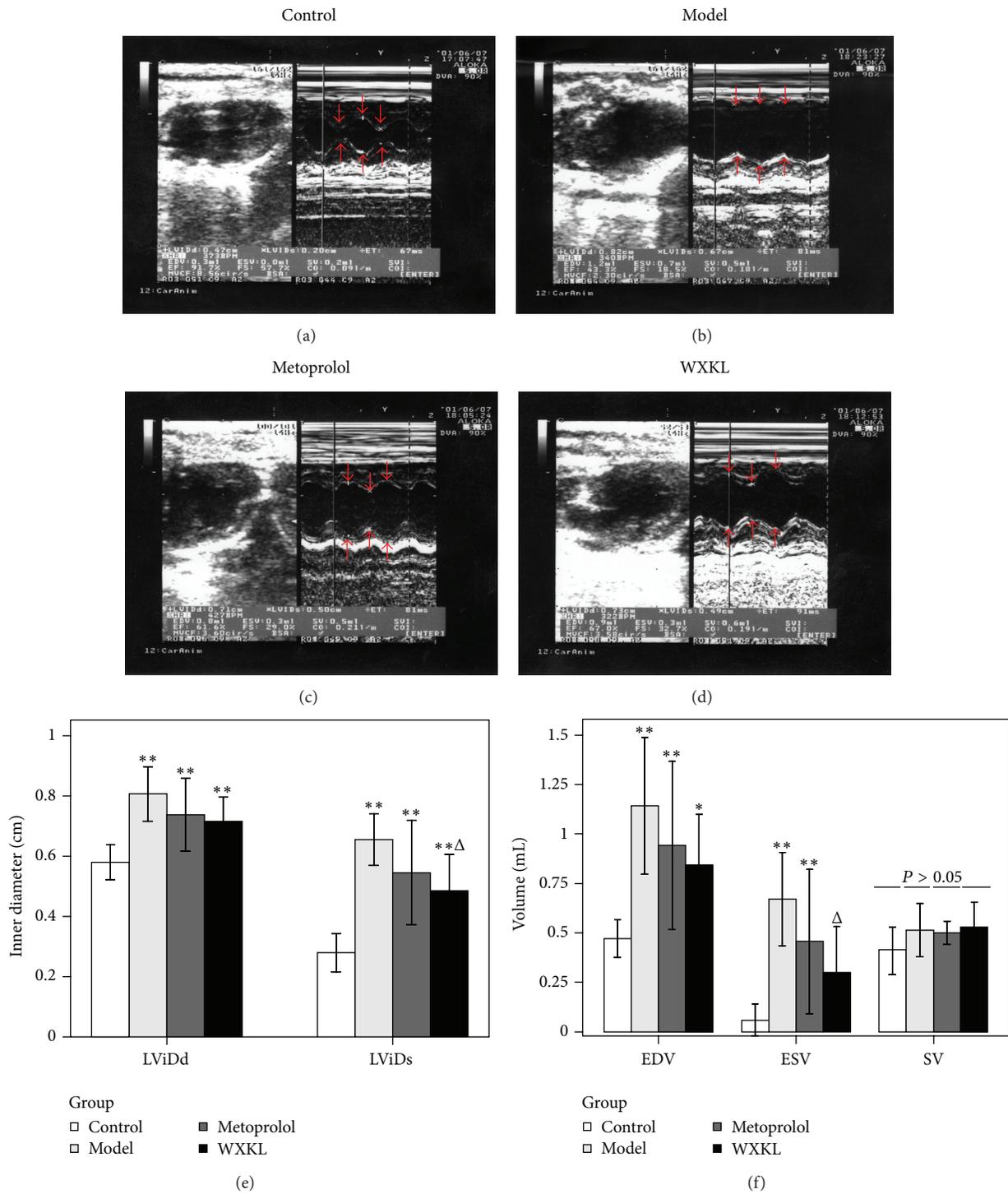


FIGURE 2: Left ventricular contraction movement, internal diameter, and volume. At 4 weeks after the coronary artery occlusion surgery, echocardiography was performed, and the typical echocardiography images were taken among different groups ((a)–(d)). Internal diameter and volume of the left ventricle show the quantitative analysis data ((e)–(f)). Values are expressed as mean  $\pm$  SD ( $n = 7$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , versus control.  $\Delta P < 0.05$ , versus model.

( $13.23 \pm 1.91\%$ ,  $P < 0.01$ ) and the WXKL group ( $14.36 \pm 0.98\%$ ,  $P < 0.01$ ) were significantly lower compared with the model group.

**3.6. Serum Ang II Concentration.** At 4 weeks after the coronary artery occlusion surgery, the serum Ang II concentration was detected by ELISA assay. As shown in Figure 6,

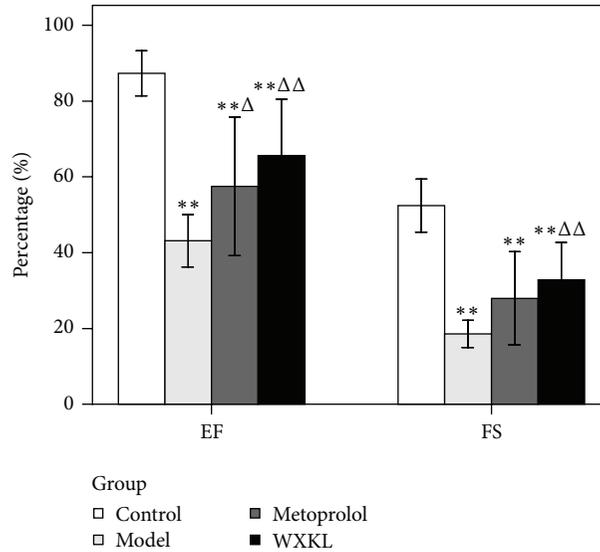


FIGURE 3: Cardiac function. The EF and FS were detected by echocardiography at 4 weeks after the coronary artery occlusion surgery. The EF and FS were significantly decreased in all groups compared with the control group. Compared with the model group, the EF in the metoprolol group and WXKL group was significantly increased, while only the FS in the WXKL group was significantly increased. Values are expressed as mean  $\pm$  SD ( $n = 7$ ). \*\* $P < 0.01$ , versus control.  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ , versus the model.

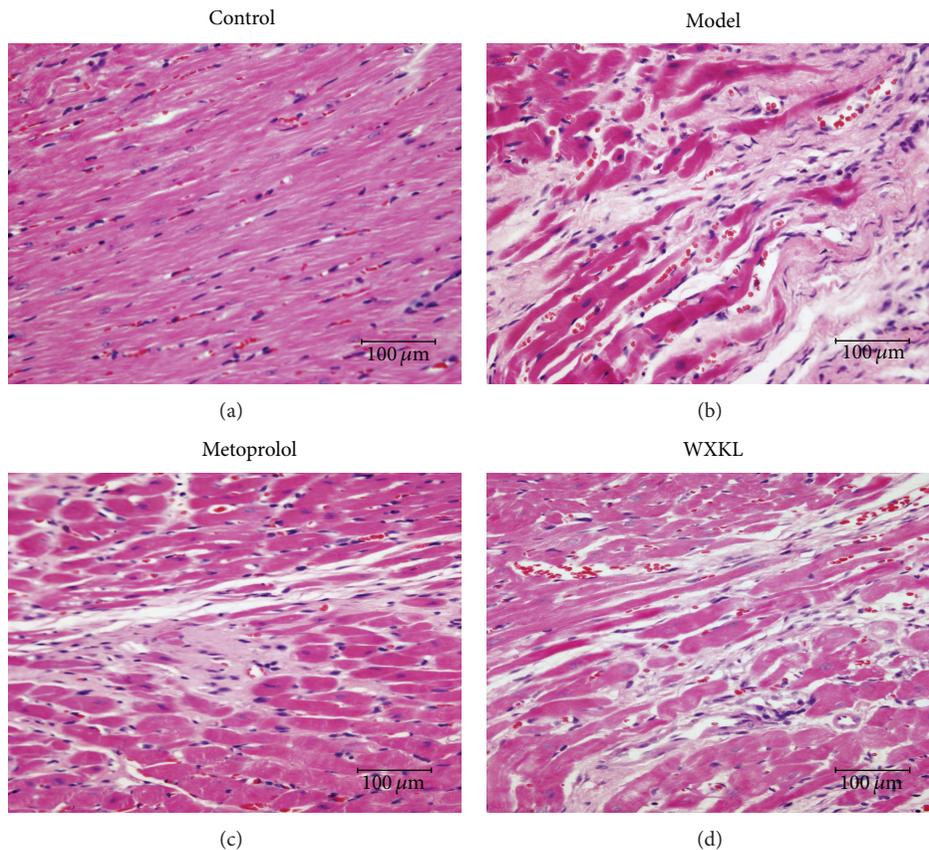


FIGURE 4: Myocardial histopathological findings. Four-micron-thick sections of myocardial tissue were H&E stained and photographed with a digital camera mounted on a light microscope ( $\times 400$  magnification; scale bar,  $100 \mu\text{m}$ ) at 4 weeks after the coronary artery occlusion surgery. (a) Control group, (b) model group, (c) metoprolol group, and (d) WXKL group.

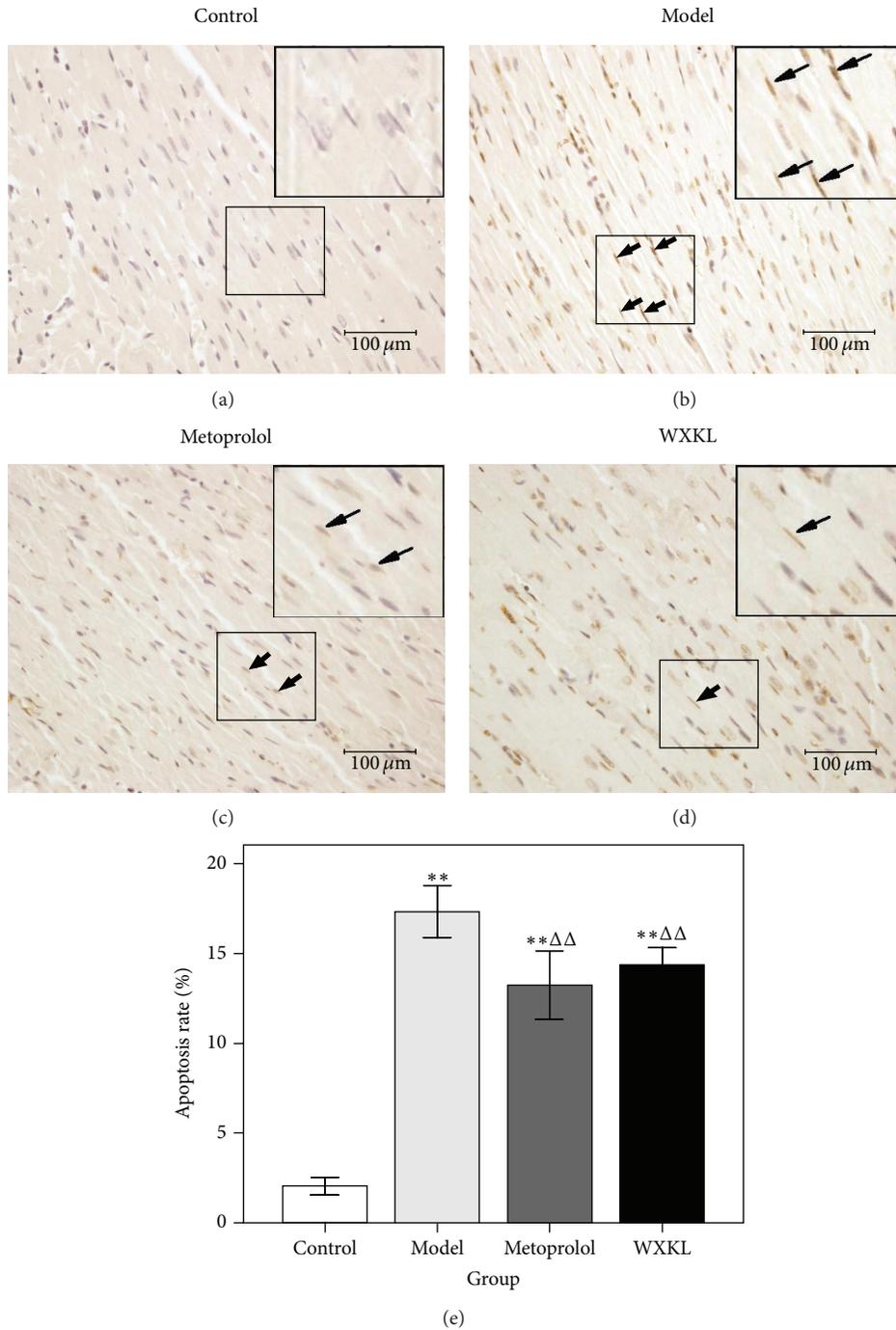


FIGURE 5: Myocardial apoptosis. Apoptotic nuclei were stained in brownish yellow, while normal nuclei were stained in blue. The typical apoptosis images (TUNEL, DAB;  $\times 400$  magnification; scale bar,  $100 \mu\text{m}$ ) were taken among different groups (a)–(d). Quantitative analysis of apoptotic rates (e). Values are expressed as mean  $\pm$  SD ( $n = 7$ ). \*\* $P < 0.01$ , versus control.  $\Delta\Delta P < 0.01$ , versus model.

the serum Ang II concentration in the model group was significantly increased compared to the control group ( $206.62 \pm 17.24$  versus  $176.19 \pm 15.24$  ng/L,  $P < 0.01$ ). The serum Ang II concentration in the WXKL group was significantly decreased compared with the model group ( $178.11 \pm 22.51$  versus  $206.62 \pm 17.24$  ng/L,  $P < 0.01$ ).

#### 4. Discussion

In modern society, there has been a growing interest in traditional Chinese Medicine (TCM) for patients due to the personalized therapy available in many countries [26, 27]. TCM in cardiovascular disease prevention and treatment is a

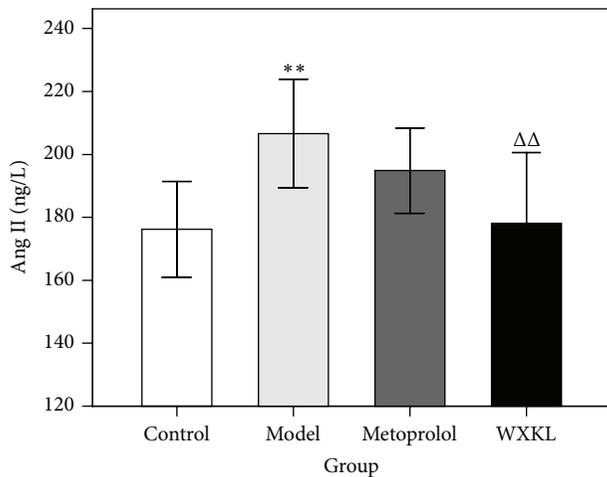


FIGURE 6: Serum Ang II concentration in the 4 experimental groups. The serum Ang II concentration was detected by ELISA assay at 4 weeks after the coronary artery occlusion surgery. Ang II in the model group was significantly increased compared with the control group. Ang II in the WXKL group was significantly decreased compared with the model group. Values are expressed as mean  $\pm$  SD ( $n = 7$ ). \*\* $P < 0.01$ , versus control.  $\Delta\Delta P < 0.01$ , versus model.

valuable and promising prospect. TCM has a history of more than 2500 years with a unique theory of diagnosis and treatment. Whilst there is no such term as MI in TCM, its symptoms can be classified into the categories of true heart pain, palpitation, thoracic obstruction, and heart-energy stagnation syndrome. In recent years, an increasing number of clinicians have successfully applied TCM drugs for supplementing Qi, nourishing Yin, and activating blood circulation in the treatment of MI [28, 29]. Nevertheless, the role of TCM in cardiovascular diseases requires further experimental evidence.

Wenxin Granule is a Chinese medicinal granule, which has effects on supplementing Qi and nourishing Yin, promoting blood circulation for removing blood stasis. Several studies have shown that Wenxin Granule can increase coronary blood flow, reduce myocardial oxygen consumption, enhance myocardial compliance, improve myocardial hypoxia tolerance, relieve anterior and posterior cardiac loading, reduce myocardial tissue damage in patients with high blood pressure, and reduce the occurrence of arrhythmia [30]. To further support previous studies, the present study aimed to provide experimental evidence of the cardioprotective effect of Wenxin Granule in the MI rat model.

The results of the current study showed that administration of Wenxin Granule could partially reverse left ventricular remodeling and improve left ventricular function to an extent. It should be noted that whilst the Wenxin Granule decreased LViDs and ESV, Wenxin Granule administration had no effects on LViDd and EDV. It is possible that 4 weeks after modeling, fibrotic scarring or a ventricular aneurysm occurred in the left ventricular infarct wall, which may be responsible for the increase in LViDd and EDV. Wenxin Granule did not completely reverse fibrotic scarring and ventricular aneurysm after MI. Consequently, the LViDd and EDV did not change significantly in the diastolic

phase. During the systolic phase, the contractile function of non-infarct myocardium in the Wenxin Granule group was stronger than that in the model group. Therefore, the Wenxin Granule could decrease LViDs and ESV markedly. This was the main difference between the metoprolol group and the WXKL groups whereby metoprolol only showed a decreasing trend on the left ventricular internal diameter and volume, but without significant difference, in either the systolic or the diastolic phase. The possible reasons for this are that the cardioprotective mechanisms of metoprolol are achieved mainly by blocking cardiac  $\beta_1$ -receptors and thereby slowing heart rate and reduction in myocardial contractility and myocardial oxygen consumption. Compared with other beta-Blockers and angiotensin converting enzyme inhibitors (ACEI), the effect of metoprolol on preventing left ventricular remodeling is relatively weak [31, 32].

In addition, the present study observed the improvement of histopathological injury and the inhibition of myocardial apoptosis after MI in the WXKL group and the metoprolol group. *Radix Notoginseng*, one of the main components of Wenxin Granule, can repair ischemia myocardial and reduce myocardial ischemic injury by decreasing oxidative stress and repressing the inflammatory cascade [33]. This may be the means by which treatment with Wenxin Granule reduced histopathologic injury after MI. Meanwhile, it was also found that Wenxin Granule was capable of reducing Ang II concentrations in the MI rat model. Myocardial ischemia induces activation of various components of the renin-angiotensin system (RAS), including angiotensinogen, renin, angiotensin-converting enzyme, angiotensins, and angiotensin receptors, in the acute phase of MI and in the postinfarction remodeling process [34]. In the RAS, Ang II is a biologically active substance, which is closely correlated with myocardial apoptosis. Several studies have investigated the relationship between Ang II and myocardial apoptosis. A study by Kajstura et al. (1997) showed that in primary cultures of adult rat ventricular myocytes exposed to  $10^{-9}$  M of Ang II for 24 h, presented with a fivefold increase in apoptosis documented by the terminal deoxynucleotidyl transferase assay, and confirmed by DNA agarose gel electrophoresis [35]. A study conducted by Leri et al. (1998) confirmed that Ang II could increase the susceptibility of myocytes to undergo apoptosis [36]. Ang II stimulation was associated with translocation of the epsilon and delta isoforms of protein kinase C. This was coupled with an increase in cytosolic  $Ca^{2+}$  in the cells which can induce apoptosis [35]. Several studies have confirmed that *Radix Codonopsis Pilosulae*, one of the main components of Wenxin Granule, can reverse  $Ca^{2+}$  influx and the increase in apoptosis. This is achieved by attenuating Ang II and the cardiac-impaired insulin-like growth factor II (IGF II) receptor pathway in myocardial cells [37]. The current study found that treatment with Wenxin Granule decreased Ang II concentrations. Consequently, Ang II could be the underlying mechanism of Wenxin Granule inhibition of apoptosis.

Based on the previous findings, the authors draw the conclusion that Wenxin Granule can partially reverse ventricular remodeling, improve heart function, alleviate the histopathological damage, inhibit myocardial apoptosis, and reduce

AngII concentrations in rats with MI. These results suggest that Wenxin Granule might be a potentially alternative and complementary medicine for the treatment of MI.

### Authors' Contribution

Aiming Wu, Jianying Zhai, Dongmei Zhang, Lixia Lou, and Haiyan Zhu contributed equally to this work.

### Conflict of Interests

The authors declare that they have no conflict of interests.

### Acknowledgments

This paper was partially supported by the National Natural Science Foundation Project of China (no. 81202685) and Beijing Municipal Commission of Education Build Project of China (2012 Beijing University of Chinese Medicine).

### References

- [1] D. D. McManus, S. M. Piacentini, D. Lessard et al., "Thirty-year (1975 to 2005) trends in the incidence rates, clinical features, treatment practices, and short-term outcomes of patients <55 years of age hospitalized with an initial acute myocardial infarction," *The American Journal of Cardiology*, vol. 108, no. 4, pp. 477–482, 2011.
- [2] N. Rumana, Y. Kita, T. C. Turin et al., "Trend of increase in the incidence of acute myocardial infarction in a Japanese population: Takashima AMI registry, 1990–2001," *The American Journal of Epidemiology*, vol. 167, no. 11, pp. 1358–1364, 2008.
- [3] S. Yi, W. Lei, and Z. Minzhou, "Progress in the epidemiological study of acute myocardial infarction," *Chinese Journal of Integrative Medicine on Cardio-/Cerebrovascular Disease*, vol. 10, no. 4, pp. 467–469, 2012.
- [4] L. Lacey and M. Tabberer, "Economic burden of post-acute myocardial infarction heart failure in the United Kingdom," *European Journal of Heart Failure*, vol. 7, no. 4, pp. 677–683, 2005.
- [5] D. V. Baklanov, L. A. Kaltenbach, S. P. Marso et al., "The prevalence and outcomes of transradial percutaneous coronary intervention for ST-segment elevation myocardial infarction: analysis from the national cardiovascular data registry (2007 to 2011)," *Journal of the American College of Cardiology*, vol. 61, no. 4, pp. 420–426.
- [6] G. Falsini, F. Liistro, K. Ducci et al., "Shifting from pharmacological to systematic mechanical reperfusion therapy for acute myocardial infarction via a cooperating network: impact on reperfusion rate and in-hospital mortality," *Journal of Cardiovascular Medicine*, vol. 9, no. 3, pp. 245–250, 2008.
- [7] S. Gupta, S. Das, R. Sahewalla et al., "A study on quality of life in patients following myocardial infarction," *Indian Journal of Physiology and Pharmacology*, vol. 56, no. 1, pp. 28–35, 2012.
- [8] W. Wang, D. R. Thompson, C. F. Ski, and M. Liu, "Health-related quality of life and its associated factors in Chinese myocardial infarction patients," *European Journal of Preventive Cardiology*. In press.
- [9] S. Mangiapane and R. Busse, "Prescription prevalence and continuing medication use for secondary prevention after myocardial infarction: the reality of care revealed by claims data analysis," *Deutsches Arzteblatt*, vol. 108, no. 50, pp. 856–862, 2011.
- [10] A. Abbate, G. G. L. Biondi-Zoccai, R. Bussani et al., "Increased myocardial apoptosis in patients with unfavorable left ventricular remodeling and early symptomatic post-infarction heart failure," *Journal of the American College of Cardiology*, vol. 41, no. 5, pp. 753–760, 2003.
- [11] J. N. Kirkpatrick and M. S. J. Sutton, "Assessment of ventricular remodeling in heart failure clinical trials," *Current Heart Failure Reports*, vol. 9, no. 4, pp. 328–336, 2012.
- [12] J. J. Gajarsa and R. A. Kloner, "Left ventricular remodeling in the post-infarction heart: a review of cellular, molecular mechanisms, and therapeutic modalities," *Heart Failure Reviews*, vol. 16, no. 1, pp. 13–21, 2011.
- [13] P. M. Kang and S. Izumo, "Apoptosis and heart failure: a critical review of the literature," *Circulation Research*, vol. 86, no. 11, pp. 1107–1113, 2000.
- [14] Y. Hojo, T. Saito, and H. Kondo, "Role of apoptosis in left ventricular remodeling after acute myocardial infarction," *Journal of Cardiology*, vol. 60, no. 2, pp. 91–92, 2012.
- [15] F. Sam, D. B. Sawyer, D. L. Chang et al., "Progressive left ventricular remodeling and apoptosis late after myocardial infarction in mouse heart," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 279, no. 1, pp. H422–H428, 2000.
- [16] A. Abbate, G. G. L. Biondi-Zoccai, and A. Baldi, "Pathophysiologic role of myocardial apoptosis in post-infarction left ventricular remodeling," *Journal of Cellular Physiology*, vol. 193, no. 2, pp. 145–153, 2002.
- [17] A. M. Shah, C. L. Hung, S. H. Shin et al., "Cardiac structure and function, remodeling, and clinical outcomes among patients with diabetes after myocardial infarction complicated by left ventricular systolic dysfunction, heart failure, or both," *The American Heart Journal*, vol. 162, no. 4, pp. 685–691, 2011.
- [18] L. Spinelli, C. Morisco, E. A. di Panzillo, R. Izzo, and B. Trimarco, "Reverse left ventricular remodeling after acute myocardial infarction: the prognostic impact of left ventricular global torsion," *International Journal of Cardiovascular Imaging*, vol. 29, no. 4, pp. 787–795, 2013.
- [19] V. Jayasankar, Y. J. Woo, T. J. Pirolli et al., "Induction of angiogenesis and inhibition of apoptosis by hepatocyte growth factor effectively treats postischemic heart failure," *Journal of Cardiac Surgery*, vol. 20, no. 1, pp. 93–101, 2005.
- [20] W. Liu, R. Jiang, S. Ding et al., "Quality assessment of randomized controlled trials on Wenxin granule for treatment of atrial fibrillation," *Zhongguo Zhong Yao Za Zhi*, vol. 37, no. 1, pp. 109–114, 2012.
- [21] X. Wang, Y. Gu, T. Wang, and C. Huang, "Wenxin Keli attenuates ischemia-induced ventricular arrhythmias in rats: involvement of L-type calcium and transient outward potassium currents," *Molecular Medicine Reports*, vol. 7, no. 2, pp. 519–524, 2013.
- [22] M. Wang, Y. B. Yu, and S. E. Huang, "Clinical observation on effect and safety of combined use of wenxin granule and amiodarone for conversion of auricular fibrillation," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 26, no. 5, pp. 445–448, 2006.
- [23] Y. Xing, Y. Gao, J. Chen et al., "Wenxin-Keli regulates the calcium/calmodulin-dependent protein kinase II signal transduction pathway and inhibits cardiac arrhythmia in rats with myocardial infarction," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 464508, 15 pages, 2013.

- [24] M. C. Fishbein, D. Maclean, and P. R. Maroko, "Experimental myocardial infarction in the rat. Qualitative and quantitative changes during pathologic evolution," *The American Journal of Pathology*, vol. 90, no. 1, pp. 57–70, 1978.
- [25] Y. C. Zhou, B. Liu, Y. J. Li et al., "Effects of Buyang Huanwu decoction on ventricular remodeling and differential protein profile in a rat model of myocardial infarction," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 385247, 11 pages, 2012.
- [26] J. Wang and X. Xiong, "Current situation and perspectives of clinical study in integrative medicine in China," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.
- [27] J. J. Park, S. Beckman-Harned, G. Cho, D. Kim, and H. Kim, "The current acceptance, accessibility and recognition of Chinese and Ayurvedic medicine in the United States in the public, governmental, and industrial sectors," *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 405–408, 2012.
- [28] S. L. Qiu, M. Jin, J. H. Yi, T. G. Zhu, X. Quan, and Y. Liang, "Therapy for replenishing qi, nourishing yin and promoting blood circulation in patients with acute myocardial infarction undergoing percutaneous coronary intervention: a randomized controlled trial," *Zhong Xi Yi Jie He Xue Bao*, vol. 7, no. 7, pp. 616–621, 2009.
- [29] Y. Q. Li, M. Jin, S. L. Qiu et al., "Effect of Chinese drugs for supplementing Qi, nourishing yin and activating blood circulation on myocardial perfusion in patients with acute myocardial infarction after revascularization," *Chinese Journal of Integrative Medicine*, vol. 15, no. 1, pp. 19–25, 2009.
- [30] N. Su, T. Xu, Y. Tang, and Z. Zhou, "Efficacy and safety of Wenxin granules in the treatment of congestive heart failure: a systematic review," *China Pharmacy*, vol. 21, no. 7, pp. 637–640, 2010.
- [31] G. Cimmino, B. Ibanez, C. Giannarelli et al., "Carvedilol administration in acute myocardial infarction results in stronger inhibition of early markers of left ventricular remodeling than metoprolol," *International Journal of Cardiology*, vol. 153, no. 3, pp. 256–261, 2011.
- [32] R. Ricci, C. Coletta, V. Ceci et al., "Effect of early treatment with captopril and metoprolol singly and together on postinfarction left ventricular remodeling," *The American Heart Journal*, vol. 142, no. 4, article E5, 2001.
- [33] S. Y. Han, H. X. Li, X. Ma et al., "Evaluation of the anti-myocardial ischemia effect of individual and combined extracts of *Panax notoginseng* and *Carthamus tinctorius* in rats," *Journal of Ethnopharmacology*, vol. 145, no. 3, pp. 722–727, 2013.
- [34] W. Dai and R. A. Kloner, "Potential role of renin-angiotensin system blockade for preventing myocardial ischemia/reperfusion injury and remodeling after myocardial infarction," *Postgraduate Medicine*, vol. 123, no. 2, pp. 49–55, 2011.
- [35] J. Kajstura, E. Cigola, A. Malhotra et al., "Angiotensin II induces apoptosis of adult ventricular myocytes in vitro," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 3, pp. 859–870, 1997.
- [36] A. Leri, P. P. Claudio, Q. Li et al., "Stretch-mediated release of angiotensin II induces myocyte apoptosis by activating p53 that enhances the local renin-angiotensin system and decreases the Bcl-2-to-Bax protein ratio in the cell," *Journal of Clinical Investigation*, vol. 101, no. 7, pp. 1326–1342, 1998.
- [37] K. H. Tsai, N. H. Lee, G. Y. Chen et al., "Dung-shen (*Codonopsis pilosula*) attenuated the cardiac-impaired insulin-like growth factor II receptor pathway on myocardial cells," *Food Chemistry*, vol. 138, no. 2-3, pp. 1856–1867, 2013.

## Research Article

# The Effect of Sodium Tanshinone IIA Sulfate and Simvastatin on Elevated Serum Levels of Inflammatory Markers in Patients with Coronary Heart Disease: A Study Protocol for a Randomized Controlled Trial

Qinghua Shang,<sup>1,2</sup> Hanjay Wang,<sup>3</sup> Siming Li,<sup>1</sup> and Hao Xu<sup>2</sup>

<sup>1</sup> Graduate School, Beijing University of Chinese Medicine, Beijing 100029, China

<sup>2</sup> Department of Cardiovascular Diseases, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>3</sup> College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Correspondence should be addressed to Hao Xu; xuhaotcm@gmail.com

Received 22 April 2013; Revised 16 June 2013; Accepted 25 June 2013

Academic Editor: Keji Chen

Copyright © 2013 Qinghua Shang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Background.** Coronary heart disease (CHD) due to atherosclerotic inflammation remains a significant threat to global health despite the success of the lipid-lowering, anti-inflammatory statins. Tanshinone IIA, a potent anti-inflammatory compound derived from Traditional Chinese Medicine (TCM), may be able to supplement statins by further reducing levels of circulating inflammatory markers correlated to cardiovascular risk. Here, we present the protocol of a randomized controlled trial (RCT) that will investigate the synergistic effect of sodium tanshinone IIA sulfate and simvastatin on reducing elevated inflammatory markers in patients with CHD. **Participants:** Seventy-two inpatients with confirmed CHD, elevated serum high-sensitivity C-reactive protein (Hs-CRP) level, and a TCM diagnosis of blood stasis syndrome will be enrolled and randomized 1:1 into the control or experimental group. **Intervention.** All subjects will receive a standard Western therapy including 20 mg simvastatin orally once per evening. Patients in the experimental group will additionally receive a daily 80 mg dose of sodium tanshinone IIA sulfate intravenously, diluted into 250 mL 0.9% NaCl solution. The treatment period will be 14 days. **Outcomes.** Primary outcome parameter: serum Hs-CRP level. Secondary outcome parameters: other circulating inflammatory markers (including IL-6, TNF $\alpha$ , VCAM-1, CD40, sCD40L, MCP-1, and MMP-9), improvement in symptoms of angina and blood stasis syndrome, and safety. This trial is registered with ChiCTR-TRC-12002361.

## 1. Background

Cardiovascular disease is the worldwide leading cause of death. Globally in 2008, over 17 million people died from cardiovascular diseases, representing 30% of total deaths around the world [1]. Among all cardiovascular diseases, coronary heart disease (CHD) is responsible for the greatest mortality, accounting for 7.3 million deaths worldwide in 2008 [1].

CHD involves narrowing of the arteries supplying oxygen to the heart, most often due to buildup of atherosclerotic plaque in the coronary vessels. The ultimate rupture of atherosclerotic plaque may lead to the onset of acute coronary syndrome (ACS), a medically emergent manifestation of CHD involving unstable angina or myocardial infarction.

The formation of atherosclerotic plaque is an inflammatory response, usually associated with elevated levels of low-density lipoprotein cholesterol (LDL-C) in the blood [2]. To this end, statins have served as a notably successful pharmacologic intervention against CHD from the standpoint of Western medicine. One meta-analysis study found that the average statin regimen reduces LDL-C levels by 35%, leading to a 60% decrease in ischemic cardiac events such as those of ACS [3]. Remarkably, as demonstrated by the PRINCE study, statins also moderate the atherosclerotic inflammatory response by reducing levels of C-reactive protein (CRP) [4], a robust marker of systemic inflammation whose concentration in the blood strongly correlates with the patient's cardiovascular risk [5]. Nevertheless, a multicenter

study involving over 4,000 ACS patients showed that 22.4% of patients receiving intensive statin therapy suffered a serious cardiovascular or cerebrovascular event within two years of initiating treatment [6], indicating that although statins may be one of Western medicine's most effective agents for CHD, the toll of this disease remains significantly high.

At East-West integrative medical centers in Asia, cardiovascular diseases may also be evaluated according to the principles of Traditional Chinese Medicine (TCM). Among patients with the Western diagnosis of CHD, the TCM diagnosis of blood stasis syndrome (BSS) is exceedingly common, and the treatment for these CHD-BSS patients via TCM frequently involves herbal therapies using the root of *Salvia miltiorrhiza* (丹参, danshen) [7].

The danshen root contains tanshinone IIA, an active biochemical compound that has been shown to possess a multitude of antiatherosclerotic properties [8]. Most noteworthy is the ability of tanshinone IIA to decrease the levels of numerous inflammatory mediators associated with the progression of atherosclerosis, such as CRP, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF $\alpha$ ), vascular cell adhesion molecule-1 (VCAM-1), CD40, monocyte chemoattractant protein-1 (MCP-1), and matrix metalloproteinase-9 (MMP-9) [8, 9]. A recent clinical trial demonstrated that giving simvastatin in combination with intravenous sodium tanshinone IIA sulfate (STS), the most widely used clinical formulation of tanshinone IIA in China, significantly decreased the levels of CRP, cholesterol, and plaque buildup in patients with peripheral vascular disease [10]. Notably, the integrative therapy was safer and more effective in treating this form of atherosclerosis than simvastatin alone. Whether there is potential for synergy between STS and statins in treating CHD, however, remains unknown.

Here, we present the protocol for a randomized, controlled clinical study that applies East-West integrative medicine to the treatment of CHD. Using standard Western therapy involving simvastatin as a foundation, we aim to explore the potential of further dampening the atherosclerotic inflammatory reaction in CHD-BSS patients through the concomitant addition of STS, utilizing the compound's potent anti-inflammatory properties as a supplement to simvastatin. In addition, we also aim to assess the integrative therapy's safety and its efficacy in improving angina and BSS symptoms relative to the simvastatin regimen without STS. Overall, this study offers an entry point for understanding and verifying the clinical applications of STS-statin integrative therapy in treating patients with CHD.

## 2. Methods/Design

**2.1. Setting and Design.** This trial is a monocentric, parallel-design, randomized, controlled, clinical pilot study that will be conducted at Xiyuan Hospital, and China Academy of Chinese Medical Sciences in Beijing, China. Subject recruitment is scheduled to begin in August 2012. This study will involve 72 patients with the diagnoses of CHD and BSS according to Western medicine and TCM, respectively. The 72 participants will be randomized 1:1 into a standard Western therapy (control) group and an East-West integrative

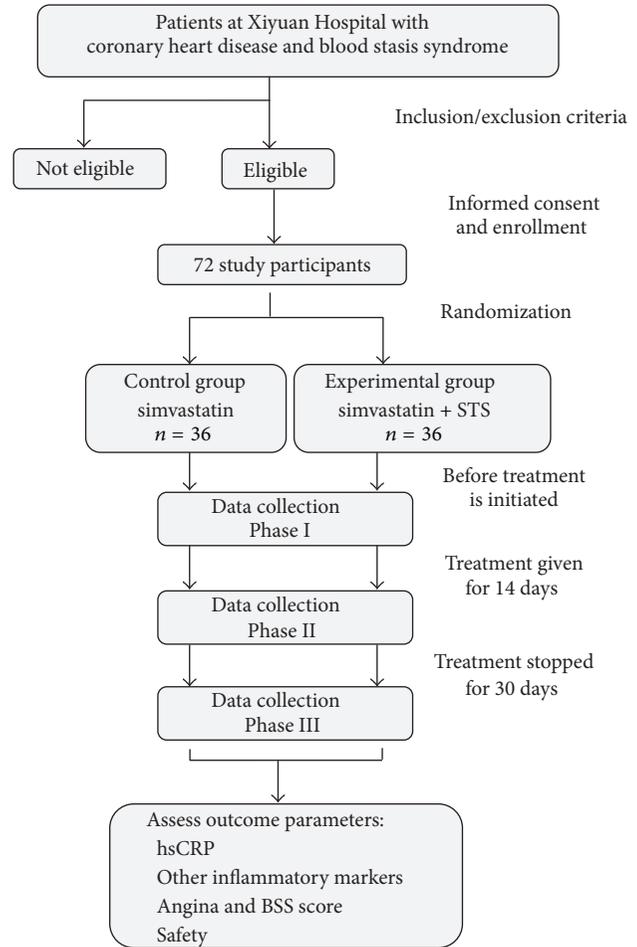


FIGURE 1: Study flowchart.

therapy (experimental) group. For 14 days, all 72 patients will be treated with a standard Western therapy involving simvastatin, and patients in the experimental group will additionally receive intravenous STS. Data will be collected before initiating treatment, immediately after the 14-day treatment period and 30 days posttreatment. Serum CRP levels measured by high-sensitivity CRP (hsCRP) testing will serve as the primary outcome parameter, whereas the levels of other inflammatory markers, the improvement of angina and BSS symptoms, and safety will serve as secondary outcome parameters.

The study design is illustrated in Figure 1 and described in detail below according to the CONSORT 2010 statement [11].

**2.2. Diagnostic Criteria.** The diagnosis of CHD will be based on the standardized criteria established in "Nomenclature and criteria for diagnosis of ischemic heart disease," a joint report published by the International Society and Federation of Cardiology and the World Health Organization [12]. In addition, following the current practice of CHD clinical research, we will require subjects to either have a previous history of myocardial infarction or have at least one coronary

artery stenosis  $\geq 50\%$  confirmed by coronary angiography in order to ensure the diagnosis of CHD.

The TCM diagnosis of BSS will follow the principles described in “Criteria for TCM syndrome differentiation of patients with coronary heart disease,” published by the China Society of Integrated Traditional Chinese and Western Medicine [13].

**2.3. Subject Inclusion and Exclusion.** To participate in this study, subjects must be 35 to 75 years of age and must either have a previous history of myocardial infarction or have at least one coronary artery stenosis  $\geq 50\%$  confirmed by coronary angiography. In addition, subjects must currently be hospitalized with either unstable angina or an acute non-ST segment elevation myocardial infarction and have taken statin medicine for at least 1 month. From a TCM perspective, study participants must have the diagnosis of BSS. Finally, subjects must have a serum hsCRP level between 3 mg/L and 15 mg/L.

The criteria for exclusion include infection, fever, trauma, burn injury, or surgery within one month prior to recruitment; a concomitant diagnosis of cancer, sexually transmitted diseases, tuberculosis, or rheumatoid arthritis or other autoimmune diseases; or a history of serious pulmonary, hepatic, renal, neurological, psychiatric, or hematological diseases. In addition, subjects must not have previously undergone or currently be planning to undergo surgical intervention for CHD. Patients with severe heart failure indicated by an ejection fraction  $< 35\%$  will be excluded as will those with a reduced platelet count or a tendency to bleed or hemorrhage. Study participants must not currently be taking antibiotics or using TCM preparations that relieve fever or clear internal heat. Finally, patients who may become noncompliant or may participate in other clinical trials will be excluded.

During the course of the study, a subject may be excluded if (1) it is discovered that a subject was misdiagnosed for either BSS or CHD or was otherwise inappropriately accepted for participation in the trial; (2) if a subject misses a significant number of treatments or is missing significant records of data; (3) if subject’s blood samples from the first two phases of the study (see Section 2.9) are contaminated or damaged in any way such that reliable data cannot be obtained; or (4) if for any reason (e.g., onset of a mid-study nosocomial infection), patient’s inflammatory marker levels may not accurately reflect atherosclerotic inflammation.

**2.4. Sample Size Estimation.** Because the data required to perform an *a priori* sample size calculation for this study is not available, we have adopted the sample size of a comparable trial for use in this pilot study. The comparable trial investigated the effects of TCM on unstable angina patients undergoing percutaneous coronary intervention and involved 60 subjects randomized into two treatment groups of 30 each [14]. For our pilot study, we will recruit 72 patients to begin the trial, assuming conservatively that 20% of the participants in each treatment group will ultimately not complete the study.

**2.5. Randomization and Blinding.** A member of the Good Clinical Practice (GCP) Clinical Center of Xiyuan Hospital who is independent of the study will use SAS 9.2 software to perform a block randomization, generating a sequence of 72 random numbers in 1:1 allocation between the two groups. In order to conceal the generation, the outcomes will be conducted by another member of GCP Clinical Center using a central randomization; once a patient meets all the criteria, a random number will then be delivered by telephone to the clinical researchers, unblinding only after data is collected for the first phase of the study (see Section 2.9). The number delivered by telephone will determine whether the subject receives the control or experimental therapy.

Because the color of the STS solution given to the experimental group is unique and challenging to emulate, blinding will be difficult to achieve at the physician and patient levels. In order to minimize biases as much as possible, all other potential sources of information that may reveal treatment allocation to patients (e.g., contents of case report form, patient’s random number) will be judiciously guarded. Additionally, the details of treatment allocation will not be disclosed to any patient until the study has concluded, and all study participants will be discouraged from discussing with one another their involvement in the trial. Finally, the clinical researchers will strictly abide by the study’s random design and will interact with the patients in each group with as few differences as possible.

Blinding will be maintained at the level of outcome assessment. The individuals performing laboratory blood analyses, data management, and statistical analyses will be independent of the clinical component of the study and will not be provided with any information that may reveal treatment allocation details.

**2.6. Ethics.** This trial has been approved by the local institutional ethics committee (Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing, China; (2012XL022-2)) and is registered at the Chinese Clinical Trial Registry (ChiCTR-TRC-12002361). All aspects of our study will be conducted with adherence to the current version of the Declaration of Helsinki, the guidelines established by the International Conference on Harmonization of Good Clinical Practice, and the laws of China. Informed written consent will be required of all study participants, none of whom will be denied a standard, accepted therapy.

**2.7. Treatment.** Each patient in the standard Western therapy (control) group will receive 20 mg simvastatin orally once per evening, in addition to any other oral or IV Western medications or TCM preparations (aside from those listed as exclusion criteria, above) that are deemed appropriate for a standard treatment of the patient’s individual condition. These other medications will be prescribed by physicians who are not associated with the trial.

In addition to receiving the treatments described above for the control group, each patient in the East-West integrative therapy (experimental) group will also receive a daily intravenous dose of 80 mg STS (10 mg per ampoule,

TABLE 1: Data Collection Phases and Scheme.

Data collection phase	Phase I: pretreatment 0 day	Phase II: posttreatment 14 days	Phase III: posttreatment 30 days
Inclusion/exclusion criteria	√		
Signed informed consent	√		
Patient interview			
General health	√		
Past medical history	√		
Signs and symptoms of present illness	√	√	√
Current medications	√	√	√
Physical exam			
Tongue and pulse examination	√	√	√
Heart rate and blood pressure	√	√	√
Laboratory tests			
Routine blood, urine, and stool (+ occult blood)	√	√	
Liver and kidney function	√	√	
Blood lipid and glucose	√		
Coagulation	√	√	
High-sensitivity CRP	√	√	√
Cardiac markers: CK-MB, cTnT, cTnI	√		
Electrocardiogram	√	√	
Inflammatory markers*	√	√	√
Assessment using TCM and Western medicine	√	√	√
Angina and BSS score	√	√	√
Randomization into treatment groups	√		
Overall evaluation			
Evaluation of efficacy		√	√
Evaluation of compliance		√	√
Evaluation of safety and side effects		√	√

\*NOTE: blood sample will be stored for analysis at the end of the study. Inflammatory markers include interleukin-6 (IL-6), tumor necrosis factor alpha (TNF $\alpha$ ), vascular cell adhesion molecule (VCAM-1), CD40 antigen and CD40 ligand (sCD40L), monocyte chemotactic protein-1 (MCP-1), and matrix metalloproteinase-9 (MMP-9).

Jiangsu Carefree Pharmaceutical Co., Ltd., national drug approval number: H31022558), diluted with 250 mL 0.9% NaCl solution.

All study participants will receive treatment as indicated above for 14 consecutive days.

**2.8. Outcome Parameters.** Serum hsCRP level will serve as the primary outcome parameter in this study. The levels of other inflammatory mediators, including IL-6, TNF $\alpha$ , VCAM-1, CD40 and soluble CD40 ligand (sCD40L), MCP-1, and MMP-9, as measured by enzyme-linked immunosorbent assay (ELISA), will constitute secondary outcome parameters. Additional secondary parameters will include safety and the extent of improvement in angina and BSS symptoms.

To assess safety, patients will be asked to report any side effects or changes in feelings that they have noticed since the previous phase of data collection. In addition, the results of routine blood, urine, and stool tests, liver and kidney

function tests, coagulation tests, and electrocardiogram tests will also be considered in the evaluation of safety. Finally, the clinical researchers will carefully monitor all patients for the potential onset of adverse events, the most severe of which may include arrhythmias, heart failure, recurrent myocardial infarction, myocardial rupture, cerebral infarction, cerebral hemorrhage, sudden cardiac death, and all-cause death.

To assess improvement in angina symptoms, a scoring system will be applied based on the frequency, duration, and intensity of angina episodes [15, 16]. Similarly, a scoring system will also be used to evaluate BSS based on changes in signs and symptoms such as angina, a pulse of choppy or knotted nature, ecchymoses, dark purple tongue, lips, and gums, and expanded sublingual veins [17].

**2.9. Data Collection Phases.** Table 1 indicates the data to be collected at each phase of the study. At Phase I, before treatment for the trial is initiated, each patient's full medical

history will be recorded, along with vital signs, symptoms of present illness, and all current medications. Tongue and pulse examination will be performed by experienced TCM physicians. Laboratory tests will include routine blood, urine, and stool tests, an electrocardiogram, an hsCRP test, and various other tests measuring cardiac markers (creatin kinase, CK-MB; troponin T, cTnT; troponin I, cTnI), liver and kidney function, blood lipid and glucose, and coagulation. A 10 mL blood sample will also be collected from each patient, centrifuged, and stored at  $-80^{\circ}\text{C}$  for subsequent measurement of other inflammatory mediators (IL-6, TNF $\alpha$ , VCAM-1, CD40, sCD40L, MCP-1, and MMP-9) at the end of the study. Finally, the severity of each patient's angina and BSS symptoms will be quantitatively scored.

Phase II marks the end of the 14-day treatment period, at which time the medications specific to this study (i.e., simvastatin and STS) will be discontinued and the patients reassessed. Again, vital signs, symptoms of present illness, tongue and pulse exam results, and all current medications will be recorded. Angina and BSS scoring will be repeated, along with all Phase I laboratory tests aside from those measuring cardiac markers and blood lipid and glucose. As in Phase I, a blood sample from each patient will be collected and preserved for subsequent analysis of secondary inflammatory factors at the end of the study. A preliminary, nonstatistical evaluation of treatment safety and efficacy will also be performed by the data collectors.

Thirty days after simvastatin and STS are discontinued, Phase III data will be recorded, including level of hsCRP, angina and BSS scores, vital signs, symptoms of present illness, tongue and pulse exam results, and all current medications. As before, a blood sample for analysis of secondary inflammatory factors will be collected, and treatment safety and efficacy will again be preliminarily evaluated.

**2.10. Data Management.** The principal investigator of this study will collaborate with the director of the GCP Clinical Center of Xiyuan Hospital to schedule and coordinate this clinical trial. The GCP Clinical Center will be responsible for randomizing subjects into treatment groups, monitoring research progress, managing the data, and performing statistical analyses. All those involved in these aspects of the trial will remain blind to the details of treatment allocation.

All patient data will be recorded by trained clinical researchers using a standardized, preprinted, and paper case report form (CRF). Collection, transportation and preservation of all blood samples will be performed by trained staff using a standardized procedure. The Laboratory of Cardiovascular Diseases at Xiyuan Hospital will analyze all blood samples and report all results to the clinical researchers for documentation. At the conclusion of the study, CRFs will be delivered to the GCP Clinical Center and examined for completeness by an individual unassociated with the data collection process. If complete, the CRF will be closed to further revision in preparation for data entry.

At the GCP Clinical Center, a data manager uninvolved with subsequent statistical analysis for the study will be responsible for overseeing data entry. To ensure the reliability of the recorded data, two individuals under the

data manager will each independently input a copy of the CRF data into a special database (EpiData 3.1 software, <http://www.epidata.dk/>). If any data in the CRF is unclear, the data manager will submit a clarification form to the principal investigator of the study, who will then issue an inquiry for the clinical researchers to resolve as soon as possible. The data manager will confirm the correct data according to the clinical researchers' response.

A third individual under the data manager will proofread the two independently completed database records to ensure that they are identical and accurately represent the data in the CRF. If the database records are not identical, the data in question will be confirmed from the original CRF. Once complete, the database records will be locked to further revision.

**2.11. Statistical Analysis.** Statistical analyses will be performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). A Student's *t*-test or Wilcoxon rank-sum test was used, as appropriate, for the analyses of intergroup differences of measurement data;  $\chi^2$  test or Fisher's exact test if necessary was used for comparison of enumeration data. All tests were two tailed and a statistical probability of  $<0.05$  was considered significant.

### 3. Discussion

Despite recent advances in the treatment and prevention of cardiovascular diseases, CHD remains a significant threat to global health. The use of intensified lipid-lowering therapy involving statins and other Western medications has been proposed in an effort to reduce residual cardiovascular risk [18]. The multinational JUPITER trial, however, clearly demonstrated that patients with healthy LDL-C levels according to standard guidelines may still be at elevated risk due to inflammation associated with atherosclerosis, as measured by hsCRP [19]. Controlling inflammation may therefore be critically important in the treatment of CHD, and to this end, our study will explore the effect of an East-West integrative therapy involving STS and simvastatin on elevated levels of inflammatory markers in CHD patients.

The inflammatory process of atherosclerosis begins when excess LDL particles accumulate in the arterial intima and undergo oxidative modification [20, 21], resulting in recruitment of circulating monocytes via factors including MCP-1 and VCAM-1 [22–24]. In the vascular subendothelium, the monocytes differentiate into macrophages and then become foam cells after endocytosis of the oxidized LDL complexes [25]. Expansion of the inflammatory reaction occurs as these lipid-laden foam cells form the necrotic core of the developing atherosclerotic lesion and release proinflammatory cytokines such as TNF $\alpha$  that recruit additional monocytes, dendritic cells, mast cells, and T cells to the area [26]. The extensive crosstalk of cytokines induces vascular smooth muscle cells to produce IL-6, resulting in CRP release by the liver via the acute-phase response [27]. The activated smooth muscle cells also migrate into the arterial intima and lay down fibrous deposits [28], ultimately forming the atherosclerotic

plaques that contribute to the development of CHD. As the disease progresses, CD40L-mediated stimulation of MMP-9 expression by vascular smooth muscle cells plays a major role in plaque destabilization [29], altogether increasing the risk of plaque rupture and the onset of complications such as ACS.

Elevated levels of the inflammatory mediators mentioned above may serve as useful tools for gauging a patient's disease state and cardiovascular risk. CRP is widely regarded as the most useful biomarker for assessing atherosclerotic diseases, not only because of its ease and reliability of measurement, its wide and dynamic range of concentrations, and its remarkable stability but also because its degree of elevation in the blood correlates significantly with the level of risk for future adverse cardiovascular events associated with advanced atherosclerosis, such as myocardial infarction and ischemic stroke [27, 30]. Notably, CRP level is not related to the risk of developing venous thrombosis, a vascular condition typically independent of atherosclerosis [30]. Moreover, Liuzzo et al. found that although CRP levels are significantly increased in patients with unstable angina, which is usually caused by CHD due to atherosclerosis, the biomarker's concentration is not elevated in patients with variant angina, which is caused not by atherosclerosis but by vasospasms of the coronary arteries [31]. These observations altogether suggest that, in the context of cardiovascular disease, the extent of CRP elevation predicts the extent of atherosclerotic inflammation, thereby making CRP level a suitable primary outcome parameter for our study.

The levels of IL-6, TNF $\alpha$ , VCAM-1, CD40, sCD40L, MCP-1, and MMP-9 were selected as secondary outcome parameters in this trial, as these inflammatory markers are less effective indicators of atherosclerotic disease status compared to CRP. Tayebjee et al. found that levels of sCD40L and MMP-9 are significantly higher in stable CHD patients than in healthy controls but also that these markers may not strongly predict the severity of CHD [32]. In addition, although Biasucci et al. found that elevated IL-6 levels predict worse prognosis in patients with unstable angina, the authors were able to detect IL-6 in only 61% of their unstable angina group [33]. Nevertheless, the analysis of these inflammatory markers in addition to CRP provides a broader perspective of the inflammatory state of each patient, allowing us to examine and interpret the effect of STS-simvastatin integrative therapy on inflammation reduction with greater depth.

It is possible that the biomarker concentrations measured in our study may reflect systemic inflammation due to a cause other than atherosclerosis. Indeed, CRP is an acute phase protein released by the liver as part of the body's immune response to nonspecific disturbances, including infection, autoimmune disorders, trauma or surgery, and cancer [34]. To diminish the effect of this possibility, we will carefully consider the past and present medical histories of all eligible patients before enrollment and exclude any patients in whom inflammatory markers may not accurately reflect the state of CHD. Additionally, we will also monitor all study participants during the course of the trial for changes that may confound interpretation of biomarker data, such as acquisition of a nosocomial infection.

The mechanisms by which tanshinone IIA and statins inhibit the inflammatory process of atherosclerosis are diverse, numerous, and in many cases shared (reviewed in [8, 35]). Using STS and simvastatin in combination may yield an additive, supplementary effect not only in the reduction of internal inflammation but also in the improvement of external symptoms associated with CHD. In this trial, we will examine the therapeutic efficacy of a STS-simvastatin integrative therapy in CHD patients by quantitatively scoring each study participant's angina and BSS symptoms and examining for any changes over the course of the trial.

There is a risk for negative interactions in any combination drug therapy, but integrative treatments involving STS appear to be relatively safe. In a systematic review of 25 randomized controlled trials, Qiu et al. found that integrative therapies involving STS for unstable angina may produce fewer side effects than the component Western therapies alone [36]. Side effects that were associated with the integrative therapies included flushing, dizziness, bruising, gum bleeding, blood in the sputum, and swelling at the STS injection site, but none were considered severe and none resulted in discontinuation of treatment. Another clinical study, which used STS and simvastatin in combination to treat peripheral vascular disease also found the integrative treatment to be safer than the statin therapy alone [10]. Our study will continue to monitor the short- and long-term side effects of STS-simvastatin integrative therapy and verify the safety observations reported by previous trials.

Several limitations affect the strength of our study design. Due to the unavailability of data needed to perform an *a priori* calculation, we determined the sample size for this study by adopting the number used in a comparable trial that we previously completed [14]. As a result, it is possible that our current study design may have insufficient power to reveal statistical significance at the level we desire. In addition, because the color of the STS solution makes double-blinding difficult, our study will be subjected to potential expectation biases from both clinical researchers and study participants. We will attempt to minimize these biases by maintaining blinding at the patient level to the fullest extent possible and by having clinical researchers interact with all subjects as similarly as possible. Full blinding will be maintained at the data analysis level. Finally, our project represents a single-center study and our results may not be wholly generalizable due to a possible selection bias. In the future, multicenter double-blinded, randomized controlled trials are necessary in order to faithfully establish the potential of STS-statin integrative therapy in treating CHD.

Nevertheless, the results of this trial are expected to clarify the potential of an East-West integrative therapy involving STS and simvastatin in reducing atherosclerotic inflammation in CHD patients. Our results will additionally confirm the safety and efficacy of this integrative therapy in treating CHD. Finally, the protocol of our study will also provide a methodological foundation upon which future clinical studies of integrative medicine may develop.

## Trial Status

This trial has begun to recruit patients from November 2012, and there has been 10 volunteers up to February 2013.

## Abbreviations

ACS:	Acute coronary syndrome
BSS:	Blood stasis syndrome
sCD40L:	Soluble CD40 ligand
CHD:	Coronary heart disease
CK-MB:	Creatine kinase
CRF:	Case report form
CRP:	C-reactive protein
hsCRP:	High-sensitivity C-reactive protein
ELISA:	Enzyme-linked immunosorbent assay
GCP:	Good Clinical Practice
IL-6:	Interleukin-6
LDL-C:	Low-density lipoprotein cholesterol
MCP-1:	Monocyte chemotactic protein-1
MMP-9:	Matrix metalloproteinase-9
STS:	Sodium tanshinone IIA sulfate
TCM:	Traditional Chinese medicine
TNF $\alpha$ :	Tumor necrosis factor alpha
cTnI:	Troponin I
cTnT:	Troponin T
VCAM-1:	Vascular cell adhesion molecule-1.

## Conflict of Interests

This trial received financial support from the Jiangsu Carefree Pharmaceutical Co., Ltd. The company is not involved in developing the study design, collecting or interpreting the data, writing the paper, or publishing the results related to this trial.

## Authors' Contributions

Qinghua Shang contributed to design of the study, registration of the trial, and drafting of the manuscript. Hanjay Wang contributed to design of the study and drafting of the manuscript. Hao Xu contributed to conceptualization and design of the study, and revision of the manuscript. Siming Li contributed to execution of the study. All the authors read and discussed the manuscript, and all gave approval for the publication of this protocol.

## Acknowledgments

This trial was supported with funding from the Jiangsu Carefree Pharmaceutical Co., Ltd.. The authors most gratefully thank the physicians and nurses of the Department of Cardiovascular Diseases at Xiyuan Hospital as well as the scientists at the Laboratory of Cardiovascular Diseases at Xiyuan Hospital, for their involvement in carrying out this trial. They also wish to thank Rui Gao and Mingjie Zi from the GCP Clinical Center of Xiyuan Hospital for their suggestions and insight regarding revision of the study protocol and case report form. Qinghua Shang and Hanjay Wang are co-first authors.

Qinghua Shang is a physician at the Department of Cardiovascular Diseases at Xiyuan Hospital, China Academy of Chinese Medical Sciences in Beijing, China, and she is also a doctorate student at the Beijing University of Chinese Medicine in Beijing, China. Hanjay Wang is a medical student from Columbia University's College of Physicians and Surgeons in New York, NY, USA, who is a summer research intern at Xiyuan Hospital, China Academy of Chinese Medical Sciences in Beijing, China. Siming Li is a doctorate student at the Beijing University of Chinese Medicine in Beijing, China. Hao Xu is the chief physician and director of the Department of Cardiovascular Diseases at Xiyuan Hospital, China Academy of Chinese Medical Sciences in Beijing, China.

## References

- [1] World Health Organization, "Cardiovascular diseases, Fact sheet No. 317," 2011, <http://www.who.int/mediacentre/factsheets/fs317/en/index.html>.
- [2] G. K. Hansson, "Inflammation, atherosclerosis, and coronary artery disease," *The New England Journal of Medicine*, vol. 352, no. 16, pp. 1685–1626, 2005.
- [3] M. R. Law, N. J. Wald, and A. R. Rudnicka, "Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis," *The British Medical Journal*, vol. 326, no. 7404, pp. 1423–1427, 2003.
- [4] M. A. Albert, E. Danielson, N. Rifai, P. M. Ridker, and PRINCE Investigators, "Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study," *The Journal of the American Medical Association*, vol. 286, no. 1, pp. 64–70, 2001.
- [5] E. Paffen and M. P. M. deMaat, "C-reactive protein in atherosclerosis: a causal factor?" *Cardiovascular Research*, vol. 71, no. 1, pp. 30–39, 2006.
- [6] C. P. Cannon, E. Braunwald, C. H. McCabe et al., "Intensive versus moderate lipid lowering with statins after acute coronary syndromes," *The New England Journal of Medicine*, vol. 350, no. 15, pp. 1495–1504, 2004.
- [7] Z. Y. Gao, H. Xu, D. Z. Shi, C. Wen, and B. Y. Liu, "Analysis on outcome of 5284 patients with coronary artery disease: the role of integrative medicine," *Journal of Ethnopharmacology*, vol. 141, no. 2, pp. 578–583, 2012.
- [8] S. Gao, Z. Liu, H. Li, P. J. Little, P. Liu, and S. Xu, "Cardiovascular actions and therapeutic potential of tanshinone IIA," *Atherosclerosis*, vol. 220, no. 1, pp. 3–10, 2012.
- [9] Q. L. Wang, X. J. Deng, X. Q. Li, and X. T. Chen, "Effect of sodium tanshinone injection on CRP and D-dimer level in patients with unstable angina," *Journal of New Chinese Medicine*, vol. 39, no. 7, pp. 16–17, 2007.
- [10] W. L. Wang, B. R. Ma, X. S. Hu, Z. Li, Y. H. Yuan, and Y. Tian, "The effect of tanshinone IIA and simvastatin treating the legs atheromatosis," *Chinese Journal of Arteriosclerosis*, vol. 18, no. 11, pp. 897–899, 2010.
- [11] K. F. Schulz, D. G. Altman, D. Moher, and The CONSORT Group, "CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials," *Trials*, vol. 11, article 32, 2010.
- [12] E. Rapaport, R. Bernard, and E. Corday, "Nomenclature and criteria for diagnosis of ischemic heart disease. Report of the joint international society and federation of cardiology/world

- health organization task force on standardization of clinical nomenclature," *Circulation*, vol. 59, no. 3, pp. 607–609, 1979.
- [13] Subcommittee of Cardiovascular Diseases of China Society of Integrated Traditional Chinese and Western Medicine, "Criteria for TCM syndrome differentiation of patients with coronary heart disease," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 11, no. 5, pp. 257–258, 1991.
- [14] H. Chen, Z. Y. Gao, H. Xu et al., "Clinical study of unstable angina patients undergoing percutaneous coronary intervention treated with Chinese medicine for activating blood circulation and detoxification," *Chinese Journal of Integrative Medicine on Cardio/Cerebrovascular Disease*, vol. 7, no. 10, pp. 1135–1137, 2009.
- [15] Bureau of Drug Administration of the People's Republic of China, *New Medicine (Western Medicine) Clinical Research Guiding Principles*, 1993.
- [16] X. Y. Zheng and D. Q. Ren, *Chinese Herbal Medicine Clinical Research Guiding Principles (for Trial Implementation)*, Chinese Medicine and Technology Publishing House, 2002.
- [17] K. J. Chen, *Study and Clinical Practice of Methods for Activating Blood to Resolve Stagnation*, Beijing Medical University and Peking Union Medical College Press, 1993.
- [18] M. Evans, A. Roberts, S. Davies, and A. Rees, "Medical lipid-regulating therapy: current evidence, ongoing trials and future developments," *Drugs*, vol. 64, no. 11, pp. 1181–1196, 2004.
- [19] P. M. Ridker, E. Danielson, F. A. H. Fonseca et al., "Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein," *The New England Journal of Medicine*, vol. 359, no. 21, pp. 2195–2207, 2008.
- [20] P. F. E. M. Nivelstein, A. M. Fogelman, G. Mottino, and J. S. Frank, "Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein: a deep-etch and immunolocalization study of ultrarapidly frozen tissue," *Arteriosclerosis and Thrombosis*, vol. 11, no. 6, pp. 1795–1805, 1991.
- [21] W. Palinski, M. E. Rosenfeld, S. Yla-Herttuala et al., "Low density lipoprotein undergoes oxidative modification in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 4, pp. 1372–1376, 1989.
- [22] J. A. Berliner, M. C. Territo, A. Sevanian et al., "Minimally modified low density lipoprotein stimulates monocyte endothelial interactions," *Journal of Clinical Investigation*, vol. 85, no. 4, pp. 1260–1266, 1990.
- [23] S. D. Cushing, J. A. Berliner, A. J. Valente et al., "Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 13, pp. 5134–5138, 1990.
- [24] Y. Huo and K. Ley, "Adhesion molecules and atherogenesis," *Acta Physiologica Scandinavica*, vol. 173, no. 1, pp. 35–43, 2001.
- [25] J. L. Goldstein, Y. K. Ho, S. K. Basu, and M. S. Brown, "Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 76, no. 1, pp. 333–337, 1979.
- [26] G. K. Hansson, P. Libby, U. Schönbeck, and Z. Q. Yan, "Innate and adaptive immunity in the pathogenesis of atherosclerosis," *Circulation Research*, vol. 91, no. 4, pp. 281–291, 2002.
- [27] R. R. S. Packard and P. Libby, "Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction," *Clinical Chemistry*, vol. 54, no. 1, pp. 24–38, 2008.
- [28] E. W. Raines and N. Ferri, "Cytokines affecting endothelial and smooth muscle cells in vascular disease," *Journal of Lipid Research*, vol. 46, no. 6, pp. 1081–1092, 2005.
- [29] U. Schönbeck, F. Mach, G. K. Sukhova et al., "Regulation of matrix metalloproteinase expression in human vascular smooth muscle cells by T lymphocytes: a role for CD40 signaling in plaque rupture?" *Circulation Research*, vol. 81, no. 3, pp. 448–454, 1997.
- [30] P. M. Ridker, M. Cushman, M. J. Stampfer, R. P. Tracy, and C. H. Hennekens, "Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men," *The New England Journal of Medicine*, vol. 336, no. 14, pp. 973–979, 1997.
- [31] G. Liuzzo, L. M. Biasucci, A. G. Rebuzzi et al., "Plasma protein acute-phase response in unstable angina is not induced by ischemic injury," *Circulation*, vol. 94, no. 10, pp. 2373–2380, 1996.
- [32] M. H. Tayebjee, G. Y. H. Lip, K. T. Tan, J. V. Patel, E. A. Hughes, and R. J. MacFadyen, "Plasma matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-2, and CD40 ligand levels in patients with stable coronary artery disease," *The American Journal of Cardiology*, vol. 96, no. 3, pp. 339–345, 2005.
- [33] L. M. Biasucci, A. Vitelli, G. Liuzzo et al., "Elevated levels of interleukin-6 in unstable angina," *Circulation*, vol. 94, no. 5, pp. 874–877, 1996.
- [34] E. Gruys, M. J. M. Toussaint, T. A. Niewold, and S. J. Koopmans, "Acute phase reaction and acute phase proteins," *Journal of Zhejiang University Science B*, vol. 6, no. 11, pp. 1045–1056, 2005.
- [35] R. S. Rosenson, "Pluripotential mechanisms of cardioprotection with HMG-CoA reductase inhibitor therapy," *The American Journal of Cardiovascular Drugs*, vol. 1, no. 6, pp. 411–420, 2001.
- [36] X. Qiu, A. Miles, X. Jiang, X. Sun, and N. Yang, "Sulfotanshinone sodium injection for unstable angina pectoris: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 715790, 11 pages, 2012.

## Research Article

# Analysis on Outcome of 3537 Patients with Coronary Artery Disease: Integrative Medicine for Cardiovascular Events

Zhu-ye Gao,<sup>1</sup> Yu Qiu,<sup>2</sup> Yang Jiao,<sup>3</sup> Qing-hua Shang,<sup>1</sup> Hao Xu,<sup>1</sup> and Da-zhuo Shi<sup>1</sup>

<sup>1</sup> Department of Cardiology, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>2</sup> Chinese Journal of Integrated Traditional and Western Medicine, Beijing 100091, China

<sup>3</sup> Beijing University of Chinese Medicine, Beijing 100029, China

Correspondence should be addressed to Hao Xu; xuhaotcm@gmail.com and Da-zhuo Shi; shidazhuo@126.com

Received 22 April 2013; Accepted 25 June 2013

Academic Editor: Ke-ji Chen

Copyright © 2013 Zhu-ye Gao et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Aims.** To investigate the treatment of hospitalized patients with coronary artery disease (CAD) and the prognostic factors in Beijing, China. **Materials and Methods.** A multicenter prospective study was conducted through an integrative platform of clinical and research at 12 hospitals in Beijing, China. The clinical information of 3537 hospitalized patients with CAD was collected from September 2009 to May 2011, and the efficacy of secondary prevention during one-year followup was evaluated. In addition, a logistic regression analysis was performed to identify some factors which will have independent impact on the prognosis. **Results.** The average age of all patients was  $64.88 \pm 11.97$ . Of them, 65.42% are males. The medicines for patients were as follows: antiplatelet drugs accounting for 91.97%, statins accounting for 83.66%,  $\beta$ -receptor blockers accounting for 72.55%, ACEI/ARB accounting for 58.92%, and revascularization (including PCI and CABG) accounting for 40.29%. The overall incidence of cardiovascular events was 13.26% (469/3537). The logistic stepwise regression analysis showed that heart failure (OR, 3.707, 95% CI = 2.756–4.986), age  $\geq$  65 years old (OR, 2.007, 95% CI = 1.587–2.53), and myocardial infarction (OR, 1.649, 95% CI = 1.322–2.057) were the independent risk factors of others factors for cardiovascular events that occurred during followup of one-year period. Integrative medicine (IM) therapy showed the beneficial tendency for decreasing incidence of cardiovascular events, although no statistical significance was found (OR, 0.797, 95% CI = 0.613~1.036). **Conclusions.** Heart failure, age  $\geq$  65 years old, and myocardial infarction were associated with an increase in incidence of cardiovascular events, and treatment with IM showed a tendency for decreasing incidence of cardiovascular events.

## 1. Introduction

According to research, the risk factors like hypertension, diabetes mellitus, dyslipidemia, and smoking have a positive correlation with CAD. If the numbers of risk factors reduced, the incidence of CAD will decrease significantly. European Society of Cardiology (ESC), American Heart Association (AHA), American College of Cardiology (ACC), and Chinese Medical Association have published in succession clinical guidelines on angina pectoris, myocardial infarction, hypertension, and dyslipidemia, which have played an important role in improving secondary prevention of CAD. However, the implementation of the previously mentioned guidelines in clinical practice IM hospitals in China and the potential benefit of IM therapy in improving CAD prognosis remain unclear. The previous study showed that the treatment of IM

can prevent restenosis after PCI [1] and potentially decrease the incidence of cardiovascular events [2]. In this study, we performed a prospective research for CAD patients who were hospitalized in cardiovascular departments in twelve hospitals in Beijing from September 2009 to May 2011 for analyzing the secondary prevention status of CAD. We also investigate the one-year following incidence of cardiovascular events with the purpose of the problem of secondary prevention and the potential role of IM.

## 2. Materials and Methods

**2.1. Patients.** 3537 patients were recruited from 12 hospitals in Beijing, China. The research followed guidelines of the Declaration of Helsinki and Tokyo for humans and was approved by

TABLE 1: Source of patients.

Hospital	Case (male/female)	Percentage of total
Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine	178 (111/67)	5.03%
Guanganmen Hospital affiliated to China Academy of Chinese Medical Science	564 (363/201)	15.95%
Huairou Hospital of Traditional Chinese Medicine	121 (77/44)	3.42%
Beijing Hospital of Traditional Chinese Medicine	220 (142/78)	6.22%
People's Hospital affiliated to Beijing University	107 (72/35)	3.03%
Tongzhou Hospital of Traditional Chinese Medicine	160 (98/62)	4.52%
Tongren Hospital affiliated to Capital University of Medical Sciences	260 (162/98)	7.35%
Wangjing Hospital affiliated to China Academy of Chinese Medical Science	93 (58/35)	2.63%
Xiyuan Hospital affiliated to China Academy of Chinese Medical Science	462 (304/158)	13.06%
China-Japan Friendship Hospital	632 (425/207)	17.87%
Beijing Hospital of Integrated Traditional Chinese with Western Medicine	124 (80/44)	3.51%
Anzhen Hospital affiliated to Capital University of Medical Sciences	616 (422/194)	17.42%

the Institutional Human Experimentation Committee, and an informed consent was obtained. The source of patients was shown in Table 1.

## 2.2. Inclusion and Diagnostic Criteria

*Inclusion Criteria.* These include the following:

- (1) a hospital with CAD (angina pectoris, myocardial infarction, heart failure, or arrhythmia);
- (2) complying with one of the following conditions:
  - (a) old myocardial infarction;
  - (b) acute myocardial infarction diagnosed in hospital;
  - (c) coronary artery stenosis >50% confirmed by coronary angiography.

*2.3. Evaluation Methods.* The diagnostic and therapeutic statuses of CAD patients were evaluated based on relevant clinical guidelines including guidelines for the diagnosis and treatment of chronic unstable angina pectoris and myocardial infarction [3] published by Chinese Medical Association Cardiovascular Society, guidelines for diagnosis and treatment of unstable angina pectoris [4], guidelines for diagnosis and treatment of myocardial infarction [5] published by American Heart Association (AHA) and American College of Cardiology (ACC), clinical guidelines about heart failure [6, 7], guidelines for diagnosis and prevention of hypertension [8, 9], diagnosis and prevention of dyslipidemia, and National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) [10, 11].

*2.4. Definition of Cardiovascular Events.* This include the following:

- (i) death due to any cause;
- (ii) acute myocardial infarction;
- (iii) revascularization is needed.

## 2.5. Materials

*2.5.1. Data Collection.* Research process in accordance with the subject of the investigator's brochure. The clinical researchers were all trained and they passed the examination. The clinical data collected by an integrative platform of clinical and research was analyzed after clearing up. The treatment of IM means that the patient accepts the conventional treatment of modern medicine and the treatment of herbal medicine including both herbal-based injection and Chinese patent medicine, as well as decoction for at least 7 days in-hospital or 3 months out of hospital.

*2.5.2. Followup.* The follow-up was mainly through telephone. Home visit will be implemented if we cannot contact patients with telephone. The latest clinical data will be used if the patient lost to followup.

*2.5.3. Observation Index.* Demographic data, general clinical condition, drug use, and outcome of followup were observed.

*2.5.4. Statistics.* Statistical analysis of the experimental data was carried out using SPSS (version 11.0). Patients' demographic and clinical characteristics were analyzed with descriptive analytic method and the chi-square test for categorical variables. Continuous variables were presented as mean with standard deviation, and categorical variables were expressed as frequencies with percentages. Logistic regression analysis using fractional polynomial modeling was conducted to determine the association between demographic and clinical information and mortality in MACES. The criterion for statistical significance was at  $P < 0.05$ .

## 3. Results

*3.1. Demographics and Clinical Characteristics.* 3537 consecutive hospitalized CAD patients were enrolled into the study, which was approved by the Local Ethic Committee. The average age in patients was  $64.88 \pm 11.97$  years (range 24–96). The number of males is 2314, accounting for 65.42%. 795

TABLE 2: Status of complicating disease.

Complicating disease	Number	Percentage of total
Hypertension	2398	67.80%
Diabetes mellitus	1162	32.85%
Dyslipidemia	1161	32.82%
Old myocardial infarction	741	20.95%
Stroke	496	14.02%
Chronic obstructive pulmonary disease (COPD)	397	11.22%
Peripheral vasculopathy	165	4.66%
Chronic kidney disease	154	4.35%

TABLE 3: Status of treatment.

Treatment	Number	Percentage of total
IM therapy	1459	41.25%
PCI or CABG	1425	40.29%
Antiplatelet agents	3253	91.97%
Statins	2959	83.66%
Nitrate medications	2709	76.59%
$\beta$ -receptor blockers	2566	72.55%
Heparin	1905	53.86%
ACEI and ARB	2084	58.92%
Calcium channel blockers	1137	32.15%
Insulin	471	13.32%

patients had a history of smoking, accounting for 22.5%. The average history of smoking was  $30.93 \pm 12.54$  years (range 1–70). 327 patients had a history of drinking.

**3.2. Subtype of CAD and Complicating Disease.** The 3537 patients were divided into four groups through the first diagnosis which contain angina pectoris, acute myocardial infarction, arrhythmia, and heart failure. The group of angina pectoris includes 2212 patients, accounting for 62.54%. The group of acute myocardial infarction includes 836 patients, accounting for 23.92%. The group of arrhythmia includes 244 patients, accounting for 6.90%. The group of heart failure includes 235 patients, accounting for 6.64%. The status of complicating diseases was shown in Table 2.

**3.3. Status of Treatment.** Displayed in Table 3 are the numbers and rates of each medication, coronary artery revascularization and Chinese herbal medicine, which patients had received treatment of IM.

### 3.4. Prognostic Analysis

**3.4.1. Prognostic Status.** 312 patients were lost to followup, accounting for 8.82%. The average age was  $70.25 \pm 10.93$  (range 30–90) in the 469 patients with cardiovascular events, accounting for 14.54%. The males are 289, accounting for 61.62%.

TABLE 4: Single-factor analysis.

Variable	Nonevent group	Event group	<i>P</i>
Myocardial infarction	39.15%	47.97%	<0.001
Heart failure	6.91%	20.47%	<0.001
Stroke	4.63%	8.53%	0.001
Arrhythmia	18.71%	24.73%	0.003
TG elevating	38.90%	30.79%	0.001
LDL-C elevating	22.27%	27.39%	0.028
TC elevating	23.97%	17.57%	0.004
Age $\geq 65$ years	49.28%	68.87%	<0.001
Diabetes mellitus	16.36%	20.68%	0.024
$\beta$ -receptor blockers	73.11%	68.87%	0.059
Insulin	12.91%	15.99%	0.068
Antiplatelet agents	92.28%	89.98%	0.1
IM therapy	40.9%	43.5%	0.291

TABLE 5: Analysis of prognostic factors using multivariate logistic stepwise regression.

Variables	$\beta$	<i>P</i>	OR	95% CI
Heart failure	1.310	<0.001	3.707	2.756–4.986
Age $\geq 65$ years	0.697	<0.001	2.007	1.587–2.539
Myocardial infarction	0.500	<0.001	1.649	1.322–2.057
TG elevating	−0.259	0.02	0.754	0.595–0.956

**3.4.2. Single-Factor Analysis.** Table 4 displayed the result of single-factor analysis, in which myocardial infarction, heart failure, stroke, arrhythmia, LDL-C elevating, age  $\geq 65$  years, and diabetes mellitus were significantly higher in event-group; meanwhile, TG elevating and TC elevating were significantly higher in nonevent group.

**3.4.3. Logistic Regression Analysis.** Based on measured indexes in which  $P < 0.1$  in the former analysis and IM therapy, a multiple regression equation was generated between cardiovascular events and indexes. Table 5 displayed the result of logistic stepwise regression analysis, in which heart failure, age  $\geq 65$  years, and myocardial infarction were the independent negative prognostic factors for cardiovascular events, while TG elevating was the independent protective factor.

Table 6 displayed the result of logistic regression analysis, in which IM therapy showed potential tendency for decreasing the incidence of cardiovascular events.

## 4. Discussions

It is shown that the range of the age of the majority of hospitalized CAD patients' is from 60 years old to 74 years old, accounting for 41.93% in this study. Event rate of one-year followup of hospitalized CAD patients is 14.54% (469 cases), the average age is  $70.25 \pm 10.93$  years (range 30–90). The probability of recurrent cardiovascular events in patients whose age is that greater than 65 years old is 2.4 times of the patients whose age is lower than 65 years old (18.5% versus

TABLE 6: Analysis of prognostic factors using multivariate logistic regression.

Variables	$\beta$	<i>P</i>	OR	95% CI
IM therapy	-0.227	0.09	0.797	0.613–1.036
Myocardial infarction	0.386	0.001	1.471	1.172–1.847
Heart failure	1.308	<0.001	3.7	2.737–5.002
Stroke	0.392	0.095	1.48	0.935–2.342
Arrhythmia	0.084	0.536	1.088	0.833–1.42
Antiplatelet agents	-0.115	0.591	0.892	0.587–1.355
$\beta$ -receptor blockers	-0.077	0.548	0.926	0.72–1.19
Insulin	0.333	0.056	1.396	0.991–1.966
TG elevating	-0.208	0.099	0.812	0.635–1.04
LDL-C elevating	0.036	0.846	1.036	0.722–1.487
TC elevating	-0.255	0.206	0.775	0.522–1.15
Age $\geq$ 65	0.574	<0.001	1.775	1.384–2.278
Different hospital	0.295	0.16	1.343	0.89–2.026
Diabetes mellitus	0.072	0.647	1.075	0.789–1.465

7.6%). It is also suggested that age is an important affecting factor of the prognosis of CAD which cannot be changed. Other studies have confirmed that age is an independent risk factor for CAD prognosis; the rate of CAD incidence and mortality is increasing with age [12–14].

Epidemiological and clinical studies have demonstrated that hypertension, diabetes, dyslipidemia, smoking, obesity, and other risk factors can aggravate atherosclerosis and increase the mortality of CAD [15]. It is shown that hypertension, diabetes, dyslipidemia, cerebrovascular disease and COPD are common complications of CAD, and with the development of CAD and the increase of age, complications are more and more serious. One research from abroad shows that once artery atherosclerosis forms, only a simple monitoring of risk factors cannot fully control the progression of disease [16]. The correlative guidelines of CAD also recommend that patients with CAD should insist on long-term drug therapies that include  $\beta$ -receptor blockers, antiplatelet agents, and statin. Overall treatment modalities in this study were similar to the suggestion of relevant guidelines. Although the treatment rate of statins,  $\beta$ -receptor blockers, and ACEI/ARB is more than 50%, neither of these treatments show beneficial effects to decrease the incidence of cardiovascular events. This result is different from previous RCTs; it may be related to the type of design and cases as well as too short follow-up period.

Therefore, as for CAD patients who also have complications, they should pay more attention to the treatment of secondary prevention drugs and to the control of the complications on the long term, for reaching the maximum limit to decrease the incidence of cardiovascular events [17].

Diabetes mellitus was a CAD risk equivalent [18, 19]. It is shown that diabetes and injection of insulin may be attributed to more incidences of cardiovascular events. It may contribute to serious complex illness and poor glycemic control usually accompanying CAD patients who also contract diabetes. It is certificated that the rate of cardiovascular events

such as morality in CAD complicating diabetes patients is greater than that in nondiabetes ones in interrelating reach.

It was found that patients in normal left ventricular ejection fraction group and lower left ventricular ejection fraction group have similar prognosis in previous epidemiological investigations and observational studies [20]. But most of those experiment objects have dilated cardiomyopathy, and hypertensive heart disease patients take low proportion in patients with CAD. Moreover most of the clinical trials will usually exclude patients with heart failure whose left ventricular ejection fraction is normal. CAD is one of the important causes of heart failure, and persistent coronary ischemia will further aggravate heart failure. This study also showed that heart failure can increase the incidence of endpoint events. It is important to effectively prevent HF readmissions and improve overall outcomes [21].

In recent years, the combination of medicines rises extensively worldwide, and it is used increasingly in clinical application. IM is a unique discipline in China; it also has a wide range of applications in China [22]. This study shows that, from the results of one-year followup, although IM has no statistical significance in affecting incidence of cardiovascular events, IM therapy showed the tendency of decreasing incidence of cardiovascular events. This also shows that a single drug or treatment could not prevent multiple-factor disease such as CAD. IM has advantages in improving the clinical symptoms especially for CAD patients having diabetes, high blood pressure, lung infection, and kidney disease. It was shown that it has greater advantages in improving the quality of life for patients in previous studies [23, 24]. There are many people willing to adopt the IM treatment for cardiovascular disease [25].

In this study, a lengthways study design was adopted, using traditional Chinese medicine clinical research integrated research platform to investigate and analogize the clinical information and cardiovascular events in patients with CAD from cardiovascular departments of 12 hospitals in Beijing. This research reflects objectively and accurately the treatment and independent prognostic factors of hospitalized CAD patients in Beijing. There are many complications of CAD: high incidence of clinical events and a gap between clinical drugs and guidelines. Therefore, we should strengthen secondary prevention degree and health education for patients with CAD. It needs to control multiple risk factors and intervene complete complex situation to reduce the incidence of complication and the rate of endpoint events.

For the limited follow-up time, this research could not make new diseases as the endpoint, such as new-onset diabetes, new-onset kidney disease, and new-onset heart failure. As IM treatments for CAD have the advantage of being multidimensional and multi-target, further analysis of research material can include factors like the patients' quality of life, degree of symptom improvement and the different situations of occurred cases. These factors could then be combinedly and systematically evaluated to define the intended population of IM treatments, and thus be used to draw up intervening programs of IM treatments for CAD.

## Conflict of Interests

The authors have no conflict of interests to declare.

## Authors' Contribution

Professor Zhu-ye Gao and Dr. Yu Qiu contributed equally to this paper.

## Acknowledgments

The authors are indebted to all the staff members of all the participating hospitals for their outstanding efforts: Li-zhi Li, Jing-chun Zhang, Xiao-chang Ma, and Feng-qin Xu, Xiyuan Hospital affiliated to China Academy of Chinese Medical Science; Li Huang, Jin-hang Du, Xiao-yan Lu, Hai-zhong Jia, and Chun-yan Li, China-Japan Friendship Hospital; Yuan-hui Hu, Ling Feng, and Qing-qiao Song, Guanganmen Hospital affiliated to China Academy of Chinese Medical Science; Yan-ming Huo, Rui-lin Zhang, and Guo-hua Yang and Wangjing Hospital affiliated to China Academy of Chinese Medical Science; Xian Wang, Yu-qing Liu, Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine; Hong-jin Wu, Beijing Hospital of Integrated Traditional Chinese with Western Medicine; Hong-xu Liu, Beijing Hospital of Traditional Chinese Medicine; Shu-zheng Lv, Anzhen Hospital affiliated to Capital University of Medical Sciences; Ming-ying Wu, Tongren Hospital affiliated to Capital University of Medical Sciences; Cheng-bin Xu, People's Hospital affiliated to Beijing University; Shu-yun Zhang, Huairou Hospital of Traditional Chinese Medicine; Yu-quan Zhang, Tongzhou Hospital of Traditional Chinese Medicine. This project was supported by Beijing Committee of Science and Technology (no. D08050703020801), the TCM Public Welfare Scientific Research Project, the State Administration of TCM of People's Republic of China (no. 201007001), Capital Foundation of Medical Developments (no. SF-2007-II-13, SF-2009-I-09), and the National Science and Technology Major Projects for "Major New Drugs Innovation and Development" (no. 2009ZX09502-031).

## References

- [1] H. Xu and K. Chen, "Integrative medicine: the experience from China," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 3–7, 2008.
- [2] Z.-Y. Gao, H. Xu, D.-Z. Shi, C. Wen, and B.-Y. Liu, "Analysis on outcome of 5284 patients with coronary artery disease: the role of integrative medicine," *Journal of Ethnopharmacology*, vol. 141, no. 2, pp. 578–583, 2012.
- [3] Chinese Society of Cardiology of Chinese Medical Association, Editorial Board of Chinese Journal of Cardiology, "Guideline for diagnosis and treatment of patients with unstable angina and non-ST-segment elevation myocardial infarction," *Chinese Journal of Cardiology*, vol. 35, no. 4, pp. 295–304, 2007.
- [4] J. L. Anderson, C. D. Adams, E. M. Antman et al., "ACC/AHA 2007 guidelines for the management of patients with unstable angina/non-ST-Elevation myocardial infarction. A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines," *Journal of the American College of Cardiology*, vol. 50, no. 7, pp. e1–e157, 2007.
- [5] E. M. Antman, M. Hand, P. W. Armstrong et al., "2007 focused update of the ACC/AHA 2004 guidelines for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines," *Journal of the American College of Cardiology*, vol. 51, no. 2, pp. 210–247.
- [6] H. Hunt et al., "ACC/AHA 2005 guideline update for the diagnosis and management of chronic heart failure in the adult: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines," *Journal of the American College of Cardiology*, vol. 46, no. 6, pp. e1–e82, 2006.
- [7] Chinese Society of Cardiology of Chinese Medical Association, Editorial Board of Chinese Journal of Cardiology, "Guideline for diagnosis and treatment of acute heart failure," *Chinese Journal of Cardiology*, vol. 35, no. 12, pp. 1076–1095, 2007.
- [8] The Drafting Committee of Chinese Guidelines for Hypertension Prevention and Treatment, "A draft of Chinese guidelines for prevention and treatment of hypertension," *Chinese Journal of Hypertension*, vol. 8, no. 1, pp. 94–102, 2000.
- [9] The Drafting Committee of Chinese Guidelines for Hypertension Prevention and Treatment, "2009 Community Version of Chinese Hypertension Prevention Guideline," *Chinese Journal of Hypertension*, vol. 18, no. 1, pp. 11–30, 2010.
- [10] Joint committee for developing Chinese guidelines on prevention and treatment of dyslipidemia in adults, "Chinese guidelines on prevention and treatment of dyslipidemia in adults," *Chinese Journal of Cardiology*, vol. 35, no. 5, pp. 390–419, 2007.
- [11] National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), "Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report," *Circulation*, vol. 106, pp. 3143–3421, 2002.
- [12] G. Jiang, D. Wang, W. Li et al., "Coronary heart disease mortality in China: age, gender, and urban-rural gaps during epidemiological transition," *Revista Panamericana de Salud Pública*, vol. 31, no. 4, pp. 317–324, 2012.
- [13] Y. L. Gao, J. T. Su, Z. H. Wei, J. L. Liu, and J. Wang, "Characteristics of out-of-hospital acute coronary heart disease deaths of Beijing permanent residents at the age of 25 or more from 2007 to 2009," *Zhonghua Xin Xue Guan Bing Za Zhi*, vol. 40, no. 3, pp. 199–203 (Chinese).
- [14] B. S. Levine and W. B. Kannel, "Coronary heart disease risk in people 65 years of age and older," *Progress in Cardiovascular Nursing*, vol. 18, no. 3, pp. 135–140, 2003.
- [15] S. Ebrahim, F. Taylor, K. Ward, A. Beswick, M. Burke, and G. Davey Smith, "Multiple risk factor interventions for primary prevention of coronary heart disease," *Cochrane Database of Systematic Reviews*, vol. 1, Article ID CD001561, 2011.
- [16] J. D. Spence and D. G. Hackam, "Treating arteries instead of risk factors: a paradigm change in management of atherosclerosis," *Stroke*, vol. 41, no. 6, pp. 1193–1199, 2010.
- [17] T. Hofmann, "Risk management of coronary artery disease—pharmacological therapy," *Wiener Medizinische Wochenschrift*, vol. 154, no. 11-12, pp. 266–281, 2004.

- [18] L. B. Daniels, D. Grady, L. Mosca et al., "Is diabetes mellitus a heart disease equivalent in women? Results from an international study of postmenopausal women in the Raloxifene Use for the Heart (RUTH) Trial," *Circulation: Cardiovascular Quality and Outcomes*, vol. 6, no. 2, pp. 164–170, 2013.
- [19] M. Chiha, M. Njeim, and E. G. Chedrawy, "Diabetes and coronary heart disease: a risk factor for the global epidemic," *International Journal of Hypertension*, vol. 2012, Article ID 697240, 7 pages, 2012.
- [20] K. Miyagishima, S. Hiramitsu, H. Kimura et al., "Long term prognosis of chronic heart failure—reduced vs preserved left ventricular ejection fraction," *Circulation Journal*, vol. 73, no. 1, pp. 92–99, 2009.
- [21] M. Gheorghade, M. Vaduganathan, G. C. Fonarow, and R. O. Bonow, "Rehospitalization for heart failure: problems and perspectives," *Journal of the American College of Cardiology*, vol. 61, no. 4, pp. 391–403, 2013.
- [22] J. Wang and X. Xiong, "Current situation and perspectives of clinical study in integrative medicine in china," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.
- [23] Q.-Y. Yang, S. Lu, and H.-R. Sun, "Clinical effect of astragalus granule of different dosages on quality of life in patients with chronic heart failure," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 146–149, 2011.
- [24] L. Wang, M. Zhang, L. Guo et al., "Clinical pathways based on integrative medicine in chinese hospitals improve treatment outcomes for patients with acute myocardial infarction: a multicentre, nonrandomized historically controlled trial," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 821641, 8 pages, 2012.
- [25] K. Prasad, V. Sharma, K. Lackore, S. M. Jenkins, A. Prasad, and A. Sood, "Use of complementary therapies in cardiovascular disease," *American Journal of Cardiology*, vol. 111, no. 3, pp. 339–345, 2013.

## Research Article

# Long-Term Exercise and Risk of Metabolic and Cardiac Diseases: The Erlangen Fitness and Prevention Study

Wolfgang Kemmler, Simon von Stengel, Michael Bebenek, and Willi A. Kalender

*Institute of Medical Physics, University of Erlangen-Nuremberg, Henkestraße 91, 91052 Erlangen, Germany*

Correspondence should be addressed to Wolfgang Kemmler; [wolfgang.kemmler@imp.uni-erlangen.de](mailto:wolfgang.kemmler@imp.uni-erlangen.de)

Received 23 February 2013; Revised 3 June 2013; Accepted 10 June 2013

Academic Editor: Hao Xu

Copyright © 2013 Wolfgang Kemmler et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In female subjects, ageing and the menopausal transition contribute to a rapid increase of metabolic and cardiac risk factors. Exercise may be an option to positively impact various risk factors prone to severe metabolic and cardiac diseases and events. This study was conducted to determine the long-term effect of a multipurpose exercise program on metabolic and cardiac risk scores in postmenopausal women. 137 osteopenic Caucasian females ( $55.4 \pm 3.2$  yrs), 1–8 years postmenopausal, were included in the study. Eighty-six subjects joined the exercise group (EG) and performed an intense multipurpose exercise program which was carefully supervised during the 12-year period, while 51 females maintained their habitual physical activity (CG). Main outcome measures were 10-year coronary heart disease risk (10 y CHD risk), metabolic syndrome Z-score (MetS Index), and 10-year myocardial infarction risk (10 y hard CHD risk). Significant between-group differences all in favor of the EG were determined for 10 y-CHD risk (EG:  $2.65 \pm 2.09\%$  versus CG:  $5.40 \pm 3.30\%$ ;  $P = 0.001$ ), MetS-Index (EG:  $-0.42 \pm 1.03\%$  versus CG:  $1.61 \pm 1.88$ ;  $P = 0.001$ ), and 10 y-hard-CHD risk (EG:  $2.06 \pm 1.17\%$  versus CG:  $3.26 \pm 1.31\%$ ;  $P = 0.001$ ). Although the nonrandomized design may prevent definite evidence, the intense multi-purpose exercise program determined the long-term efficacy and feasibility of an exercise program to significantly impact metabolic and cardiac risk scores in postmenopausal women. This trial is registered with ClinicalTrials.gov NCT01177761.

## 1. Introduction

Multimorbidity of the elderly is an increasing problem in the western world. With respect to community dwelling subjects, in Germany, two thirds of the female population, 55–69 years old, showed two to four diseases [1]. Beside musculoskeletal problems, metabolic and cardiac diseases largely contribute to the high morbidity of our elderly population [2, 3]. Due to the unfavorable demographic development this dilemma will not only increasingly stress our health systems but also impact the subject's quality of life and independence [4]. Thus, effective strategies to prevent diseases largely related with increasing age are of high priority. Unlike dedicated pharmaceutical agents, exercise represents a complex agent that affects most, if not all, of the relevant risk factors and diseases of the elderly [5–7]. However, although evidence for the positive effect of exercise per se on various risk factors and diseases is quite convincing [5–7], multiple purpose exercise protocols that focus on more than one or two relevant risk

factors and diseases of the elderly are scarce. Further, most exercise trials were rather short, rarely exceeding 6 months. Moreover, even when these protocols were effective during the first months of exercise, this does not guarantee general effectiveness. Consequently, the consistent long-term effects of exercise on various endpoints still have to be determined.

To adequately address these issues, the goal of the Erlangen Fitness and Prevention Study (EFOPS) is to validate a long-term general purpose exercise program with reasonable training volume that could be adopted by other health sports institutions or organizations. In this paper the authors focus on the effect of this ongoing 12-year exercise program on metabolic and cardiac risk scores. The hypothesis was tested that changes among the exercisers for Framingham based 10-year CHD risk [8], metabolic syndrome Z-score [9, 10], and 10-year hard CHD risk (i.e., risk of myocardial infarction or coronary death) [10] were significantly more favorable compared with nontraining controls.

## 2. Materials and Methods

The Erlangen Fitness and Osteoporosis Prevention Study (EFOPS) is an ongoing controlled exercise trial that determines the long-term effects of exercise on various risk factors with a particular focus on fractures and bone mineral density in (early) postmenopausal women with osteopenia. The study protocol was approved by the ethics committee of the Friedrich-Alexander University of Erlangen-Nuremberg (Ethikantrag 905 and 4209) and the Bundesamt für Strahlenschutz (S9108-202/97/1). The EFOPS study was initiated and headed by the Institute of Medical Physics, University of Erlangen, Germany. The study reported here started in October 1998. The 12-year follow-up assessments were performed in September/October, 2010, and corresponding analyses were conducted up to April, 2011. All study participants gave written informed consent.

**2.1. Subjects.** Figure 1 gives the participant flow of the EFOPS study. Briefly, 1,100 Caucasian women 48–60 years old responded to personal mails and were checked for eligibility. In a first step 618 females were excluded by interview for reasons given in the flow chart (Figure 1). The remaining subjects were invited to check further eligibility criteria. 223 women did not meet the inclusion criteria of osteopenia and two subjects were excluded due to (very) low physical activity (<75 Watt) at cycle ergometry. Finally, 137 eligible women agreed to participate in the trial. Based on their own decision, 86 subjects participated in the exercise group (EG) and 51 subjects joined the control group (CG). The EG underwent the exercise program described later, while participants of the CG were requested to continue their normal lifestyle and habitual physical activity. During the 12 years of the study course, 27 subjects of the EG and 3 subjects of the CG were lost for reasons given in Figure 1. Baseline characteristic of the subgroups (EG, CG) did not differ between the cohort presented here and the initial cohort included in 1998. Of relevance, although all subjects of the EG that quit the program for the reasons given in Figure 1 were invited to the 12-year follow-up, but all subjects refused to attend the assessment.

Thus, in 2010, 59 subjects of the EG and 48 subjects of the CG were assessed within the 12-year follow-up. However, 8 subjects of the EG and 5 subjects of the CG were excluded from the statistical analysis by protocol due to medication directly affecting the primary and secondary endpoints. Thus, 51 subjects of the EG and 43 subjects of the CG were included in the 12-year follow-up analysis. Table 1 shows baseline characteristics of the exercise and control group. No significant differences between both groups were determined.

**2.2. Study Intervention.** A block periodization scheme with 12 weeks of high intensity exercise specifically dedicated to muscle and bone was intermitted by 6 week periods of exercise with increased volume/lower intensity. High intensity blocks were structured into 3 cycles of 4 weeks using linear periodization.

All group sessions were closely supervised by certified instructors who monitored and controlled compliance of

the subjects. After the end of each 6 or 12 week period, training logs of the participants were analyzed to determine compliance and to check the rate of perceived exertion listed by the participants. Importantly, no sanctions were imposed on participants who did not regularly exercise at home in order to reduce potential motivations to cheat with the training logs. Apart from three weeks of holiday (Christmas, Easter) the group exercise program was rigorously maintained throughout the year. Total amount of exercise classes (2 sessions/week) and home training (2 sessions/week) averaged around 200 sessions per year (3.85 sessions/week).

**2.2.1. Exercise Program.** The complex exercise protocol of the EFOPS study was described in more details in earlier publications [11, 12]; thus only a summary will be given here.

Briefly, the program consists of two supervised group sessions ( $\approx 60$  min) performed on nonconsecutive days of the week and two home training sessions ( $\approx 20$ – $25$  min).

**2.2.2. Supervised Group Session.** Generally, the group exercise sessions were structured into three main sequences: (1) 20–25 min of warmup/endurances exercises, (2) 3–5 min of jumping exercises, and (3) a 35–40 min resistance exercise training sequence.

(1) During the endurance sequence 5–10 minutes of different running exercises and 10–15 minutes of low and high impact aerobic exercises with a progressively increasing amount of high impact exercises were conducted. Heart rates (HR) averaged 70%–85% HR<sub>max</sub> (as assessed during stepwise treadmill test to a voluntary maximum) during this phase.

(2) The jumping exercise section started after 6 months of initial conditioning and was specifically dedicated to impact bone. After 2 min rope skipping exercise, 4 different jumping exercises with 1 set of 15 repetitions with 30 sec of rest between each exercise were performed. HR averaged between 65% and 90% HF<sub>max</sub> during this section.

(3) The strength and power section consisted of one session/week on resistance machines (Techno Gym, Gambettola, Italy) and one session/week using isometric exercises, elastic bands, and free weights. During the session on machines 9–13 exercises affecting all the main muscle groups were performed. Twelve weeks of periodized high intensity resistance training (9–10 exercises with 1–4 sets and  $\approx 12$ –4 reps with 70%–92.5% 1RM) were interleaved with 6-week transitional periods of lower intensity ( $\approx 55\%$  1RM) but higher volume (13 exercises with 2–3 sets and 20–25 reps). Beside one 12-week “power” block per year with explosive concentric movements [13], time under tension was structured in a 2 s concentric—1 s isometric—2 sec eccentric movement mode. Initially individual resistance training plans of the subjects based on 1RM tests. After five years of this procedure, load prescription given by the training plans was based on repetition number combined with the rate of perceived exertion which was assessed to be comparably effective [14].

During the second resistance training session/week 12–15 isometric exercises (2–4 sets, 6–10 sec) for trunk flexors/extensors, hip extensors/flexors, and leg abductors/adductors

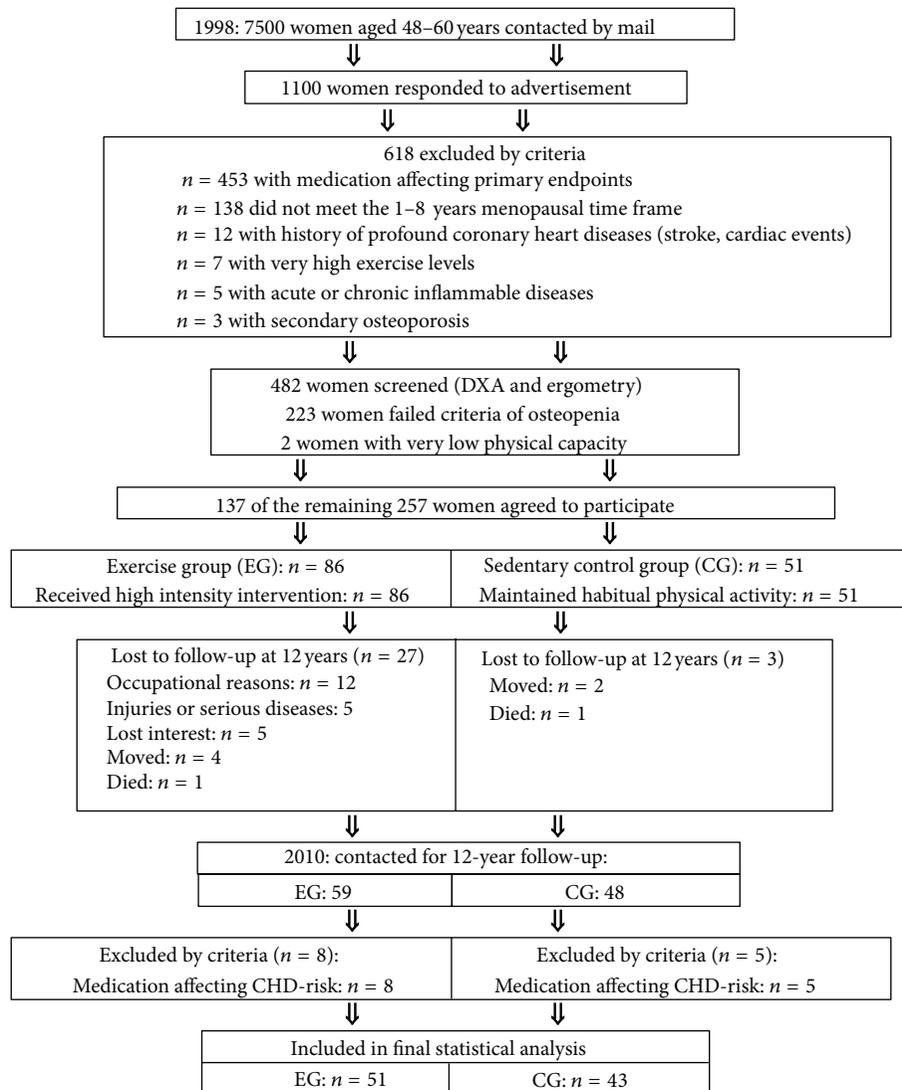


FIGURE 1: Extended flow chart of the EFOPS study.

were performed with maximum effort followed by 20–30 s of active (stretching) rest. Additionally 3 elastic band exercises (2–4 sets and 15–20 reps.) dedicated to shoulder and upper back were carried out. Finally, three resistance exercises using free weights and weighted vests (squat/dead lift, one hand dumbbell rowing, and dumbbell chest press) were performed according to the periodized protocol described earlier.

**2.2.3. Home Training Session.** After a brief warmup (3 min of LI-Aerobic-routine) the nonsupervised home training consisted of 2–3 min of different rope skipping exercises, 6–8 isometric floor exercises, and 2 elastic band exercises with 2 sets each described earlier. Four to five stretching exercises were carried out during the corresponding rest periods. Each 12–24 weeks new home training routines replaced the existing protocols.

In summary, total intensity of the present exercise protocol was rather high; however, it was not focused on complete exhaustion of the subjects, which was notably manifested by the approach of prescribing a submaximal number of repetitions per load.

**2.3. Measurements.** Measurements were carried out by research assistants blinded to the status of the participant. Further all baseline and follow-up tests described here were performed by the same assistants at the same time of day ( $\pm 1$  h). Testing procedures described later were performed at baseline and after years 1, 2, 3, 4, 5, and 12. However, primary endpoints of this contribution were retrospectively calculated for baseline and year 12.

**2.3.1. Primary Outcome Measures.** The primary outcome measures of this study were “10-year CHD risk” according to Wilson et al. [8] and the MetS-Z-Score proposed by Johnson et al. [9] based on the NCEP Adult Treatment Panel (ATP) III MetS-Definition [10]. Secondary outcome was “10-year hard CHD risk” (i.e., risk of myocardial infarction and cardiac death; [10]).

**2.3.2. Anthropometry.** Body weight and body height were always determined with the same standardized devices and procedures. Body composition was measured using the bioimpedance technique (Tanita BF 305, Tanita, Japan). Waist

TABLE 1: Baseline characteristics of the exercise and the control groups.

Variable	Exercise EG (n = 51)	Control CG (n = 43)
Age (years)	54.8 ± 3.6	55.8 ± 3.2
Body mass index (kg/m <sup>2</sup> )	24.8 ± 3.0	25.6 ± 3.6
Body fat (%) <sup>b</sup>	35.8 ± 4.6	35.2 ± 5.8
Age at menarche (years) <sup>a</sup>	13.1 ± 1.4	13.3 ± 1.6
Age at menopause (years) <sup>a</sup>	50.6 ± 3.1	50.6 ± 3.3
Volume of exercise (min/week) <sup>a</sup>	89 ± 84	75 ± 66
Physical activity (Index) <sup>a,c</sup>	4.1 ± 1.2	4.0 ± 1.3
VO <sub>2</sub> peak (mL/min/kg) <sup>d</sup>	25.3 ± 6.3	25.3 ± 5.9
Energy intake (MJ/d) <sup>e</sup>	7.75 ± 1.39	7.69 ± 1.85
Fat intake (% of energy intake) <sup>e</sup>	35 ± 8	36 ± 7
Smokers (%) <sup>a</sup>	14	12
Diabetes (%) <sup>a</sup>	4	5
Prevalence metabolic syndrome (%) <sup>f</sup>	12	12

No significant between-group differences were determined. <sup>a</sup>As determined by questionnaire; <sup>b</sup>as determined by bioimpedance analysis (Tanita BF 305, Tokyo, Japan); <sup>c</sup>as determined by physical activity questionnaire (1: very low to 7: very high) [15]; <sup>d</sup>stepwise treadmill test to voluntary maximum; <sup>e</sup>5-day dietary analysis (Prodi-4.5/03, Wissenschaftlicher Verlag, Freiburg, Germany); <sup>f</sup>according to NCEP-ATP III.

circumference was determined as the minimum circumference between the distal end of the rib cage and the top of the iliac crest along the midaxillary line.

**2.3.3. Blood Parameter.** After an overnight fast, blood was sampled in the morning (7:00 to 9:00) in a sitting position from an antecubital vein. Serum samples were centrifuged at 3000 RPM for 20 minutes and analyzed by the “Zentrallabor” of the Medical Department I University of Erlangen-Nuremberg. Glucose, total cholesterol, HDL- and LDL cholesterol, and triglycerides (Olympus Diagnostica GmbH, Hamburg, Germany) were determined.

Blood pressure was determined in a sitting position after 5 minutes rest with an automatic oscillometric device (Bosco, Bosch, Jungingen, Germany). Measurements were taken in a nonfasting condition. Subjects refrained from coffee or tea for at least two hours prior to testing.

**2.3.4. Questionnaires.** To adequately assess physical activity and exercise at baseline and during the intervention, a questionnaire specifically developed to assess physical activity and exercise with impact on bone in this cohort was used [16]. Follow-up questionnaires additionally asked for corresponding changes during the intervention period, in particular with respect to diseases and medication, additional sport activities, and changes of physical activity and dietary intake. The good reproducibility of the questionnaires had been determined in an earlier stage of the study [15, 17].

In order to control nutritional changes, nutritional behavior was assessed using 5-day dietary protocols. The analysis of the protocols was performed using Prodi-4.5/03 Expert software (Wissenschaftlicher Verlag, Freiburg, Germany).

**2.3.5. 10-Year CHD Risk and 10-Year Hard CHD Risk.** Parameters constituting 10-year CHD risk in women [8] were age, diabetes status, smoking status, total cholesterol categories, LDL-C categories, and blood pressure categories while specific risk factors for 10-year hard CHD risk (myocardial infarction or cardiac death [10]) were age, total cholesterol, HDL-C, treatment for hypertension, and smoking status.

Based on score sheets, the corresponding 10-year risk (CHD risk/hard CHD risk) for each subject was given as a percentage value.

**2.3.6. Metabolic Syndrome Z-Score.** MetS Z-score was calculated according to the formula proposed by Johnson et al. [9] based on the NCEP-ATP III Criteria of the MetS. According to this criteria the MetS is prevalent if three out of the five risk factors were present: (1) raised triglyceride (TriGly) levels ( $\geq 150$  mg/dL); (2) reduced HDL-C ( $< 50$  mg/dL for females, or specific treatment for previously detected hypertriglyceridaemia/reduced HDL-C); (3) raised blood pressure ( $\geq 130/85$  mmHG, or specific treatment); (4) raised fasting plasma glucose ( $\geq 100$  mg/dL); (5) waist circumference (WC  $> 88$  cm for females).

According to Johnson et al. [9] for each parameter (i.e., HDL-C, Triglycerides) of the individual data, the ATP-III cut-point for a female population and the corresponding baseline standard deviation (SD) of the entire EFOPS-cohort were used. In detail, the Z-score was calculated:  $[(50 - \text{HDL-C}) / \text{SD HDL-C}] + [(\text{TriGly} - 180) / \text{SD TriGly}] + [(\text{Glucose} - 100) / \text{SD Glucose}] + [(\text{WC} - 88) / \text{SD WC}] + [(\text{Mean arterial pressure (MAP)} - 100) / \text{SD MAP}]$ .

**2.4. Statistical Procedures.** The sample size calculation of the present study was based on “10-year CHD risk.” In order to detect a 50% difference (i.e., changes of CHD risk in the EG half as high compared with the CG) between exercisers and control, 40 subjects per group were required for a 5% error probability with 80% statistical power. A per protocol analysis (PPA) was performed that excluded all subjects who underwent therapy with medications that relevantly affected primary endpoints after study start. Additionally, an intention to treat (ITT) analysis including all the subjects independently of adherence to the protocol or lost to follow-up was performed for primary and secondary endpoints (10-year CHD risk; MetS Z-score; 10-year hard CHD risk) using the “last observation carried forward” (LOCF) principle.

Baseline characteristics were reported as means with standard deviations. Between-group differences of parameters presented in Tables 1 and 3 were calculated using Mann-Whitney *U* tests. Primary and secondary endpoints were log-transformed to obtain normally distributed data required for the analysis of variance with repeated measurements. Between-group differences are given as absolute difference with 95% confidence intervals (Tables 2 and 3). Within-group differences were analyzed with paired *t*-tests (text). Effect sizes (ES) based on the absolute difference ( $\pm$ standard deviation) between baseline and 12-year follow-up in the EG and CG were calculated using Cohen’s *d*. SPSS 18.0 (SPSS Inc, Chicago, IL) was used for all statistical procedures.

TABLE 2: Per protocol analysis: changes in the exercise and control groups for primary and secondary study endpoints.

	Exercise MV (SD) <i>n</i> = 51	Control MV (SD) <i>n</i> = 43	Absolute difference MV (95% CI)	<i>P</i>	Effect size ( <i>d</i> )
10-year CHD risk					
Baseline (%)	8.39 ± 3.27	7.42 ± 2.58	—	—	—
12 y follow-up (%)	11.04 ± 3.16	12.81 ± 3.08	3.89	—	—
Risk changes (%)	2.65 ± 2.09***	5.40 ± 3.30***	2.75 (1.61 to 3.89)	0.001	0.90
Metabolic syndrome Z-score					
Baseline (%)	-2.44 ± 2.39	-3.65 ± 2.84	—	—	—
12 y follow-up (%)	-2.88 ± 3.01	-2.04 ± 3.77	—	—	—
Changes (%)	-0.42 ± 1.03**	1.61 ± 1.88***	2.03 (1.42 to 2.64)	0.001	1.36
10-year myocardial infarction/cardiac death risk (hard 10-year CHD risk)					
Baseline (%)	1.78 ± 1.44	1.60 ± 1.26	—	—	—
12 y follow-up (%)	3.84 ± 1.41	4.86 ± 2.01	—	—	—
Risk changes (%)	2.06 ± 1.17***	3.26 ± 1.31***	1.20 (0.68 to 1.71)	0.001	0.97

Significance (*P*) for within-group effects: \*\**P* < 0.01, \*\*\**P* < 0.001. Exact significance values are listed in the Result Section.

### 3. Results

Figure 1 gives the participant flow during the EFOPS study between 1998 and 2010. Briefly 59 subjects of the EG still exercised after 12 years, and 48 subjects of the nontraining control were willing to perform the 12-year follow-up. Looking at the 27 dropouts of the EG, 5 subjects cited study-related reasons for their withdrawal (too intensive: *n* = 2; too frequent: *n* = 3). Twelve reported quitting the program for occupational reasons, 5 subjects developed serious diseases (e.g., asthma, cancer), 4 women moved, and one participant died.

Attendance rate was 73% for the group session and 36% (reported performance rate) for the home training session. Thus, on average, participants of the EG exercised with an exercise frequency of 2.2 sessions/week (range: 1.4 to 3.0 sessions/week) representing an average weekly exercise volume of 92 min/week (range: 72 to 137 min/week). During the ≈600 participant years of exercise one hairline fracture of the os pubis, three strain traumas, and two muscle fiber ruptures were recorded.

Apart from changes of medication affecting primary endpoints which resulted in exclusion, no significant differences regarding changes of parameters that may impact study results (i.e., physical activity, additional exercise, diet, life style) were determined between EG and CG.

Table 2 shows the results for primary and secondary study endpoints. Taken together, changes of 10-year CHD risk (EG: 2.65 ± 2.09%, *P* = 0.001 versus CG: 5.40 ± 3.30%, *P* = 0.001), MetS Z-score (EG: -0.42 ± 1.03%, *P* = 0.003 versus CG: 1.61 ± 1.88%, *P* = 0.001), and 10-year risk of myocardial infarction/cardiac death (EG: 2.06 ± 1.17%; *P* = 0.001 versus CG: 3.26 ± 1.31%; *P* = 0.001) were significantly (all *P* = 0.001) more favorable in the EG compared with the CG. Ignoring the subjects' increasing age, which was, however, considered as a core risk factor by both CHD risk scores, changes of 10-year CHD risk and CHD risk were no more significantly negative in the EG, contrarily to the CG.

Since the per protocol analysis presented in Table 2 may provide too positive study effects, we also analyzed our data with the intention to treat principle using the last observation carried forward (OCF) method. Although the ITT-analysis presented in Table 3 resulted in slightly lower effect sizes, all the differences remained highly significant.

Thus, the hypothesis was clearly supported that changes of 10-year CHD risk, MetS Z-score, and 10-year risk of myocardial infarction/coronary death were significantly more favorable in the training group compared with non-training controls.

With respect to the underlying mechanisms, Table 4 shows changes of modifiable, continuously scaled risk factors constituting metabolic and cardiac risk scores among the EG and CG. Briefly, significant negative changes (all *P* < 0.007) in the CG were determined for waist circumference (WC), RR-MAP, triglycerides, and total and LDL-cholesterol. In the EG significant negative changes were detected for WC and total cholesterol (both *P* = 0.001), while changes of RR-MAP, Glucose, and HDL-C were significantly positive. As given in Table 4, between-group differences with more favorable changes in the EG were assessed for WC, RR-MAP, triglycerides, LDL-C, and HDL-C.

Diabetes status (EG: *n* = 2 versus CG: *n* = 2) did not change during the study period; however, 3 out of 7 subjects of the EG stopped smoking and one exerciser markedly decreased her tobacco abuse, whereas the number of smokers in the CG (*n* = 5) increased by one subject.

### 4. Discussion

The central aim of the EFOPS study is to determine the comparative effects of increasing age/menopause versus regular exercise on health risk factors in women 1–8 years postmenopausal at baseline. In this contribution, the authors clearly demonstrated the positive long-term effect of an ambulatory multipurpose exercise program on metabolic and cardiac risk factor scores. There are many exercise trials that

TABLE 3: Intention to treat analysis: changes in the exercise and control groups for primary and secondary study endpoints.

	Exercise MV (SD) <i>n</i> = 86	Control MV (SD) <i>n</i> = 51	Absolute difference MV (95% CI)	<i>P</i>	Effect size ( <i>d</i> )
10-year CHD risk					
Baseline (%)	8.42 ± 3.14	7.49 ± 2.72	—	—	—
12 y follow-up (%)	10.24 ± 3.22	11.69 ± 2.80	3.89	—	—
Risk changes (%)	1.80 ± 2.16***	4.23 ± 3.10***	2.44 (1.55 to 3.33)	0.001	0.91
Metabolic syndrome Z-score					
Baseline (%)	-2.47 ± 2.39	-3.54 ± 2.60	—	—	—
12 y follow-up (%)	-2.72 ± 3.09	-2.07 ± 3.44	—	—	—
Changes (%)	-0.25 ± 1.44 <sup>n.s.</sup>	1.47 ± 1.76***	1.72 (1.17 to 2.27)	0.001	1.07
10-year myocardial infarction/cardiac death risk (hard 10-year CHD risk)					
Baseline (%)	1.71 ± 1.11	1.62 ± 1.20	—	—	—
12 y follow-up (%)	3.02 ± 1.56	4.18 ± 1.61	—	—	—
Risk changes (%)	1.31 ± 1.35***	2.55 ± 1.40***	1.23 (0.76 to 1.72)	0.001	0.90

Significance (*P*) for within-group effects: \*\*\* *P* < 0.001; n.s.: nonsignificant.

TABLE 4: Changes of modifiable CHD risk factors constituting the metabolic and cardiac risk scores.

Parameter	Exercise MV (SD) <i>n</i> = 51	Control MV (SD) <i>n</i> = 43	Absolute difference MV (95% CI)	<i>P</i>	Effect size ( <i>d</i> )
Waist circumference (cm)	7.43 ± 5.42	11.33 ± 5.26	3.90 (1.70 to 6.09)	0.001	0.73
RR-MAP (mmHg)	-3.22 ± 5.11	4.65 ± 6.31	7.87 (5.53 to 10.21)	0.001	1.37
Glucose (mg/dL)	-2.80 ± 6.40	0.09 ± 7.89	2.90 (-0.03 to 5.82)	0.052	0.40
Triglycerides (mg/dL)	-0.1 ± 22.7	10.9 ± 24.9	11.1 (1.34 to 20.85)	0.026	0.46
Total cholesterol (mg/dL)	11.9 ± 22.1	18.5 ± 22.2	6.6 (3.9 to 27.5)	0.157	0.30
HDL-cholesterol (mg/dL)	7.37 ± 6.96	1.07 ± 8.22	6.30 (2.3 to 10.30)	0.001	0.83

assessed the effect of various types of exercise on metabolic and cardiac risk factors [18–21]. However, this study extended the existing data by establishing that high intensity, multipurpose exercise programs with reasonable training volume positively impact metabolic and cardiac risk scores in the critical (early) postmenopausal years persistently over 12 years. Of methodological importance, highly validated cardiac risk scores [8, 10] could be used that included factors (i.e., diabetes status, smoking) hard to influence within short-term interventions.

With respect to the primary and secondary study endpoints, an apparent discrepancy between positive changes for MetS Z-score and negative developments for both CHD risk factors may prevent a proper interpretation of the results of the EG. However, taking into account that “age” is a highly weighted risk factor within the 10-year CHD/hard CHD risk score concept [8, 10], the clinically moderate but statistically highly significant CHD risk changes in the EG (Tables 2 and 3) can be largely attributed to the increased age of the females.

Unfortunately, the short study duration of most exercise studies along with the paucity of studies that take 10-year CHD risk as a study endpoint prevents a distinct discussion with respect to the present literature. Regarding 10-year CHD risk, two studies focus on the effect of exercise on 10 y CHD risk in postmenopausal women with an intervention ≥12

months. The Senior Fitness and Prevention study (SEFIP) [22] prescribed a comparable multipurpose exercise program for 18 months with independent living females 65 years and older. Not strictly in line with the present finding, the authors reported significant positive changes in the EG (-2.0 ± 3.8%) and in the active CG (-1.2 ± 2.8%) with no significant between-group differences. However, beside the study duration and subject age, the main methodological difference between both studies is the implementation of an active CG that exercised 4 × 10 weeks during the SEFIP study course with one session of 60 min/week of (very) low exercise intensity. Of interest, the favorable changes of 10 y CHD risk in the SEFIP-CG are mainly based on significant decreases of diastolic (8 ± 6%) and systolic blood pressure (5 ± 4%). With respect to the exercise protocol and the baseline status (early-postmenopausal) most closely corresponding to the present study, the Training and Cimicifuga Racemosa (TRACE) study [23] did not show significant differences between the EG (+0.2 ± 1.9%, *P* = 0.603) and the CG (+1.1 ± 2.1%; *P* = 0.007) which also performed the low intensity/low volume exercise protocol described earlier. Although one may argue that the menopausal transition along with its hormonal and metabolic turbulences is a nonrepresentative phase in female life, this result could not be anticipated. Considering that the exercise protocol of the TRACE study focuses even more

on CHD risk factors compared with the EFOPS exercise program, exercise effects on 10-year CHD risk were expected to be much more pronounced in the TRACE study. To which degree this result could be due to the different stages of the menopausal transition (TRACE: 1–3 versus EFOPS: 1–8 year postmenopausal) is debatable. However, in summary, readers and researchers should notice that the effect of identical (CG: SEFIP versus Trace) or comparable (EG: SEFIP versus Trace versus EFOPS) exercise protocols on CHD risk profoundly varied although the female cohorts were quite homogeneous concerning age, menopausal status, and physical fitness.

Contrary to the more static 10-year CHD risk score, many more exercise trials focus on MetS prevalence (i.e., [24–29]), number of MetS risk factors (i.e., [9, 30, 31]), or continuously scaled MetS scores [9, 32]. With respect to the latter parameter, the results of this study largely confirmed the positive results of two studies [9, 32], which included postmenopausal female cohorts (40–65 y) with elevated LDL-C or decreased HDL-C-levels, however. Although the isolated endurance exercise programs of Camhi et al. (3 × 45–60 min/week aerobic exercise at 60–85% HRmax for 12 months) and Johnson et al. (aerobic exercise: 179 min/week at 40%–55% VO<sub>2</sub> peak versus 114 min at 65%–80% VO<sub>2</sub> peak versus 175 min at 65%–80% VO<sub>2</sub> peak for 8 months) widely differ from the present exercise protocol, in line with our data, 3 out of 4 exercise protocols/study arms resulted in significant positive MetS Z-score changes among the EG and significant differences compared with a nontraining control group (with slight, nonsignificant changes) while results of one study arm (114 min at 65%–80% VO<sub>2</sub> peak; [9]) did not reach statistical significance.

With respect to the underlying mechanisms, changes of isolated risk factors constituting 10-year CHD/hard CHD risk and MetS according to IDF [33] or NCEP ATP III [10] were analyzed in the present study. As the main result of this analysis, changes among the EG were far from uniform with significant negative changes for waist circumference (WC) and total cholesterol and significant positive changes for RR-MAP, Glucose, and HDL-C. Most interestingly, although it is frequently reported particularly for female cohorts that WC and CHD risk is closely related [34, 35] and some authors [32, 34] attributed positive changes of CHD risk and MetS status to corresponding changes of WC, the present result does not support a high longitudinal correlation of both factors. However, despite the general cross-sectional validity of waist circumference to represent visceral adipose tissue (VAT) [36, 37], exercise induced changes of visceral fat as assessed by MRT/CT were not relevantly reflected by changes of WC [18]. On the other hand, it is hard to believe that WC increments of almost 10% in the EFOPS-EG were not due to relevantly raised VAT.

Judging the relevance of the present study, strength and limitations of EFOPS must be adequately considered. Starting with the strong points, the central facet of this study is its closely supervised exercise program for 12 years so far. To derive adequate exercise recommendations and robust results, trial interventional period should at least exceed the period of initial adaptation to exercise. Revisiting

basic exercise principles, strains that initially increase system thresholds may become ineffective after adaptation of the system addressed [38, 39]. Translated into clinical practice this suggested that even when protocols are effective during the first months of exercise, this does not guarantee general effectiveness. Further strong points were (a) a homogenous group of (early) postmenopausal females, for which CHD risk is increasingly relevant. (b) The exercise protocol was progressively increased and regularly adjusted to subjects' capability during the entire intervention period. (c) Group sessions were closely supervised by certified trainers. (d) Covariates (diseases, medication, nutrition, life style) affecting study endpoints were strictly requested and controlled throughout the study in order to exclusively relate results to the given intervention (see also limitations). (e) Low drop-out rates and high overall attendance/compliance rates (at least with respect to the study length of 12 years so far) [40] indicate the attractiveness of the exercise program. (f) Two different methods of analysis (PPA and ITT) resulted in congruent results indicating the high reliability of our findings.

However, a number of limitations decrease the evidence of the study: (a) from a strictly methodological/statistical point of view, the nonrandomized group allocation may considerably reduce the evidence of our study results. We were aware of this problem; however, with respect to the intended long study duration, three main arguments encouraged our decision to allow the participants to join the study arm of their choice. (1) Though negligible in short-term studies, study allocation to the “wrong” (i.e., undesired) study arm of (very) long-term studies may produce a severe bias with respect to adherence. The most serious risk is that subjects who are willing to exercise but are randomized into the (life-long) inactive CG would exercise on their own without disclosing the fact and thus have a negative impact on the validity of the findings. (2) With respect to dropout and loss to follow-up, randomization into the (wrong) study will relevantly increase withdrawal of the subjects. Besides the loss of statistical power and a subsequent attrition bias, the fact that drop-out rates higher than 20% for short-term and 30% for long-term studies may break the random assignment to the study arms [41]. (3) In order to provide a realistic insight into long-term compliance and attendance of motivated participants, we decided to dispense with randomization. With respect to the issue of whether our results are generalizable to “random” osteopenic females, from a pragmatic point of view this question may be inappropriate and irrelevant since the majority of these females would never perform an intense 12-year exercise program, thus making any comparison impossible.

(b) Despite consistent monitoring of confounders, it cannot be claimed that all the determinants during these 12 years were perfectly controlled. This may be especially true changes of physical activity. Although a great deal of effort went into monitoring physical activity, our physical activity questionnaires [15, 16] are “bone specific,” and, hence, it may not have picked up slight changes of physical activities that impact the metabolic and/or cardiac system.

(c) Due to the nonsupervised home exercise program, exercise frequency may not be perfectly recorded for this component of the exercise training.

## 5. Conclusion

Although the nonrandomized design may prevent a definite conclusion, in summary EFOPS provides a large body of evidence that multipurpose exercise programs significantly and consistently address relevant (early) postmenopausal risk factors as determined for CHD risk and bone loss/fracture risk [12], at least for the minority of postmenopausal subjects willing to exercise [42].

## Conflict of Interests

None of the authors had any advisory board or financial interests that constitute a conflict of interests for this paper.

## Acknowledgments

The authors acknowledge support by Deutsche Forschungsgemeinschaft and Friedrich-Alexander-Universität Erlangen-Nürnberg within the funding programme “Open Access Publishing.” They are further grateful for the support of the Staedtler Foundation (Nuremberg, Germany) and the Behinderten- und Rehabilitations-Sportverband Bayern (Munich, Germany).

## References

- [1] C. Tesch-Römer, H. Engstler, and S. Wurm, *Altwerden in Deutschland. Sozialer Wandel und individuelle Entwicklung in der zweiten Lebenshälfte* VS-Verlag für Sozialwissenschaften, Wiesbaden, Germany, 2006.
- [2] H. Löwel, “Koronare Herzkrankheit und akuter Myokardinfarkt,” in *Gesundheitsberichterstattung des Bundes*, Robert Koch Institut. Statistisches Bundesamt, Ed., Robert-Koch-Institut, Berlin, Germany, 2006.
- [3] V. L. Roger, A. S. Go, D. M. Lloyd-Jones et al., “Executive summary: heart disease and stroke statistics—2012 update: a report from the American Heart Association,” *Circulation*, vol. 125, no. 1, pp. 188–197, 2012.
- [4] C. Scheidt-Nave, S. Richter, J. Fuchs, and A. Kuhlmeier, “Herausforderung an die Gesundheitsforschung für eine alternde Gesellschaft am Beispiel ‘Multimorbidität,’” *Bundesgesundheitsblatt—Gesundheitsforschung—Gesundheitsschutz*, vol. 53, no. 5, pp. 441–450, 2010.
- [5] M. Börjesson, M. L. Hellenius, E. Jansson et al., *Physical Activity in the Prevention and Treatment of Disease*, Professional Association for Physical Activity, Stockholm, Sweden, 2010.
- [6] W. J. Chodzko-Zajko, D. N. Proctor, M. A. Fiatarone Singh et al., “Exercise and physical activity for older adults,” *Medicine and Science in Sports and Exercise*, vol. 41, no. 7, pp. 1510–1530, 2009.
- [7] B. K. Pedersen and B. Saltin, “Evidence for prescribing exercise as therapy in chronic disease,” *Scandinavian Journal of Medicine and Science in Sports*, vol. 16, no. 1, pp. 3–63, 2006.
- [8] P. W. F. Wilson, R. B. D’Agostino, D. Levy, A. M. Belanger, H. Silbershatz, and W. B. Kannel, “Prediction of coronary heart disease using risk factor categories,” *Circulation*, vol. 97, no. 18, pp. 1837–1847, 1998.
- [9] J. L. Johnson, C. A. Slentz, J. A. Houmard et al., “Exercise training amount and intensity effects on metabolic syndrome (from studies of a targeted risk reduction intervention through defined exercise),” *American Journal of Cardiology*, vol. 100, no. 12, pp. 1759–1766, 2007.
- [10] NCEP, “Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III),” *Journal of the American Medical Association*, vol. 285, no. 19, pp. 2486–2497, 2001.
- [11] W. Kemmler, K. Engelke, D. Lauber, J. Weineck, J. Hensen, and W. A. Kalender, “Exercise effects on fitness and bone mineral density in early postmenopausal women: 1-year EFOPS results,” *Medicine and Science in Sports and Exercise*, vol. 34, no. 12, pp. 2115–2123, 2002.
- [12] W. Kemmler, S. von Stengel, M. Bebenek, K. Engelke, C. Hentschke, and W. A. Kalender, “Exercise and fractures in postmenopausal women: 12-year results of the Erlangen Fitness and Osteoporosis Prevention Study (EFOPS),” *Osteoporosis International*, vol. 23, no. 4, pp. 1267–1276, 2012.
- [13] S. von Stengel, W. Kemmler, D. Lauber et al., “Power training is more effective than strength training for maintaining bone mineral density in postmenopausal women,” *Journal of Applied Physiology*, vol. 99, no. 1, pp. 181–188, 2005.
- [14] W. Kemmler, D. Lauber, S. von Stengel, and K. Engelke, “Developing maximum strength in older adults—a series of studies,” in *Current Results of Strength Training Research*, J. Gießing, M. Fröhlich, and P. Preuss, Eds., pp. 114–133, Cuvillier, Göttingen, Germany, 2005.
- [15] W. Kemmler, J. Weineck, W. A. Kalender, and K. Engelke, “The effect of habitual physical activity, non-athletic exercise, muscle strength, and  $VO_{2max}$  on bone mineral density is rather low in early postmenopausal osteopenic women,” *Journal of Musculoskeletal Neuronal Interactions*, vol. 4, no. 3, pp. 325–334, 2004.
- [16] I. Schöffl, W. Kemmler, S. von Stengel, K. Engelke, and W. Kalender, “Physical activity, strength and  $VO_{2max}$  have no significant influence on bone parameters in elderly women,” *Journal of Musculoskeletal Neuronal Interactions*, vol. 8, pp. 363–374, 2008.
- [17] W. Kemmler, D. Lauber, J. Weineck, J. Hensen, W. Kalender, and K. Engelke, “Benefits of 2 years of intense exercise on bone density, physical fitness, and blood lipids in early postmenopausal osteopenic women: results of the Erlangen Fitness Osteoporosis Prevention Study (EFOPS),” *Archives of Internal Medicine*, vol. 164, no. 10, pp. 1084–1091, 2004.
- [18] S. J. Kay and M. A. Fiatarone Singh, “The influence of physical activity on abdominal fat: a systematic review of the literature,” *Obesity Reviews*, vol. 7, no. 2, pp. 183–200, 2006.
- [19] G. A. Kelley and K. S. Kelley, “Progressive resistance exercise and resting blood pressure: a meta-analysis of randomized controlled trials,” *Hypertension*, vol. 35, no. 3, pp. 838–843, 2000.
- [20] G. A. Kelley, K. S. Kelley, and Z. V. Tran, “Aerobic exercise and lipids and lipoproteins in women: a meta-analysis of randomized controlled trials,” *Journal of Women’s Health*, vol. 13, no. 10, pp. 1148–1164, 2004.
- [21] G. A. Kelley and K. S. Kelley, “Efficacy of aerobic exercise on coronary heart disease risk factors,” *Preventive Cardiology*, vol. 11, no. 2, pp. 71–75, 2008.
- [22] W. Kemmler, S. von Stengel, K. Engelke, L. Häberle, and W. A. Kalender, “Exercise effects on bone mineral density, falls, coronary risk factors, and health care costs in older women: the randomized controlled senior fitness and prevention (SEFIP) study,” *Archives of Internal Medicine*, vol. 170, no. 2, pp. 179–185, 2010.

- [23] M. Bebenek, W. Kemmler, S. von Stengel, K. Engelke, and W. A. Kalender, "Effect of exercise and *Cimicifuga racemosa* (CR BNO 1055) on bone mineral density, 10-year coronary heart disease risk, and menopausal complaints: the randomized controlled Training and *Cimicifuga racemosa* Erlangen (TRACE) study," *Menopause*, vol. 17, no. 4, pp. 791–800, 2010.
- [24] P. A. Ades, P. D. Savage, M. J. Toth et al., "High-calorie-expenditure exercise: a new approach to cardiac rehabilitation for overweight coronary patients," *Circulation*, vol. 119, no. 20, pp. 2671–2678, 2009.
- [25] J. S. Green, P. R. Stanforth, T. Rankinen et al., "The effects of exercise training on abdominal visceral fat, body composition, and indicators of the metabolic syndrome in postmenopausal women with and without estrogen replacement therapy: the HERITAGE family study," *Metabolism*, vol. 53, no. 9, pp. 1192–1196, 2004.
- [26] P. T. Katzmarzyk, A. S. Leon, J. H. Wilmore et al., "Targeting the metabolic syndrome with exercise: evidence from the HERITAGE family study," *Medicine and Science in Sports and Exercise*, vol. 35, no. 10, pp. 1703–1709, 2003.
- [27] K. Kukkonen-Harjula, R. Laukkanen, I. Vuori et al., "Effects of walking training on health-related fitness in healthy middle-aged adults—a randomized controlled study," *Scandinavian Journal of Medicine and Science in Sports*, vol. 8, no. 4, pp. 236–242, 1998.
- [28] T. J. Orchard, M. Temprosa, R. Goldberg et al., "The effect of metformin and intensive lifestyle intervention on the metabolic syndrome: the diabetes prevention program randomized trial," *Annals of Internal Medicine*, vol. 142, no. 8, pp. 611–619, 2005.
- [29] K. J. Stewart, A. C. Bacher, K. Turner et al., "Exercise and risk factors associated with metabolic syndrome in older adults," *American Journal of Preventive Medicine*, vol. 28, no. 1, pp. 9–18, 2005.
- [30] W. Kemmler, S. von Stengel, K. Engelke, and W. A. Kalender, "Exercise decreases the risk of metabolic syndrome in elderly females," *Medicine and Science in Sports and Exercise*, vol. 41, no. 2, pp. 297–305, 2009.
- [31] A. E. Tjønnå, S. J. Lee, Ø. Rognmo et al., "Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: a pilot study," *Circulation*, vol. 118, no. 4, pp. 346–354, 2008.
- [32] S. M. Camhi, M. L. Stefanick, P. T. Katzmarzyk, and D. R. Young, "Metabolic syndrome and changes in body fat from a low-fat diet and/or exercise randomized controlled trial," *Obesity*, vol. 18, no. 3, pp. 548–554, 2010.
- [33] K. G. M. M. Alberti and P. Zimmet, "Metabolic syndrome—a new world-wide definition. A consensus statement from the International Diabetes Federation," *Diabetic Medicine*, vol. 23, no. 5, pp. 469–480, 2006.
- [34] D. Canoy, "Distribution of body fat and risk of coronary heart disease in men and women," *Current Opinion in Cardiology*, vol. 23, no. 6, pp. 591–598, 2008.
- [35] A. Hernández-Ono, G. Monter-Carreola, J. Zamora-González et al., "Association of visceral fat with coronary risk factors in a population-based sample of postmenopausal women," *International Journal of Obesity and Related Metabolic Disorders*, vol. 26, no. 1, pp. 33–39, 2002.
- [36] E. G. Kamel, G. McNeill, T. S. Han et al., "Measurement of abdominal fat by magnetic resonance imaging, dual-energy X-ray absorptiometry and anthropometry in non-obese men and women," *International Journal of Obesity and Related Metabolic Disorders*, vol. 23, no. 7, pp. 686–692, 1999.
- [37] R. Ross, J. Rissanen, and R. Hudson, "Sensitivity associated with the identification of visceral adipose tissue levels using waist circumference in men and women: effects of weight loss," *International Journal of Obesity and Related Metabolic Disorders*, vol. 20, no. 6, pp. 533–538, 1996.
- [38] T. O. Bumpa, *Periodization. Theorie and Methodology of Training*, Human Kinetics Books, Champaign, Ill, USA, 1999.
- [39] J. Weineck, *Sportbiologie*, Spitta, Balingen, Germany, 2009.
- [40] E. A. Marques, J. Mota, and J. Carvalho, "Exercise effects on bone mineral density in older adults: a meta-analysis of randomized controlled trials," *Age*, vol. 34, no. 6, pp. 1493–1515, 2012.
- [41] M. Müllner, *Erfolgreich wissenschaftlich arbeiten in der Klinik. Evidence Based Medicine*, Springer, Wien, Austria, 2005.
- [42] Robert-Koch-Institut, "Sportliche Aktivität," in *Daten und Fakten: Ergebnisse der Studie, Gesundheit in Deutschland aktuell 2010*, RKI, Ed., pp. 123–126, Beiträge zur Gesundheitsberichterstattung des Bundes RKI, Berlin, Germany, 2012.

## Research Article

# Panax Quinquefolius Saponin of Stem and Leaf Attenuates Intermittent High Glucose-Induced Oxidative Stress Injury in Cultured Human Umbilical Vein Endothelial Cells via PI3K/Akt/GSK-3 $\beta$ Pathway

Jingshang Wang,<sup>1</sup> Huijun Yin,<sup>1</sup> Ye Huang,<sup>1</sup> Chunyu Guo,<sup>1</sup>  
Chengdong Xia,<sup>2</sup> Qian Liu,<sup>1</sup> and Lu Zhang<sup>1</sup>

<sup>1</sup> Department of Cardiovascular Disease, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>2</sup> Department of Endocrinology, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

Correspondence should be addressed to Huijun Yin; [huijunyin@aliyun.com](mailto:huijunyin@aliyun.com)

Received 19 November 2012; Revised 17 June 2013; Accepted 26 June 2013

Academic Editor: Ke-ji Chen

Copyright © 2013 Jingshang Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Panax quinquefolius saponin of stem and leaf (PQS), the effective parts of American ginseng, is widely used in China as a folk medicine for diabetes and cardiovascular diseases treatment. In our previous studies, we have demonstrated that PQS could improve the endothelial function of type II diabetes mellitus (T2DM) rats with high glucose fluctuation. In the present study, we investigated the protective effects of PQS against intermittent high glucose-induced oxidative damage on human umbilical vein endothelial cells (HUVECs) and the role of phosphatidylinositol 3-kinase kinase (PI3K)/Akt/GSK-3 $\beta$  pathway involved. Our results suggested that exposure of HUVECs to a high glucose concentration for 8 days showed a great decrease in cell viability accompanied by marked MDA content increase and SOD activity decrease. Moreover, high glucose significantly reduced the phosphorylation of Akt and GSK-3 $\beta$ . More importantly, these effects were even more evident in intermittent high glucose condition. PQS treatment significantly attenuated intermittent high glucose-induced oxidative damage on HUVECs and meanwhile increased cell viability and phosphorylation of Akt and GSK-3 $\beta$  of HUVECs. Interestingly, all these reverse effects of PQS on intermittent high glucose-cultured HUVECs were inhibited by PI3K inhibitor LY294002. These findings suggest that PQS attenuates intermittent-high-glucose-induced oxidative stress injury in HUVECs by PI3K/Akt/GSK-3 $\beta$  pathway.

## 1. Introduction

Vascular disorders, especially cardiovascular disorders, are major causes of morbidity and mortality in diabetic patients [1]. Intermittent high glucose (IHG) and constant high glucose are two general phenomena in diabetes. Recent studies have shown that IHG may be more dangerous for the development of diabetes-related complications including cardiovascular disorders, and thus for diabetic patients [2].

Although the precise mechanism underlying the action of IHG remains unclear, significant progress has been made. Recent studies have shown that IHG could induce an increased rate of apoptosis, protein kinase C activation,

nicotinamide adenine dinucleotide phosphate oxidase activation in cultured endothelial cells, and monocytes adhesion to endothelial cells in diabetic rats. These effects are even more pronounced than those of constant high glucose [3–7]. Moreover, there is growing evidence that an acute increase of glycemia is accompanied by oxidative stress that may contribute to the generation of vascular endothelial dysfunction [8]. Meanwhile, clinical evidence suggests that *in vivo* glucose fluctuations may be damaged for endothelial cells, which could be mediated by oxidative stress [9, 10]. And enhanced oxidative damage after diverse stimuli has been confirmed to be an initial event in the development of cardiovascular diseases [11, 12]. It is, therefore, thought that

prevention of intermittent high glucose-induced oxidative damage on endothelial cells may have important implications for pharmacologic attempts to prevent these complications.

PQS is the effective parts of American ginseng, a herb widely used in clinical Chinese medicine for diabetes and cardiovascular diseases treatment. In fact, there is growing evidence demonstrating the significant beneficial effects of PQS consumption on diabetic patients, including lowering blood sugar, keeping blood sugar stable, improving insulin resistance, regulating lipid metabolism, and cardioprotective effects such as antimyocardial ischemia, reducing myocardium oxygen consumption, ameliorating myocardial remodeling, and inhibiting platelet aggregation [13–16]. A multicenter, double-blind, randomized control clinical trial organized by Ministry of Public Health of the People's Republic of China showed the similar results [17]. Our previous study had demonstrated that PQS could improve endothelial function in diabetes mellitus in experimental rats with high glucose fluctuation [18]. However, as far as we know, little evidence exists concerning the effect of PQS on oxidative damage in endothelial cells induced by intermittent high glucose.

PI3K and Akt are downstream effectors of insulin signaling [19], as well as important signaling molecules in the regulation of glycogen metabolism in myocytes, lipocytes, and hepatocytes [20, 21]. Uncoupling of insulin signaling at PI3K-Akt in response to high glucose concentrations in these cell types has been implicated in the pathogenesis of insulin resistance and T2DM [22]. By regulating angiogenesis, proliferation, microvascular permeability, survival, cellular transformation, and embryonic differentiation, PI3K-Akt also plays an important role in regulation of endothelial cell (EC) function [23, 24]. Cells respond via PI3K-Akt signaling to a variety of cytokines, G protein-coupled receptor ligands, and growth factors as well as to cellular stresses, including heat shock, hypoxia, and oxidative stress [25]. It has been reported that prolonged exposure of ECs to high glucose concentrations would result in reduced proliferation and survival through altered PI3K-Akt signaling [26]. The nitric oxide production, followed by Akt activation, had been confirmed to prevent steady high glucose-induced endothelial cell injury [27]. Our previous study had observed that PQS could improve insulin sensitivity by increasing the tyrosine phosphorylation of insulin receptor and IRS1 and the Ser473 phosphorylation of Akt [15]. A potential target of PQS may also be the serine/threonine kinase Akt.

Therefore, the aims of our present study were to (1) determine whether treatment with PQS attenuates intermittent high glucose-induced stress injury in HUVECs and, if so, (2) investigate the signaling pathway involved.

## 2. Materials and Methods

**2.1. Chemicals and Reagents.** PQS was provided by Jilin Jian Yisheng Pharmaceutical Co. Ltd. Dulbecco's-Modified Eagle's Medium (DMEM) was purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone. The antibodies against Akt, phosphorylated-Akt

(Ser473), GSK3 $\beta$ , GSK3 $\beta$ , phosphorylated-GSK3 $\beta$  (Ser9) and  $\beta$ -Actin were purchased from Cell Signaling Technology (USA). The LY294002 and 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, USA). Malonyldialdehyde (MDA) and superoxide dismutase (SOD) assay kits were obtained from Jian Cheng Biological Engineering Institute (Nanjing, China). All other biochemicals used were of the highest purity available.

**2.2. Isolation and Culture of Human Umbilical Vein Endothelial Cells (HUVECs).** HUVECs were isolated and pooled from umbilical cords obtained from normal vaginal deliveries by the procedure described by Jaffe et al. [28]. The cells were cultured in gelatin-coated 60 mm Petri dishes (Corning) and grown in DMEM, supplemented with 20% heat-inactivated fetal bovine serum, 20 mM glutamine (Sigma-Aldrich), 10 ng/mL endothelial cell growth supplement (Sigma-Aldrich), 40 U/mL heparin (Gibco), 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin (Gibco), 20 mM HEPES (Sigma-Aldrich), and 0.11 mg/mL sodium pyruvate (Sigma-Aldrich). The Petri dishes were incubated at 37°C, in 5% CO<sub>2</sub>-95% air gas mixture. Primary cultures were fluid-changed 24 h after seeding and were subcultured on reaching confluence by the use of 0.25% Trypsin-EDTA, inactivated by dilution. More than 99% HUVECs were identified to be endothelial cells by their characteristic cobblestone morphology (Figure 1(a)) under an inverted microscope (Leica DMIRB, Germany) and characterized by brown granules in cytoplasm using immunocytochemical staining of factor VIII (Figure 1(b)). Only HUVECs of the second passage were used in the study to avoid age-dependent cellular modification. HUVECs were seeded at equal density in gelatin-coated 60 mm Petri dishes or plates, allowed to attach overnight, and then exposed to the following experimental conditions for 8 days: (1) continuous DMEM containing normal (5.56 mmol/L) glucose (NG), (2) continuous DMEM containing high (25 mmol/L) glucose (HG), (3) alternating normal and high-glucose media every 24 h (IHG), (4) as (3), with the addition of 0.05 mg/mL PQS, and (5) as (3), with the addition of 0.1 mg/mL PQS. To further examine the role of the PI3K/Akt/GSK3 $\beta$  pathway on the effect of PQS, another two groups of HUVECs were pretreated with the specific PI3K inhibitor LY294002 (20  $\mu$ mol/L; Sigma) for 30 min before PQS was added.

**2.3. Cell Viability Measurement (MTT Assay).** Cell viability was determined by MTT assay. HUVECs were seeded in 96-well culture plates at a density of  $1 \times 10^5$  cells with 200  $\mu$ L culture medium per well. Four hour before the culture was terminated, 10  $\mu$ L assay medium containing 5 mg/mL MTT was added to each well. After 4 h of incubation at 37°C, the medium was removed and the cells lysed by addition of 150  $\mu$ L DMSO. The optical density of each sample was measured in an ELISA microplate reader using test and reference wavelengths of 490 nm.

**2.4. Preparation of Cell Lysates.** The cells were seeded at a density of  $1 \times 10^5$  cells/mL in 24-well plates and were allowed to attach for 24 h before treatment. Upon completion of

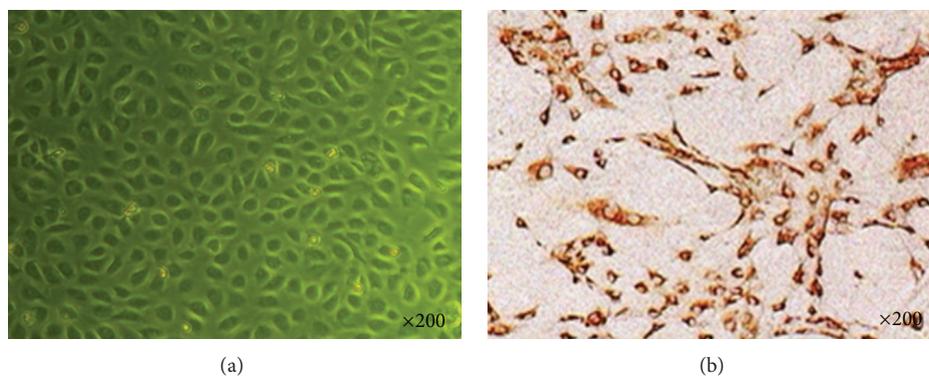


FIGURE 1: Morphology and immunocytochemical staining of HUVEC. (a) Characteristic cobblestone morphology of HUVEC under an inverted microscope. (b) Immunocytochemical staining of HUVEC.

the incubation studies, the cells were scraped from the plates into ice-cold RIPA lysis buffer (50 mM Tris with pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 0.05 mM EDTA), and protein concentration was determined by the bicinchoninic acid method, using BSA as a reference standard. Aliquots were stored at  $-80^{\circ}\text{C}$  until detection for MDA and SOD activity.

**2.5. Assay for Intracellular Contents of SOD and MDA.** The activities of SOD and the concentration of MDA were both determined by using commercially available kits. All procedures completely complied with the manufacturer's instructions. The activities of SOD were expressed as units per milligram protein. MDA was measured at a wavelength of 532 nm by reacting with thiobarbituric acid to form a stable chromophoric production. Values of MDA level were expressed as nanomoles per milligram protein.

**2.6. Western Blot Analysis.** Cells were lysed in iced lysis buffer. Total protein (50 mg/lane) was separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After incubation in blocking solution (5% nonfat milk) (Sigma), membranes were incubated with primary antibodies for Akt, phosphorylated-Akt, GSK3 $\beta$ , phosphorylated-GSK3 $\beta$ , or  $\beta$ -Actin for overnight at  $4^{\circ}\text{C}$ . Membranes were washed and then incubated with 1:2000 dilution horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, Beijing, China). The relative density of each protein band was normalized to that of  $\beta$ -Actin. All results were representative of at least 3 independent experiments.

**2.7. Statistical Analysis.** All data were expressed as mean  $\pm$  SD. The SPSS Statistics 15.0 package was utilized to analyze the data. Differences among groups were analyzed using the one-way analysis of variance (ANOVA), followed by multiple comparisons by LSD test. The  $P < 0.05$  was considered statistically significant.

### 3. Results

**3.1. Effects of PQS on Intermittent High Glucose-Induced Loss of HUVEC Viability.** After 8 days of experiment, we observed

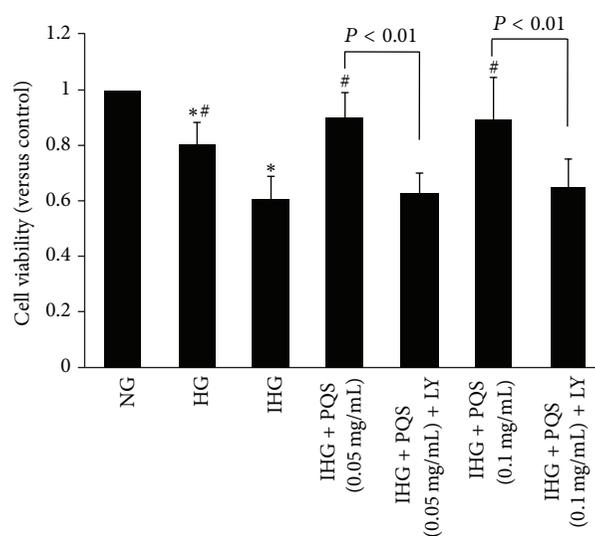


FIGURE 2: Effects of PQS on intermittent high glucose-induced loss of HUVEC viability. Cell viability was determined by MTT assay. Cell viability was expressed as a percentage of cytoprotection versus control group set at 100%. Data were presented as means  $\pm$  SD. ( $n = 5$ ). \* $P < 0.01$  versus NG; # $P < 0.01$  versus IHG.

that the cell viability in HG ( $0.8 \pm 0.12$ ) decreased significantly in comparison with NG, and this decrease was even more marked in IHG ( $0.61 \pm 0.08$ ). Two different concentrations (0.05 or 0.1 mg/mL) of PQS improved cell viability significantly ( $0.9 \pm 0.11$  or  $0.89 \pm 0.15$ ). However, pretreatment with LY294002 (PI3K inhibitor) abolished PQS's effect on cell viability in cultured endothelial cells exposed to intermittent high glucose ( $0.63 \pm 0.07$  or  $0.65 \pm 0.13$ ) (Figure 2).

**3.2. Effects of PQS on SOD and MDA Levels.** As shown in Table 1 and Figure 3(a), after 8 days of experiment, the SOD level significantly decreased in IHG compared with either NG or HG. Pretreatment HUVECs with PQS (0.05 mg/mL or 0.1 mg/mL) inhibited the decreased SOD level induced by intermittent high glucose, which was abrogated by LY294002.

TABLE 1: Effects of PQS on SOD and MDA levels ( $n = 5$ ).

Group	SOD	MDA
	U/mg protein	nmol/mg protein
NG	53.8 ± 7.62	24 ± 2.41
HG	30.81 ± 6.97 <sup>#</sup>	39.9 ± 7.18 <sup>*#</sup>
IHG	20.73 ± 3.75 <sup>*</sup>	47.16 ± 7.77 <sup>*</sup>
IHG + PQS (0.05 mg/mL)	32.69 ± 2.66 <sup>#</sup>	39.15 ± 6.86 <sup>#</sup>
IHG + PQS (0.05 mg/mL) + LY	21.41 ± 2.05 <sup>▲</sup>	52 ± 4.72 <sup>▲</sup>
IHG + PQS (0.1 mg/mL)	35.9 ± 3.37 <sup>#</sup>	33.11 ± 3.07 <sup>#</sup>
IHG + PQS (0.1 mg/mL) + LY	17.66 ± 5.93 <sup>▲</sup>	44.2 ± 3.66 <sup>▲</sup>

Note: <sup>\*</sup> $P < 0.01$  versus NG; <sup>#</sup> $P < 0.01$  versus IHG. <sup>▲</sup> $P < 0.01$  versus IHG + PQS (0.05 mg/mL or 0.1 mg/mL).

The content of MDA in the medium was increased significantly after treatment with intermittent high glucose for 48 h, compared with either normal or stable high glucose condition. Pretreatment HUVECs with PQS (0.05 mg/mL or 0.1 mg/mL) inhibited the elevation of MDA concentration elicited by intermittent high glucose significantly, which was abolished by LY294002 (Table 1 and Figure 3(b)).

Together, these results showed that blood glucose fluctuation produced higher suppression of antioxidant capacity and more oxidative damage than stable high glucose alone. However, pretreated with PQS, all these were reversed.

**3.3. Effects of PQS on Decreased Akt and GSK3 $\beta$  Phosphorylation Levels Induced by Intermittent High Glucose.** To investigate the underlying mechanism for protective effects of PQS, we examined the effect of PQS (0.1 mg/mL) on Akt and GSK3 $\beta$  level in intermittent high glucose-treated HUVECs. As shown in Figure 4(a), HG significantly reduced the phosphorylation of Akt without alteration of total Akt expression in comparison with conditioning, and this decrease was even more marked in IHG. Pretreatment of HUVECs with PQS led to a significant increase in the phosphorylation of Akt in endothelial cells exposed to intermittent high glucose. And PQS had no effect on the Akt protein level. The specific PI3K inhibitor LY294002 markedly suppressed the effects of PQS on Akt activity.

Furthermore, similar to the effects of HG/IHG and PQS on Akt phosphorylation, IHG inhibited GSK3 $\beta$  phosphorylation without alteration of total GSK3 $\beta$  expression, compared with NG or HG. PQS treatment significantly attenuated the decreased phosphorylation of GSK3 $\beta$  induced by intermittent high glucose, which was abolished by LY294002 (Figure 4(b)).

## 4. Discussion

There are two novel observations in our present experiment. Firstly, we have provided direct *in vitro* evidence that treatment with PQS attenuates intermittent high glucose-induced oxidative stress injury in HUVECs. Secondly, we have demonstrated that the protective effect of PQS on HUVECs was PI3K/Akt/GSK3 $\beta$ -dependent.

Hyperglycemia is generally regarded as one of the major causes of pathological consequences of both type I and type II diabetes [29]. Much of this damage is thought to be a consequence of elevated production of ROS and oxidative stress has recently been proposed as the unifying explanation of the hyperglycemia-related diabetic complications [30, 31]. In normal subjects, blood glucose is strictly controlled within a narrow range, while blood glucose in diabetic patients often changes obviously within a single day. Though there is still an extensive debate about glucose variability as a risk factor for complications independent of HbA1c in diabetes [32, 33], more and more lines of evidence have found that glucose fluctuations may play a significant role in the pathogenesis of diabetic complications. According to *in vitro* experimental settings and animal studies, fluctuating glucose levels display a more deleterious effect on endothelial cells than constantly high glucose exposure and that this effect should be mediated by an oxidative stress [3–7]. Moreover, human studies have shown that acute and chronic blood glucose fluctuations in T2DM levels could increase oxidative stress significantly [9, 10], although short-term glucose variability is not associated with raised oxidative stress markers in healthy volunteers [34].

In this study, we employed a cellular experimental model in which primary cultures of human endothelial cells were exposed to intermittent high glucose, a condition that partly mimics glucose excursions occurring in diabetes *in vivo*. As known to all, MDA is a by-product of lipid peroxidation induced by excessive ROS and widely used as a biomarker of oxidative stress [35]. On the other hand, SOD, as an endogenous antioxidant, plays a pivotal role in preventing cellular damage caused by ROS [36]. Enhanced oxidative damage after diverse stimuli has been confirmed to be an initial event in the development of cardiovascular diseases. In agreement with previous studies [6, 7], we confirmed that intermittent high glucose, as seen in diabetic patients, was more deleterious than those of stable high glucose and that oxidative stress was convincingly involved. In our present study, a more obvious decrease of cell viability was observed in HUVECs exposed to intermittent high glucose for 8 days, which was associated with an elevation of MDA production and a significant decrease in SOD. Nonetheless, when HUVECs were preincubated with PQS, these intermittent high glucose-induced cellular events were blocked to a great extent. These results together suggested that enhancement of endogenous antioxidant preservation and attenuation of lipid peroxidation may represent a major mechanism of cellular protection by PQS.

The underlying mechanism by which PQS protects HUVECs from intermittent high glucose-induced oxidative damage is an important question raised by the results presented in this study.

Akt, downstream of PI3K, is thought to be one of the important factors in cell survival. In endothelial cells, Akt activation has been reported to promote cell survival [37, 38]. And evidence has shown that the PI3K/Akt pathway plays an important role in preventing endothelial cell injury induced by high glucose. Our previous study had confirmed that PQS could increase insulin sensitivity by increasing the tyrosine

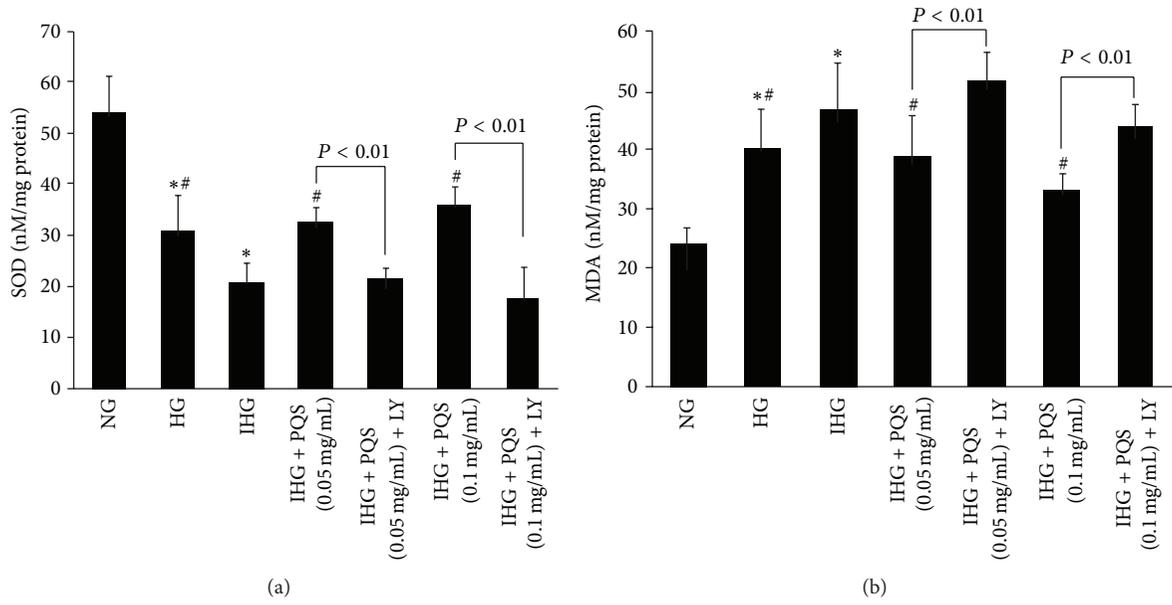


FIGURE 3: Effects of PQS on SOD (a) and MDA (b) content in HUVECs exposed to intermittent high glucose. Data were presented as means  $\pm$  SD. ( $n = 5$ ). \* $P < 0.01$  versus NG, # $P < 0.01$  versus IHG.

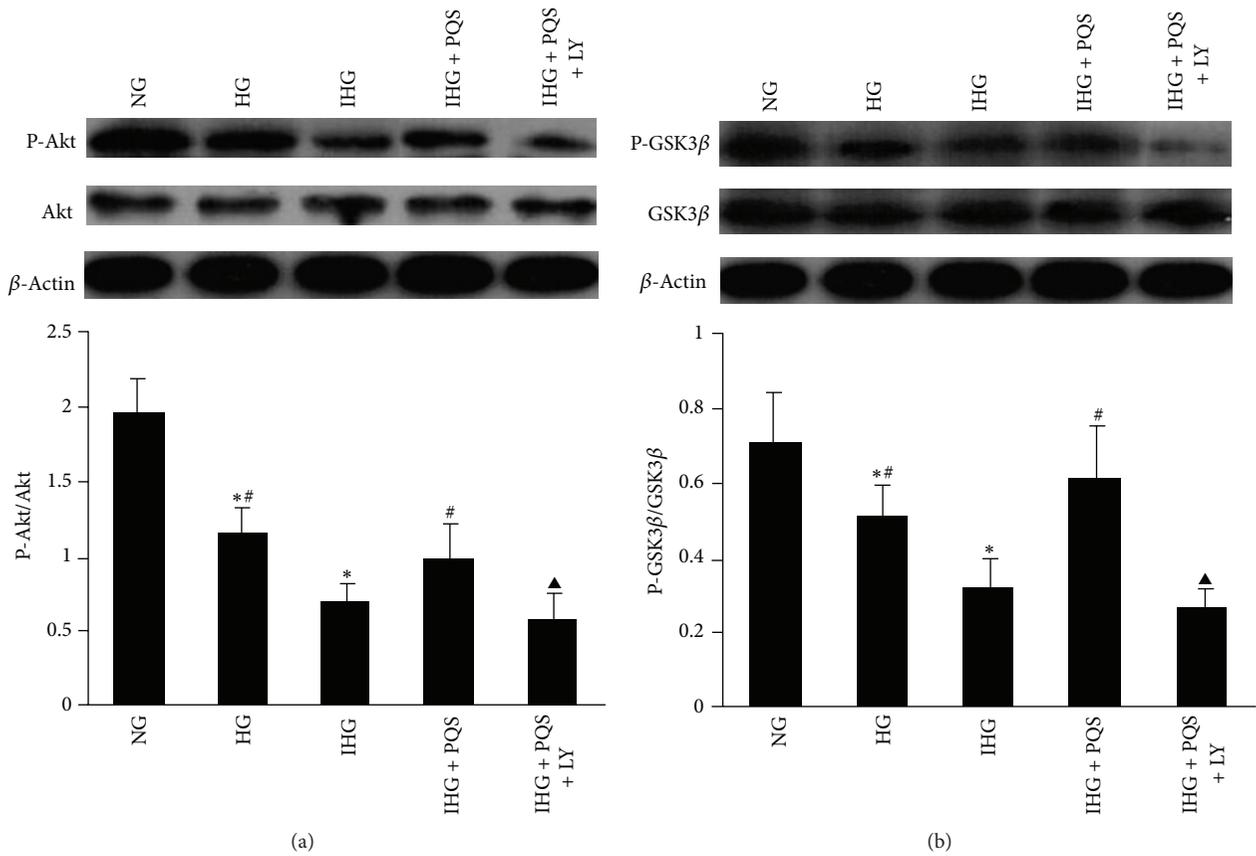


FIGURE 4: Phosphorylation of Akt (a) and GSK3 $\beta$  (b) in cultured human umbilical vein endothelial cells determined by western blot. Data obtained from quantitative densitometry were presented as mean  $\pm$  SD. ( $n = 3$ ). Before PQS was added, HUVECs were pretreated with LY294002 for 30 min. \* $P < 0.01$  versus NG, # $P < 0.01$  versus IHG;  $\blacktriangle P < 0.01$  versus IHG + PQS. PQS, panax quinquefolius saponin of stem and leaf (0.1 mg/mL).

phosphorylation of insulin receptor and IRS1 and the Ser473 phosphorylation of Akt. Based on these observations, we examined the contribution of the PI3K/Akt pathway to the protective effect of PQS. In the present study, we confirmed the inhibitory effect of high glucose on Akt activation as previous report [39] and meanwhile observed a more obvious inhibitory effect in intermittent high glucose condition. We also demonstrated that PQS treatment attenuated the decrease in Akt phosphorylation induced by intermittent high glucose, which was abolished by PI3K inhibitor. Furthermore, LY294002 significantly abolished the protective effect of PQS on oxidative damage induced by intermittent high glucose, which indicated that PQS exerted its protective effect through PI3K/Akt pathway.

Among the various intracellular downstream effectors of Akt, GSK-3 $\beta$  phosphorylation and inactivation are considered important mechanisms of cell survival [40]. In the present study, we confirmed for the first time that decreased GSK3 $\beta$  phosphorylation level was involved in high glucose-induced oxidative damage. And the decrease was even more obvious in intermittent high glucose condition. Pretreatment with PQS significantly improved the decreased GSK3 $\beta$  phosphorylation levels induced by intermittent high glucose, which was also blocked by LY294002, indicating that PQS-promoted GSK3 $\beta$  phosphorylation depends on PI3K-Akt activation. Taken together, these results provide strong evidence that the PI3K/Akt/GSK3 $\beta$  pathway is involved in the antioxidative damage effect of PQS.

In summary, the present study shows that PQS inhibits intermittent high glucose-induced oxidative damage in cultured HUVECs through the PI3K/Akt/GSK3 $\beta$  pathway. It provides further strong evidence that PQS, as well as traditional Chinese herb, might offer an alternative strategy for diabetic cardiovascular complications prevention.

## Conflict of Interests

The authors declare no conflict of interests.

## Acknowledgment

This work was supported by grants from the National Natural Science Foundation of China (no. 81041038 and no. 81202777).

## References

- [1] M. K. Ali, K. M. V. Narayan, and N. Tandon, "Diabetes & coronary heart disease: current perspectives," *Indian Journal of Medical Research*, vol. 132, no. 11, pp. 584–597, 2010.
- [2] M. Brownlee and I. B. Hirsch, "Glycemic variability: a hemoglobin A1c-independent risk factor for diabetic complications," *Journal of the American Medical Association*, vol. 295, no. 14, pp. 1707–1708, 2006.
- [3] H. Watada, K. Azuma, and R. Kawamori, "Glucose fluctuation on the progression of diabetic macroangiopathy—new findings from monocyte adhesion to endothelial cells," *Diabetes Research and Clinical Practice*, vol. 77, no. 3, supplement, pp. S58–S61, 2007.
- [4] T. Mita, A. Otsuka, K. Azuma et al., "Swings in blood glucose levels accelerate atherogenesis in apolipoprotein E-deficient mice," *Biochemical and Biophysical Research Communications*, vol. 358, no. 3, pp. 679–685, 2007.
- [5] L. Piconi, L. Quagliaro, R. Da Ros et al., "Intermittent high glucose enhances ICAM-1, VCAM-1, E-selectin and interleukin-6 expression in human umbilical endothelial cells in culture: the role of poly(ADP-ribose) polymerase," *Journal of Thrombosis and Haemostasis*, vol. 2, no. 8, pp. 1453–1459, 2004.
- [6] L. Piconi, L. Quagliaro, R. Assaloni et al., "Constant and intermittent high glucose enhances endothelial cell apoptosis through mitochondrial superoxide overproduction," *Diabetes/Metabolism Research and Reviews*, vol. 22, no. 3, pp. 198–203, 2006.
- [7] E. M. Horváth, R. Benko, L. Kiss et al., "Rapid 'glycaemic swings' induce nitrosative stress, activate poly(ADP-ribose) polymerase and impair endothelial function in a rat model of diabetes mellitus," *Diabetologia*, vol. 52, no. 5, pp. 952–961, 2009.
- [8] F. Giacco and M. Brownlee, "Oxidative stress and diabetic complications," *Circulation Research*, vol. 107, no. 9, pp. 1058–1070, 2010.
- [9] C.-M. Chang, C.-J. Hsieh, J.-C. Huang, and I.-C. Huang, "Acute and chronic fluctuations in blood glucose levels can increase oxidative stress in type 2 diabetes mellitus," *Acta Diabetologica*, vol. 49, supplement 1, pp. S171–S177, 2012.
- [10] L. Monnier, E. Mas, C. Ginet et al., "Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes," *Journal of the American Medical Association*, vol. 295, no. 14, pp. 1681–1687, 2006.
- [11] S. V. V. Lakshmi, G. Padmaja, P. Kuppusamy, and V. K. Kutala, "Oxidative stress in cardiovascular disease," *Indian Journal of Biochemistry and Biophysics*, vol. 46, no. 6, pp. 421–440, 2009.
- [12] M. M. Elahi, Y. X. Kong, and B. M. Matata, "Oxidative stress as a mediator of cardiovascular disease," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 5, pp. 259–269, 2009.
- [13] H.-J. Yin, Y. Zhang, Y.-R. Jiang et al., "The effect of panax quinquefolium saponins on blood lipid level in Alloxan-Induced hyperglycemia rat model," *Chinese Journal of Integrative Medicine on Cardio/Cerebrovascular Disease*, vol. 2, no. 11, pp. 647–648, 2004 (Chinese).
- [14] H.-J. Yin, Y. Zhang, L.-H. Yang, G.-R. Bai, D.-Z. Shi, and K.-J. Chen, "The effects of PQS on glucose transport, GLUT4 translocation and CAP mRNA expression of adipocytes," *Chinese Pharmacological Bulletin*, vol. 23, no. 10, pp. 1332–1337, 2007 (Chinese).
- [15] Y. Zhang, K. J. Chen, L.-H. Yang et al., "Effects of panax quinquefolium saponin of stem and leaf on glucose-lipid metabolism and insulin signal transduction in insulin resistant model adipocytes," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 30, no. 7, pp. 748–751, 2010 (Chinese).
- [16] B.-J. Fan, F. Fei, and X.-Z. Zhao, "Effects of panax quinquefolium saponin (PQS) on the vascular endothelial function of the myocardial hypertrophied rats," *Chinese Journal of Gerontology*, vol. 29, no. 7, pp. 811–812, 2009 (Chinese).
- [17] Y. Zhang, *Effect of Panax Quinquefolius Saponin on Insulin Sensitivity in Patients of Coronary Heart Disease and Its Mechanism*, China Academy of Chinese Medical Sciences, 2006.
- [18] J.-S. Wang, H.-J. Yin, C.-Y. Guo et al., "Influence of high blood glucose fluctuation on the endothelial function of type 2

- diabetes mellitus rats and the effects of panax quinquefolius saponin of stem and leaf," *Chinese Journal of Integrative Medicine*, vol. 19, no. 3, pp. 217–222, 2013.
- [19] I. Galetic., M. Andjelkovic., R. Meier, D. Brodbeck, J. Park, and B. A. Hemmings, "Mechanism of protein Kinase B activation by insulin/Insulin-like growth Factor-1 revealed by specific inhibitors of phosphoinositide 3-kinase-significance for diabetes and cancer," *Pharmacology and Therapeutics*, vol. 82, no. 2-3, pp. 409–425, 1999.
- [20] R. Hernandez, T. Teruel, and M. Lorenzo, "Akt mediates insulin induction of glucose uptake and up-regulation of GLUT4 gene expression in brown adipocytes," *FEBS Letters*, vol. 494, no. 3, pp. 225–231, 2001.
- [21] F. Tremblay, C. Lavigne, H. Jacques, and A. Marette, "Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (zeta/lambda) activities," *Diabetes*, vol. 50, no. 8, pp. 1901–1910, 2001.
- [22] K. Vosseller, L. Wells, M. D. Lane, and G. W. Hart, "Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 8, pp. 5313–5318, 2002.
- [23] N. Gousseva, K. Kugathasan, C. N. . Chesterman, and L. M. . Khachigian, "Early growth response factor-1 mediates insulin-inducible vascular endothelial cell proliferation and regrowth after injury," *Journal of Cellular Biochemistry*, vol. 81, no. 3, pp. 523–534, 2001.
- [24] T. Shioi, J. R. McMullen, P. M. Kang et al., "Akt/protein kinase B promotes organ growth in transgenic mice," *Molecular and Cellular Biology*, vol. 22, no. 8, pp. 2799–2809, 2002.
- [25] A. De Luca, M. R. Maiello, A. D'Alessio, M. Pergameno, and N. Normanno, "The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches," *Expert Opinion on Therapeutic Targets*, vol. 16, supplement 2, pp. S17–S27, 2012.
- [26] S. Varma, B. K. Lal, R. Zheng et al., "Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction," *American Journal of Physiology*, vol. 289, no. 4, pp. H1744–H1751, 2005.
- [27] W. Zhang, R. Wang, S.-F. Han et al., " $\alpha$ -Linolenic acid attenuates high glucose-induced apoptosis in cultured human umbilical vein endothelial cells via PI3K/Akt/eNOS pathway," *Nutrition*, vol. 23, no. 10, pp. 762–770, 2007.
- [28] E. A. Jaffe, R. L. Nachman, C. G. Becker, and C. R. Minick, "Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria," *The Journal of Clinical Investigation*, vol. 52, no. 11, pp. 2745–2756, 1973.
- [29] H. Shamon, H. Duffy, N. Fleischer et al., "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus," *The New England Journal of Medicine*, vol. 329, no. 14, pp. 977–986, 1993.
- [30] A. Ceriello, K. Esposito, L. Piconi et al., "Oscillating glucose is more deleterious to endothelial function and oxidative stress than mean glucose in normal and type 2 diabetic patients," *Diabetes*, vol. 57, no. 5, pp. 1349–1354, 2008.
- [31] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.
- [32] S. E. Siegelar, F. Holleman, J. B. L. Hoekstra, and J. H. DeVries, "Glucose variability; does it matter?" *Endocrine Reviews*, vol. 31, no. 2, pp. 171–182, 2010.
- [33] E. S. Kilpatrick, A. S. Rigby, and S. L. Atkin, "Glucose variability and diabetes complication risk: we need to know the answer," *Diabetic Medicine*, vol. 27, no. 8, pp. 868–871, 2010.
- [34] A. Wakil, K. A. Smith, S. L. Atkin, and E. S. Kilpatrick, "Short-term glucose variability in healthy volunteers is not associated with raised oxidative stress markers," *Diabetes, Obesity & Metabolism*, vol. 14, no. 11, pp. 1047–1049, 2012.
- [35] M. Cini, R. G. Fariello, A. Bianchetti, and A. Moretti, "Studies on lipid peroxidation in the rat brain," *Neurochemical Research*, vol. 19, no. 3, pp. 283–288, 1994.
- [36] T. Luo and Z. Xia, "A small dose of hydrogen peroxide enhances tumor necrosis factor-alpha toxicity in inducing human vascular endothelial cell apoptosis: reversal with propofol," *Anesthesia and Analgesia*, vol. 103, no. 1, pp. 110–116, 2006.
- [37] I. Kim, H.-G. Kim, J.-N. So, J. H. Kim, H. J. Kwak, and G. Y. Koh, "Angiopietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway," *Circulation Research*, vol. 86, no. 1, pp. 24–29, 2000.
- [38] D. Fulton, J. P. Gratton, T. J. McCabe et al., "Regulation of endothelium-derived nitric oxide production by the protein kinase Akt," *Nature*, vol. 399, no. 6376, pp. 597–601, 1999.
- [39] F. M. Ho, W. W. Lin, B. C. Chen et al., "High glucose-induced apoptosis in human vascular endothelial cells is mediated through NF- $\kappa$ B and c-Jun NH2-terminal kinase pathway and prevented by PI3K/Akt/eNOS pathway," *Cellular Signalling*, vol. 18, no. 3, pp. 391–399, 2006.
- [40] K.-W. Park, H.-M. Yang, S.-W. Youn et al., "Constitutively active glycogen synthase kinase-3 $\beta$  gene transfer sustains apoptosis, inhibits proliferation of vascular smooth muscle cells, and reduces neointima formation after balloon injury in rats," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 8, pp. 1364–1369, 2003.

## Research Article

# Qi-Shen-Yi-Qi Dripping Pills for the Secondary Prevention of Myocardial Infarction: A Randomised Clinical Trial

Hongcai Shang,<sup>1</sup> Junhua Zhang,<sup>1</sup> Chen Yao,<sup>2</sup> Baoyan Liu,<sup>3</sup> Xiumei Gao,<sup>1</sup> Ming Ren,<sup>1</sup>  
Hongbao Cao,<sup>1</sup> Guohua Dai,<sup>4</sup> Weiliang Weng,<sup>5</sup> Sainan Zhu,<sup>2</sup> Hui Wang,<sup>1</sup>  
Hongjuan Xu,<sup>1</sup> and Boli Zhang<sup>1</sup>

<sup>1</sup> Tianjin University of Traditional Chinese Medicine, 312 Anshanxi Road, Nankai District, Tianjin 300193, China

<sup>2</sup> Peking University First Hospital, Beijing 100034, China

<sup>3</sup> Chinese Academy of Traditional Chinese Medicine, Beijing 100700, China

<sup>4</sup> Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250011, China

<sup>5</sup> Xiyuan Hospital, Beijing 100091, China

Correspondence should be addressed to Hongcai Shang; [shanghongcai@foxmail.com](mailto:shanghongcai@foxmail.com) and Boli Zhang; [zhangbolipr@163.com](mailto:zhangbolipr@163.com)

Received 20 March 2013; Accepted 25 May 2013

Academic Editor: Hao Xu

Copyright © 2013 Hongcai Shang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Background.** Several types of drugs have been recommended for the secondary prevention of myocardial infarction (MI). However, these conventional strategies have several limitations, such as low adherence, high cost, and side effects during long time use. Novel approaches to this problem are still needed. This trial aimed to test the effectiveness and safety of Qi-Shen-Yi-Qi Dripping Pills (QSYQ), a multi-ingredient Chinese patent medicine, for the secondary prevention of MI. **Methods and Findings.** A total of 3505 eligible patients were randomly assigned to QSYQ group (1746 patients) or aspirin group (1759). Patients took their treatments for 12 months. The final follow-up visit took place 6 months after the end of the trial drugs. The 12-month and 18-month estimated incidences of the primary outcome were 2.98% and 3.67%, respectively, in the QSYQ group. The figures were 2.96% and 3.81% in the aspirin group. No significant difference was identified between the groups. **Conclusions.** This trial did not show significant difference of primary and secondary outcomes between aspirin and QSYQ in patients who have had an MI. Though inconclusive, the result suggests that QSYQ has similar effects to aspirin in the secondary prevention of MI.

## 1. Introduction

Acute myocardial infarction is a leading cause of death worldwide [1]. More than 7 million people a year have a myocardial infarction (MI) [2]. Over the past three decades, MI has emerged from an illness seen predominantly in developed countries to more common in developing countries [2–4]. Progresses in emergency management have led to substantial reductions in the mortality rate of acute MI [2]. However, survivors from acute MI remain at greatly increased risk of serious vascular events [2, 5]. Thus, secondary prevention aimed to decrease mortality and morbidity in survivors after acute MI is of great, and increasing, significance around the world.

Platelets play a key role in the development of thrombotic and ischemic diseases [6]. Antiplatelet therapy is a major

strategy for treating and preventing MI. Anti-platelet drugs have been shown to have definite and substantial net benefits for people who have occlusive vascular disease [7]. Aspirin is a safety and most cost-effective one of the anti-platelet drugs [8]. Current guidelines recommend low-dose aspirin (75–150 mg daily) for the secondary prevention of MI in many countries. However, there are several limitations related to this drug. Long-term therapy with aspirin is associated with an increase in the incidence of symptomatic peptic ulcer, duodenal ulcers, and gastrointestinal and intracranial haemorrhage, even when used at low doses or in buffered or enteric-coated formulations [9, 10]. In a population-based cohort with 4.1 million citizens in Italy, aspirin increased the risk of major gastrointestinal or cerebral bleeding episodes; patients with diabetes had a high rate of bleeding [11]. Furthermore,

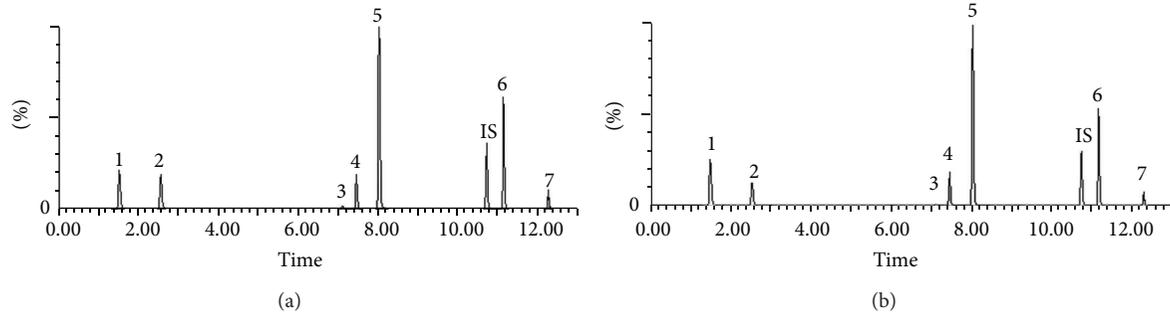


FIGURE 1: The typical chromatograms of mixed standard solution (a) and sample solution of “Qi-Shen-Yi-Qi” Dripping Pills (b). Numbers 1–7 represent danshensu, protocatechuic-aldehyde, salviolic acid B, notoginsenoside R1, ginsenoside Rg1, ginsenoside Rb1, and astragaloside IV, respectively. IS represents digoxin. (Redrew for publication).

aspirin resistance has become a notable problem. Several studies have found that about 1 in 4 individuals may express biochemically defined aspirin resistance [12–14]. Patients who are resistant to aspirin are at greater risks of recurrent serious vascular events than those who are sensitive to aspirin [13, 14].

Current anti-platelet therapies are generally based on a specific signaling pathway in platelet activation, that is, single agent acting on single target [6]. Hence, the limitations associated with aspirin also exist for other anti-platelet agents, such as clopidogrel [6]. Agents with multiple ingredients acting on multiple targets may be more effective and less harmful [15].

In Traditional Chinese Medicine (TCM), the key pathogenesis of MI is mainly “Qixu” (vital energy deficiency) and “Xueyu” (blood stagnation), that is, degradation of body function and thrombosis. Qi-Shen-Yi-Qi Dripping Pills (QSYQ), a Chinese patent medicine for adding “qi” and resolving stasis, was approved for clinical use for coronary heart disease and MI rehabilitation by the State Food and Drug Administration of China in 2003. QSYQ is made of extractions from Danshen (*Radix Salviae Miltiorrhizae*), Sanqi (*radix notoginseng*), Jiangxiang (*Lignum Dalbergiae Odoriferae*), and Huangqi (*radix astragali*). The quality control of QSYQ is good. Herbal materials were cultivated according to the Good Agriculture Practices, and manufacturing processes strictly followed the standard of Good Manufacturing Practices. UPLC-MS/MS was used to analyze seven quality control markers of QSYQ (Figure 1). There was good consistency of the active markers among different batches [16]. Pharmacological studies revealed that constituents of QSYQ could inhibit the platelet aggregation and the overrelease of  $\beta$ -TG [17]. Clinical studies have suggested that QSYQ had similar effect to aspirin in inhibiting platelet aggregation [18]. Currently, QSYQ is widely used for the secondary prevention of MI in China. However, there is insufficient evidence to know whether QSYQ can be used as an alternative to aspirin. This multicenter randomized clinical trial aimed to test whether the regular administration of QSYQ would result in a significant reduction in total serious vascular events in patients who had experienced at least one documented MI.

## 2. Methods

**2.1. Trial Design.** The clinical trial is a multi-center, randomised, double-blind, parallel controlled study. Approval was obtained from the State Administration of Traditional Chinese Medicine of China in 2004. The study protocol was reviewed and approved by the Ethics Committee in Tianjin University of Traditional Chinese Medicine. This trial was registered in the WHO Clinical Trial Registering Platform, number ChiCTR-TRC-00000002 (<http://apps.who.int/trial-search/>). The study protocol was summarized here.

**2.2. Participates.** Patients with a definite diagnosis of ST-elevation or non-ST-elevation MI [19] were potentially eligible for this study if they met the following criteria: (1) the last documented MI was 4 weeks to 24 months earlier; (2) Traditional Chinese Medicine symptoms were “Qixu-Xueyu” (vital energy deficiency combining with blood stagnation); (3) age from 18 years to 75 years (the maximum age was adjusted from 65 years to 75 years in July 2006 because of inadequate recruitment).

Patients were required to be free of other life-threatening diseases or problems which might have limited the ability to obtain long-time followup and to be free of any condition which would mean that regular use of the trial drugs was contraindicated. Patients with either of the following conditions were excluded: (1) a history of percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG); (2) pregnant women or those who were breast feeding; (3) contraindication to aspirin (e.g., asthma, active phase peptic ulcer, and hemorrhagic disease); (4) heart function of grade IV (NYHA grade); (5) uncontrolled systemic hypertension (contractive pressure  $\geq 180$  mmHg or diastolic pressure  $\geq 110$  mmHg); (6) uncontrolled serious cardiac arrhythmias (e.g., atrial fibrillation and supraventricular tachycardia); (7) serious primary disease of liver, kidney, and hemopoietic system, or psychosis, or malignant tumor; (8) allergic history to study drugs; (9) participation in other clinical trials during the previous three months.

After a comprehensive medical evaluation, patients were given a full explanation of the study by investigators. Each patient was asked for their written informed consent before joining the study. Patients were registered if they voluntarily

signed the informed consent. There was no economic compensation for participating patients. Recruitment took place in 88 hospitals throughout the east, west, south, north, and center of China.

**2.3. Intervention.** Patients were randomly assigned to two groups (ratio 1:1): QSYQ group and aspirin group. In the QSYQ group, patients took 0.5 g (one package) QSYQ for three times per day and 100 mg (in four tablets) simulated enteric-coated aspirin once a day. Patients in the aspirin group took 0.5 g (one package) simulated QSYQ three times per day and 100 mg (in four tablets) enteric-coated aspirin daily.

Patients were prohibited from taking other anti-platelet drugs or “*Yiqi-Huoxue*” Chinese medicines during the treatment period. Concomitant medications, such as antihypertensive (e.g.,  $\beta$ -blockers and ACE inhibitors), hypoglycemic agents, and lipid-lowering drugs, could be prescribed at the discretion of the attending physicians and had to be recorded in detail (including drug name, beginning-stopping time, dosage, and purpose).

The treatment period for the trial drugs was 12 months. After this time (or if the trial drugs were stopped for some other reason), patients could be prescribed treatments by their physicians without any limitation.

**2.4. Outcome Measures.** The primary endpoint was a composite of cardiovascular death, nonfatal reinfarction (documented by ECG and enzyme changes), and nonfatal stroke (diagnosed by CT or MRI). The secondary outcomes were the events of serious cardiac arrhythmias, heart failure, cardiac shock, revascularization (PCI or CABG), pulmonary embolism, and deep vein thrombosis.

All reported adverse events during the trial were recorded to allow an assessment of the safety of the treatments. Intracranial bleedings and gastrointestinal complications that have been associated with aspirin were monitored carefully. Haemorrhagic stroke was also considered as an adverse event, although it was included in the composite primary outcome.

**2.5. Followup.** After a first visit for collecting baseline data after randomization, enrolled patients, their dependents, or both were asked to visit clinical centers monthly. If no primary endpoints occurred, there were 12 visits during the treatment period and a final visit (6 months after the termination of the trial drugs). When a patient had one of the primary endpoints, the case was considered completed and there was no further follow-up visit. At each visit, investigators were required to retrieve and provide trial drugs; complete case report forms recording the patient’s condition, endpoints, adverse events, and concomitant drugs; and remind patients not to take prohibited drugs. If a patient did not visit the clinical center at a defined time, the investigators contacted the patient or their dependents to find the reasons.

**2.6. Quality Control Procedures.** The trial was designed, executed, and analyzed by a steering committee, a clinical monitoring center, an endpoints committee, a drug management

center, a data management center, and a biostatistics center. Investigators in each clinical center were trained before study beginning.

Concealment of the random allocation was achieved by using a Clinical Research Interactive Voice Respond System (CRIVRS). Investigators connected to the CRIVRS by telephone when a patient was ready to be randomised and the CRIVRS then provided a subject number, randomisation code, and drug number to the investigator by voice, email, and SMS. Because different dosage forms of two study drugs, a double-dummy design was used with placebos to blind patients and their healthcare providers. Placebos for QSYQ and aspirin were developed which had the same appearance, color, and taste as the relevant drug. One statistician who generated the blinding code was aware of the drug allocation, but patients, investigators, and other practitioners in this trial were all blinded during the whole study period.

**2.7. Statistical Analyses.** Due to a paucity of clinical data of QSYQ for the prevention of MI, the sample size for this study was estimated on the basis of practical considerations. The incident of recurred serious cardiovascular and cerebrovascular events was about 6.5% in patients who suffered from MI and used aspirin for secondary prevention [8]. It was supposed that QSYQ might have equal effectiveness compared with aspirin. The margin of equivalence ( $\Delta$ ) was 2.5% for primary endpoint. The sample size of 3600 subjects was calculated to be sufficient to establish equivalence (two sides  $\alpha = 0.05$  and 80% power; less than 20% loss to followup).

Statistical analyses were conducted with SAS 9.1.3. The trial database was blind reviewed before the data were locked and unblinded. Data analyses were mainly based on the intention-to-treat (ITT) principle: with all randomized patients and all endpoints being analysed in accordance with the patients’ allocated treatment group. A per-protocol (PP) analysis was performed to test the robust of the trial’s results.

The baseline characteristics of the two treatment groups were compared by the *t*-test (for continuous data) and the chi-square test (for categorical data). Cox proportional-hazards regression model was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) for the primary endpoint. Cumulative primary endpoints curves were constructed by Kaplan-Meier methods and differences between the curves were tested using the log-rank method. A  $P < 0.05$  level (two sided) was adopted as the test for statistical significance. No subgroup analysis was prescheduled. Secondary outcome events and adverse events were analysed, and the intergroup comparisons were analyzed by using chi-square test.

### 3. Results

A total of 3505 patients from 88 clinical centers (hospitals) throughout China were recruited and randomly assigned. There were 1746 patients in the QSYQ group and 1759 in the aspirin group (Figure 2, according to CONSORT 2010). All randomised patients were included in ITT analyses and the 2956 patients who complied with the trial protocol were included in the PP analyses.

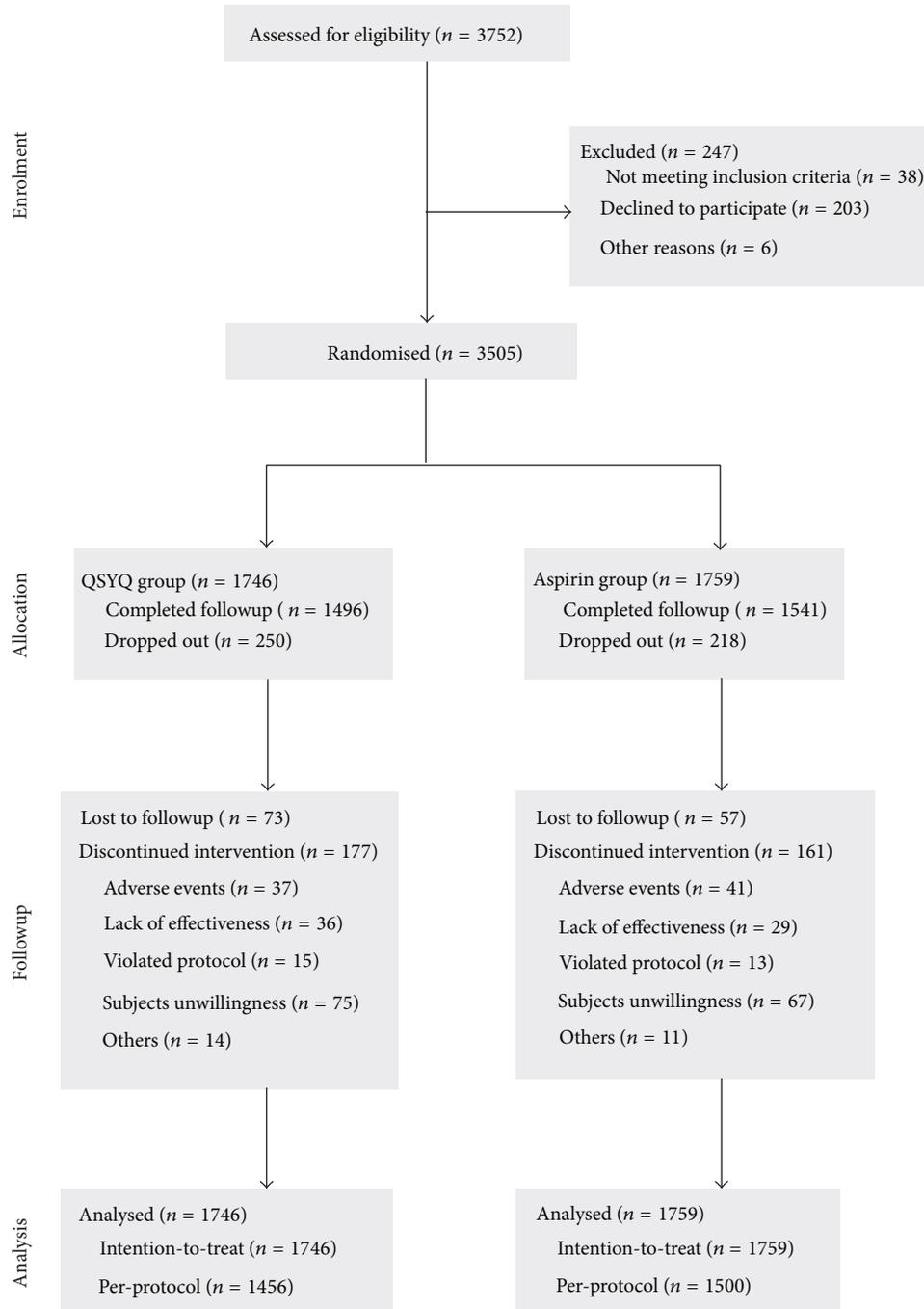


FIGURE 2: Flow diagram of participants through each stage of the trial.

**3.1. Patients' Characteristics.** Clinical characteristics of the included patients were shown in Table 1. In general, these baseline characteristics were similar in the two groups. The mean age of included patients was 58 years. Most of the patients were more than 12 months since their latest MI (3287 patients, 93.8%). Most of the patients had an ST-segment-elevation MI, including 1109 (31.6%) anterior MIs and 1558 (44.45%) inferior MIs. About a third of patients had a body

mass index (BMI) greater than 25 Kg/m<sup>2</sup> and were considered to be overweight. About 70% of the participants were males. The aspirin group has a higher proportion of men than the QSYQ group which achieved statistical significance ( $P = 0.027$ ). This imbalance for gender also led to differences in the history of smoking ( $P = 0.048$ ) and alcohol consumption ( $P = 0.036$ ). Therefore, gender was adjusted for sensitivity analysis, by using Cox regression analysis. There were no

TABLE 1: Baseline characteristics of included patients.

Proportion	QSYQ (n = 1746)	Aspirin (n = 1759)
Age at entry (years)		
Mean (SD)	58.35 (9.02)	58.28 (8.99)
≤60	910 (52.12%)	921 (52.36%)
>60	836 (47.88%)	838 (47.64%)
Gender—male*	1191 (68.21%)	1260 (71.63%)
Smoking*	773 (44.27%)	840 (47.75%)
Alcohol consumption*	505 (28.92%)	568 (32.29%)
Nationality-Han	1682 (96.33%)	1680 (95.5%)
BMI (Kg/m <sup>2</sup> )		
Mean (SD)	24.24 (2.82)	24.28 (2.86)
<25	1125 (64.43%)	1122 (63.79%)
≥25	621 (35.57%)	637 (36.21%)
Time since latest MI (months)		
<6	13 (0.74%)	12 (0.68%)
6–12	91 (5.21%)	102 (5.80%)
>12	1642 (94.04%)	1645 (93.52%)
Type of MI		
Non-ST-elevation MI	90 (5.15%)	69 (3.92%)
Mainly anterior MI	529 (30.30%)	580 (32.97%)
Mainly inferior MI	766 (43.87%)	792 (45.03%)
Medical history <sup>#</sup>		
Hyperlipidemia	683 (39.12%)	680 (38.66%)
Hypertension	753 (43.13%)	758 (43.09%)
Diabetes mellitus	219 (12.54%)	227 (12.91%)
Stroke	121 (6.93%)	106 (6.03%)
Gastritis	227 (13.00%)	212 (12.05%)
Medications before entry <sup>†</sup>		
Anti-platelet agents	1417 (81.16%)	1398 (79.48%)
Beta-blockers	786 (45.02%)	800 (45.48%)
ACE inhibitors	666 (38.14%)	669 (38.03%)
Lipid-lowering agents	723 (41.41%)	705 (40.08%)
Calcium blockers	238 (13.63%)	231 (13.13%)
Diuretics	107 (6.13%)	90 (5.12%)
Cardiotonic agents	40 (2.29%)	40 (2.27%)
Antiarrhythmia agents	26 (1.49%)	29 (1.65%)
Nitrates	1268 (72.62%)	1255 (71.35%)
Chinese patent medicines <sup>§</sup>	620 (35.51%)	636 (36.16%)

SD: standard deviation; MI: myocardial infarction; BMI: body mass index.

\*The intergroup difference is statistically significant ( $P < 0.05$ ).

<sup>#</sup>If a patient had more than one condition, they were counted for each of the different diseases.

<sup>†</sup>If a patient took more than one drug, they were counted for each of the different drugs.

<sup>§</sup>Chinese patent medicines were mainly used for heart disease.

inter-group differences in important prognostic factors such as medical histories, concomitant medications before entry, and medications during the treatment period.

**3.2. Assessment of Patient Adherence.** Of the included 3505 patients, 468 patients (218 in aspirin group and 250 in QSYQ group) without any recorded primary endpoint events did not complete their scheduled follow-up visits during the 12-month treatment period (Figure 2). Fifteen patients in the QSYQ group and 10 patients in the aspirin group used anti-platelet drugs for more than one week in treatment period. The figures for prohibited Chinese herbal medicines were 33 in the QSYQ group and 28 in the aspirin group. These patients who withdrew during the treatment period or who took prohibited drugs were excluded from the PP analyses. Pill counting was used to assess patients' adherence to study prescriptions and revealed an average adherence of 95% (95% of the trial drugs were taken). This suggested that patients' adherence to the trial protocol was good.

**3.3. Primary Outcomes.** Table 2 presents the primary outcome (composite endpoints of cardiovascular death, non-fatal MI, and non-fatal stroke) at the two scheduled evaluation points: after completion of 12 months of treatment and at the 6 months followup following the end of study treatments. There are a total of 104 composite endpoints after 12-month treatment, including 51 cardiovascular deaths, 37 non-fatal MIs, and 16 non-fatal strokes. When the 6-month posttreatment followup was completed, the composite endpoints increased to 131 cases, including 61 cardiovascular deaths, 52 non-fatal MIs, and 18 strokes. The 12-month and 18-month incidences of the primary outcome were 2.98% and 3.67% in the QSYQ group, respectively. The figures were 2.96% and 3.81% in the aspirin group. The differences between the two treatment groups were not statistically significant. Although minor changes to the results occurred after adjustment for gender, these changes did not make any meaningful difference to the results.

Cumulative analysis of the primary endpoints showed that the two curves were close during the 18-month followup (Figure 3). The hazard ratio (HR) for the primary outcome (QSYQ group versus aspirin group) was 0.977 (95% CI: 0.694, 1.377;  $P = 0.895$ ). The PP analysis generated a similar result (HR: 0.970; 95% CI: 0.682, 1.379;  $P = 0.865$ ).

**3.4. Secondary Outcomes.** Secondary outcome measures were shown in Table 3. During the 12-month treatment, there were a total of 42 (2.41%) secondary endpoints in the QSYQ group and 43 events (2.44%) in the aspirin group. After the 18-month followup had been completed, these incidences of secondary endpoints increased to 2.98% and 3.30%, respectively. There was no significant difference between the two groups at either of the two evaluation points.

**3.5. Adverse Effects of Trial Drugs.** Some potential adverse effects of aspirin and QSYQ were reported by patients as complaints or by the judgment of the investigator (Table 4). Nine patients in the aspirin group had a minor hemorrhage (two hemorrhagic strokes) compared to 2 patients (one hemorrhagic stroke) in the QSYQ group. There was no serious bleeding episode which required transfusion or caused death. Patients in the aspirin group had more gastric

TABLE 2: Composite endpoints of cardiovascular death, nonfatal myocardial infarction, and nonfatal stroke (intention-to-be-treat analyses).

Composite endpoints	QSYQ ( <i>n</i> = 1746)	Aspirin ( <i>n</i> = 1759)	HR (95% CI)	<i>P</i> value	HR (95% CI) Adjusted for gender	<i>P</i> value Adjusted for gender
12-month treatment completed						
Composite endpoints	52 (2.98%)	52 (2.96%)	1.02 (0.69, 1.50)	0.928	1.03 (0.70, 1.52)	0.872
Cardiovascular death	28 (1.60%)	23 (1.31%)	1.24 (0.71, 2.15)	0.450	1.26 (0.73, 2.19)	0.411
Nonfatal MI	18 (1.03%)	19 (1.08%)	0.97 (0.51, 1.84)	0.917	0.98 (0.51, 1.87)	0.952
Nonfatal stroke	6 (0.34%)	10 (0.57%)	0.61 (0.22, 1.68)	0.340	0.61 (0.22, 1.68)	0.339
6-month followup after study drugs terminated						
Composite endpoints	64 (3.67%)	67 (3.81%)	0.98 (0.69, 1.38)	0.895	0.99 (0.70, 1.40)	0.957
Cardiovascular death	31 (1.78%)	30 (1.71%)	1.05 (0.64, 1.74)	0.836	1.07 (0.65, 1.77)	0.782
Nonfatal MI	26 (1.49%)	26 (1.48%)	1.03 (0.60, 1.77)	0.925	1.04 (0.60, 1.79)	0.891
Nonfatal stroke	7 (0.40%)	11 (0.63%)	0.65 (0.25, 1.68)	0.373	0.65 (0.25, 1.68)	0.377

TABLE 3: Secondary outcome measure after 12-month treatment and 6-month followup after study drugs terminated.

Secondary endpoints	12-month treatment completed			18-month followup accomplished		
	QSYQ ( <i>n</i> = 1746)	Aspirin ( <i>n</i> = 1759)	HR (95% CI)	QSYQ ( <i>n</i> = 1746)	Aspirin ( <i>n</i> = 1759)	HR (95% CI)
Revascularization	21 (1.20%)	18 (1.02%)	1.18 (0.63, 2.20)	23 (1.32%)	26 (1.48%)	0.89 (0.51, 1.56)
Aggravated heart failure	20 (1.15%)	23 (1.31%)	0.88 (0.48, 1.59)	28 (1.60%)	28 (1.59%)	1.01 (0.60, 1.69)
Cardiac shock	1 (0.06%)	1 (0.06%)	1.01 (0.06, 16.09)	1 (0.06%)	2 (0.11%)	0.50 (0.05, 5.55)
Deep vein thrombosis	0 (0)	1 (0.06%)	—	0 (0)	2 (0.11%)	—
Sum up	42 (2.41%)	43 (2.44%)	0.98 (0.65, 1.50)	52 (2.98%)	58 (3.30%)	0.90 (0.62, 1.31)

TABLE 4: Adverse effects potentially associated with aspirin and Qi-Shen-Yi-Qi Dripping Pills.

Adverse effects	QSYQ group ( <i>n</i> = 1746)	Aspirin group ( <i>n</i> = 1759)	<i>P</i> value
Hemorrhage	2 (0.11%)	9 (0.51%)	0.06
Intracranial bleeding	1 (0.06%)	2 (0.11%)	—
Gastrointestinal bleeding	0 (0)	4 (0.23%)	—
Subcutaneous hemorrhage	1 (0.06%)	3 (0.17%)	—
Stomach pain	38 (2.18%)	37 (2.1%)	0.88
Gastric acid reflux	14 (0.80%)	33 (1.88%)	0.007
Anaphylaxis	11 (0.63%)	7 (0.40%)	0.34

acid reflux (1.88%) than those in the QSYQ group (0.8%). This difference is statistically significant ( $P = 0.007$ ). The incidences of stomach pain and merely allergic reactions were similar between the two groups. These data confirm that both study drugs are of good safety, and QSYQ appears to be slightly safer than aspirin.

#### 4. Discussion

In this trial, QSYQ shows similar effects to aspirin for the prevention of recurrent vascular events in patient with a previous MI. The rate of composite endpoints (cardiovascular death, non-fatal MI, and non-fatal stroke) after 12-month treatment was 2.98% in QSYQ group and 2.96% in aspirin group. The incidence of serious vascular events of this trial is lower than previous studies of secondary prevention for MI [8], which may be due to several factors.

First, this study included low-risk patients compared to previous studies: patients who had experienced PCI and

CABG were excluded, and the time since latest MI was more than 12 months in most (93.8%) of the patients in this trial. Thus, the risk of recurrent vascular events might be decreased. Second, although anti-platelet agents and “Yiqi-Huoxue” Chinese herbal medicines were prohibited during the trial, other drugs including beta-blockers, ACE inhibitors, lipid-lowering drugs, and drugs for hypertension and diabetes were allowed. Third, the follow-up period is short. The three factors are likely to be the main reasons for the low incidence of serious vascular events. In the future, a study with a more broadly inclusive eligibility criteria and longer study duration should be adopted.

Modern management of myocardial infarction is built on a high quality, clinical evidence base. Anti-platelet, beta-blockers, ACE inhibitors, and statins have been recommended for clinical practice based on the results of randomized clinical trials and their systematic review and have substantially reduced mortality and morbidity associated with MI. However, most of these conventional drugs are

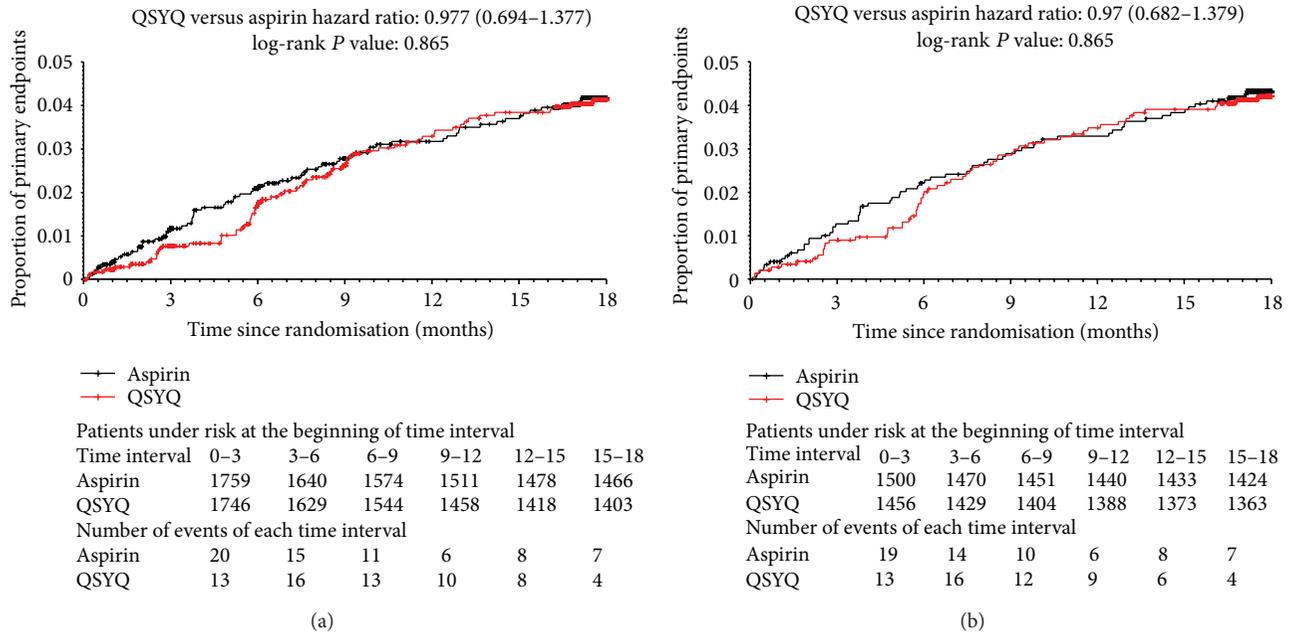


FIGURE 3: Cumulative incidence curves of the primary outcome composed of cardiovascular death, non-fatal myocardial infarction, and non-fatal stroke ((a) intention-to-treat analysis; (b) per-protocol analysis).

based on specific pathways, mainly single drug acting on single target [6, 20]. This means that a patient might need to take several drugs concurrently, which leads to new problems, such as low adherence, high cost, and more adverse effects [21]. A new strategy for the management of patients following a myocardial infarction is needed.

The concept of a “polypill” was developed about 10 years ago, with a compound pill including several recommended drugs [22, 23]. The Indian Polycap Study showed that a Polypill, composed of hydrochlorothiazide, atenolol, ramipril, simvastatin, and aspirin, had the desired effects and was safe as the individual pills [24]. Recently, a clinical trial in Sri Lanka, sponsored by the World Health Organization, has shown high acceptability of the Polypill to patients and physicians [25]. The Polypill is a new concept in western medicine but is not new in eastern medicine. QSYQ, which contains several kinds of active ingredient, is a classical polypill [26]. Over the past five years, in vivo and in vitro studies have revealed the integrated effects of QSYQ for MI, including protecting cardiac muscle cells [27], preventing cardiac ischemia-reperfusion injury via energy modulation [28], antagonizing ventricular remodeling [29], inhibiting inflammatory reaction and preventing the progress of atherosclerosis [30], and stabilizing atherosclerotic plaque by changing histological constitution [31]. A series of published studies suggested that QSYQ was a promising drug for secondary prevention of MI. However, the effects and mechanisms are not conclusive and need further studies to confirm and resolve uncertainties.

This study has some limitations. First, patients who had experienced PCI and CABG were excluded from this trial. This criterion limited the size of the sample and its representativeness. Second, biochemical measures (e.g., blood lipids) were not scheduled in this trial, in order to improve

patient’s adherence to followup. As a consequence, the effects of study drugs on these intermediate outcome measures were unknown. Third, our study was undertaken in China; whether the relative effects of the trial drugs would be similar in other ethnic groups is unknown.

Our study has several strengths. This is the first multi-center trial sponsored by the national funding of China and organized by researchers of Traditional Chinese Medicine to evaluate a Chinese patent medicine for the secondary prevention of MI. Randomization and blinding were well performed by using CRIVIS. Organization, execution, and evaluation were managed by different committees which worked independently.

## 5. Conclusion

This large sample clinical trial shows that QSYQ has similar effects to aspirin in the secondary prevention of MI and has fewer adverse effects. However, the low event rates of outcome measures were insufficient to generate a confirmatory conclusion. In addition, the polypill-like effect of QSYQ should be researched step by step. Further rigorously designed studies are warranted.

## Conflict of Interests

There is no conflict of interests.

## Authors’ Contribution

All authors were involved in the trial design, the interpretation of the results, and in making revisions and corrections to the paper. The steering committee designed and revised

the trial protocol. The coordinating center selected clinical centers and investigators and ensured that these departments worked together smoothly. The drug management center was in charge of producing, distributing, and monitoring study drugs and placebos. The clinical monitoring center checked the quality of trial documents. The endpoints committee verified all endpoints and adverse events, according to previously defined criteria. The biostatistics center was responsible for data analysis. The data management center was in charge of data capture, input, and preservation. *MISPS collaborative group members are as follows. Steering committee*—Tianjin University of Traditional Chinese Medicine (B. L. Zhang, X. M. Gao); Peking University First Hospital (C. Yao); The First Teaching Hospital of Tianjin University of TCM (J. P. Zhang, J. Y. Mao). *Coordinating center*—Tianjin University of Traditional Chinese Medicine (H. C. Shang, J. H. Zhang, H. Wang, H. J. Xu, W. K. Zheng, L. Zhang). *Data management center*—Chinese Academy of Traditional Chinese Medicine (B. Y. Liu, T. C. Wen). *Biostatistics center*—Peking University First Hospital (C. Yao, S. N. Zhu). *Clinical monitoring center*—Xiyuang Hospital (W. L. Weng); Tianjin University of Traditional Chinese Medicine (H. B. Cao). *End-points committee*—The Second Affiliated Hospital of Tianjin University of TCM (L. J. Sun). *Drug management center*—Tianjin University of Traditional Chinese Medicine (M. Ren).

## Acknowledgments

The Ministry of Science and Technology of China (no. 2004BA716B01) and the State Administration of Traditional Chinese Medicine of China provided funding for this study (no. 200807004). They had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The authors are grateful to the participants in the study and to the doctors, nurses, and administrative staff in the recruiting hospitals. *Investigators who recruited at least 10 patients, by region and hospital, are as follows: Tianjin*—The First Teaching Hospital of Tianjin University of TCM (J. Y. Mao, Z. Q. Zhao); The Second Affiliated Hospital of Tianjin University of TCM (W. X. Du, Y. Q. Zhao, T. Wang, C. Y. Liu); Tianjin Union Medicine Centre (K. Q. Liu, R. Wang); Tianjin Nankai Chinese Medicine Hospital (S. Gao, R. Y. Yuan); Tianjin WuQing Chinese Medicine Hospital (L. Q. Zhang, X. F. Liu); Tianjin Beichen Chinese Medicine Hospital (L. Shi); Tianjin Chinese Medicine Hospital (R. H. Fan, J. Zhang); The Second Hospital of Tianjin Medical University (L. F. Li, G. P. Li); Jixian People's Hospital (R. C. Xu, X. M. Zhou); Tianjin Dagang Hospital (X. Z. Zhou). *Shandong*—The Affiliated Hospital of Shandong University of TCM (H. J. Lin); Rizhao Hospital of TCM (X. G. Wang, S. M. Li); Qindao Hospital of TCM (L. B. Wei); Zibo Hospital of TCM (S. Q. Wang); Weifang Traditional Chinese Medicine Hospital (K. Q. Tang, K. G. Luan); The Affiliated Hospital of Medical College Qingdao University (Y. Li); Shandong Provincial Hospital (A. Y. Li, G. M. Si); Laiwu People's Hospital (Z. Y. Liang); Jinan Municipal Hospital of Traditional Chinese Medicine (H. Xu, X. J. Feng). *Heilongjiang*—First Affiliated Hospital of Heilongjiang University of Traditional Chinese Medicine

(Y. B. Zhou, B. Chen); Haerbin Hospital of Traditional Chinese Medicine (X. K. Liu, H. H. Wu); Qiqihaer Hospital of Traditional Chinese Medicine (B. Qi, J. Y. Li); The Third Affiliated Hospital of Qiqihaer Medical College (H. M. Liu, S. Sun); Jiamusi Hospital of Traditional Chinese Medicine (B. Sun); Mudanjiang Hospital of Traditional Chinese Medicine (S. K. Wang, X. F. Zhang). *Beijing*—Peking University First Hospital (X. M. Wang, G. X. Liu); Beijing Fu Wai Hospital (J. Z. Qu, L. H. Ma). *Gansu*—Gansu College of Traditional Chinese Medicine (Z. S. Jin, Y. K. Zhang); Gansu Province Hospital of Traditional Chinese Medicine (H. D. Deng, S. Zhou); Medical Department of Jiayuguan First Hospital; Wuwei People's Hospital (C. Y. Li, P. J. Wang); Tianshui Traditional Chinese Medical Hospital (J. C. Zhang, B. G. Cao); Jiuquan City People's Hospital (Y. F. Yuan); Jiayuguan City Jiugang Hospital (Y. J. Zhao); Gansu Province Cadres Medical Care Hospital (W. Huang, R. J. Yang); First Affiliated Hospital of Medical College of Shihezi University (W. G. Bian); Qingyang City People's Hospital (Z. Z. Liu, Y. F. Li); People's Hospital of Zhangye Municipality (G. Y. An, Z. Y. Yan). *Liaoning*—The Affiliated Hospital of Liaoning University of TCM (G. L. Yang, J. Zhang, F. R. Wang); The Second Affiliated Hospital of Liaoning University of TCM (B. Dong, D. H. Wang); Anshan Traditional Chinese Medical Hospital (J. T. Song, L. Cao); Dalian Traditional Chinese Medical Hospital (Y. L. Du, X. X. Yin); Dandong Traditional Chinese Medical Hospital (E. Wang, Z. R. Han). *Jilin*—The First Hospital of Jilin University (X. Z. Zhao); Meihekou Hospital of Traditional Chinese Medicine (Z. H. Cai, G. J. Wang). *Xian*—Xijing Hospital (Z. X. Dai, G. D. Shen); The 323 Liberation Army Hospital (H. M. Li); The 451 Liberation Army Hospital (B. X. Tuo, Y. Y. Che); Xi'an Jungong Hospital (B. X. Liu, L. K.). *Shanxi*—Shanxi Traditional Chinese Medical Hospital (T. F. Niu, H. X. Qi); Taiyuan City Centre Hospital (X. P. Chen); The Third Hospital of Shanxi Traditional Chinese Medical College (L. X. Ji, T. S. Zhang). *Inner Mongolia*—Inner Mongolia Medical College (X. P. Yang); Inner Mongolia Chinese-Mongolian Hospital (C. F. Liu, Y. Gao); Inner Mongolia Hospital (Z. Wu, A. G. Li); The Affiliated People's Hospital of Inner Mongolia Medical College (G. Liu, S. G. Zhang); Baogang Hospital (M. Q. Zhao, M. Sun). *Hunan*—Affiliated Hospital of Hunan Institute of Traditional Chinese Medicine (X. J. Hu, H. X. Zhu); The Fourth Hospital of Changsha (J. F. Li, Y. M. Yi); Wangyue Subdistrict Community Health Service Centre (S. H. Wen, L. Y. Liu). *Hebei*—Hebei Traditional Chinese Medical Hospital (Z. Q. Chen, H. W. Miao); Baiqiu Peace Hospital (Z. B. Li); Shijiazhuang Traditional Chinese Medical Hospital (Z. Liu); Qinhuangdao Traditional Chinese Medical Hospital (L. R. Chen, H. Y. Wang). *Henan*—Nanyang Centre Hospital (B. Y. Mao); The First Nanyang People's Hospital (X. D. Zhu); Nanyang Traditional Chinese Medical Hospital (L. Q. Liu, D. X. Xie); Nanzhao Traditional Chinese Medical Hospital (Y. F. Zhang, J. C. Li); Henan Nanyang Zhangzhongjing Hospital (R. Ma). *Anhui*—Linquan Traditional Chinese Medical Institute (L. P. Jiang, Q. L. Zhou). *Guangxi*—Guangxi University of Traditional Chinese Medical (X. M. Fang); The First Affiliated Hospital of Guangxi Traditional Chinese Medical University (X. B. He); Ruikang Hospital of Guangxi Traditional Chinese Medical University (J. S. He, J. H. Yue).

## References

- [1] World Health Organization, "The top ten causes of death," Fact sheet, no. 310, 2008.
- [2] H. D. White and D. P. Chew, "Acute myocardial infarction," *The Lancet*, vol. 372, no. 9638, pp. 570–584, 2008.
- [3] J. He, D. Gu, X. Wu et al., "Major causes of death among men and women in China," *New England Journal of Medicine*, vol. 353, no. 11, pp. 1124–1134, 2005.
- [4] J. E. Sanderson, B. Mayosi, S. Yusuf et al., "Global burden of cardiovascular disease," *Heart*, vol. 93, no. 10, p. 1175, 2007.
- [5] M. Moher, "Evidence of the effectiveness of interventions for secondary prevention and treatment of coronary heart disease in primary care—a review of the literature," NHS Executive, Anglia and Oxford Regional Health Authority, Oxford, UK, 1995.
- [6] Y.-Z. Xiang, Y. Xia, X.-M. Gao, H.-C. Shang, L.-Y. Kang, and B.-L. Zhang, "Platelet activation, and antiplatelet targets and agents: current and novel strategies," *Drugs*, vol. 68, no. 12, pp. 1647–1664, 2008.
- [7] C. Baigent, C. Sudlow, R. Collins, and R. Peto, "Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients," *British Medical Journal*, vol. 324, no. 7329, pp. 71–86, 2002.
- [8] Antithrombotic Trialists' Collaboration, "Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials," *The Lancet*, vol. 373, no. 9678, pp. 1849–1860, 2009.
- [9] L. A. García Rodríguez, S. Hernández-Díaz, and F. J. de Abajo, "Association between aspirin and upper gastrointestinal complications: systematic review of epidemiologic studies," *British Journal of Clinical Pharmacology*, vol. 52, pp. 563–571, 2001.
- [10] S. Derry and Y. K. Loke, "Risk of gastrointestinal haemorrhage with long term use of aspirin: meta-analysis," *British Medical Journal*, vol. 321, no. 7270, pp. 1183–1187, 2000.
- [11] G. De Berardis, G. Lucisano, A. D'Etorre et al., "Association of aspirin use with major bleeding in patients with and without diabetes," *Journal of the American Medical Association*, vol. 307, no. 21, pp. 2286–2294, 2012.
- [12] M. M. C. Hovens, J. D. Snoep, J. C. J. Eikenboom, J. G. van der Bom, B. J. A. Mertens, and M. V. Huisman, "Prevalence of persistent platelet reactivity despite use of aspirin: a systematic review," *American Heart Journal*, vol. 153, no. 2, pp. 175–181, 2007.
- [13] J. D. Snoep, M. M. C. Hovens, J. C. J. Eikenboom, J. G. Van Der Bom, and M. V. Huisman, "Association of laboratory-defined aspirin resistance with a higher risk of recurrent cardiovascular events: a systematic review and meta-analysis," *Archives of Internal Medicine*, vol. 167, no. 15, pp. 1593–1599, 2007.
- [14] G. Krasopoulos, S. J. Brister, W. S. Beattie, and M. R. Buchanan, "Aspirin "resistance" and risk of cardiovascular morbidity: systematic review and meta-analysis," *British Medical Journal*, vol. 336, no. 7637, pp. 195–198, 2008.
- [15] Y.-Z. Xiang, L.-Y. Kang, X.-M. Gao, H.-C. Shang, J.-H. Zhang, and B.-L. Zhang, "Strategies for antiplatelet targets and agents," *Thrombosis Research*, vol. 123, no. 1, pp. 35–49, 2008.
- [16] L. Yunfei, Q. Haibin, and C. Yiyu, "Identification of major constituents in the traditional Chinese medicine "QI-SHEN-YI-QI" dropping pill by high-performance liquid chromatography coupled with diode array detection-electrospray ionization tandem mass spectrometry," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 47, no. 2, pp. 407–412, 2008.
- [17] Y. Wang, J. Wang, L. Guo, and X. Gao, "Antiplatelet effects of qishen yiqi dropping pill in platelets aggregation in hyperlipidemic rabbits," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 205451, 5 pages, 2012.
- [18] J. Chen, D. Z. Qu, and R. Y. Wang, "Evaluation of Qishenyiqi Dripping Pills for aspirin resistance in patients with coronary heart disease," *Chinese Traditional and Herbal Drugs*, vol. 39, pp. 96–97, 2008.
- [19] J. S. Alpert, K. Thygesen, E. Antman, and J. P. Bassand, "Myocardial infarction redefined—a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction," *Journal of the American College of Cardiology*, vol. 36, pp. 959–969, 2000.
- [20] Y. Z. Xiang, H. C. Shang, and B. L. Zhang, "Secondary prevention of myocardial infarction and applicable prospect of traditional Chinese medicine," *Medical Recapitulate*, vol. 13, pp. 263–265, 2007.
- [21] G. Sanz, V. Fuster, M.-J. Kravis, H. R. Kravis, and R. Gorlin, "Fixed-dose combination therapy and secondary cardiovascular prevention: rationale, selection of drugs and target population," *Nature Clinical Practice Cardiovascular Medicine*, vol. 6, no. 2, pp. 101–110, 2009.
- [22] N. J. Wald and M. R. Law, "The Indian Polycap Study (TIPS)," *The Lancet*, vol. 374, p. 781, 2009.
- [23] N. J. Wald and M. R. Law, "A strategy to reduce cardiovascular disease by more than 80%," *British Medical Journal*, vol. 326, no. 7404, pp. 1419–1423, 2003.
- [24] S. Yusuf, P. Pais, R. Afzal et al., "Effects of a polypill (Polycap) on risk factors in middle-aged individuals without cardiovascular disease (TIPS): a phase II, double-blind, randomised trial," *The Lancet*, vol. 373, pp. 1341–1351, 2009.
- [25] E. Z. Soliman, S. Mendis, W. P. Dissanayake et al., "A Polypill for primary prevention of cardiovascular disease: a feasibility study of the World Health Organization," *Trials*, vol. 12, article 3, 2011.
- [26] J. Gu, G. Yuan, Y. Zhu, and X. Xu, "Computational pharmacological studies on cardiovascular disease by Qishenyiqi Diwan," *Science in China B*, vol. 39, pp. 1415–1423, 2009.
- [27] S. Q. Lin, X. H. Wei, P. Huang, Y. Y. Liu et al., "QiShenYiQi Pills prevents cardiac ischemia-reperfusion injury via energy modulation," *International Journal of Cardiology*, 2012.
- [28] C. Hong, Y. Wang, J. Lou, Q. Liu, H. Qu, and Y. Cheng, "Analysis of myocardial proteomic alteration after Qishenyiqi formula treatment in acute infarcted rat hearts," *Zhongguo Zhongyao Zazhi*, vol. 34, no. 8, pp. 1018–1021, 2009.
- [29] W. X. Du, M. D. Zhu, L. M. Feng et al., "Intervention effect of Qishenyiqi dripping pills on early ventricular remodeling after acute myocardial infarction," *Chinese Journal of Evidence-Based Cardiovascular Medicine*, vol. 28, pp. 41–43, 2008.
- [30] F. F. Yan, Y. Liu, Y. F. Liu, and Y. X. Zhao, "Effects of Qishenyiqi Dripping pills on high sensitivity C reactive protein in experimental atherosclerosis rabbits," *Shanghai Journal of Traditional Chinese Medicine*, vol. 41, pp. 59–60, 2007.
- [31] F. F. Yan, Y. Liu, Y. F. Liu, and Y. X. Zhao, "Effect of Qishenyiqi dripping pills on histology of experimental atherosclerotic plaque," *Journal of Nanjing University of Traditional Chinese Medicine*, vol. 23, pp. 295–297, 2007.

## Research Article

# Combination of Chinese Herbal Medicines and Conventional Treatment versus Conventional Treatment Alone in Patients with Acute Coronary Syndrome after Percutaneous Coronary Intervention (5C Trial): An Open-Label Randomized Controlled, Multicenter Study

Shao-Li Wang,<sup>1</sup> Cheng-Long Wang,<sup>2</sup> Pei-Li Wang,<sup>2</sup> Hao Xu,<sup>2</sup> Hong-Ying Liu,<sup>3</sup> Jian-Peng Du,<sup>2</sup> Da-Wu Zhang,<sup>2</sup> Zhu-Ye Gao,<sup>2</sup> Lei Zhang,<sup>2</sup> Chang-Geng Fu,<sup>2</sup> Shu-Zheng Lü,<sup>4</sup> Shi-Jie You,<sup>5</sup> Jun-Bo Ge,<sup>3</sup> Tian-Chang Li,<sup>6</sup> Xian Wang,<sup>7</sup> Guan-Lin Yang,<sup>8</sup> Hong-Xu Liu,<sup>9</sup> Jing-Yuan Mao,<sup>10</sup> Rui-Jie Li,<sup>11</sup> Li-Dian Chen,<sup>12</sup> Shu Lu,<sup>13</sup> Da-Zhuo Shi,<sup>2</sup> and Ke-Ji Chen<sup>2</sup>

<sup>1</sup>Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100053, China

<sup>2</sup>Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>3</sup>Zhongshan Hospital, Fudan University, Shanghai 200032, China

<sup>4</sup>Beijing Anzhen Hospital, Beijing Institute of Respiratory Medicine, Capital Medical University, Beijing 10029, China

<sup>5</sup>Cardiovascular Institute & Fuwai Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100037, China

<sup>6</sup>Beijing Tongren Hospital, Capital Medical University, Beijing 100730, China

<sup>7</sup>Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing 100007, China

<sup>8</sup>The Affiliated Hospital of Liaoning Traditional Chinese Medicine University, Shenyang 110033, China

<sup>9</sup>Beijing Chinese Medicine Hospital, Capital Medical University, Beijing 100010, China

<sup>10</sup>First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China

<sup>11</sup>Beijing Chuiyangliu Hospital, Beijing 100022, China

<sup>12</sup>The Second People's Hospital of Fujian Province, Fuzhou 350100, China

<sup>13</sup>Wuxi Traditional Chinese Medicine Hospital, Nanjing University of Traditional Chinese Medicine, Wuxi 214001, China

Correspondence should be addressed to Da-Zhuo Shi; [shidazhuo@126.com](mailto:shidazhuo@126.com) and Ke-Ji Chen; [keji.chen@yahoo.com](mailto:keji.chen@yahoo.com)

Received 13 March 2013; Accepted 16 June 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Shao-Li Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Aims.** To evaluate the efficacy of Chinese herbal medicines (CHMs) plus conventional treatment in patients with acute coronary syndrome (ACS) after percutaneous coronary intervention (PCI). **Methods and Results.** Participants ( $n = 808$ ) with ACS who underwent PCI from thirteen hospitals of mainland China were randomized into two groups: CHMs plus conventional treatment group (treatment group) or conventional treatment alone group (control group). All participants received conventional treatment, and participants in treatment group additionally received CHMs for six months. The primary endpoint was the composite of cardiac death, nonfatal recurrent MI, and ischemia-driven revascularization. Secondary endpoint was the composite of readmission for ACS, stroke, or congestive heart failure. The safety endpoint involved occurrence of major bleeding events. The incidence of primary endpoint was 2.7% in treatment group versus 6.2% in control group (HR, 0.43; 95% CI, 0.21 to 0.87;  $P = 0.015$ ). The incidence of secondary endpoint was 3.5% in treatment group versus 8.7% in control group (HR, 0.39; 95% CI, 0.21 to 0.72;  $P = 0.002$ ). No major bleeding events were observed in any participant. **Conclusion.** Treatment with CHMs plus conventional treatment further reduced the occurrence of cardiovascular events in patients with ACS after PCI without increasing risk of major bleeding.

## 1. Introduction

Acute coronary syndrome (ACS), encompassing unstable angina (UA) and acute myocardial infarction (AMI, non-ST elevation, and ST elevation), is one of the leading causes of morbidity and mortality. There has been a steady decline in mortality from coronary artery disease (CAD) in most developed countries over the last three decades [1], primarily due to dramatic advances in revascularization procedures such as percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG), as well as pharmacological treatments [2]. Approximately 10% of ACS survivors after PCI, however, will ultimately suffer a second AMI, stroke, or cardiovascular death [3, 4] despite the availability of timely, appropriate treatments. Therefore, reducing the risk of recurrent cardiovascular events in patients with ACS after PCI remains a great challenge in the foreseeable future [5].

Chinese herbal medicines (CHMs) have been widely used in clinical practice for thousands of years. In the past few decades, CHMs have shown beneficial effects in improving clinical symptoms and clinical outcomes in CAD patients. Xinyue Capsule and Fufang *Chuanxiong* Capsule are commonly prescribed in mainland China, and both have been approved by the State Food and Drug Administration (SFDA) of China for clinical use in CAD patients. The major therapeutic effects of these drugs, as documented in previous trials, include relieving myocardial ischemia, decreasing symptoms, improving myocardial reperfusion after PCI, regulating blood lipids, and reducing recurrent angina [6–9]. However, no study has yet to focus on the efficacy of the two capsules in reducing recurrence of cardiovascular events in patients with ACS after PCI. Therefore, in this multicenter, open-label, randomized controlled trial (chictr.org number: ChiCTR-TRC-00000021), we evaluated the efficacy of Xinyue Capsule and Fufang *Chuanxiong* Capsule plus conventional treatment on cardiovascular events in patients with ACS after PCI.

## 2. Method

**2.1. Design Overview.** This study was conducted at thirteen hospitals in five provinces of mainland China. The participants were recruited from April 2008 to October 2009, and follow-up was completed by October 2010. The study protocol was approved by the ethics review board of Xiyuan Hospital, China Academy of Chinese Medical Sciences (CACMS), in accordance with the principles described in the *Declaration of Helsinki* [10], and all participants signed informed consent forms before enrollment.

**2.2. Setting and Participants.** Recruitment, intervention, and data collection were performed at the thirteen participating hospitals. Patients between 18 and 75 years of age were eligible for inclusion if they were hospitalized for ACS [11, 12] involving either AMI (with or without ST segment elevation) or UA and also underwent successful PCI (defined as the target vessel with TIMI grade 3 flow). The exclusion criteria were as follows: (1) concomitant affliction with severe

complications including hepatic, renal, and hematopoietic dysfunction, psychiatric disorders, or cancers; (2) absence of written informed consent, unwillingness to participate in follow-up, or refusal to receive treatment with study drugs; (3) pregnancy or breastfeeding; and (4) concurrent enrollment in other clinical studies.

**2.3. Randomization and Intervention.** An independent, off-site clinical trials statistician at CACMS used a computer-generated random allocation sequence to randomize the trial in blocks of four, stratified with each recruiting center. The details of the sequence remained unknown to any investigator or coordinator and were contained in sequentially numbered, opaque, sealed envelopes (SNOSE), bearing only the hospital name and a number on the outside. A pharmacist at each center who was independent of the clinical study kept the allocation sequence, took responsibility for the allocation, and prepared the treatment medication. After completing the baseline visit, participants who met the enrollment criteria were randomly assigned in a 1:1 ratio to receive either CHMs (Xinyue Capsule and Fufang *Chuanxiong* Capsule) plus conventional treatment or conventional treatment alone. Participants and investigators were masked to the treatment allocation until interventions were assigned. Data collectors and outcome adjudicators were masked until all data were entered into the database. Data management and statistical analyses were performed solely by data handlers and data analysts at Beijing Jiaotong University who were masked to the treatment assignments until the statistical report was completed. The study was open-label because the unique aroma and taste of Fufang *Chuanxiong* Capsule and Xinyue Capsule significantly challenged the successful blinding. In addition, even if we designed placebo capsules for the present study, the participants could easily distinguish between placebo and true capsules by the specific aftertaste left from oral intake of the true capsules.

All participants received conventional treatment in accordance with current guidelines [11, 12], including aspirin (100 mg/day indefinitely), clopidogrel (75 mg/day for at least 12 months), and statins. All other medications were decided by physicians at each center who were not involved in the study. After participants were discharged, medication decisions and the option of revascularization were made by the responsible clinician without restriction. Angiographic follow-up was performed during the follow-up period, but it was not required in this study.

In addition to the conventional treatment, participants in the treatment group received Xinyue Capsule (two capsules orally, three times daily) and Fufang *Chuanxiong* Capsule (two capsules orally, three times daily) for six successive months. The Xinyue Capsule (SFDA Registry number: Z20030073; manufacturer: Jilin Jian Yisheng Pharmaceutical Co., Ltd., Jian City, Jilin Province, China) is an extract from leaves and stems of *Panax quinquefolius* L., containing 50 mg total ginsenosides. The Fufang *Chuanxiong* Capsule (SFDA Registry number: 0802205; manufacturer: Shandong Phoenix Pharmaceutical Co., Ltd., Dongying City, Shandong

Province, China) is made from *Chuanxiong* and *Ligusticum*, with each capsule containing 3.20 mg/gm ligustrazine and 1.73 mg/gm ferulic acid. The quality of the two CHMs met the Chinese Medicine Standards of the SFDA. Capsules were distributed to the thirteen study sites with the same batch number. The companies that provided the two CHMs had no role in the design, analysis, or interpretation of the study.

From the baseline visit to the end of the study, the other CHMs used in the treatment of ACS after PCI, which might complicate the pharmacological effectiveness of Xinyue Capsule or Fufang *Chuanxiong* Capsule, were prohibited. A member of the executive committee in the study was responsible for monitoring quality control with respect to the management of all participants. The adherence of participants to study medication was assessed by independent nurses at each site.

**2.4. Outcomes and Follow-Up.** At the baseline visit, investigators assessed the following characteristics which might have an impact on treatment: body mass index (BMI), heart rate, the number of diseased vessels, target vessels, smoking history, presence or absence of diabetes, hypertension, hyperlipidemia, CAD family history and medications, and so forth. The primary and secondary endpoints were adjudicated at 30 days, as well as at 3, 6, 9, and 12 months after the baseline visit.

The primary endpoint was the composite of cardiac death, nonfatal recurrent MI, or ischemia-driven revascularization. The secondary endpoint was the composite of readmission for ACS, stroke, or congestive HF. The safety endpoint concerned major bleeding events, defined as any intracranial bleeding, or any clinically relevant bleeding necessitated a blood transfusion judged by the investigators. All deaths were considered cardiac unless an unequivocal noncardiac cause was identified. Ischemia-driven revascularization was defined as repeat revascularization with either PCI or CABG because of recurrent myocardial ischemic events. Repeat PCI was defined as revascularization of target lesions or target vessels. Stroke was defined as the development of disabling neurologic symptoms with objective findings lasting at least 24 hours. Recurrent MI was diagnosed based on reappearance of symptoms, and/or new electrocardiographic changes in association with a reelevation of creatine kinase-MB (CK-MB) to levels greater than three times the upper limit of the reference level. Congestive HF was defined as a new diagnosis of congestive HF requiring hospitalization.

Subjects were followed up at each study center. The endpoint data were collected and recorded in a case report form (CRF) by the investigators at each visit (either a direct visit or telephone interview). For remote participants interviewed by telephone, local medical reports were collected by mail and a direct visit was performed at least once during the one-year follow-up period. All clinical outcomes were adjudicated by independent outcome committees whose members were blinded to treatment assignment with review of original documentation.

**2.5. Statistical Analysis.** Sample size calculations were based on evidence from previous studies, which showed that

the one-year composite incidence of cardiac death, nonfatal recurrent MI, or ischemia-driven revascularization in patients with ACS after PCI treated by conventional treatment was 8% to 18% [13, 14]. Thus the incidence of the primary endpoint in this study during one-year in the control group was estimated at 13.5%, and treatment with additional CHMs reduced it to 7% [15]. For our study, a total of 676 participants would provide 80% power to test a difference in the primary endpoint at the 5%, two-sided level of significance. Allowing for a 20% dropout rate and adding power for analysis of the secondary endpoint, we recruited 808 total participants.

All participants were subject to baseline analysis as well as efficacy and safety evaluations. All data analysis was conducted according to a preestablished analysis plan. For categorical variables, the data were presented in a frequency table and expressed as percentages, and intergroup differences were compared by Chi-square or Fisher exact tests. For continuous variables, mean and standard deviation was used for normally distributed data, and median with interquartile range was calculated for not normally distributed data. A Student's *t*-test or Wilcoxon Rank-sum test was used, as appropriate, for the analyses of intergroup differences. The difference in cumulative incidence of the primary or secondary endpoints at one-year between groups was estimated by the Kaplan-Meier method with the log-rank test. The treatment efficacy, as measured by the hazard ratio (HR) and its associated 95% confidence interval (CI), was estimated with the Cox proportional hazards regression. For the calculation of an adjusted HR with 95% CI for the primary or secondary endpoints, Cox proportional hazards regression was performed with 11 preidentified covariates of interest: age, gender, number of diseased vessels, target vessels, final diagnosis, smoking history, CAD family history, BMI, number of randomization centers, presence or absence of diabetes, hypertension, and hyperlipidemia. Participants who were lost to follow-up were censored at their last visit. The intention-to-treat method was applied in the analysis.

A two-sided *P* value less than 0.05 was considered to be statistically significant. The statistical analysis was performed with SPSS statistical software, Version 17.0 for Windows.

### 3. Results

**3.1. Participant Characteristics.** In this study, 808 participants with ACS after successful PCI from April 2008 to October 2010 were assigned to the control (404 participants) and treatment (404 participants) groups randomly. During follow-up, three participants died of cardiac events (two in the treatment group and one in the control group) and two participants in the control group died of cancer (both due to lung cancer). Thirty eight participants (4.7%) were classified as dropout with no significant difference between the two groups [16 (4.0%) in the treatment group versus 22 (5.4%) in the control group,  $P = 0.319$ ]. Among the dropouts, five declined to participate in the follow-up, two had noncardiac adverse events (cancer), twenty-eight were unreachable for

TABLE 1: Baseline characteristics of participants.

Characteristic	Treatment group (n = 404)	Control group (n = 404)
Demographics		
Male, n (%)	322 (79.7)	281 (69.6)
Age, median (interquartile ranges)	60 (53, 67.75)	61 (53, 68)
Final diagnosis <sup>‡</sup> , n (%)		
NSTE-ACS	287 (71.0)	296 (73.3)
STE-ACS	117 (29.0)	108 (26.7)
Number of diseased vessels, n (%)		
One	104 (25.7)	110 (27.2)
Two	131 (32.4)	115 (28.5)
Three	169 (41.8)	179 (44.3)
Target vessels <sup>§</sup> , n (%)		
LAD	322 (79.7)	319 (79.0)
LCX	226 (55.9)	209 (51.7)
RCA	231 (57.2)	234 (57.9)
LM	30 (7.4)	36 (8.9)
Risk factors, n (%)		
Hypertension	247 (61.1)	262 (64.9)
Diabetes mellitus	111 (27.5)	123 (30.4)
Hyperlipidemia	163 (40.3)	159 (39.4)
Smoking history	234 (57.9)	225 (55.7)
Family history of CAD	103 (25.5)	95 (23.5)
BMI** mean (SD)	25.31 (3.01)	25.60 (2.88)
Medication, n (%)		
Beta-blocker	157 (38.9)	160 (39.6)
ACEI	125 (30.9)	123 (30.4)
ARB	74 (18.3)	80 (19.8)
CCB	96 (23.8)	102 (25.2)
Statin	195 (48.3)	192 (47.5)

<sup>‡</sup> NSTE-ACS: non-ST-segment elevation ACS; STE-ACS: ST-segment elevation ACS.

<sup>§</sup> LAD: left anterior descending artery; LCX: left circumflex artery; RCA: right coronary artery; LM: left main coronary artery.

\*\* BMI: body mass index (kg/m<sup>2</sup>).

data collection, and three in the control group received CHMs were excluded. A total of 765 participants completed the one-year follow-up (Figure 1). CHMs were administered to 378 (93.6%) participants in the treatment group for the six months. The baseline characteristics of the participants are shown in Table 1, and the two groups were well matched, except for the proportion of male participants.

**3.2. Primary Endpoint.** During the follow-up period, the cumulative incidence of the primary endpoint in the treatment group was significantly lower than that in the control group [11 (2.7%) versus 25 (6.2%); unadjusted HR 0.43, 95% CI 0.21 to 0.87,  $P = 0.015$ ]. After adjusting for the effects of covariates, the combination of CHMs with conventional treatment was associated with a significant reduction in

the primary endpoint compared to conventional treatment alone (adjusted HR 0.44, 95% CI 0.21 to 0.92,  $P = 0.028$ ) (Table 2 and Figure 2(a)). Among the components of the primary endpoint, cardiac death and recurrent MI did not differ significantly between the treatment and control groups (1.0% versus 2.0%, unadjusted HR 0.49, 95% CI 0.15 to 1.64, adjusted HR 0.35, 95% CI 0.09 to 1.33,  $P = 0.238$ ) (Table 2 and Figure 2(b)). Ischemia-driven revascularization, however, was significantly reduced in the treatment group compared to the control group (2.0% versus 5.4%, unadjusted HR 0.35, 95% CI 0.16 to 0.80, adjusted HR 0.36, 95% CI 0.16 to 0.82,  $P = 0.008$ ) (Table 2 and Figure 2(c)).

**3.3. Secondary Endpoint.** The secondary endpoints occurred in 14 (3.5%) in the treatment group and 35 (8.7%) in the control group (unadjusted HR 0.39, 95% CI 0.21 to 0.72,  $P = 0.002$ ). After adjusting for the effects of covariates, the addition of CHMs to conventional treatment was associated with a significant reduction in the secondary endpoint compared with conventional treatment alone (adjusted HR 0.37, 95% CI 0.21 to 0.72,  $P = 0.002$ ) (Table 2 and Figure 3(a)). Among the components of the endpoint, the cumulative incidence of readmission for ACS in the treatment group was lower than that in the control group (2.0% versus 5.9%, unadjusted HR 0.33, 95% CI 0.15 to 0.72, adjusted HR 0.29, 95% CI 0.13 to 0.65,  $P = 0.004$ ) (Table 2 and Figure 3(b)). However, the incidence of stroke (0.7% versus 1.5%, unadjusted HR 0.49, 95% CI 0.12 to 0.97, adjusted HR 0.69, 95% CI 0.16 to 3.02,  $P = 0.307$ ) and congestive HF (0.7% versus 1.2%, unadjusted HR 0.59, 95% CI 0.14 to 2.48, adjusted HR 0.52, 95% CI 0.12 to 2.36,  $P = 0.469$ ) did not differ between the two groups.

**3.4. Safety.** Major bleeding events were not observed in all participants. Aside from the cardiovascular events defined as primary and secondary endpoints in this study, four participants in the control group were afflicted with cancer. One dropped out due to esophageal cancer, one due to thyroid cancer, and two died of lung cancer. In the treatment group, no cancer-related events occurred, but slight stomach bloating was noted in two (0.5%) participants at one or three months after enrollment. The symptom of stomach bloating was relieved after extending the time interval between taking food and medicines.

## 4. Discussion

In this study, CHMs plus conventional treatment led to a more favorable outcome for patients with ACS after successful PCI compared to conventional treatment alone. The benefits included a reduction in the incidence of the primary endpoint and secondary endpoint, as well as incidence of ischemia-driven revascularization in components of primary endpoint and readmission for ACS in components of secondary endpoint. The safety of CHMs plus conventional treatment was also confirmed in the study.

We had searched the MEDLINE (1966 to 2012), OVID (1946 to 2012), and Cochrane libraries (last search done on May 15, 2012) using the terms “Chinese herbal medicine,”

TABLE 2: Clinical outcomes at 1 year<sup>††</sup>.

Endpoint	Treatment group ( <i>n</i> = 404) <sup>††</sup>	Control group ( <i>n</i> = 404) <sup>††</sup>	Unadjusted HR (95% CI)	Adjusted HR (95% CI)	<i>P</i> value <sup>§§</sup>
Primary endpoint	11 (2.7)	25 (6.2)	0.43 (0.21 to 0.87)	0.44 (0.21 to 0.92)	0.015
Death/MI	4 (1.0)	8 (2.0)	0.49 (0.15 to 1.64)	0.35 (0.09 to 1.33)	0.238
Revascularization	8 (2.0)	22 (5.4)	0.35 (0.16 to 0.80)	0.36 (0.16 to 0.82)	0.008
Secondary end point	14 (3.5)	35 (8.7)	0.39 (0.21 to 0.72)	0.37 (0.20 to 0.71)	0.002
Readmission for ACS	8 (2)	24 (5.9)	0.33 (0.15 to 0.72)	0.29 (0.13 to 0.65)	0.004
Stroke	3 (0.7)	6 (1.5)	0.49 (0.12 to 1.97)	0.69 (0.16 to 3.02)	0.307
Congestive HF	3 (0.7)	5 (1.2)	0.59 (0.14 to 2.48)	0.52 (0.12 to 2.36)	0.469

<sup>††</sup> Values are expressed as *n* (%).

<sup>††</sup> Kaplan-Meier estimate.

<sup>§§</sup> *P* value derived from log-rank test.

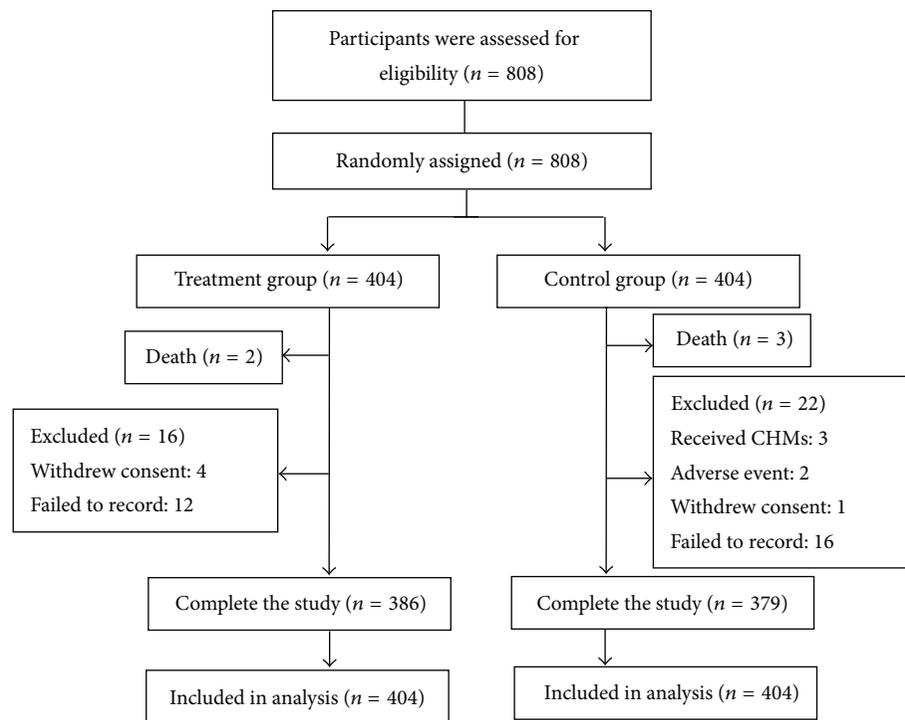


FIGURE 1: Study flow diagram.

“percutaneous coronary intervention,” and “coronary artery disease” to identify all randomized controlled clinical trials that had compared the efficacy of CHMs plus conventional treatment versus conventional treatment alone on cardiovascular events for CAD after PCI. Four trials met the selection criteria [15–18], but none of these addressed patients with a full spectrum of ACS after PCI. Thus, to our knowledge, our trial is the first randomized, controlled study in mainland China to assess the efficacy of CHMs plus conventional treatment versus conventional treatment alone on ACS after PCI, as evaluated by cardiovascular events. Of the four trials that met selection criteria, three [15, 17, 18] demonstrated an association between CHMs pharmacologically similar in effect to Fufang *Chuanxiong* Capsule and reduction of restenosis in post-PCI patients. The benefits of CHMs in

restenosis lend support to our finding of a reduction in ischemia-driven revascularizations in the treatment group.

This study did not demonstrate a significant impact of CHMs on mortality or recurrent MI, a result that might be ascribed to the relatively small sample size of our trial. The reduction in the incidence of the composite primary endpoint in the treatment group was largely attributed to the benefits of CHMs plus conventional treatment in reducing ischemia-driven revascularization.

Our results also demonstrated a significant reduction in the secondary endpoint. The reduced incidence of the composite secondary endpoint was largely derived from a significant decrease in readmission for ACS in particular. However, we cannot exclude the possibility that CHMs might also provide benefits regarding stroke or congestive HF,

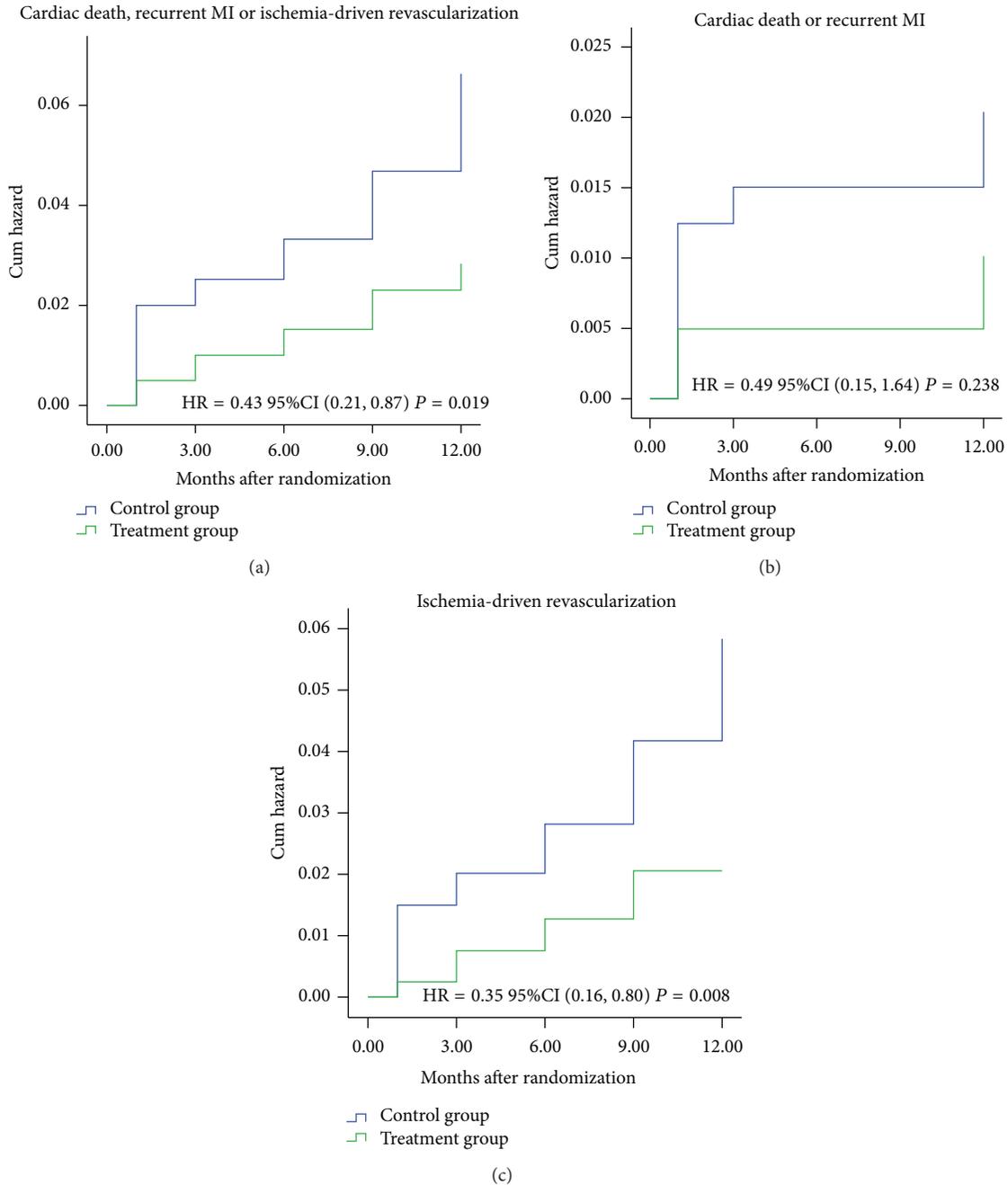


FIGURE 2: Kaplan-Meier time to event curve for primary endpoint.

because previous experimental studies have demonstrated that the active ingredients contained in Xinyue Capsule and Fufang *Chuanxiong* Capsule exert cardiovascular benefits in myocardial ischemia, myocardial hypertrophy, myocardial remodeling, HF, and thrombosis [19–21]. In recent randomized controlled trials, the benefits of CHMs on myocardial perfusion, infarcted area, and ventricular wall movement were demonstrated in patients with ST-segment elevation MI after PCI [22]. Given that no previous study has investigated the effects of CHMs on cardiovascular events in ACS patients after PCI, our trial provides the first evidence that Xinyue

Capsule and Fufang *Chuanxiong* Capsule in combination with conventional treatment may further improve clinical outcomes in ACS patients after PCI by reducing cardiovascular events.

In consideration of the potential pharmacological interplay between CHMs and antiplatelet agents, the major side effect of Xinyue Capsule and Fufang *Chuanxiong* Capsule in combination with conventional treatment in this study was predicted to be a possible increase in hemorrhagic events. Because no major bleeding events occurred in either of the two groups, our results suggest that CHMs plus conventional

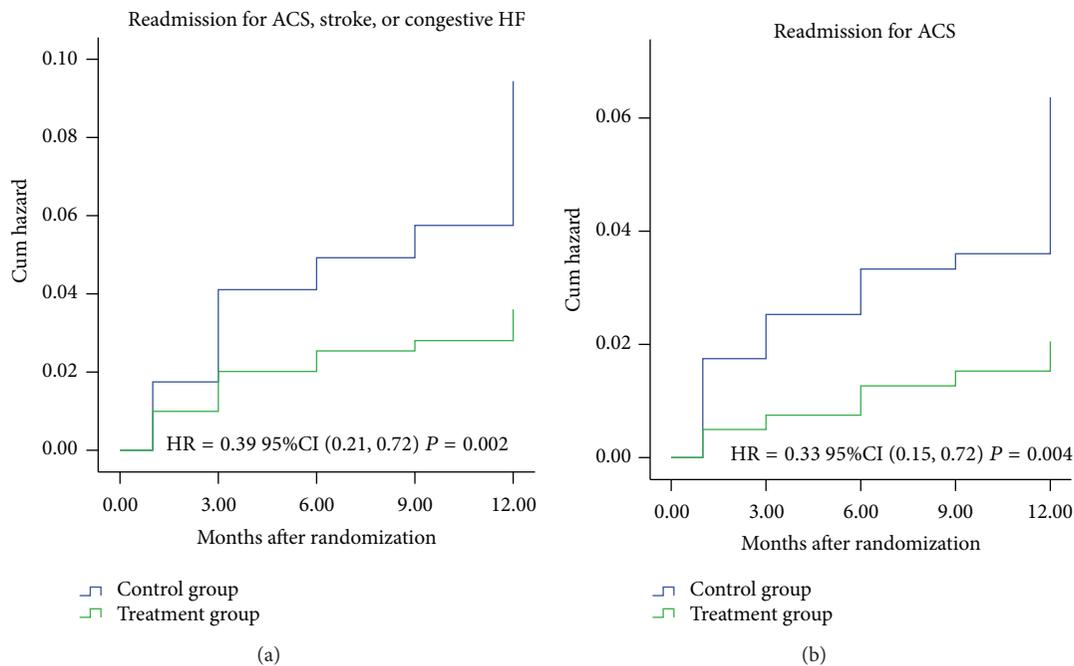


FIGURE 3: Kaplan-Meier time to event curve for secondary endpoint.

treatment with antiplatelet agents did not increase the risk of major bleeding events in patients with ACS after PCI.

Data from a previous meta-analysis [23] indicated a statistically increased risk of noncardiac mortality (cancer, stroke, or infectious diseases, etc.) in CAD patients treated with sirolimus-eluting stents (SES) versus bare metal stents, 40% of which was cancer-related death, implicating an association between the use of SES and an increase in cancer-related mortality. Our study found that four subjects were afflicted with cancer in the control group, and all had an implanted SES. No patients in the treatment group, however, suffered from cancer. The association between cancer-related events and the use of SES or CHMs require further investigation owing to the small number of cancer-related events observed in the present study and the lack of statistical evidence provided in previous reports.

Some limitations in this study should be noted. First, our study was not a blind, placebo-controlled study. To reduce biases from observation, data collection, and efficacy evaluation, all data collectors, outcome adjudicators, data handlers, and data analysts involved in this study were not knowledgeable of the study group assignment. Second, the number of enrolled participants was relatively small and the follow-up period was only one-year. As a result, the study may not have sufficient power to detect a statistically significant difference in each endpoint between the two groups. Finally, our trial was only conducted in mainland China and all participants were Chinese; thus, our findings may not be applicable to patients from different races or other countries.

However, our findings shed light on the benefits and safety of CHMs plus conventional treatment for ACS patients after PCI, thereby offering potential implications for clinical practice. As more evidence related to the benefits and safety of

CHMs emerges from large-scale and long-term trials, CHMs may serve as an adjunctive therapy to conventional treatment for ACS after PCI in the future.

In conclusion, this study demonstrated that CHMs in combination with conventional treatment further reduced cardiovascular events in patients with ACS after PCI without an increased risk of major bleeding.

## Conflict of Interests

The authors have no conflict of interests to declare.

## Authors' Contribution

Dr. Shao-Li Wang and Professor Cheng-Long Wang contributed equally to this paper.

## Funding

The authors would like to thank Professor Michael Shen and Hanjay for comments on earlier versions of this paper. The authors are also grateful to Drs. Yu-Jie Zeng and Zi-Xiang Lin, for the collection of clinical data, and Drs. Qiao-Ning Yang and Qiang Wang for their participation in data loading. The authors would also like to thank Xue-Zhong Zhou and Shuai Li for the contribution in creating the database.

## Acknowledgments

This work was supported by the Grant from the Supporting Program of the "Eleventh Five-year Plan" for Sci & Tech Research of China (no. 2006BA104A01). The sponsor of

the study had no role in the study design, data collection, data analysis, data interpretation, or manuscript preparation. All authors contributed to the data collection, manuscript writing, and final approval of this study.

## References

- [1] J. Iqbal and K. A. A. Fox, "Epidemiological trends in acute coronary syndromes: understanding the past to predict and improve the future," *Archives of Medical Science*, vol. 6, no. 1A, pp. S3–S14, 2010.
- [2] D. M. Kolansky, "Acute coronary syndromes: morbidity, mortality, and pharmacoeconomic burden," *The American Journal of Managed Care*, vol. 15, no. 2, pp. S36–S41, 2009.
- [3] N. B. Norgard and M. Abu-Fadel, "Comparison of prasugrel and clopidogrel in patients with acute coronary syndrome undergoing percutaneous coronary intervention," *Vascular Health and Risk Management*, vol. 5, pp. 873–882, 2009.
- [4] S. D. Wiviott, E. Braunwald, C. H. McCabe et al., "Prasugrel versus clopidogrel in patients with acute coronary syndromes," *The New England Journal of Medicine*, vol. 357, no. 20, pp. 2001–2015, 2007.
- [5] S. Yusuf, F. Zhao, S. R. Mehta, S. Chrolavicius, G. Tognoni, and K. K. Fox, "Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation," *The New England Journal of Medicine*, vol. 345, no. 7, pp. 494–502, 2001.
- [6] Y.-Q. Li, M. Jin, and S.-L. Qiu, "Effect of Chinese herbal medicine for benefiting qi and nourishing yin to promote blood circulation on ventricular wall motion of AMI patients after revascularization," *Zhongguo Zhong Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi*, vol. 29, no. 4, pp. 300–304, 2009.
- [7] S. L. Qiu, M. Jin, T. G. Zhu et al., "Effect of replenishing Qi and nourishing Yin to promote the blood circulation on 103 patients with acute myocardial infarction after reperfusion," *Journal of Capital Medical University*, vol. 30, no. 4, pp. 426–428, 2009.
- [8] G. Y. Sheng, D. M. Niu, S. B. Wu, J. B. Deng, and W. Y. Qiang, "Influence of complex *Chuanxiong* capsule on the blood fat of coronary and the heart function," *Journal of Chinese Modern Medicine*, vol. 6, no. 6, pp. 401–404, 2009.
- [9] W. Zhang, "Clinical Study of Fufang *Chuanxiong* capsule on angina pectoris," *Medical Innovation of China*, vol. 8, no. 11, pp. 57–58, 2011.
- [10] World Medical Association declaration of Helsinki, "Recommendations guiding physicians in biomedical research involving human subjects," *Journal of the American Medical Association*, vol. 277, pp. 925–926, 1997.
- [11] Chinese Society of Cardiology of Chinese Medical Association, Editorial Board of Chinese Journal of Cardiology, "Guideline for diagnosis and treatment of patients with unstable angina and non-ST-segment elevation myocardial infarction," *Chinese Journal of Cardiology*, vol. 35, no. 4, pp. 295–304, 2007.
- [12] E. M. Antman, M. Hand, P. W. Armstrong et al., "2007 Focused update of the ACC/AHA 2004 guidelines for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association task force on practice guidelines," *Circulation*, vol. 117, no. 2, pp. 296–329, 2008.
- [13] Y. Li, R. Torguson, A. I. Syed et al., "Effect of drug-eluting stents on frequency of repeat revascularization in patients with unstable angina pectoris or non-st-elevation myocardial infarction," *American Journal of Cardiology*, vol. 104, no. 12, pp. 1654–1659, 2009.
- [14] C. Spaulding, P. Henry, E. Teiger et al., "Sirolimus-eluting versus uncoated stents in acute myocardial infarction," *The New England Journal of Medicine*, vol. 355, no. 11, pp. 1093–1104, 2006.
- [15] K.-J. Chen, D.-Z. Shi, H. Xu et al., "XS0601 reduces the incidence of restenosis: a prospective study of 335 patients undergoing percutaneous coronary intervention in China," *Chinese Medical Journal*, vol. 119, no. 1, article 6, 2006.
- [16] X. Y. Cui, Y. Wu, Y. B. Nong et al., "Effect of Liangxue Shengji recipe on incidence of post-percutaneous coronary intervention restenosis and adverse cardiovascular events," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 30, no. 1, pp. 30–32, 2010.
- [17] X.-Y. Lu, D.-Z. Shi, and H. Xu, "Clinical study on effect of xiongshao capsule on restenosis after percutaneous coronary intervention," *Zhongguo Zhong Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi*, vol. 26, no. 1, pp. 13–17, 2006.
- [18] Q.-H. Shang, H. Xu, X.-Y. Lu, C. Wen, D.-Z. Shi, and K.-J. Chen, "A multi-center randomized double-blind placebo-controlled trial of Xiongshao Capsule in preventing restenosis after percutaneous coronary intervention: a subgroup analysis of senile patients," *Chinese Journal of Integrative Medicine*, vol. 17, no. 9, pp. 669–674, 2011.
- [19] C.-L. Wang, D.-Z. Shi, and H.-J. Yin, "Effect of panax quinquefolius saponin on angiogenesis and expressions of VEGF and bFGF in myocardium of rats with acute myocardial infarction," *Zhongguo Zhong Xi Yi Jie He Za Zhi Zhongguo Zhongxiyi Jiehe Zazhi*, vol. 27, no. 4, pp. 331–334, 2007.
- [20] M. Karmazyn, M. Moey, and X. T. Gan, "Therapeutic potential of ginseng in the management of cardiovascular disorders," *Drugs*, vol. 71, no. 15, pp. 1989–2008, 2011.
- [21] L.-W. Qi, C.-Z. Wang, and C.-S. Yuan, "Ginsenosides from American ginseng: chemical and pharmacological diversity," *Phytochemistry*, vol. 72, no. 8, pp. 689–699, 2011.
- [22] H.-T. Zhang, Z.-H. Jia, J. Zhang et al., "No-reflow protection and long-term efficacy for acute myocardial infarction with Tongxinluo: a randomized double-blind placebo-controlled multicenter clinical trial (ENLEAT trial)," *Chinese Medical Journal*, vol. 123, no. 20, pp. 2858–2864, 2010.
- [23] A. J. Nordmann, M. Briel, and H. C. Bucher, "Mortality in randomized controlled trials comparing drug-eluting vs. bare metal stents in coronary artery disease: a meta-analysis," *European Heart Journal*, vol. 27, no. 23, pp. 2784–2814, 2006.

## Research Article

# The Expression of CD14<sup>+</sup>CD16<sup>+</sup> Monocyte Subpopulation in Coronary Heart Disease Patients with Blood Stasis Syndrome

Ye Huang,<sup>1</sup> Jing-Shang Wang,<sup>2</sup> Hui-jun Yin,<sup>2</sup> and Ke-ji Chen<sup>2</sup>

<sup>1</sup>Emergency Department, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 10091, China

<sup>2</sup>Department of Cardiovascular Disease, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

Correspondence should be addressed to Hui-jun Yin; [huijunyin@aliyun.com](mailto:huijunyin@aliyun.com)

Received 6 March 2013; Revised 31 May 2013; Accepted 9 June 2013

Academic Editor: Hao Xu

Copyright © 2013 Ye Huang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Blood stasis syndrome (BSS), a comprehensive pathological state, is one of the traditional Chinese medicine syndromes of coronary heart disease (CHD). In our previous study, we investigated that FcγRIIIA (also called CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation) is one of the differentially expressed genes related to CHD patients and its possible role in the atherosclerotic formation and plaque rupture. However, whether or not the deregulation of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation expression is implicated in the pathogenesis of CHD patients with BSS has not yet been elucidated. In this study, we found that there was no significant difference between CHD patients with BSS and non-BSS in CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation at gene level. Moreover, the protein level of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation in CHD patients with BSS was increased significantly when compared to the CHD patients with non-BSS. Additionally, the level of inflammatory cytokines downstream of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation such as TNF-α and IL-1 in sera was much higher in CHD patients with BSS than that in CHD patients with non-BSS. Taken together, these results indicated that CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation was implicated in the pathogenesis of CHD patients with BSS, which may be one of the bases of the essence of BSS investigation.

## 1. Introduction

The way of disease-syndrome combination is an important style to diagnose and treat disease in traditional Chinese medicine (TCM) clinical practice today. Study on the blood stasis syndrome (BSS) is the most active field of integration of traditional and western medicine research in China [1]. To normalize and standardize the BSS, the way of disease-syndrome combination is used to explore the essence of BSS, which will be the inevitable tendency in the future. Study on CHD with BSS initiated by research team of Chen keji is the model of the way of disease-syndrome combination. In our previous study, we found that Fc receptor III A of immunoglobulin G (FcγRIIIA, also called CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation) is one of the differentially expressed genes related to coronary heart disease (CHD) patients using the oligonucleotide microarray technique [2], and high level of FcγRIIIA in CHD patients observed previously was verified by both mRNA level and its protein content [3]. Our recent study suggested an important

role of FcγRIIIA in the atherosclerotic formation by elevating the adhesive efficiency of monocytes to HUVECs *in vitro*, by increasing expression of inflammatory cytokines and also by kindling the atherosclerotic plaque destabilization in ApoE<sup>-/-</sup> mouse [4]. Additionally, we also investigated that traditional Chinese medicine of activation of blood and dissolving stasis, effective components of Chuanxiong Rhizome and Red Peony Root, could stabilize the atherosclerotic plaque by suppressing inflammation, and its target was relative with FcγRIIIA [5]. However, whether or not the deregulation of the expression of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation is implicated in the pathogenesis of CHD patients with BSS has not yet been elucidated.

## 2. Methods

**2.1. Patients and Healthy Control.** All patients with coronary heart disease were the inpatients of Beijing Anzhen Hospital, from May 2010 to December 2010, diagnosed by a diameter

TABLE 1: Characteristics of the CHD patients with BSS/non-BSS and healthy individuals participated in the study.

	CHD patients			P value
	BSS patients (n = 50)	Non-BSS patients (n = 50)	Healthy control (n = 40)	
Age (year)	53.00 ± 6.43	52.92 ± 6.03	49.50 ± 8.71	0.507
Sex (male/female)	36/14	33/17	28/12	0.804
BMI (kg/m <sup>2</sup> )	25.50 ± 2.77	25.10 ± 2.57	24.29 ± 1.57	0.509
SAP (n)	12	13	—	—
ACS (n)	38	37	—	—
UAP (n)	31	31	—	—
AMI (n)	7	6	—	—
Hypercholesterolemia (>230 mg/dL) (yes/no)	7/43	5/45	—	—
Hypertension (yes/no)	31/19	30/20	—	—
Diabetes (yes/no)	20/30	20/30	—	—
Monocyte count (mmol/L)	0.38 ± 0.11	0.37 ± 0.20	0.36 ± 0.11	0.891

BMI: body mass index; SAP: stable angina pectoris; ACS: acute coronary syndrome; UAP: unstable angina; AMI: acute myocardial infarction. Data are expressed as mean ± SD.

stenosis of at least 50% from standard selective coronary angiography [6, 7]. These CHD patients were selected into blood stasis syndrome (BSS) group and non-BSS group based on the standard diagnostic criteria established by the Special Committee of Promoting Blood Circulation and Removing Blood Stasis, Chinese Association of Integrative Medicine [8]. Patients with severe valvulopathy, serious primary diseases such as liver or kidney dysfunction, malignant tumors, medication history of antiplatelet therapy, and women in pregnancy or lactation stage were excluded from enrollment. Forty age- and sex-matched healthy individuals from the physical examination center of Xiyuan Hospital were selected as a control group. These individuals were without any history of chest pain or evidence of cardiac or other systemic disease verified by history examination, chest film, electrocardiogram, and blood routine examination, and none was taking any medication. Our study was in accordance with the Helsinki Declaration, with ethical approval granted by the Ethics Committee at Xiyuan Hospital, China Academy of Chinese Medical Sciences, and a written informed consent was obtained from all study participants. The clinical characteristics of the participants are shown in Table 1.

**2.2. Blood Samples.** Peripheral blood was collected from the CHD patients with BSS/non-BSS and healthy subjects under standardized conditions. Blood from CHD patients was drawn before coronary angiography was performed. For the analyses of cytometry or mRNA expression or flow cytometry, 2 mL ethylenediaminetetraacetate- (EDTA-) anticoagulated blood was taken and immediately analyzed. For the detection of inflammatory cytokines by ELISA assay, blood was centrifuged at 3,000 rpm for 20 min, and serum was frozen at  $-80^{\circ}\text{C}$  until analysis.

**2.3. RT-PCR.** FcγRIIIA mRNA expression was investigated by the quantitative real-time polymerase chain reaction (PCR) assay. Total RNA samples were extracted from leukocytes using Trizol reagent (Invitrogen, USA) according to

the manufacturer's protocol. The purity and integrity of RNA were determined on a UV spectrophotometer (Eppendorf, Germany) by 260–280 nm absorbance ratio and agarose gel electrophoresis (1.5%) and ethidium bromide staining, respectively. cDNA preparations were performed at  $42^{\circ}\text{C}$  for 1 h, with reverse transcriptase, 2 μL RNase, 2 μL of an oligo (dT) primer, and 10 mM of each dNTP in a total volume of 50 μL of 1x first strand cDNA synthesis buffer, incubated at  $70^{\circ}\text{C}$  for 10 min. PCR assays were carried out in a PCR (ABI 7500, USA). 1.5 μL of cDNA mixture was subjected to amplification in a 20 μL mixture. The following primer sequences with the predicted size were used for amplification: FcγRIIIA: forward, 5'-TGTTCAAGGAGGAAG-ACCCT-3', reverse, 5'-GAAGTAGGAGCCGCTGTCTT-3'; GAPDH: forward, 5'-GGGTGTGAACCATGAGAAGT-3', reverse, 5'-GGCATGGACTGTGGTCATGA-3'. PCR conditions were as follows: initial denaturation at  $94^{\circ}\text{C}$  for 15 min followed by 40 cycles of denaturation for 15 s at  $94^{\circ}\text{C}$ , annealing at  $60^{\circ}\text{C}$  for 34 sec, extending at  $72^{\circ}\text{C}$  for 15 sec, and a final extension at  $72^{\circ}\text{C}$  for 10 min. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal control in all PCR reactions. The PCR products were subjected to 2% agarose gel electrophoresis. The relative mRNA expression level of the target gene in each individual was calculated using the comparative cycle time ( $C_t$ ) method [9].

**2.4. Flow Cytometry.** FcγRIIIA protein level was assessed by flow cytometry. Ethylenediaminetetraacetic-Acid- (EDTA-) anticoagulated peripheral blood (PB) samples were collected from all patients and healthy controls for flow cytometric analysis performed previously described [10]. Briefly, PB samples were stained with saturating concentrations of fluorescein-isothiocyanate- (FITC-) conjugated anti-CD14 monoclonal antibody (mAb) (BD Biosciences, Lot: 74003) and phycoerythrin- (PE-) conjugated anti-CD16 mAb (BD Biosciences, Lot: 73903) or isotype-matched control mAb for 20 min at room temperature in the dark. After erythrocytes were lysed by incubation with lysing solution for 8 min, PB

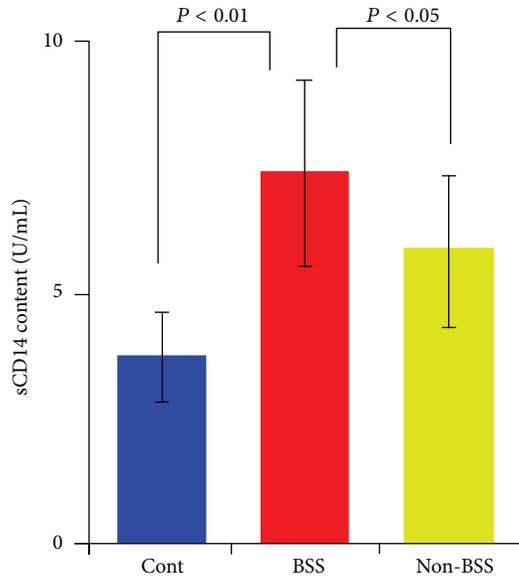


FIGURE 1: The significant level of soluble CD14 in sera in CHD patients with BSS by ELISA assay. Results were presented as mean  $\pm$  SD.

mononuclear cells were resuspended in PBS with 1% fetal calf serum. The surface expression of CD14 and CD16 on PB monocytes was performed by a fluorescence-activated cell sorter (FACS) cytometer (Becton Dickinson). The test data were obtained by FS versus SS gate and analyzed by Expo32 special software. Monocytes were identified by gating CD14<sup>+</sup> events, and all additional analyses were performed on this population. FcγRIIIA protein content defined by the percentage of CD16 on the monocyte population (CD14<sup>+</sup>/CD16<sup>+</sup>%) was measured.

**2.5. Enzyme-Linked Immunosorbent Assay (ELISA).** Concentrations of TNF- $\alpha$  (R&D, USA, Lot: 1007143), IL-1 (R&D, USA, Lot: 1007155), and soluble CD14 (sCD14) (R&D, USA, Lot: 1010179) in sera were determined by double-antibody sandwich avidin-biotin peroxidase complex enzyme-linked immunosorbent assay (ABC-ELISA), according to manufacturer's instructions.

**2.6. Statistical Analysis.** All data are expressed as mean  $\pm$  SD. The SPSS Statistics 15.0 package was utilized to analyze the data. Differences among groups were analyzed using the one-way analysis of variance (ANOVA), followed by multiple comparisons by LSD test. Difference was considered significant at  $P < 0.05$ .

### 3. Results and Discussion

BSS is a pathological state, which is the outward manifestation of some certain pathological stage of various diseases. Due to lack of objective diagnosis criteria, the essence of BSS is studied into the bottleneck stage. Recently, based on the way of disease-syndrome combination, much effective

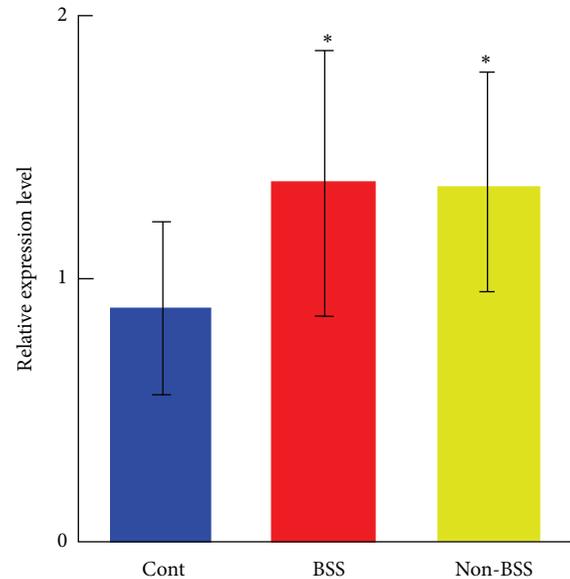


FIGURE 2: The mRNA level of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation in leukocytes in CHD patients with BSS by qRT-PCR. \* $P < 0.01$  compared to the control group. Results were presented as mean  $\pm$  SD.

exploration of the essence of BSS was the foundation of BSS objective diagnosis criteria construction. During the past 50 years, we found a correlation between CHD with BSS and inflammatory, hemodynamics, platelet, and microcirculation [11].

Atherosclerosis, a chronic inflammatory immune state, is chiefly responsible for the development of CHD. Various leukocytes have been shown to influence atherogenesis. Monocytes and their descendant macrophages are central protagonists in the development of atherosclerosis [12]. Monocyte migration to the vessel wall is an initial event in the growth of atherosclerotic lesions [13]. Once monocytes are activated, adhesion to endothelial cells was induced by the transform of phenotype, which led to myocardium injury, inducing the proinflammatory cytokines such as TNF- $\alpha$  and IL-1 synthesis to initiate the inflammatory cascade reaction and oxidative stress injury, producing matrix metalloproteinase (MMP) and releasing many media to induce plaque instability and even fracture [14, 15]. Therefore, monocytes played the key role in the chronic inflammation-immunoreaction of the arterial vessels. In our study, we investigated that there was no significant difference of monocyte count based on CBC count among CHD patients with BSS, non-BSS, and healthy control (Table 1). However, the level of sCD14 which is the indicator of activated monocyte [16] was obviously increased in CHD patients with BSS, compared to non-BSS and healthy control (Figure 1). The increased level of sCD14 in sera in CHD patients with BSS indicated monocytes activation. To demonstrate the correlation between deregulated expression of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation and pathogenesis of CHD with BSS, its mRNA expression at the leukocyte level was assessed. As shown in Figure 2, relative expression level of CD14<sup>+</sup>CD16<sup>+</sup>

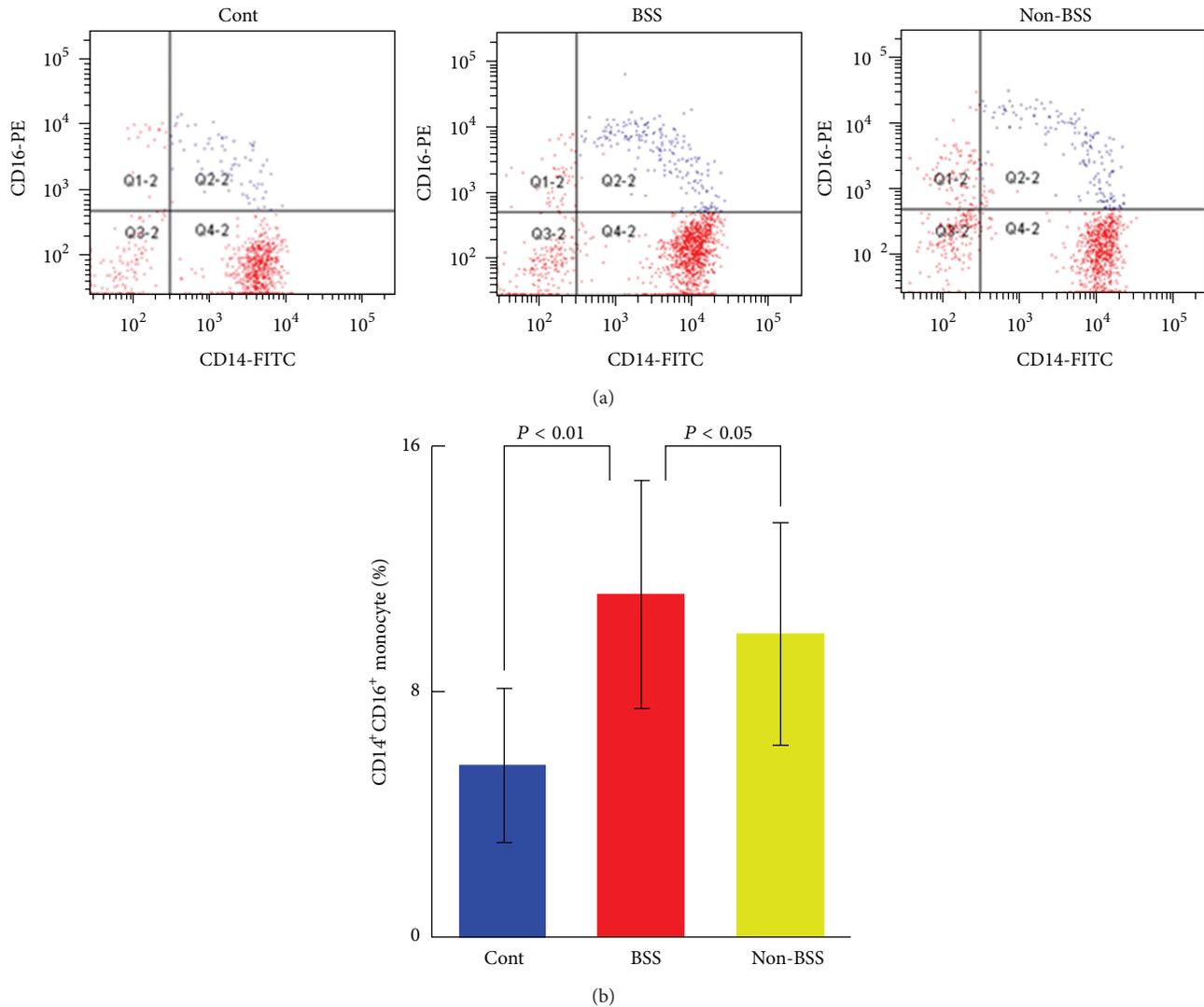


FIGURE 3: The protein level of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation in CHD patients with BSS by FACS analysis. (a) Representation data of FACS analysis of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation. Whole peripheral blood samples from patients and healthy individuals were stained with FITC-conjugated anti-CD14 antibody and PE-conjugated anti-CD16 antibody, followed by FACS. (b) The percentage of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation in the whole CD14-positive cells. Results were presented as mean  $\pm$  SD.

monocyte subpopulation in both CHD patients with BSS and non-BSS was largely increased by 99% and 77%, respectively, compared to the healthy control ( $P < 0.01$ ). However, there was no significant difference of this relative expression level between CHD patients with BSS and non-BSS (Figure 2). The expression of biological traits was controlled by gene, and the biological traits were reflected by protein. To investigate whether or not the expression change of CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation in both CHD patients with BSS and non-BSS at its protein level, therefore, we further analyzed the protein level of CD14<sup>+</sup>CD16<sup>+</sup> on monocyte member using 2-color immunofluorescent staining (Figure 3(a)). The FACS results showed that the protein level of CD14<sup>+</sup>CD16<sup>+</sup> on monocyte member was significantly increased in the CHD patients with BSS, when compared to the CHD patients with non-BSS and the healthy control ( $P < 0.01$  or  $P < 0.05$ , Figure 3(b)).

TNF- $\alpha$  is one of the cytokines with various biological activation, and one previous study confirms that increased level of TNF- $\alpha$  was existed in the monocyte/macrophage, smooth muscle cells, and endothelial cells in the atherosclerotic plaque, and this high level was correlated to the severity of atherosclerosis [17]. IL-1 was released by active monocyte/macrophage, which induced the releasing of many cytokines and growth factors by monocyte/macrophage and the expression of adhesion molecules such as ICAM-1. Additionally, IL-1 could stimulate vascular endothelium to produce many inflammatory factors such as TNF- $\alpha$ , aggravated local inflammatory reaction, and promote the development of atherosclerosis [18, 19]. Additionally, previous studies indicated that the circulation of CD14<sup>+</sup>CD16<sup>+</sup> monocytes could spontaneously produce TNF and IL-1 [20], which could provoke cell proliferation and migration of smooth muscle cells and macrophages in the atherosclerotic plaque [21].

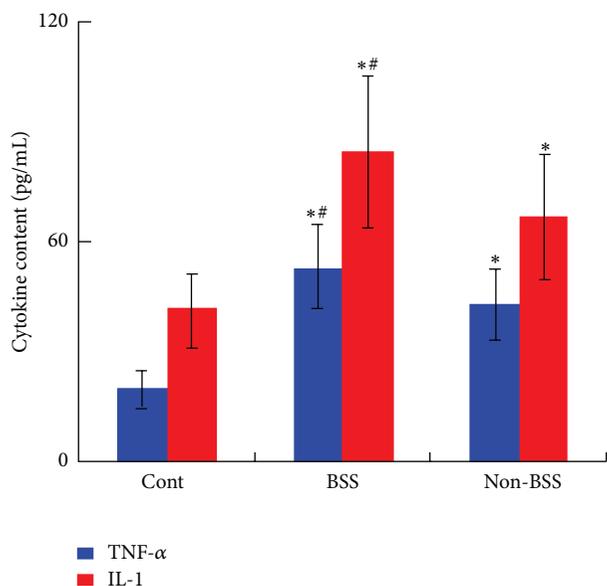


FIGURE 4: The changes of inflammatory cytokines of TNF- $\alpha$  and IL-1 in sera in CHD patients with BSS. \* $P < 0.01$  compared to the control group. # $P < 0.05$  compared to the CHD patients with non-BSS. Results were presented as mean  $\pm$  SD.

Therefore, protein level of TNF- $\alpha$  and IL-1 in sera was also assessed in our study by ELISA. The significant increased serum of TNF- $\alpha$  and IL-1 level was observed in CHD patients with BSS and non-BSS compared to the healthy control ( $P < 0.01$ , Figure 4), and the level of TNF- $\alpha$  and IL-1 in CHD patients with BSS was much higher than that in CHD patients with non-BSS ( $P < 0.05$ , Figure 4).

In the current study, we investigated that there were monocyte activation and an increased CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation at protein level and its downstream inflammatory cytokines such as TNF- $\alpha$  and IL-1 in sera in CHD patients with BSS. Herein, we presumed that monocyte activation in the CHD patient with BSS induced the phenotype of monocyte member transforming to CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation which was involved in the pathogenesis of CHD with BSS.

#### 4. Conclusion

Overall, the present work confirmed the correlation between increased CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation at protein level and CHD patients with based on the way of disease-syndrome combination. Thus, the increased CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation and its downstream inflammatory cytokines in CHD patient with BSS indicated that CD14<sup>+</sup>CD16<sup>+</sup> monocyte subpopulation was one of the sensitive markers in the pathogenesis of CHD with BSS.

#### Authors' Contribution

Ye Huang and Jingshang Wang contributed equally to this work.

#### Acknowledgments

This work was supported by the Chinese National Key Basic Research and Development Program under Grant no. 81073086 and the Major Research Plan of the Chinese National Key Basic Research & Development Program under Grant no. 90409021. The authors thank Dr. Xin Chen and Hua Chen the for assistance with CHD patient selection, Dr. Changgeng Fu for assistance with CHD patient with BSS selection, and Dr. Yonggang Xu for assistance with flow cytometric analysis.

#### References

- [1] Y. Liu, H.-J. Yin, D.-Z. Shi, and K.-J. Chen, "Chinese herb and formulas for promoting blood circulation and removing blood stasis and antiplatelet therapies," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 184503, 8 pages, 2012.
- [2] X.-J. Ma, H.-J. Yin, and K.-J. Chen, "Differential gene expression profiles in coronary heart disease patients of blood stasis syndrome in traditional Chinese medicine and clinical role of target gene," *Chinese Journal of Integrative Medicine*, vol. 15, no. 2, pp. 101–106, 2009.
- [3] Y. Huang, H.-J. Yin, J.-S. Wang, X.-J. Ma, Y. Zhang, and K.-J. Chen, "The significant increase of Fc $\gamma$ RIIIA (CD16), a sensitive marker, in patients with coronary heart disease," *Gene*, vol. 504, no. 2, pp. 284–287, 2012.
- [4] Y. Huang, H.-J. Yin, J.-S. Wang, Q. Liu, C.-F. Wu, and K.-J. Chen, "Aberrant expression of Fc $\gamma$ RIIIA (CD16) contributes to the development of atherosclerosis," *Gene*, vol. 498, no. 1, pp. 91–95, 2012.
- [5] Y. Huang, H.-J. Yin, X.-J. Ma et al., "Correlation between Fc $\gamma$ RIII a and aortic atherosclerotic plaque destabilization in ApoE knockout mice and intervention effects of effective components of *Chuanxiong Rhizome* and *Red Peony Root*," *Chinese Journal of Integrative Medicine*, vol. 17, no. 5, pp. 355–360, 2011.
- [6] E. Braunwald, E. M. Antman, J. W. Beasley et al., "ACC/AHA 2002 guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction—summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (committee on the management of patients with unstable angina)," *Journal of the American College of Cardiology*, vol. 40, no. 7, pp. 1366–1374, 2002.
- [7] R. J. Gibbons, K. Chatterjee, J. Daley et al., "ACC/AHA/ACP-ASIM guidelines for the management of patients with chronic stable angina: executive summary and recommendations. A report of the American College of Cardiology/American Heart Association task force on practice guidelines (committee on management of patients with chronic stable angina)," *Circulation*, vol. 99, no. 21, pp. 2829–2848, 1999.
- [8] Society of Cardiology, Chinese Association of the Integrative Medicine, "The diagnostic criteria of Chinese medicine in coronary heart disease," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 11, p. 257, 1991.
- [9] J. P. P. Meijerink, C. Mandigers, L. Van De Loch, E. Tönnessen, F. Goodsaid, and J. Raemaekers, "A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR," *Journal of Molecular Diagnostics*, vol. 3, no. 2, pp. 55–61, 2001.

- [10] T. Gremmel, C. W. Kopp, D. Seidinger et al., "The formation of monocyte-platelet aggregates is independent of on-treatment residual agonists'-inducible platelet reactivity," *Atherosclerosis*, vol. 207, no. 2, pp. 608–613, 2009.
- [11] K.-J. Chen, "Exploration on the possibility of reducing cardiovascular risk by treatment with Chinese medicine recipes for promoting blood-circulation and relieving blood-stasis," *Zhong Guo Zhong Xi Yi Jie He Za Zhi*, vol. 28, no. 5, p. 389, 2008.
- [12] M. J. Pittet and F. K. Swirski, "Monocytes link atherosclerosis and cancer," *European Journal of Immunology*, vol. 41, no. 9, pp. 2519–2522, 2011.
- [13] K. J. Woollard and F. Geissmann, "Monocytes in atherosclerosis: subsets and functions," *Nature Reviews Cardiology*, vol. 7, no. 2, pp. 77–86, 2010.
- [14] B. Pamukcu, G. Y. H. Lip, A. Devitt, H. Griffiths, and E. Shantsila, "The role of monocytes in atherosclerotic coronary artery disease," *Annals of Medicine*, vol. 42, no. 6, pp. 394–403, 2010.
- [15] P. Saha, B. Modarai, J. Humphries et al., "The monocyte/macrophage as a therapeutic target in atherosclerosis," *Current Opinion in Pharmacology*, vol. 9, no. 2, pp. 109–118, 2009.
- [16] C. Kruger, C. Schutt, U. Obertacke et al., "Serum CD14 levels in polytraumatized and severely burned patients," *Clinical and Experimental Immunology*, vol. 85, no. 2, pp. 297–301, 1991.
- [17] P. Barath, M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester, "Detection and localization of tumor necrosis factor in human atheroma," *American Journal of Cardiology*, vol. 65, no. 5, pp. 297–302, 1990.
- [18] F. Merhi-Soussi, B. R. Kwak, D. Magne et al., "Interleukin-1 plays a major role in vascular inflammation and atherosclerosis in male apolipoprotein E-knockout mice," *Cardiovascular Research*, vol. 66, no. 3, pp. 583–593, 2005.
- [19] K. Isoda, S. Sawada, N. Ishigami et al., "Lack of interleukin-1 receptor antagonist modulates plaque composition in apolipoprotein E-deficient mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 6, pp. 1068–1073, 2004.
- [20] M. Frankenberger, T. Sternsdorf, H. Pechumer, A. Pforte, and H. W. L. Ziegler-Heitbrock, "Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis," *Blood*, vol. 87, no. 1, pp. 373–377, 1996.
- [21] N. de Bont, M. G. Netea, C. Rovers et al., "LPS-induced release of IL-1 $\beta$ , IL-1Ra, IL-6, and TNF- $\alpha$  in whole blood from patients with familial hypercholesterolemia: no effect of cholesterol-lowering treatment," *Journal of Interferon and Cytokine Research*, vol. 26, no. 2, pp. 101–107, 2006.

## Review Article

# Trends in the Treatment of Hypertension from the Perspective of Traditional Chinese Medicine

Xingjiang Xiong,<sup>1</sup> Xiaochen Yang,<sup>1</sup> Wei Liu,<sup>1</sup> Fuyong Chu,<sup>2</sup>  
Pengqian Wang,<sup>3</sup> and Jie Wang<sup>1</sup>

<sup>1</sup> Department of Cardiology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beixiange NO. 5, Xicheng District, Beijing 100053, China

<sup>2</sup> Department of Cardiology, Traditional Chinese Medicine Hospital of Beijing, Beijing 100010, China

<sup>3</sup> Department of Endocrinology, Traditional Chinese Medicine Hospital of Mentougou District, Beijing 102300, China

Correspondence should be addressed to Xingjiang Xiong; [xiongxingjiangtcm@163.com](mailto:xiongxingjiangtcm@163.com) and Jie Wang; [wangjie0103@yahoo.cn](mailto:wangjie0103@yahoo.cn)

Received 9 October 2012; Revised 3 June 2013; Accepted 10 June 2013

Academic Editor: Keji Chen

Copyright © 2013 Xingjiang Xiong et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Hypertension is a major public-health issue. Much consensus has been reached in the treatment, and considerable progress has been made in the field of antihypertensive drugs. However, the standard-reaching rate of blood pressure is far from satisfaction. Considering these data and the seriousness of the effects of hypertension on the individual and society as a whole, both economically and socially, physicians must look for more effective and alternative ways to achieve the target blood pressure. Could treatment of hypertension be improved by insights from traditional Chinese medicine? As one of the most important parts in complementary and alternative therapies, TCM is regularly advocated for lowering elevated blood pressure. Due to the different understanding of the pathogenesis of hypertension between ancient and modern times, new understanding and treatment of hypertension need to be reexplored. Aiming to improve the efficacy of Chinese herbal medicine in treating hypertension, the basis of treatment is explored through systematically analyzing the literature available in both English and Chinese search engines. This paper systematically reviews the trends in emerging therapeutic strategies for hypertension from the perspective of traditional Chinese medicine.

## 1. Introduction

Hypertension is an increasingly important medical and public-health issue [1]. The prevention and management of hypertension are major public-health challenges. Many cardiovascular diseases (CVDs), cerebrovascular diseases, and hypertension would be preventable if the rise in blood pressure (BP) with age can be prevented or diminished [2]. Complementary and alternative medicine (CAM) therapies are increasingly popular and frequently used by patients with CVDs [3, 4]. Many CAM therapeutic options exist, and >95 CAM therapies have been recommended for hypertension [5]. Recent researches have shown that numerous CAM therapies are advocated for lowering elevated BP [5, 6]. Traditional Chinese medicine (TCM), including herbal medicine and acupuncture, is an important component of CAM therapies [7].

TCM played an important role in the provision of healthcare. Although there is no diagnosis of hypertension at ancient time in China, physicians attempted to treat hypertension-related symptoms by using TCM principles in clinical practice for centuries [8]. It is noteworthy that the poor antihypertensive efficacy of TCM has always been a major emerging problem, which has led to a high degree of consensus that Chinese medicines cannot independently treat hypertension [9–13]. However, considerable progress has been made in lowering BP by TCM recently [14–16]. Could treatment of hypertension be improved by insights from traditional Chinese medicine? Aiming to improve the efficacy of Chinese herbal medicine in treating hypertension, we explored the basis of treatment by systematically analyzing the literature available through both English and Chinese search engines, including the Cochrane Central Register of Controlled Trials (CENTRAL) in the Cochrane Library

(September, 2012), MEDLINE (1959–2012), PUBMED (1959–2012), EMBASE, Chinese National Knowledge Infrastructure (CNKI) (1980–2012), Chinese Scientific Journal Database (VIP) (1989–2012), Chinese Biomedical Literature Database (CBM) (1978–2012), and WANFANG (1998–2012) databases, that discuss the potential uses of Chinese medicine therapies to treat hypertension. It was found that the aspects described below should be considered for the effective treatment of hypertension, and it is possible for TCM to be mainstream in health care system. The paper systematically reviews trends in emerging therapeutic strategies for hypertension from the perspective of traditional Chinese medicine.

## 2. Disease-Syndrome Combination Is a New Mode for Treatment of Hypertension

Syndrome (also known as “pattern” or “zheng”) is the basic unit and a key concept in TCM theory, which has been used in China for over 2,500 years [17]. It is different from disease or symptom. TCM syndrome is the abstraction of a major disharmonious pathogenesis, which is identified from a comprehensive analysis of all symptoms and signs (tongue appearance and pulse feeling included) from four main diagnostic TCM methods: observation, listening, questioning, and pulse analyses [18, 19]. It is the generalization of cause, location, nature, and trend of disease in certain stage. In brief, all diagnostic and therapeutic methods in TCM are based on the differentiation of TCM syndrome [20, 21]. In ancient China, due to technological backwardness and lack of testing methods, Chinese practitioners explored the etiology and pathogenesis of a disease by conjecturing on the interior by observing the exterior; exploring the intrinsic etiology from extrinsic appearances; testing the state of the internal organs; and differentiating symptoms and signs. In general, it is believed that syndrome differentiation is a special feature and priority of TCM. Thus, syndrome differentiation plays an important role in the therapeutic process and affects the therapeutic result of certain diseases [22–24].

However, as mentioned above, syndrome is the outcome of differentiation of symptoms and signs, and definitely syndrome is relatively generous, vague, uncertain, and abstractive, which has brought great difficulties to the clinical and scientific research of TCM [17]. The following example can be used to explain the shortage of syndrome information. Hypertension, angioneurotic headache, and intracranial hypertension all could show similar symptoms and primary signs such as headache and dizziness, suggesting that they could be diagnosed as the same syndrome in TCM and could be treated by the same TCM approach. The effect, there is no doubt, should be different since the severity and prognosis of the above diseases are different. Thus, the differentiation of syndrome would not give any good effect when disease is not clarified based on biomedical diagnosis. More attentions have been paid to the treatment and the objective indicators of diseases. For example, BP, and blood levels of glucose and lipids as well as other objective indicators have become the focus of concern and assessment of therapeutic effects. Therefore, disease-syndrome combination, also called combination of syndrome classification and biomedical diagnosis, is a new

mode for syndrome research involving the idea and theory of disease differentiation in western medicine as well as syndrome differentiation in TCM [25]. Currently, it has become a common model in the diagnosis and treatment of TCM clinical practice [26]. The mode of disease-syndrome combination has been successfully and widely applied to a variety of clinical diseases including restenosis after percutaneous coronary intervention [27], unstable angina [28], cancer [29], nonerosive reflux disease [30], rheumatoid arthritis [31], posthepatitis B liver cirrhosis [32], liver fibrosis [33], poststroke dysphagia [34], menopausal symptoms [35], and psoriasis vulgaris [36].

The new mode has changed the treatment philosophy of hypertension and thus improved the antihypertensive efficacy of TCM. It included two cases. The first one is diagnosis based on disease-syndrome combination. As compared with TCM syndrome criteria or BP criteria alone, hypertension could be diagnosed more accurately by using the combination of TCM syndrome and BP. When referring to Chinese medicine treatment alone based on diagnosis of disease-syndrome combination, Zhong et al. [37] conducted a randomized controlled trial to observe the effect of Chinese herbal medicine for calming *Gan* and suppressing hyperactive *yang* (CGSHY) on arterial elasticity function and the circadian rhythm of blood pressure in patients with essential hypertension (EH). The authors studied 64 patients comparing Chinese herbal medicine with enalapril to a control. After 12 weeks of treatment, significant difference was found in the ratio of T/P of SBP & DBP and in the levels of NO and ET-1 between treatment and control groups after treatment. The trial showed that Chinese herbal medicine for CGSHY may lower the blood pressure smoothly, recover the circadian rhythm of BP in EH patients, and improve the carotid elasticity of EH patients, which is similar to that of enalapril.

The second one is treatment based on disease-syndrome combination. Previous studies have shown that TCM can improve syndromes and symptoms such as headache, vertigo, and fatigue (in particular for patients with liver *yang* hyperactivity syndrome or liver-kidney *yin* deficiency syndrome), whereas it cannot effectively improve objective indicators and pathological mechanisms such as BP, blood pressure variability (BPV), target organ damage, lipid disorders, insulin resistance, and leptin resistance [38–40]. Thus, it was concluded that Chinese herbs and formulas had poor efficacy for improving the objective indicators and pathological mechanisms of hypertension, leading to Chinese herbs and formulas being considered as “adjunctive treatment methods” [8]. Therefore, achieving the optimal target BP is a crucial issue in hypertensive patients currently. Numerous studies have confirmed that Chinese herbs and formulas could contribute to lowering BP smoothly [16]. While referring to combination therapy of Chinese medicine and western medicine based on treatment of disease-syndrome combination, another multicenter, randomized, double-blinded controlled trial was conducted by Li et al. [41]. A total of 270 cases were included to observe the effects of Chinese medical regimen and integrative medical regimen on quality of life and early renal impairment in elderly patients with isolated systolic hypertension (EISH). 3 groups were divided as Chinese medicine

group (CM), combination group, and western medicine group (WM). After 12 weeks of treatment, the combination group was superior to CM or WM group in depressing systolic blood pressure, improving integral of quality of life, and decreasing the levels of mALB and  $\beta$ 2-MG in urine. It was concluded that combination therapy has affirmative effect in treating EISH patient, and deserves further study.

The disease-syndrome combination is not only the cornerstone of hypertensive treatment in TCM, but also an important way of TCM “merging” into modern clinical treatment. It is noteworthy that one must pay considerable attention to syndromes as well as the objective indicators for the treatment of TCM syndrome and disease together. However, clinical evidence of the efficacy of TCM on the mortality and morbidity of hypertension still needs to be strengthened in future researches.

### 3. A Different Understanding between “Vertigo and Headache” and Hypertension Is the Ideological Basis for the Treatment of Hypertension

According to TCM theory, hypertension belongs to the category of “vertigo and headache,” which has been recorded in the ancient Chinese medical literature such as *The Canon of Internal Medicine* and *Synopsis of Golden Chamber*. Therefore, TCM principles, which have been used to treat “vertigo and headache” in clinical practice for centuries, have been applied to the treatment of hypertension by physicians in China. However, it cannot be neglected that some new problems have arisen in treating hypertension due to the different understanding between “vertigo and headache” and hypertension. Previous treatment experience cannot solve these problems perfectly. Both specific differences between them and new understanding about the etiology, pathogenesis, diagnosis, and treatment of hypertension are needed to be explored.

Firstly, “vertigo and headache” is not the same as hypertension. It is due to the following four reasons: (a) diagnostic criteria: as the concept of BP has not yet appeared in ancient China, the diagnosis, clinical evaluation, and treatment of “vertigo and headache” were all based on “signs and subjective symptoms instead of BP” according to the unique concept of “wholism” in the understanding and treatment of the disease [42, 43]. While in modern medicine, BP and blood pressure variability (BPV) are regarded as the diagnostic gold standard of hypertension [44]. Although we cannot rule out the possibility that most of the patients with “vertigo and headache” have hypertension, but the diagnostic basis in TCM is incompletely understood. Patients who are diagnosed with vertigo and headache in TCM may possibly not meet the diagnosis of hypertension actually. (b) Diagnosis time: “Vertigo and headache” could be only diagnosed when uncomfortable symptoms appear. It cannot be excluded that some hypertensive patients are asymptomatic. However, as the measurement of BP was so simple and easy to operate, hypertension is diagnosed much earlier, even in asymptomatic period or prehypertension stage. (c) Usage of antihypertensive drugs: there is no intervention of conventional

medicine for “vertigo and headache” in ancient time. However, hypertension is generally treated by antihypertensive drugs. (d) Control of BP: during the treatment of “vertigo and headache” in ancient time, the control of BP was still unclear. However, it could be well controlled by antihypertensive drugs in hypertension treatment. Therefore, we cannot structurally be consistent with the previous experience of “vertigo and headache” for hypertension [45].

Secondly, the pathogenesis of “vertigo and headache” has been changed and different pathogenesis between “vertigo and headache” and hypertension should also be paid attention to. In the ancient TCM literature, *Basic Questions of the Yellow Emperor’s Inner Classic: Truth and Importance by the Theory*, the author states “the wind syndrome, such as convulsion, vertigo, and dizziness, all belong to the liver,” which showed that vertigo is closely related to the “liver and wind” according to TCM theory. Therefore, it has become the precursor of treating hypertension from the perspective of liver-yang and liver-wind [46]. Recently, studies also indicated that liver yang hyperactivity syndrome is a crucial syndrome of hypertension [47, 48]. Consequently, it is widely accepted that hypertension should be treated based on the theory of liver-wind until now [49]. Famous prescriptions for calming liver-wind including *Zhen gan xi feng decoction* (decoction for tranquilizing liver-wind) in *Yi Xue Zhong Zhong Can Xi Lu* (*Records of Chinese Medicine with Reference to Western Medicine*), *Tianma Gouteng Yin* (“decoction of *Gastrodia* and *Uncaria*”) in *Za Bing Zheng Zhi Xin Yi* (*New Meanings in Syndrome and Therapy of Miscellaneous Diseases*), and *Lingjiao gouteng decoction* (decoction of *Antelope Horn* and *Uncaria*) in *Chong Ding Tong Su Shang Han Lun* (revised popular guide to *Treatise on Febrile Diseases*) are all widely used today. However, some new issues have emerged due to great changes have been taken place for the treatment of vertigo. And considerable attention should be paid on the different pathogenesis between “vertigo and headache” and hypertension. These differences are discussed below. (a) The natural progression of “vertigo and headache” has been changed due to the following three reasons in modern medicine: early diagnosis, early intervention, and the continual optimization of medical methods. In particular, the old transformational principle of pathogenesis about “vertigo and headache,” that is, “the changes from excess of liver fire to liver yang hyperactivity, then to liver-kidney yin deficiency, finally to deficiency of both yin and yang,” has been interrupted [50, 51]. (b) Therapeutic function of antihypertensive drugs has potential influence on the pathogenesis of “vertigo and headache.” For example, beta blockers can be used to decrease heart rate; however, this action could also purge liver fire and heart fire to significantly improve fire syndrome in TCM. Therefore, it can lead to deficiency syndrome more easily due to the negative chronotropic action and negative inotropic effects [52]. It is worth noting that the number of patients with liver yang hyperactivity syndrome, hyperactivity of heart fire, and liver fire syndrome is decreasing, whereas the number of patients with deficiency syndrome (such as *qi* deficiency syndrome and kidney deficiency syndrome) is increasing [16, 51]. (c) The adverse effects of antihypertensive drugs can affect the pathogenesis of “vertigo and headache” directly. For

instance, calcium-channel blockers have adverse effects such as hypotension, headache, facial flushing, polyuria, constipation, tibia, and ankle edema;  $\beta$ -blockers and diuretic could affect sexual function [53–56]. What is more, it was found out that patients who have used oral antihypertensive drugs for several years are more prone to suffer from waist soreness, tiredness in the loins and legs, and erectile dysfunction, which belongs to the category of fluid retention syndrome or kidney deficiency syndrome in TCM [57]. (d) Owing to changes of the disease spectrum, hypertension is associated with more comorbidities, thus leading to multisystem and multiorgan damage. With the increasing incidence of metabolic diseases, hypertension combined with diabetes mellitus and lipid abnormalities becomes quite common now [58, 59].

In summary, a different understanding between “vertigo and headache” and hypertension is the ideological basis for the treatment of hypertension. Due to the differences between “vertigo and headache” and hypertension, the pathogenesis law of hypertension is still unknown and should be reevaluated.

#### 4. Application of Herbal Pharmacological Achievements in Clinical Treatment Is an Important Reference for Lowering BP by Chinese Herbs and Formulas

In recent years, researchers have carried out several herbal pharmacology studies [60–62]. With continuous enrichment of achievements in herbal pharmacology, the effective components and pharmacological effects of many Chinese herbs and formulas have been illustrated [63], which provides evidence for the treatment of modern diseases [64]. Chinese herbs such as *Tianma* (*Gastrodia*) [65], *Gouteng* (*Uncaria*) [66], *Shijueming* (Abalone Shell), *Zhenzhumu* (Nacre or mother of pearl), *Daizheshi* (Ruddle), *Cishi* (Magnetite), *Juhua* (*Chrysanthemum*) [67], *Juemingzi* (Cassia seed), *Xixiancao* (*Herba Siegesbeckiae*) [68], *Baijili* (*Tribulus Terrestris* L.) [69], *Xiakucao* (*Prunella vulgaris* L.), *Luobuma* (*Apocynum venetum* L.), *Duzhong* (*Eucommia ulmoides*) [70–72], *Niuxi* (*Achyranthes* root), *Sangjisheng* (*Loranthus*) [73, 74], *Huangqi* (*Astragalus membranaceus*) [75–77], *Shengdihuang* (*Radix rehmanniae*), *Chuanxiong* (*Ligusticum Chuanxiong* Hort) [78], *Gegen* (*Kudzu* root) [79, 80], *Chishao* (red peony root), *Danshen* (*Salvia miltiorrhiza*) [81], *Yimucao* (*Leonurus japonicus*), *Shenghuaihua* (*Sophora* flower), *Chongweizi* (*Leonurus artemisia* (Lour.) S.Y. Hu seed), *Shanzha* (Hawthorn) [82, 83], *Laifuzi* (radish seed) [67], *Huanglian* (*Coptis chinensis*), *Huangqin* (*Scutellaria baicalensis* Georgi), *Huangbai* (*Phellodendron* bark), *Zhizi* (*Gardenia*), *Xuanshen* (*Radix scrophulariae*), and *Lianzixin* (lotus Germ) can lower BP. In addition, other Chinese herbs such as *Dahuang* (*Rheum officinale* Baill.), *Heshouwu* (*Polygonum multiflorum* Thunb.), *Nvzhenzi* (*Ligustrum lucidum* Ait.), *Jinyingzi* (Cherokee rose), *Zexie* (*Alisma orientale*), *Juemingzi* (Cassia seed), and *Shanzha* (Hawthorn) lower levels of blood lipids to prevent atherosclerosis and coronary heart disease.

Currently, two attitudes emerged about the application of herbal pharmacological achievements. The first one is

rejecting. It is partially believed that TCM should lay special stress on the importance of “tradition” instead of “modern” in China. They reject all modern scientific achievements, including achievements of modern medicine. Therefore, due to the traditional habit of thinking, most achievements in herbal pharmacology have not been widely used in clinic treatment. Treatment remains reliant mainly on traditional clinical experience under the guidance of syndrome differentiation by TCM physicians. The second one is accepting. It is advocated by modern TCM practitioners that modern scientific achievements, such as modern medicine, biology, chemistry, physics, and genetics, should be made full use of in the process of modernization of TCM [50]. We are in favor of the latter view. Why Chinese herbs and formulas have poor antihypertensive efficacy? An important reason is that achievements in herbal pharmacology cannot be effectively converted to appropriate clinical use. It is thought that syndrome differentiation, the traditional therapy, has perfect effects in improving the signs, symptoms, and syndrome in TCM, but poor effects in treating disease (and even no effects in improving the key indicators of diseases) [84]. Therefore, application of the achievements of herbal pharmacology in clinical treatment is an important reference for lowering BP using Chinese herbs and formulas.

However, some new problems have risen in the application of these achievements. Although some physicians have already consciously taken advantage of achievements in clinical treatment, most prescriptions are simple additions of these achievements, which violate the theory of syndrome differentiation in TCM. Therefore, how to appropriately apply herbal pharmacology achievements in the clinic without violating TCM theory should be considered carefully [50]. Our strategy is to combine syndrome differentiation and the achievements of herbal pharmacology together in clinical treatment. Clinical efficacy could be greatly improved if suitable herbs and formulas with clear therapeutic effects against disease were applied on the basis of the syndrome differentiation theory in TCM. It is noteworthy that some crucial Chinese herbs in these classical formulas (recommended for hypertension treatment in Table 1) definitely possess certain antihypertensive effect. All of these will be discussed as below.

According to our previous studies, hypertension could be divided into the following three major types on the basis of the stage and symptoms of the disease [8, 16]. Firstly, fire syndrome (e.g., liver fire, heart fire, stomach fire, and intestinal fire) can be found in various stages of hypertension, especially if target-organ damage is not found [16, 51]. When aiming to counteract liver fire syndrome, *Tianma Gouteng Yin* (decoction of *Gastrodia* and *Uncaria*), a famous prescription noted in *Za Bing Zheng Zhi Xin Yi* (*New Meanings in Syndrome and Therapy of Miscellaneous Diseases*), was recommended. It could suppress liver yang hyperactivity, clear heat, activate blood, and nourish the kidney. One systematic review (SR) also revealed that *Tianma Gouteng Yin* could contribute to lowering BP smoothly [85]. Moreover, Chinese herbs such as *Tianma* (*Gastrodia*), *Gouteng* (*Uncaria*), *Duzhong* (*Eucommia ulmoides*), and *Niuxi* (*Achyranthes* root) in the formula had good antihypertensive effects in pharmacological

TABLE 1: Crucial Chinese herbs in classical formulas recommended for hypertension treatment.

Syndrome	Formulas	Components	TCM efficacy	Crucial Chinese herbs
Fire syndrome	<i>Tianma Gouteng Yin</i> (decoction of <i>Gastrodia</i> and <i>Uncaria</i> )	<i>Tianma</i> ( <i>Gastrodia</i> ), <i>Gouteng</i> ( <i>Uncaria</i> ), <i>Shijueming</i> ( <i>Abalone shell</i> ), <i>Duzhong</i> ( <i>Eucommia ulmoides</i> ), <i>Niuxi</i> ( <i>Achyranthes</i> root), <i>Sangjisheng</i> ( <i>Loranthus</i> ), <i>Zhizi</i> ( <i>Gardenia</i> ), <i>Huangqin</i> ( <i>Scutellaria Baicalensis Georgi</i> ), <i>Yimucao</i> ( <i>Leonurus japonicus</i> ), <i>Yejiaoteng</i> ( <i>Caulis polygoni multiflori</i> ), and <i>Fushen</i> ( <i>Poria cocos</i> ).	Suppressing liver yang hyperactivity, clearing heat, activating blood, and nourishing the kidney.	<i>Tianma</i> ( <i>Gastrodia</i> ), <i>Gouteng</i> ( <i>Uncaria</i> ), <i>Duzhong</i> ( <i>Eucommia ulmoides</i> ), and <i>Niuxi</i> ( <i>Achyranthes</i> root).
	<i>Huanglian Jie Du Tang</i> (detoxicant decoction of <i>Coptis</i> )	<i>Huanglian</i> ( <i>Coptis Chinensis</i> ), <i>Huangqin</i> ( <i>Scutellaria Baicalensis Georgi</i> ), <i>Huangbai</i> ( <i>Phellodendron</i> bark), and <i>Zhizi</i> ( <i>Gardenia</i> ).	Counteracting heart fire syndrome, clearing heat and toxic materials, and relieving headache and dizziness.	<i>Huanglian</i> ( <i>Coptis Chinensis</i> ), <i>Huangqin</i> ( <i>Scutellaria Baicalensis Georgi</i> ), <i>Huangbai</i> ( <i>Phellodendron</i> bark), and <i>Zhizi</i> ( <i>Gardenia</i> ).
	<i>Zeng Ye Tang</i> (fluid-increasing decoction)	<i>Xuanshen</i> ( <i>Radix scrophulariae</i> ), <i>Maidong</i> ( <i>Dwarf lilyturf tuber</i> ), and <i>Shengdihuang</i> ( <i>Radix rehmanniae</i> ).	Counteracting stomach fire and intestinal fire syndrome and nourishing yin to relieve the symptoms of dryness.	<i>Xuanshen</i> ( <i>Radix scrophulariae</i> ) and <i>Shengdihuang</i> ( <i>Radix rehmanniae</i> ).
Fluid retention syndrome	<i>Wuling powder</i>	<i>Fuling</i> ( <i>Poria cocos</i> ), <i>Guizhi</i> ( <i>Cassia twig</i> ), <i>Zhuling</i> ( <i>Polyporus</i> ), <i>Zexie</i> ( <i>Alisma</i> ), and <i>Baizhu</i> ( <i>Atractylodes</i> ).	Removing dampness by promoting diuresis.	<i>Zexie</i> ( <i>Alisma</i> ) and <i>Baizhu</i> ( <i>Atractylodes</i> ).
	<i>Zexie Tang</i> (decoction of <i>Alisma</i> )	<i>Zexie</i> ( <i>Alisma</i> ) and <i>Baizhu</i> ( <i>Atractylodes</i> ).	Counteracting fluid retention syndrome.	<i>Zexie</i> ( <i>Alisma</i> ) and <i>Baizhu</i> ( <i>Atractylodes</i> ).
	<i>Banxia Baizhu Tianma Tang</i> (decoction of <i>Pinellia ternata</i> , <i>Atractylodes macrocephala</i> , and <i>Gastrodia elata</i> )	<i>Banxia</i> ( <i>Pinellia ternata</i> ), <i>Baizhu</i> ( <i>Atractylodes</i> ), <i>Tianma</i> ( <i>Gastrodia</i> ), <i>Chenpi</i> ( <i>Tangerine peel</i> ), <i>Fuling</i> ( <i>Poria cocos</i> ), <i>Gancao</i> ( <i>Glycyrrhiza</i> ), <i>Shengjiang</i> ( <i>Ginger</i> ), and <i>Hongzao</i> ( <i>Red jujube</i> ).	Calming the liver, strengthening the spleen, and dissipating excessive fluid.	<i>Tianma</i> ( <i>Gastrodia</i> ).
Deficiency syndrome	<i>Liu Wei Dihuang Wan</i> (pill of <i>Rehmannia</i> )	<i>Dihuang</i> ( <i>Radix rehmanniae</i> ), <i>Shanyuou</i> ( <i>Pulp of cornus</i> ), <i>Shanyao</i> ( <i>Yam</i> ), <i>Fuling</i> ( <i>Poria cocos</i> ), <i>Zexie</i> ( <i>Alisma</i> ), and <i>Danpi</i> ( <i>Cortex moutan</i> ).	Replenishing kidney yin.	<i>Dihuang</i> ( <i>Radix rehmanniae</i> ) and <i>Danpi</i> ( <i>Cortex moutan</i> ).
	<i>Shen qi Wan</i> (kidney <i>qi</i> pill)	<i>Guizhi</i> ( <i>Cassia twig</i> ), <i>Fuzi</i> ( <i>Prepared aconite root</i> ), <i>Dihuang</i> ( <i>Radix rehmanniae</i> ), <i>Shanyuou</i> ( <i>Pulp of cornus</i> ), <i>Shanyao</i> ( <i>Yam</i> ), <i>Fuling</i> ( <i>Poria cocos</i> ), <i>Zexie</i> ( <i>Alisma</i> ), and <i>Danpi</i> ( <i>Cortex moutan</i> ).	Recuperating the kidney yang.	<i>Dihuang</i> ( <i>Radix rehmanniae</i> ) and <i>Danpi</i> ( <i>Cortex moutan</i> ).

studies. Modern studies also showed that *Tianma Gouteng Yin* could improve memory, regulate the secretions of vasoactive substances, increase the serum concentration of nitric oxide (NO) and nitric oxide synthase (NOS), decrease levels of endothelin and angiotensin II, reduce the left ventricular mass index and collagen content, and improve left ventricular remodeling [86–88]. When aiming to counteract heart fire

syndrome, *Huanglian Jie Du Tang* (detoxicant decoction of *Coptis*) was recommended [8]. It could clear heat and toxic materials and relieve headache and dizziness quickly. In addition, all the component drugs such as *Huanglian* (*Coptis chinensis*), *Huangqin* (*Scutellaria baicalensis Georgi*), *Huangbai* (*Phellodendron* bark), and *Zhizi* (*Gardenia*) could lower BP. Current researches also showed that *Huanglian Jie Du*

*Tang* could control BP and improve the prothrombotic state in spontaneously hypertensive rats (SHRs) by decreasing the content of thromboxane (TX) A<sub>2</sub>, endothelin (ET)-1, homocysteine, and von Willebrand factor (vWF) and increasing the contents of 6-keto-prostaglandin and NO [89]. When aiming to counteract stomach fire and intestinal fire syndrome, *Zeng Ye Tang* (fluid-increasing decoction) was recommended [8]. It could not only nourish yin to relieve the symptoms of dryness but also lower BP by *Xuanshen* (*Radix scrophulariae*) and *Shengdihuang* (*Radix rehmanniae*) in the formula.

Secondly, phlegm-fluid retention syndrome is another important type of hypertension in TCM, which is characterized by dizziness aggravated by change in body position; thirst without a desire to drink, or not being thirsty; chest distress; palpitation; gastric distension; abdominal distension; nausea; vomiting; poor appetite; lumbar heaviness; low back pain; weakness and heaviness in the lower extremities; edema; daytime sleepiness; abnormal leucorrhea; dysuria; and greasy fur [51]. It could be also divided into 2 syndromes: fluid retention syndrome and upward going of phlegm turbidity syndrome (UPTS). The former one could be treated by *Wuling* powder, *Zexie Tang* (decoction of *alisma*), and so forth. *Wuling* powder, a famous classical prescription recorded in *Shang Han Lun* (*Treatise on Febrile Diseases*) by *Zhang Zhongjing* in the Han Dynasty, was recommended [8]. It could not only remove dampness by promoting diuresis but also had satisfying therapeutic effects in increasing the discharge of urine, decreasing BP, and maintaining the balance of serum electrolyte contents in rats with renal hypertension [90]. *Zexie Tang* is another famous classical prescription used for treating fluid retention syndrome. It is effective in improving the construction and function of kidney injuries of rats with hypertension induced by a high-salt diet. This effect may be associated with increasing renin activity and angiotensin-II level in renal tissues, which thereby prevent kidney injuries [91]. *Zexie* (*Alisma*) and *Baizhu* (*Atractylodes*), the two component drugs in *Zexie Tang* (decoction of *alisma*), can lower the blood levels of lipids and glucose, respectively. While referring to the later one, fluid retention syndrome and liver-yang hyperactivity syndrome often appear simultaneously, which is known as UPTS. *Banxia Baizhu Tianma Tang* (decoction of *Pinellia ternata*, *Atractylodes macrocephala*, and *Gastrodia elata*) was recommended [8]. It could calm the liver, strengthen the spleen, and dissipate excessive fluid. One SR demonstrated that *Banxia Baizhu Tianma Tang* could contribute to lowering BP [92]. Among them, *Tianma* (*Gastrodia*), the monarch drug of the formula, is an important Chinese herb for lowering BP. Modern studies also showed that it can improve clinical symptoms, promote a stable decrease in BP, improve insulin resistance and salt sensitivity, decrease serum levels of total cholesterol (TC), triglycerides (TGs), and low-density lipoprotein-cholesterol (LDL-C), and regulate the renin-angiotensin system (RAS) [93–95].

Deficiency syndrome is very common in hypertension [16, 51]. It is found that the longer the duration of illness, the higher the incidence rate of deficiency syndrome. When aiming to counteract kidney yin deficiency syndrome, *Liu Wei Dihuang Wan* (pill of *Rehmannia*) was recommended

[8]. It could replenish kidney yin. One SR showed that *Liu Wei Dihuang Wan* could also contribute to hypertension treatment [96]. Among them, *Dihuang* (*Radix rehmanniae*) and *Danpi* (*Cortex moutan*) could lower BP. Studies have also shown that it can lessen damage to the heart and kidney in hypertensive patients by reducing the myocardial oxygen consumption index, reversing myocardial hypertrophy, improving left ventricular function, protecting the endothelium of blood vessels, preventing arteriosclerosis, increasing the glomerular filtration rate, and decreasing microalbuminuria [97, 98]. When aiming to counteract kidney yang deficiency syndrome, *Shen qi Wan* (kidney qi pill) was recommended [8]. It could not only recuperate the kidney yang but also improve the sexual function of hypertensive patients, reduce urinary levels of albumin, and protect renal function [99, 100].

Although application of herbal-pharmacology achievements to clinical treatment could contribute to disease-targeted treatment in TCM, the following aspects must be addressed. Firstly, as “formulas corresponding to syndromes” is a basic principle of TCM treatment, it is advocated that Chinese herbs and formulas which can not only treat the disease in western medicine but also TCM syndromes could be preferentially selected [50]. Hence, we cannot select Chinese herbs and formulas solely on the basis of their pharmacological effects in clinical treatment. For instance, there are many Chinese herbs for treating kidney deficiency syndrome; however, only *Duzhong* (*Eucommia ulmoides*), *Niuxi* (*Achyranthes* root), and *Sangjisheng* (*Loranthus*) should be given prior consideration for having a clear effect of treating hypertension and kidney deficiency syndrome together. Secondly, studies on the bioactive constituents of Chinese herbs cannot represent all the characteristics of Chinese herbs (especially the rules for clinical application).

## 5. Reasonable Drug Dosage Is the Effective Way to Improve the Antihypertensive Efficacy of Chinese Herbs and Formulas

There exists an interesting phenomenon in clinical practice. That is, although some TCM physicians choose certain Chinese herbs and formulas with clear antihypertensive effects, antihypertensive efficacy remains difficult to improve. It may be related to the limitation of drug dose.

There are many factors that affect the clinical efficacy of TCM. These include an accurate diagnosis, accurate prescriptions, quality and dose of Chinese herbs, water decoction, and administration method. Among them, dosage remains the key issue restricting the use of ancient formulas today [45]. As the saying goes, “the secret of Chinese medicine is in the dosage,” the dosage of Chinese herbs has always been difficult to study. It can be influenced by mistakes in the calculation of the dose in the original historical texts, the production area and effective fraction of Chinese herbs, and the formulation of the preparation. Due to differences of weights and measures between ancient and modern times, it is generally agreed that one “*Liang*” in ancient China is equal to 3 g. Irrespective of *Zhang Zhongjing*’s classical prescriptions

TABLE 2: Drug dosage of crucial Chinese herbs recommended for hypertension treatment.

Chinese herbs	TCM efficacy	Indications	Routine dosage	Recommended dosage
<i>Chuanxiong</i> ( <i>Ligusticum Chuanxiong</i> Hort)	Promoting <i>qi</i> , activating blood circulation, and relieving pain.	Hypertension with severe headache, hypertensive crisis, and hypertensive encephalopathy.	3–10 g	≥30 g
<i>Niuxi</i> ( <i>Achyranthes</i> root)	Nourishing liver and kidney, strengthening muscles and bones, promoting blood circulation to remove blood stasis, making fire and blood downstream, promoting diuresis, and relieving stranguria.	Hypertension with lower-limb swelling and tiredness in the loins and knees.	5–12 g	60–120 g
<i>Tianma</i> ( <i>Gastrodia</i> )	Calming liver, suppressing liver-yang hyperactivity, activating collaterals, and relieving spasm.	Hypertension with dizziness.	3–10 g	≥30 g
<i>Juhua</i> ( <i>Chrysanthemum</i> )	Clearing away heat, dispelling wind, calming the liver, and improving eyesight.	Hypertension with dizziness.	5–10 g	≥30 g

or current prescriptions, the dose conversion refers to the criteria used today. This leads to the limitation of doses of many Chinese herbs except for natural minerals of Chinese herbs in the *Pharmacopoeia of the People's Republic of China* (2010 edition). It greatly limits flexible application of drug dose by TCM physicians. Also, some physicians have paid insufficient attention to the dosage problem, especially for monarch drugs such as *Tianma* (*Gastrodia*), *Chuanxiong* (*Ligusticum Chuanxiong* Hort), *Juhua* (*Chrysanthemum*), *Shigao* (*Gypsum fibrosum*), and *Niuxi* (*Achyranthes* root) in the treatment of hypertension [51].

Routine dosages of those monarch drugs in *Pharmacopoeia of the People's Republic of China* (2010 edition) are shown in Table 2. However, in our previous studies, we demonstrated the relationship between Chinese herbs and dosages through the literature review and mining [45, 51]. The result indicated that although routine dosage such as 3 to 10 g could control BP in some extent, higher dosage of certain monarch Chinese herbs may show better antihypertensive effects in hypertension treatment. The dose-effect relationship is obvious in a certain range. It is worth noting that the conclusions have been confirmed in randomized controlled trials of type 2 diabetes in China. And the recommended dosages of those monarch Chinese herbs are also shown in Table 2.

Taking *Chuanxiong* (*Ligusticum Chuanxiong* Hort) as an example, it is often used in the treatment for hypertension with severe headache. The dose must be greater if the headache is more severe with high BP, whereas the routine dose (3–10 g) is usually ineffective [101]. Also, the recommended dose must be ≥30 g if treating hypertensive crisis and hypertensive encephalopathy [51]. *Niuxi* (*Achyranthes* root) is another important drug often used in the treatment for hypertension with lower-limb swelling and tiredness in the loins and knees. The recommended dose of *Niuxi* (*Achyranthes* root) must be ≥60 g for lowering BP [51], whereas the routine dosage is “5–12 g” [101]. It is also found that the higher

the BP, the greater the dosage. *Chuan Niuxi* (*Achyranthes bidentata*) and *Huai Niuxi* (*Achyranthes bidentata* Blume) are used simultaneously when treating hypertensive crisis and hypertensive encephalopathy. Also, the dose of these herbs can start from 30 g to a maximum dose of ≤120 g, respectively [51]. *Tianma* (*Gastrodia*) and *Juhua* (*Chrysanthemum*) are two classical Chinese herbs used for treating hypertension with dizziness. The routine dosages of these two herbs are “3–10 g” and “5–10 g,” respectively [101]. It was also found that the recommended dose of these herbs must be ≥30 g when lowering BP [51, 102]. Therefore, it could be concluded that reasonable dosage of Chinese herbs is closely related to clinical outcomes. However, further randomized, placebo-controlled clinical trials with strict design and adequate sample size are warranted to elaborate the dose-effect relationship and mechanism of Chinese herbs and formulas on treating hypertension. Moreover, due to the certain toxic and adverse effects of these herbs, indications of herbs must be followed strictly [101].

## 6. Discussion and Perspectives

CAM has been frequently used and is gaining popularity worldwide, especially in the most developed countries such as North America, Europe, and Australia. Due to aging of population and prevalence of chronic diseases and stress-related diseases as well as concern about the adverse reaction of chemical drugs, CAM is increasingly popular [103–107]. Recently, Nature has published a special issue of traditional Asian medicine (vol. 480 no. 7378\_supp ppS81–S121, 2011). The topic was derived from TCM, also including Kampo medicine and Korean medicine. It traced its origin to today's modernization. As the editorial department comments, it “plays an important role in health maintenance for the peoples of Asia, and is becoming more frequently used in countries in the

West” [108]. Some perspectives in the special edition are very insightful and very heuristic.

The merits of traditional medicine to people’s healthcare have been proven by its age-old legacy, its clinical efficacy in curing diseases, and improving quality of life from the ancient times to the present day [109–112]. TCM has long been used in the treatment of a wide variety of illnesses including hypertension. And a series of medical practices were originated including Chinese herbs and formulas, acupuncture, moxibustion, cupping, qigong, Tai Chi (shadow boxing exercise), diet, and exercise therapy [113–116]. Recently, many RCT trials and SRs showed that acupuncture is useful for lowering BP level and improving the circadian rhythm of BP in patients with hypertension [117–120]. Among them, Chinese herbal therapy is the most commonly used. Current researches showed that great progress has been made in Chinese herbs and formulas for the treatment of hypertension from theory, experiments to clinic fields both in vitro and vivo [121–126]. In our previous studies, we also demonstrated the potential effect of Chinese herbs and formulas on decreasing blood pressure, protecting target organs, regulating rennin-angiotensin-aldosterone system, reversing risk factors, and improving endothelial function, although their effects on BP were inferior to CCB, ACEI, ARB, and other common used western medicines [16, 51, 127]. Furthermore, as a special form of Chinese herbs and formulas, Chinese patent drugs are commonly used in the treatment of hypertension in clinic, including Niu Huang Jiangya Pill, Qing Gan Jiang Ya Capsule, and Yangxue Qingnao Granule [128, 129]. They have also played an important role in the treatment of hypertension. However, due to the generally low methodological quality and small sample size, single-center of TCM clinical trials, there is little conclusive clinical evidence to support the antihypertensive efficacy of the vast majority of Chinese medicines [8, 130]. Therefore, although the popularity and expenditure of TCM have been increased dramatically, the potential role of TCM in modern hypertension clinical practice and health care system seems to be limited and have even been questioned.

In the light of our previous studies, it is not difficult to find that the etiology, pathogenesis, and control strategies of hypertension have been changed quietly. The concept highlighting the combination of syndrome and disease, reunderstanding the etiology and pathogenesis, and applying pharmacological achievement, as well as paying attention to dosage, has been gradually and widely accepted, which happens to coincide with the concept and treatment of modern medicine based on biomedical diagnosis. Only by facing these aspects rigorously, the clinical efficacy of Chinese herbs and formulas on hypertension could be improved. What is more, the emergence of evidence-based medicine (EBM) had provided objective therapeutic evaluation of TCM or integrative medicine with new thinking and method [131]. Eugene Braunwald, a famous cardiologist, also pointed out that current cardiology practice is evidence-based and global in scope [132]. Thus, it is urgent to formulate a scientific way to evaluate the efficacy and safety. Unfortunately, due to the poor methodological quality in the majority of current controlled studies, confirmative conclusions on the beneficial

effect of TCM for essential hypertension could not be drawn conclusively, and more rigorous trials are needed. We firmly believe that applying these findings to clinical researches will play an important role in hypertension prevention and public health practice.

Additionally, making good use of these findings and achievements could contribute to modern new drug discovery for hypertension in TCM [16, 133]. Due to the inadequate effectiveness and concerns of safety of current antihypertensive western drugs, a great need has arisen to develop both efficacious and pharmaceutical medicines to fight against this disease. Screening the high efficiency and fewer adverse effects of antihypertensive drugs from Chinese herbs and formulas for hypertension attracts great attention of researchers. From the 1950s, Chinese physicians had been concentrating on effective prevention strategies for hypertension using TCM, and considerable progress had been achieved. Alkaloids of *Rauwolfia verticillata* (the first antihypertensive drug independently invented in China) consist of *reserpine* and ingredients such as  $\alpha$ -receptor blockers. Total puerarin flavonoids and tetramethylpyrazine are effective components extracted from *Gegen* (*Kudzu* root) and *Chuanxiong* (*Ligusticum chuanxiong* Hort), respectively, by scientists from the Chinese Academy of Medical Sciences; both of which could not only lower BP but also improve signs and symptoms in hypertensive patients [134]. It is noteworthy that due to the unique experience in treating hypertension and its related symptoms in TCM, effective drug screening would be more targeted [135]. The development of modern new drugs for hypertension will be full of challenge and opportunity, but we have full confidence.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Author’s Contribution

X. Xiong and X. Yang contributed equally to this paper.

## Acknowledgments

This work was supported in part by the National Basic Research Program of China (973 Program, 2003CB517103) and the National Natural Science Foundation Project of China (90209011).

## References

- [1] K. Sliwa, S. Stewart, and B. J. Gersh, “Hypertension: a global perspective,” *Circulation*, vol. 123, no. 24, pp. 2892–2896, 2011.
- [2] H. Redwood, “Hypertension, society, and public policy,” *European Heart Journal*, vol. 9, pp. B13–B18, 2007.
- [3] K. J. Chen, K. K. Hui, M. S. Lee, and H. Xu, “The potential benefit of complementary/alternative medicine in cardiovascular diseases,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 125029, 1 page, 2012.

- [4] G. Y. Yeh, R. B. Davis, and R. S. Phillips, "Use of complementary therapies in patients with cardiovascular disease," *American Journal of Cardiology*, vol. 98, no. 5, pp. 673–680, 2006.
- [5] E. Ernst, "Complementary/alternative medicine for hypertension: a mini-review," *Wiener Medizinische Wochenschrift*, vol. 155, no. 17–18, pp. 386–391, 2005.
- [6] R. Nahas, "Complementary and alternative medicine approaches to blood pressure reduction: an evidence-based review," *Canadian Family Physician*, vol. 54, no. 11, pp. 1529–1533, 2008.
- [7] H. Xu and K. Chen, "Integrating traditional medicine with biomedicine towards a patient-centered healthcare system," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 83–84, 2011.
- [8] J. Wang and X. J. Xiong, "Evidence-based Chinese medicine for hypertension," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 978398, 12 pages, 2013.
- [9] H. W. Zhang, J. Tong, G. Zhou, H. Jia, and Y. Jiang, "Tianma gouteng yin formula for treating primary hypertension," *Cochrane Database of Systematic Reviews*, no. 6, Article ID CD008166, 2012.
- [10] P. M. Stavro, M. Woo, T. F. Heim, L. A. Leiter, and V. Vuksan, "North American ginseng exerts a neutral effect on blood pressure in individuals with hypertension," *Hypertension*, vol. 46, no. 2, pp. 406–411, 2005.
- [11] P. M. Stavro, M. Woo, L. A. Leiter, T. F. Heim, J. L. Sievenpiper, and V. Vuksan, "Long-term intake of North American ginseng has no effect on 24-hour blood pressure and renal function," *Hypertension*, vol. 47, no. 4, pp. 791–796, 2006.
- [12] J. J. Li, Z. L. Lu, W. R. Kou et al., "Long-term effects of xuezhikang on blood pressure in hypertensive patients with previous myocardial infarction: data from the Chinese coronary secondary prevention study (CCSPS)," *Clinical and Experimental Hypertension*, vol. 32, no. 8, pp. 491–498, 2010.
- [13] J. J. Li, Z. L. Lu, W. R. Kou et al., "Impact of Xuezhikang on coronary events in hypertensive patients with previous myocardial infarction from the China Coronary Secondary Prevention Study (CCSPS)," *Annals of Medicine*, vol. 42, no. 3, pp. 231–240, 2010.
- [14] J. S. Janicki and S. P. Levick, "The convergence of ancient chinese medicine with modern therapeutics to prevent cardiac fibrosis," *American Journal of Hypertension*, vol. 25, no. 2, p. 139, 2012.
- [15] J. Wang, B. Feng, and X. J. Xiong, "Chinese herbal medicine for the treatment of obesity-related hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 757540, 11 pages, 2013.
- [16] X. J. Xiong, X. C. Yang, Y. M. Liu, Y. Zhang, P. Q. Wang, and J. Wang, "Chinese herbal formulas for treating hypertension in traditional Chinese medicine: perspective of modern science," *Hypertension Research*, 2013.
- [17] J. Wang, P. Q. Wang, and X. J. Xiong, "Current situation and re-understanding of syndrome and formula syndrome in Chinese medicine," *Internal Medicine*, 2012.
- [18] K. J. Chen and L. Li, "Study of traditional Chinese medicine—which is after all the right way?" *Chinese Journal of Integrative Medicine*, vol. 11, no. 4, pp. 241–242, 2005.
- [19] F. Cheung, "TCM: made in China," *Nature*, vol. 480, supplement 7378, pp. S82–S83, 2011.
- [20] P. Tian, "Where West meets East," *Nature*, vol. 480, supplement 7378, pp. S84–S86, 2011.
- [21] K. J. Chen, "Clinical service of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 14, no. 3, pp. 163–164, 2008.
- [22] L. Liu, "The clinical trial barriers," *Nature*, vol. 480, supplement 7378, p. S100, 2011.
- [23] G. Dobos and I. Tao, "The model of Western integrative medicine: the role of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 11–20, 2011.
- [24] X. Xiong, F. Chu, H. Li, and Q. He, "Clinical application of the TCM classic formulae for treating chronic bronchitis," *Journal of Traditional Chinese Medicine*, vol. 31, no. 1, pp. 69–72, 2011.
- [25] J. Wang and X. J. Xiong, "Current situation and perspectives of clinical study in integrative medicine in China," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.
- [26] K. J. Chen and H. Xu, "The integration of traditional Chinese medicine and Western medicine," *European Review*, vol. 11, no. 2, pp. 225–235, 2003.
- [27] Q. H. Shang, H. Xu, X. Y. Lu, C. Wen, D. Shi, and K. Chen, "A multi-center randomized double-blind placebo-controlled trial of Xiongshao Capsule in preventing restenosis after percutaneous coronary intervention: a subgroup analysis of senile patients," *Chinese Journal of Integrative Medicine*, vol. 17, no. 9, pp. 669–674, 2011.
- [28] F. Y. Chu, J. Wang, K. W. Yao, and Z. Z. Li, "Effect of Xuefu Zhuyu Capsule on the symptoms and signs and health-related quality of life in the unstable angina patients with blood-stasis syndrome after percutaneous coronary intervention: a randomized controlled trial," *Chinese Journal of Integrative Medicine*, vol. 16, no. 5, pp. 399–405, 2010.
- [29] Z. Y. Tang and J. Wang, "Fighting against acquired immunodeficiency syndrome with Chinese medicine: a perspective from China," *Chinese Journal of Integrative Medicine*, vol. 17, no. 5, pp. 323–324, 2011.
- [30] B. S. Li, Z. H. Li, X. D. Tang et al., "A randomized, controlled, double-blinded and double-dummy trial of the effect of Tongjiang Granule on the nonerosive reflux disease of and Gan-Wei incoordination syndrome," *Chinese Journal of Integrative Medicine*, vol. 17, no. 5, pp. 339–345, 2011.
- [31] C. Zhang, M. Jiang, and A. Lu, "A traditional Chinese medicine versus Western combination therapy in the treatment of rheumatoid arthritis: two-stage study protocol for a randomized controlled trial," *Trials*, vol. 12, p. 137, 2011.
- [32] X. Deng, J. Liang, F. S. Wu, Y. B. Li, Y. P. Zhang, and Y. F. Tang, "Influence of Fuzheng Huayu Tablet on mental state and social function of patients with post-hepatitis B liver cirrhosis," *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 466–472, 2012.
- [33] P. Liu, "Fuzheng Huayu Capsule in the treatment of liver fibrosis: clinical evidence and mechanism of action," *Chinese Journal of Integrative Medicine*, vol. 18, no. 5, pp. 398–400, 2012.
- [34] X. G. Feng, W. J. Hao, Z. Ding, Q. Sui, H. Guo, and J. Fu, "Clinical Study on Tongyan Spray for post-stroke dysphagia patients: a randomized controlled trial," *Chinese Journal of Integrative Medicine*, vol. 18, no. 5, pp. 345–349, 2012.
- [35] C. Shou, J. Li, and Z. Liu, "Complementary and alternative medicine in the treatment of menopausal symptoms," *Chinese journal of integrative medicine*, vol. 17, no. 12, pp. 883–888, 2011.
- [36] C. J. Lu, Y. Xiang, X. L. Xie, M. L. Xuan, and Z. H. He, "A randomized controlled single-blind clinical trial on 84 outpatients with psoriasis vulgaris by auricular therapy combined with optimized Yinxieling Formula," *Chinese Journal of Integrative Medicine*, vol. 18, no. 3, pp. 186–191, 2012.

- [37] G. W. Zhong, M. J. Chen, Y. h. Luo et al., "Effect of Chinese herbal medicine for calming Gan and suppressing hyperactive yang on arterial elasticity function and circadian rhythm of blood pressure in patients with essential hypertension," *Chinese Journal of Integrative Medicine*, vol. 17, no. 6, pp. 414–420, 2011.
- [38] A. Benetos, P. Salvi, and P. Lacolley, "Blood pressure regulation during the aging process: the end of the "hypertension era"?" *Journal of Hypertension*, vol. 29, no. 4, pp. 646–652, 2011.
- [39] K. Yin and C. K. Tang, "Inflammation, lipid metabolism dysfunction, and hypertension: active research fields in atherosclerosis-related cardiovascular disease in China," *Science China Life Sciences*, vol. 54, no. 10, pp. 976–979, 2011.
- [40] R. E. Katholi and D. M. Couri, "Left ventricular hypertrophy: major risk factor in patients with hypertension: update and practical clinical applications," *International Journal of Hypertension*, vol. 2011, Article ID 495349, 10 pages, 2011.
- [41] H. Li, L. Liu, W. Zhao et al., "Effect of traditional and integrative regimens on quality of life and early renal impairment in elderly patients with isolated systolic hypertension," *Chinese Journal of Integrative Medicine*, vol. 16, no. 3, pp. 216–221, 2010.
- [42] H. Xu and K. Chen, "Integrative medicine: the experience from China," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 3–7, 2008.
- [43] K. J. Chen, "Where are we going?" *Chinese Journal of Integrative Medicine*, vol. 16, no. 2, pp. 100–101, 2010.
- [44] G. Mancia, G. De Backer, A. Dominiczak et al., "Guidelines for the management of arterial hypertension: the task force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)," *Journal of Hypertension*, vol. 25, no. 6, pp. 1105–1187, 2007.
- [45] X. J. Xiong and J. Wang, "Application of classic formulae in treatment of hypertension," *Zhongguo Zhong Yao Za Zhi*, vol. 38, no. 11, pp. 27–31, 2013.
- [46] W. Liu, X. J. Xiong, and J. Wang, "Clinical application of therapy eliminating turbidity and calming liver in the treatment of essential hypertension with metabolic disorders," *Zhongguo Zhong Yao Za Zhi*, vol. 38, no. 8, pp. 1251–1254, 2013.
- [47] X. J. Xiong, X. C. Yang, B. Feng et al., "Zhen gan xi feng decoction, a traditional Chinese herbal formula, for the treatment of essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 982380, 9 pages, 2013.
- [48] A. B. Luiz, I. Cordovil, J. B. Filho, and A. S. Ferreira, "Zangfu zheng (patterns) are associated with clinical manifestations of zang shang (target-organ damage) in arterial hypertension," *Chinese Medicine*, vol. 6, p. 23, 2011.
- [49] W. L. Gu, Z. X. Shi, Y. X. Yu, Y. Wu, B. Lu, and K. Hui, "Distribution characteristics of syndrome types in essential hypertension," *Journal of Chinese Integrative Medicine*, vol. 8, no. 9, pp. 842–847, 2010.
- [50] H. Xu and K. J. Chen, "Progress, difficulty and countermeasure in treating hypertensive disease with integrated Chinese and western medicine," *Shi Jie Zhong Yi Yao*, vol. 2, no. 1, pp. 3–5, 2007.
- [51] J. Wang and X. J. Xiong, "Control strategy on hypertension in Chinese medicine," *Evidence-based Complementary and Alternative Medicine*, vol. 2012, Article ID 284847, 6 pages, 2012.
- [52] W. Gu, Y. Cao, Z. Shi, and K. Hui, "Potential of using pattern diagnosis of traditional Chinese medicine to improve the clinical use of antihypertensive agents," *Journal of Chinese Integrative Medicine*, vol. 5, no. 3, pp. 255–258, 2007.
- [53] H. A. Feldman, C. B. Johannes, C. A. Derby et al., "Erectile dysfunction and coronary risk factors: prospective results from the Massachusetts Male Aging Study," *Preventive Medicine*, vol. 30, no. 4, pp. 328–338, 2000.
- [54] L. C. Keene and P. H. Davies, "Drug-related erectile dysfunction," *Adverse Drug Reactions and Toxicological Reviews*, vol. 18, no. 1, pp. 5–24, 1999.
- [55] P. Buranakitjaroen, M. Phoojaroenchanachai, and S. Saravich, "Prevalence of erectile dysfunction among treated hypertensive males," *Journal of the Medical Association of Thailand*, vol. 89, supplement 5, pp. S28–S36, 2006.
- [56] M. Baumhäkel, N. Schlimmer, and M. Böhm, "Effect of irbesartan on erectile function in patients with hypertension and metabolic syndrome," *International Journal of Impotence Research*, vol. 20, no. 5, pp. 493–500, 2008.
- [57] J. Wang, X. J. Xiong, and W. Liu, "Discussion on the treatment of hypertension by tonifying kidney," *Zhongguo Zhong Yao Za Zhi*, vol. 38, no. 9, pp. 1277–1279, 2013.
- [58] P. Anagnostis, A. Karagiannis, K. Tziomalos, V. G. Athyros, M. Kita, and D. P. Mikhailidis, "Endocrine hypertension: diagnosis and management of a complex clinical entity," *Current Vascular Pharmacology*, vol. 8, no. 5, pp. 646–660, 2010.
- [59] K. Masuo, M. L. Tuck, and G. W. Lambert, "Hypertension and diabetes in obesity," *International Journal of Hypertension*, vol. 2011, Article ID 695869, 2 pages, 2011.
- [60] B. H. Lee and T. M. Pan, "Benefit of Monascus-fermented products for hypertension prevention: a review," *Applied Microbiology and Biotechnology*, vol. 49, no. 5, pp. 1151–1161, 2012.
- [61] H. C. Shih, T. H. Lee, S. C. Chen, C. Li, and T. Shibuya, "Anti-hypertension effects of traditional Chinese medicine Ju-Ling-Tang on renal hypertensive rats," *American Journal of Chinese Medicine*, vol. 33, no. 6, pp. 913–921, 2005.
- [62] J. Talha, M. Priyanka, and A. Akanksha, "Hypertension and herbal plants," *International Research Journal of Pharmacy*, vol. 2, no. 8, pp. 26–30, 2011.
- [63] J. T. Cheng, "Review: drug therapy in Chinese traditional medicine," *Journal of Clinical Pharmacology*, vol. 40, no. 5, pp. 445–450, 2000.
- [64] E. Chan, M. Tan, J. Xin, S. Sudarsanam, and D. E. Johnson, "Interactions between traditional Chinese medicines and Western therapeutics," *Current Opinion in Drug Discovery and Development*, vol. 13, no. 1, pp. 50–65, 2010.
- [65] O. H. Lee, K. I. Kim, C. K. Han, Y. Kim, and H. Hong, "Effects of acidic polysaccharides from *Gastrodia* rhizome on systolic blood pressure and serum lipid concentrations in spontaneously hypertensive rats fed a high-fat diet," *International Journal of Molecular Sciences*, vol. 13, no. 1, pp. 698–709, 2012.
- [66] J. Y. Zhou and S. W. Zhou, "Antihypertensive and neuroprotective activities of rhynchophylline: the role of rhynchophylline in neurotransmission and ion channel activity," *Journal of Ethnopharmacology*, vol. 132, no. 1, pp. 15–27, 2010.
- [67] S. H. Chen, G. Y. Lv, X. D. Zhang et al., "Anti-hypertensive effects of laiju extract in two different rat models," *Asia Pacific Journal of Clinical Nutrition*, vol. 16, no. 1, pp. 309–312, 2007.
- [68] J. P. Wang, J. L. Ruan, Y. L. Cai, and Y. X. Wu, "Antiinflammatory and analgesic activity of topical administration of *Siegesbeckia pubescens*," *Pakistan Journal of Pharmaceutical Sciences*, vol. 21, no. 2, pp. 89–91, 2008.
- [69] O. A. Phillips, K. T. Mathew, and M. A. Oriowo, "Antihypertensive and vasodilator effects of methanolic and aqueous extracts of *Tribulus terrestris* in rats," *Journal of Ethnopharmacology*, vol. 104, no. 3, pp. 351–355, 2006.

- [70] J. Gu, J. J. Wang, J. Yan et al., "Effects of lignans extracted from *Eucommia ulmoides* and aldose reductase inhibitor epalrestat on hypertensive vascular remodeling," *Journal of Ethnopharmacology*, vol. 133, no. 1, pp. 6–13, 2011.
- [71] L. Li, J. Yan, K. Hu et al., "Protective effects of *Eucommia* lignans against hypertensive renal injury by inhibiting expression of aldose reductase," *Journal of Ethnopharmacology*, vol. 139, no. 2, pp. 454–461, 2012.
- [72] F. Greenway, Z. Liu, Y. Yu, and A. Gupta, "A clinical trial testing the safety and efficacy of a standardized *Eucommia ulmoides* oliver bark extract to treat hypertension," *Alternative Medicine Review*, vol. 16, no. 4, pp. 338–347, 2011.
- [73] M. Ouedraogo, M. Ruiz, E. Vardelle et al., "From the vasodilator and hypotensive effects of an extract fraction from *Agelanthus dodoneifolius* (DC) *Danser* (Loranthaceae) to the active compound dodoneine," *Journal of Ethnopharmacology*, vol. 133, no. 2, pp. 345–352, 2011.
- [74] M. Radenkovic, V. Ivetic, M. Popovic, S. Brankovic, and L. Gvozdenovic, "Effects of mistletoe (*Viscum Album L., Loranthaceae*) extracts on arterial blood pressure in rats treated with atropine sulfate and hexocycline," *Clinical and Experimental Hypertension*, vol. 31, no. 1, pp. 11–19, 2009.
- [75] R. L. Simeonova, V. B. Vitcheva, M. S. Kondeva-Burdina, I. N. Krasteva, S. D. Nikolov, and M. K. Mitcheva, "Effect of purified saponin mixture from *Astragalus corniculatus* on enzyme- and non-enzyme-induced lipid peroxidation in liver microsomes from spontaneously hypertensive rats and normotensive rats," *Phytomedicine*, vol. 17, no. 5, pp. 346–349, 2010.
- [76] B. Xue, J. Li, Q. Chai, Z. Liu, and L. Chen, "Effect of total flavonoid fraction of *Astragalus complanatus* R. Brown on angiotensin II-induced portal-vein contraction in hypertensive rats," *Phytomedicine*, vol. 15, no. 9, pp. 759–762, 2008.
- [77] L. M. Yao, T. W. Liu, W. F. Wu, and G. Q. Zhong, "Effects of *Astragalus* injection in reversing left ventricular hypertrophy induced by renal hypertension in rats," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 29, no. 10, pp. 918–921, 2009.
- [78] Y. Z. Hou, G. R. Zhao, Y. J. Yuan, G. Zhu, and R. Hiltunen, "Inhibition of rat vascular smooth muscle cell proliferation by extract of *Ligusticum chuanxiong* and *Angelica sinensis*," *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 140–144, 2005.
- [79] N. B. Zhang, Z. G. Huang, W. D. Cui, and B. P. Ding, "Effects of puerarin on expression of cardiac Smad3 and Smad7 mRNA in spontaneously hypertensive rat," *Journal of Ethnopharmacology*, vol. 138, no. 3, pp. 737–740, 2011.
- [80] C. F. Ng, C. M. Koon, D. W. S. Cheung et al., "The anti-hypertensive effect of *Danshen* (*Salvia miltiorrhiza*) and *Gegen* (*Pueraria lobata*) formula in rats and its underlying mechanisms of vasorelaxation," *Journal of Ethnopharmacology*, vol. 137, no. 3, pp. 1366–1372, 2011.
- [81] D. D. Kim, F. A. Sánchez, R. G. Durán, T. Kanetaka, and W. N. Durán, "Endothelial nitric oxide synthase is a molecular vascular target for the Chinese herb *Danshen* in hypertension," *American Journal of Physiology*, vol. 292, no. 5, pp. H2131–H2137, 2007.
- [82] W. T. Chang, J. Dao, and Z. H. Shao, "Hawthorn: potential roles in cardiovascular disease," *American Journal of Chinese Medicine*, vol. 33, no. 1, pp. 1–10, 2005.
- [83] A. F. Walker, G. Marakis, A. P. Morris, and P. A. Robinson, "Promising hypotensive effect of hawthorn extract: a randomized double-blind pilot study of mild, essential hypertension," *Phytotherapy Research*, vol. 16, no. 1, pp. 48–54, 2002.
- [84] T. Sun, H. Xu, and F. Q. Xu, "*Astragalus* injection for hypertensive renal damage: a systematic review," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 929025, 6 pages, 2012.
- [85] J. Wang, B. Feng, X. C. Yang et al., "*Tianma gouteng* yin as adjunctive treatment for essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 706125, 18 pages, 2013.
- [86] S. J. Wang, Y. Chen, D. D. He et al., "Inhibition of vascular smooth muscle cell proliferation by serum from rats treated orally with *Gastrodia* and *Uncaria* decoction, a traditional Chinese formulation," *Journal of Ethnopharmacology*, vol. 114, no. 3, pp. 458–462, 2007.
- [87] S. C. Ho, Y. F. Ho, T. H. Lai, T. Liu, S. Su, and R. Wu, "Effect of *Tianma gouteng* Decoction with subtractive ingredients and its active constituents on memory acquisition," *American Journal of Chinese Medicine*, vol. 36, no. 3, pp. 593–602, 2008.
- [88] S. X. Xian, S. Y. Hu, X. H. Liu, L. C. Zhao, and N. Y. Li, "Study on effect of *Tianma gouteng* decoction in intervening myocardial collagen reconstruction in renovascular hypertensive rats," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 23, no. 6, pp. 21–24, 2003.
- [89] Z. W. Zhang, G. H. YUE, and Y. Luo, "Effects of *Huanglian jie du* decoction on prothrombotic state in spontaneous hypertension rats," *Zhongguo Shi Yan Fang Ji Xue Za Zhi*, vol. 17, no. 2, pp. 105–108, 2011.
- [90] Y. P. Han, N. S. Wang, S. Q. Mi, and Q. Liu, "Effect of *Wuling* Powder on rats with renal hypertension," *Zhong xi yi Jie he Xue Bao*, vol. 1, no. 4, pp. 285–288, 2003.
- [91] J. Y. Chen, H. L. Fan, and S. F. Zhang, "Effect of modified *Zexie* decoction on prevention of kidney injuries of rats with hypertension induced by high-salt diet," *Zhong Yi Za Zhi*, vol. 53, no. 3, pp. 234–237, 2012.
- [92] X. J. Xiong, X. C. Yang, W. Liu et al., "*Banxia baizhu tianma* decoction for essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 271462, 10 pages, 2012.
- [93] J. Y. Jiang, X. Z. Wang, S. S. Luo, X. Wang, K. Bian, and Y. Yan, "Effect of *Banxia baizhu tianma* decoction on the left ventricular hypertrophy of hypertrophied myocardium in spontaneously hypertensive rat," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 30, no. 10, pp. 1061–1066, 2010.
- [94] Q. F. Wu, M. X. Wen, and D. H. Lan, "Effect of *Banxia baizhu tianma* decoction on insulin resistance and blood lipid in hypertensive patients with abundant phlegm-dampness syndrome," *Fujian Zhong Yi Xue Yuan Xue Bao*, vol. 17, no. 2, pp. 8–10, 2007.
- [95] Q. F. Wu, M. X. Wen, and D. H. Lan, "Effects of *Banxia baizhu tianma* decoction on salt sensitivity and blood lipid in hypertensive patients with abundant phlegm-dampness syndrome," *Fujian Yi Yao Za Zhi*, vol. 29, no. 1, pp. 146–148, 2007.
- [96] J. Wang, K. W. Yao, X. C. Yang et al., "Chinese patent medicine *liu wei di huang wan* combined with antihypertensive drugs, a new integrative medicine therapy, for the treatment of essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 714805, 7 pages, 2012.
- [97] F. X. Huang, Y. Ye, P. Yan, and M. H. Chen, "Therapeutic observation of using *Liuweidihuang Pills* and pancreatic kininogenase interfering kidney damage by hypertensive disease," *Fujian Zhong Yi Xue Yuan Xue Bao*, vol. 14, no. 5, pp. 9–11, 2004.

- [98] L. L. Gao, B. C. Wang, and Q. Z. Lin, "Protective effect of *Liuweidihuang pill* combined with *Shengmai capsule* on heart of patients with hypertension," *Zhong Hua Zhong Yi Yao Za Zhi*, vol. 28, no. 7, pp. 85–88, 2008.
- [99] X. W. Li, "The effect of Shenqi Pill on the sexual function of young and middle-aged male hypertensive patients," *Zhejiang Zhong Xi Yi Jie He Za Zhi*, vol. 18, no. 4, pp. 214–215, 2008.
- [100] Y. L. Liu, "The effect of Golden Chamber Pill for Reinforcing Kidney qi combined with enalapril on urinary albumin in hypertensive patients," *Xin Zhong Yi*, vol. 40, no. 8, pp. 37–38, 2008.
- [101] National Pharmacopoeia Committee, *Pharmacopoeia of People's Republic of China. Part 1*, vol. 38 of *Medicinal Materials and Decoction Pieces*, China Medical Science Press, Beijing, China, 2010.
- [102] Y. H. Xie, J. C. Zhang, Y. R. Jiang et al., "Data-mining of clinical hypertensive cases from Dr. Chen Keji," *Zhong Xi Yi Jie He Xin Nao Xue Guan Bing Za Zhi*, vol. 6, no. 2, pp. 135–136, 2008.
- [103] H. Xu and K. J. Chen, "Complementary and alternative medicine: is it possible to be mainstream?" *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 403–404, 2012.
- [104] A. Weil, "The state of the integrative medicine in the U.S. and Western World," *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 6–10, 2011.
- [105] L. Jia, "Cancer complementary and alternative medicine research at the US National Cancer Institute," *Chinese Journal of Integrative Medicine*, vol. 18, no. 5, pp. 325–332, 2012.
- [106] J. J. Park, S. Beckman-Harned, G. Cho, D. Kim, and H. Kim, "The current acceptance, accessibility and recognition of Chinese and Ayurvedic medicine in the United States in the public, governmental, and industrial sectors," *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 405–408, 2012.
- [107] H. Abolhassani, M. Naseri, and S. Mahmoudzadeh, "A survey of complementary and alternative medicine in Iran," *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 409–416, 2012.
- [108] J. M. Crow, "That healthy gut feeling," *Nature*, vol. 480, supplement 7378, pp. S88–S89, 2011.
- [109] M. Y. Liu and K. J. Chen, "Convergence: the tradition and the modern," *Chinese Journal of Integrative Medicine*, vol. 18, no. 3, pp. 164–165, 2012.
- [110] X. Sun, W. Wu, and Z. Lu, "Chinese integrative medicine: translation toward person-centered and balanced medicine," *Chinese Journal of Integrative Medicine*, vol. 18, no. 1, pp. 3–6, 2012.
- [111] N. Robinson, "Integrative medicine—traditional Chinese medicine, a model?" *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 21–25, 2011.
- [112] D. Eisenberg, "Reflections on the past and future of integrative medicine from a lifelong student of the integration of Chinese and Western medicine," *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 3–5, 2011.
- [113] M. S. Lee, H. J. Lim, and M. S. Lee, "Impact of qigong exercise on self-efficacy and other cognitive perceptual variables in patients with essential hypertension," *Journal of Alternative and Complementary Medicine*, vol. 10, no. 4, pp. 675–680, 2004.
- [114] M. S. Lee, M. H. Pittler, R. Guo, and E. Ernst, "Qigong for hypertension: a systematic review of randomized clinical trials," *Journal of Hypertension*, vol. 25, no. 8, pp. 1525–1532, 2007.
- [115] J. I. Kim, J. Y. Choi, H. Lee, M. S. Lee, and E. Ernst, "Moxibustion for hypertension: a systematic review," *BMC Cardiovascular Disorders*, vol. 10, p. 33, 2010.
- [116] M. S. Lee, T. Choi, B. Shin, J. Kim, and S. Nam, "Cupping for hypertension: a systematic review," *Clinical and Experimental Hypertension*, vol. 32, no. 7, pp. 423–425, 2010.
- [117] D. D. Kim, F. A. Sanchez, M. P. Boric, and W. N. Duran, "Mechanisms of Acupuncture and herbal medicine in hypertension," *Asian Biomedicine*, vol. 2, no. 4, pp. 257–274, 2008.
- [118] F. A. Flachskampf, J. Gallasch, O. Gefeller et al., "Randomized trial of acupuncture to lower blood pressure," *Circulation*, vol. 115, no. 24, pp. 3121–3129, 2007.
- [119] R. Zhao, L. X. Fu, J. Xiong, S. Li, and Z. L. Wang, "The effect of acupuncture therapy on essential hypertension: a systematic review of long-term effect," *Zhen Jiu Lin Chuang Za Zhi*, vol. 27, no. 3, pp. 46–51, 2011.
- [120] N. M. Kaplan, "Acupuncture for hypertension: can 2500 years come to an end?" *Hypertension*, vol. 48, no. 5, p. 815, 2006.
- [121] Y. Li, K. Higashiura, N. Ura et al., "Effects of the Chinese medicine TSJN on insulin resistance and hypertension in fructose-fed rats," *Hypertension Research*, vol. 23, no. 2, pp. 101–107, 2000.
- [122] H. Li, L. Liu, W. Zhao et al., "Traditional Chinese versus integrative treatment in elderly patients with isolated systolic hypertension: a multicenter, randomized, double-blind controlled trial," *Journal of Chinese Integrative Medicine*, vol. 8, no. 5, pp. 410–416, 2010.
- [123] Q. Zhou, D. K. Rowlands, Y. L. Gou, L. L. Tsang, Y. W. Chung, and H. C. Chan, "Cardiovascular protective effects of traditional Chinese medicine Bak Foong Pills in spontaneously hypertensive rats," *Biological and Pharmaceutical Bulletin*, vol. 26, no. 8, pp. 1095–1099, 2003.
- [124] B. Wang, J. Zhang, J. Feng, H. Yin, F. Liu, and Y. Wang, "Improvement of vascular remodeling in spontaneous hypertensive rats with traditional Chinese medicine," *Clinical and Experimental Hypertension*, vol. 29, no. 5, pp. 345–355, 2007.
- [125] B. Wang, J. Zhang, J. Feng, H. Yin, F. Liu, and Y. Wang, "Effect of traditional Chinese medicine *Qin-Dan-Jiang-Ya-Tang* on remodeled vascular phenotype and osteopontin in spontaneous hypertensive rats," *Journal of Ethnopharmacology*, vol. 110, no. 1, pp. 176–182, 2007.
- [126] P. Ye, C. Wu, L. Sheng, and H. Li, "Potential protective effect of long-term therapy with *Xuezhikang* on left ventricular diastolic function in patients with essential hypertension," *Journal of Alternative and Complementary Medicine*, vol. 15, no. 7, pp. 719–725, 2009.
- [127] X. J. Xiong and J. Wang, "TCM understanding of hypertension and the control strategies by classic formulas," *Zhong Yi Za Zhi*, vol. 52, no. 23, pp. 1985–1989, 2011.
- [128] L. Fu, Z. X. Mao, J. Wang, T. R. Zheng, and S. L. Wang, "Effects of *Song ling xue mai kang* capsule on ambulatory blood pressure in treatment of essential hypertension: a single-blind randomized controlled trial," *Zhong Xi Yi Jie He Xue Bao*, vol. 7, no. 6, pp. 509–513, 2009.
- [129] J. Wang, X. C. Yang, B. Feng et al., "Is Yangxue Qingnao Granule combined with antihypertensive drugs, a new integrative medicine therapy, more effective than antihypertensive therapy alone in treating essential hypertension?" *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 540613, 8 pages, 2013.
- [130] J. Wang and X. J. Xiong, "Outcome measures of Chinese herbal medicine for hypertension: an overview of systematic reviews," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 697237, 7 pages, 2012.

- [131] H. Xu and K. Chen, "Making evidence-based decisions in the clinical practice of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 6, pp. 483–485, 2010.
- [132] E. Braunwald, "The rise of cardiovascular medicine," *European Heart Journal*, vol. 33, no. 7, pp. 838–845, 2012.
- [133] N. J. White, "Qinghaosu (artemisinin): the price of success," *Science*, vol. 320, no. 5874, pp. 330–334, 2008.
- [134] L. S. Liu, M. Q. Chen, G. Y. Zeng, and B. F. Zhou, "A forty-year study on hypertension," *Zhongguo Yi Xue Ke Xue Yuan Xue Bao*, vol. 24, no. 4, pp. 401–408, 2002.
- [135] J. Li, C. Lu, M. Jiang et al., "Traditional Chinese medicine-based network pharmacology could lead to new multicomponent drug discovery," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 149762, 11 pages, 2012.

## Review Article

# Regulation of DDAH1 as a Potential Therapeutic Target for Treating Cardiovascular Diseases

Xiaoyu Liu,<sup>1</sup> John Fassett,<sup>2</sup> Yidong Wei,<sup>1</sup> and Yingjie Chen<sup>2</sup>

<sup>1</sup> Shanghai Tenth People's Hospital, Tongji University, Shanghai, China

<sup>2</sup> Cardiovascular Division and Lillehei Heart Institute, University of Minnesota, Minneapolis, MN 55455, USA

Correspondence should be addressed to Yingjie Chen; [chenx106@umn.edu](mailto:chenx106@umn.edu)

Received 13 April 2013; Accepted 29 May 2013

Academic Editor: Keji Chen

Copyright © 2013 Xiaoyu Liu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Asymmetric dimethylarginine (ADMA) is an endogenous nitric oxide synthase inhibitor that blocks nitric oxide production, while congestive heart failure is associated with increased plasma and tissue ADMA content. Increased plasma ADMA is a strong and independent predictor of all-cause mortality in the community and the strongest predictor of mortality in patients after myocardial infarction. Recent studies demonstrated that dimethylarginine dimethylaminohydrolase-1 (DDAH1) is the critical enzyme for ADMA degradation and thereby plays an important role in maintaining cardiovascular nitric oxide bioavailability. Interestingly, activation of the farnesoid X receptor (FXR) through the bile acid ursodeoxycholic acid (UDCA) or synthetic FXR agonists, such as GW4064, can increase DDAH1 expression. Thus, modulating DDAH1 activity through FXR receptor agonists such as UDCA could be a therapeutic target for treating reduced nitric oxide bioavailability in congestive heart failure and other cardiovascular diseases.

## 1. Introduction

Congestive heart failure (CHF) is a major cardiovascular disease of epidemic proportion that has increased in prevalence in the past few decades. Nitric oxide (NO) activates soluble guanylyl cyclase, and the resultant increase of cGMP and activation of cGMP-dependent protein kinases (PKG) regulate vasomotor tone, blood flow, angiogenesis, vascular endothelial cell growth/proliferation, and injury repair. NO is known to exert protective effects on the cardiovascular system. Impaired NO signaling is a hallmark of CHF and many other cardiovascular diseases such as hypertension, stroke, coronary disease, atherosclerosis, and diabetes. Thus, responses to agonists or shear stress that rely on NO to cause coronary or systemic vasodilatation are attenuated in CHF, indicating decreased NO bioavailability [1–4]. Reduced NO bioavailability causes hypertension [5], coronary disease, atherosclerosis and aging-dependent CHF in experimental animals [6], indicating an important role of NO in attenuating CHF and other cardiovascular diseases causing CHF.

Asymmetric dimethylarginine (ADMA) is an endogenous NO synthase (NOS) inhibitor that blocks NO production and increases NOS-derived ROS generation. CHF is associated with increased ADMA levels in the heart and plasma. Increased plasma ADMA is a strong and independent predictor of all-cause mortality in the community [7] and the strongest predictor of mortality in patients with CHF [8]. Dimethylarginine dimethylaminohydrolase-1 (DDAH1) degrades ADMA and thereby enhances NO/cGMP signaling (Figure 1). Recent studies have demonstrated that DDAH1 is essential for ADMA degradation, indicating that DDAH1 plays an important role in maintaining cardiovascular NO bioavailability. Thus, elevated DDAH1 activity could be an important therapeutic target for increasing NO bioavailability in CHF and other cardiovascular diseases.

*1.1. Reduced NO Bioavailability Contributes to CHF Development.* NO synthesis is catalyzed by a family of proteins, the NO synthases (NOS). At least three NOS isoforms exist in mammalian cells: endothelial NOS (eNOS), neuronal NOS

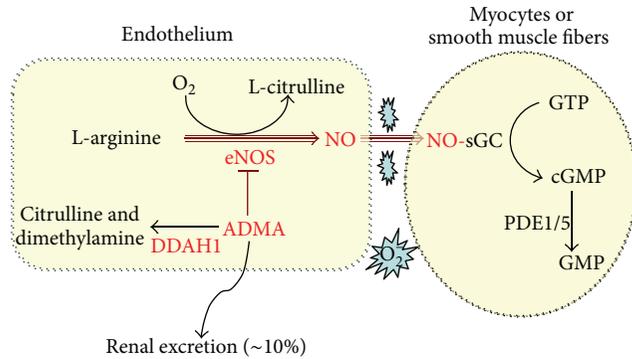


FIGURE 1: DDAH1 regulates NO production through degrading ADMA.

(nNOS), and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed (cNOS) in many cell types, and produce NO in response to increased cytosolic Ca<sup>++</sup> (Ca<sup>++</sup>-dependent NOS). In the normal heart, eNOS is highly expressed in coronary endothelial cells and also moderately expressed on the sarcolemma of cardiac myocytes. Myocardial nNOS expression is low and mainly expressed on sarcoplasmic reticulum of cardiac myocytes, where it acts to regulate Ca<sup>++</sup> dynamics. iNOS is expressed in response to inflammation or cytokine stimulation and can produce much larger quantities of NO for a sustained period of time in the absence of elevated Ca<sup>++</sup> (Ca<sup>++</sup>-independent NOS).

Loss of NO bioavailability and cGMP production is a key feature of endothelial dysfunction in diseases such as hypertension and heart failure. One contribution to loss of NO-cGMP signaling is decreased NO production by NOS. Under conditions of oxidative stress or reduced substrate availability, NOS activity can become disrupted (NOS uncoupling; further described later), so that NOS produces superoxide, rather than NO [9]. In addition, elevated superoxide in cardiomyocytes or endothelial cells can interact with and scavenge NO before it can beneficially activate guanylate cyclase to produce cGMP. Furthermore, heart failure is associated with elevated expression of phosphodiesterase 5 (PDE5), which degrades cGMP and further reduces NO-cGMP dependent signaling. Thus NO-cGMP signaling is reduced through several mechanisms in the failing heart. Several studies using transgenic or knockout mouse models have now confirmed that NO-cGMP signaling significantly influences the development of myocyte hypertrophy and dysfunction during aging [6, 10, 11] and in response to myocardial injury or overload [12, 13]. Thus, transgenic mice overexpressing eNOS are protected from myocardial infarct-induced LV remodeling and the development of CHF [12]. Conversely, progressive cardiomyocyte hypertrophy, interstitial fibrosis, LV dilation, and dysfunction, that develops in the residual surviving tissue after myocardial infarction, are exacerbated in eNOS KO mice as compared with control wild-type mice [13]. Several laboratories have also reported that eNOS KO exacerbated LV dysfunction in response to left ventricular pressure overload (transverse aortic constriction; TAC) [14, 15]. Moreover, cardiomyocyte-restricted restoration of

eNOS (overexpressing eNOS in eNOS KO mice) attenuates TAC-induced ventricular remodeling [16], suggesting a cardiomyocyte specific protective effect of eNOS expression under conditions of pressure overload. Cardiomyocyte specific eNOS overexpression in a wild-type background also attenuated TAC-induced LV remodeling [17]. These studies indicate that cardiomyocyte-restricted eNOS expression can protect the overloaded heart from ventricular dysfunction. These data demonstrated that the cNOS-derived NO-cGMP signaling pathway exerts cardioprotective effects against the development of LV hypertrophy and CHF. In contrast to the cardioprotective effect of eNOS, cardiac-specific iNOS expression can contribute to the development of CHF [18]. iNOS expression is increased in failing hearts [19, 20], and we [21] demonstrated that iNOS KO or selective pharmacologic iNOS inhibition with 1400 W protected the heart from TAC-induced LV hypertrophy and dysfunction. The finding that eNOS and iNOS have opposite influences on LV adaptation to pressure overload is explained by differences in the cell type and subcellular location of these NOS isoforms and by the fact that iNOS can produce much larger quantities of NO for a sustained period of time in the absence of elevated Ca<sup>++</sup>.

*1.2. ADMA Accumulation Is Associated with CHF and Other Cardiovascular Diseases.* The effect of endogenous NOS inhibitors on NO bioavailability has been an area of intense research in recent years. There are two compounds that can inhibit NOS, N-monomethyl-arginine (NMMA), and ADMA, which both reduce NO synthesis by competing with arginine for NOS binding [22]. Plasma NMMA concentrations are much lower compared with plasma ADMA concentrations. NMMA is formed when protein-incorporated arginine is methylated by the enzymes protein arginine methyltransferases- (PRMT-) 1 or PRMT-2. PRMT-1 can subsequently methylate NMMA, resulting in the formation of ADMA, whereas PRMT-2 can methylate NMMA into symmetric dimethylarginine (SDMA). After proteolysis, the methylated arginines are released as unbound forms in the cytosol where NMMA and ADMA are able to inhibit NOS. SDMA is not able to inhibit NOS. Because only small amounts of NMMA are found in the plasma, ADMA is considered the major endogenous NOS inhibitor. ADMA and L-NMMA are eliminated principally by DDAH with a small contribution from renal excretion.

Plasma ADMA levels are elevated in CHF [23, 24], hypertension [25], diabetes [26], and atherosclerosis [27, 28], and increased ADMA is believed to contribute to endothelial dysfunction in these conditions. In agreement with ADMA accumulation in CHF patients, we demonstrated that rapid ventricular pacing-induced CHF in dogs was associated with increased plasma ADMA that was accompanied by the development of progressive coronary endothelial dysfunction [29]. In addition, administration of ADMA attenuated acetylcholine induced coronary vessel dilation in normal animals [29]. Several investigators have reported that increased plasma ADMA levels are associated with an increased risk for developing angina pectoris, myocardial infarction, or cardiac

death [7]. However, despite the association between increased levels of ADMA and CHF or other cardiovascular diseases, it is uncertain whether chronic ADMA accumulation can actually cause or exacerbate the development of myocardial dysfunction or CHF.

*1.3. ADMA Enhances NOS-Derived  $O_2^-$  and Peroxynitrite ( $ONOO^-$ ).* Although the most obvious consequence of increased levels of ADMA and L-NMMA is to inhibit NO production, recent reports indicate that the endogenous NOS inhibitors may cause NOS to generate  $O_2^-$  rather than NO. Normally, NOS transfers electrons from NADPH, via the flavins FAD and FMN in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxygenase domain, where the substrate L-arginine is oxidized to L-citrulline and NO. The flow of electrons within NOS is normally tightly regulated but, if disturbed, oxygen reduction and NO generation can become uncoupled so that  $O_2^-$  is generated by the oxygenase domain. This uncoupling can occur when NOS is exposed to oxidant stress (including peroxynitrite), when it is deficient of the reducing cofactor BH4 [30, 31] or when it is deprived of its substrate L-arginine [32, 33]. BH4 is required for iNOS dimerisation [34, 35] and stabilizes the dimeric forms of eNOS, nNOS, and iNOS once formed [34]. Thus, BH4 depletion (or oxidation of BH4 to BH2) can induce NOS-derived  $O_2^-$  generation [9, 30, 34, 35]. Deprivation of the substrate L-arginine can also induce NOS to generate  $O_2^-$  and  $ONOO^-$  [32, 33, 36]. We recently found that both iNOS and eNOS monomer were increased in failing hearts from wild-type mice in response to TAC, and this was associated with increased myocardial superoxide production [21]. We found that iNOS KO or the selective iNOS inhibitor 1400 W protected the heart against TAC-induced LV dysfunction and oxidative stress [21]. However, it is not fully clear whether iNOS-derived ROS is due to substrate deficiency. In a manner similar to substrate deficiency, several *in vitro* studies have demonstrated that the addition of ADMA or L-NMMA, which act as a competitive inhibitors of L-arginine, caused  $O_2^-$  generation by purified NOS protein [22, 37], in cultured human endothelial cells [38, 39], isolated arterioles from rat gracilis muscle [40], and in a murine lung epithelial cell line LA-4 [41]. *In vitro* studies have demonstrated that the NOS inhibitor N-monomethyl-L-arginine (L-NMMA) is also capable of inducing NOS uncoupling through multiple mechanisms such as heme loss [37]. Importantly, administration of tetrahydrobiopterin, which prevents NOS uncoupling, can significantly attenuate ROS production, pressure overload-induced cardiac hypertrophy, and heart failure, indicating that the loss of NO production, as well as the increased ROS production that results from NOS uncoupling, is important in the development of heart failure.

Thus, ADMA inhibition of NO production, and possibly ADMA-induced NOS uncoupling and production of superoxide, may act as a double-edged sword in endothelial and cardiomyocyte pathophysiology. Identification and understanding of the mechanisms that reduce ADMA accumulation are therefore, clinically important.

*1.4. DDAH1 Is Essential for ADMA Degradation.* DDAH1 was originally identified by Ogawa et al. in 1987 [42]. DDAH2 was identified in 1999 [43]. Previous existing concepts regarding tissue or cell-specific DDAH1/2 biology and their function in regulating NO production in various tissues are based on the reports that DDAH1 and DDAH2 have comparable activities for degrading ADMA and L-NMMA [43], as well as the report that DDAH1 is minimally expressed in the heart [43, 44], vessels [43], and vascular endothelial cells [45]. Accordingly, it was originally accepted that DDAH2 plays the major role in regulating ADMA and L-NMMA levels in the heart and vessels, while DDAH1 plays the major role in degrading ADMA and L-NMMA in neuronal tissues. However, recent studies clearly demonstrated that DDAH1 is important in regulating systemic ADMA and L-NMMA and cardiovascular NO bioavailability [46]. Thus, using endothelial specific DDAH1 gene deficient mice (endo-DDAH1 KO), we found that endo-DDAH1 KO caused significant decreases of DDAH1 in vascular tissues, increased tissue and plasma ADMA, reduced acetylcholine-induced NO production and vessel dilatation, and resulted in systemic hypertension [46]. Consistent with the above findings, studies from Cooke and Associates demonstrated that a moderate 2-3-fold overexpression of DDAH1 in transgenic mice was sufficient to significantly decrease plasma ADMA and to cause a moderate decrease of aortic pressure [47]. These findings imply that DDAH1 in vascular endothelial cells plays an important role in degrading the NOS inhibitors and regulating vascular tone. The increased systemic hypertension observed in DDAH1 KO mice in which ADMA levels were increased and NO production was decreased suggests that DDAH1 degradation of ADMA is physiologically important and may help protect against cardiovascular diseases in which ADMA levels are elevated.

Most importantly, we demonstrated that DDAH activity was totally abolished in all tissues obtained from global DDAH1 deficient mice while the expression of DDAH2 was unaffected in these tissues [48]. In other words, tissues obtained from our global DDAH1 KO mice are unable to degrade ADMA or NMMA [48]. Consistent with our findings, Dr. Leiper et al. also demonstrated that DDAH activity was reduced ~50% in tissues obtained from heterozygous DDAH1 KO mice [49]. Furthermore, we found that selective gene silencing of DDAH1, but not DDAH2, caused accumulation of ADMA and decreased NO production in cultured endothelial cells [46], while overexpression of DDAH1 (but not DDAH2) decreased ADMA content in cultured endothelial cells [50]. These findings clearly indicate that DDAH1 is the critical enzyme for ADMA and L-NMMA degradation, while DDAH2 has no detectable role in ADMA degradation. Technical limitation and inappropriate experimental methods used for detecting ADMA degradation are likely the culprit in defining DDAH2 as an enzyme for ADMA degradation. Because DDAH activity is reduced in the failing heart [29] and this likely contributes to increased ADMA levels and endothelial dysfunction, identifying new mechanisms to increase DDAH1 expression or activity may be clinically relevant in the treatment of heart failure.

1.5. *The Potential Role of Ursodeoxycholic Acid (UDCA) in Vascular NO Bioavailability and Blood Flow.* Bile acid ursodeoxycholic acid (UDCA) is a major component of bear bile, which has been used extensively in traditional Chinese medicine. UDCA has been on the market in Japan since the 1950s and in Western countries since the mid-1980s [51]. UDCA or bile acids contribute to several essential functions, including cholesterol catabolism and intestinal lipid emulsification. In addition to their role as detergents, bile acids can also act as endocrine signaling molecules via activation of nuclear receptors, including farnesoid X receptor (FXR) and pregnane X receptor [52] to achieve profound effects on hepatic lipid and glucose metabolism [53]. FXR is activated by compounds such as UDCA, chenodeoxycholic acid, and cholic acid [54, 55] and by the synthetic compound GW4064 [56]. FXR plays an important role in maintaining cholesterol, triglyceride, and glucose homeostasis [56–58]. Activation of FXR with bile acids or GW4064, or hepatic expression of constitutively active FXR, significantly lowers plasma triglyceride, cholesterol, and glucose levels [56, 57]. Conversely, FXR gene deletion increased plasma cholesterol and triglyceride levels [58]. UDCA is currently employed in the clinical treatment of diverse hepatobiliary disorders, including primary biliary cirrhosis [59] and other liver diseases. Interestingly, genetic disruption of eNOS or nNOS has been shown to alter lipid metabolism, resulting in increased fat deposition in the liver [60, 61]. It would be interesting to find out whether FXR-mediated increase of DDAH1 activity influences lipid metabolism through preservation of NOS activity. UDCA can also attenuate ER stress [62], a phenomena often observed in CHF. One study demonstrated that 6-week UDCA therapy improved endothelium-dependent vasodilatation and arterial blood flow in patients with HF under conditions of impaired nitric oxide production [55]. In a double-blind, randomized, and placebo-controlled clinical trial, von Haehling et al. demonstrated that UDCA significantly improved peak postischemic blood flow in the arm and that there was a trend towards improved peak postischemic blood flow in the leg, while liver function was also improved [63]. Most interestingly, a recent report demonstrated that DDAH1 is a downstream target gene of FXR [64]. Thus, activation of FXR with GW4064 dose dependently increased DDAH1 gene transcription in the liver through a FXR response element, and this was associated with a decrease of plasma ADMA [64]. A separate study by a different group also recently reported that activation of FXR with GW4064 increased DDAH1 gene expression in the liver and kidney and decreased plasma ADMA [65]. Activation of FXR with bile acids was also found to enhance tumor angiogenesis [58]. Interestingly, FXR is expressed in cardiomyocytes [66] and endothelial cells [67], but whether activation of FXR is able to increase DDAH1 gene expression in the cardiovascular system, or can attenuate pressure overload-induced ventricular dysfunction, has not been studied. The effects of UDCA and/or other bile acids in attenuating plasma cholesterol and triglyceride levels [58] and potential for increasing vascular NO bioavailability through modulating DDAH1 expression suggest that UDCA (and/or other bile acids) may exert cardiac protective effect in the failing heart.

Collectively, the current scientific literature in the field indicates that ADMA attenuates vascular NO bioavailability in the cardiovascular system, that DDAH1 plays the major role in ADMA degradation, and that activation of FXR increases DDAH1 expression. Together these findings suggest that increasing DDAH1 expression through FXR activation could be an important therapeutic target for treating reduced NO bioavailability in CHF and other cardiovascular diseases.

## Acknowledgments

This study was supported by US Public Health Service Grants HL021872, HL098669, HL098719, HL102597, HL089249, and R01HL105406 from the National Institutes of Health, research Grants 30500681 and 30973845 from the National Natural Science Foundation of China, and Grant 09dZ1975000 from TCM Modernization Programs of Shanghai Science and Technology Commission.

## References

- [1] C.-A. Chen, L. J. Druhan, S. Varadaraj, Y.-R. Chen, and J. L. Zweier, "Phosphorylation of endothelial nitric-oxide synthase regulates superoxide generation from the enzyme," *Journal of Biological Chemistry*, vol. 283, no. 40, pp. 27038–27047, 2008.
- [2] Y. Chen, J. H. Traverse, R. Du, M. Hou, and R. J. Bache, "Nitric oxide modulates myocardial oxygen consumption in the failing heart," *Circulation*, vol. 106, no. 2, pp. 273–279, 2002.
- [3] Y. Chen, J. H. Traverse, M. Hou, Y. Li, R. Du, and R. J. Bache, "Effect of PDE5 inhibition on coronary hemodynamics in pacing-induced heart failure," *The American Journal of Physiology*, vol. 284, no. 5, pp. H1513–H1520, 2003.
- [4] J. H. Traverse, P. Melchert, G. L. Pierpont, B. Jones, M. Crampton, and R. J. Bache, "Regulation of myocardial blood flow by oxygen consumption is maintained in the failing heart during exercise," *Circulation Research*, vol. 84, no. 4, pp. 401–408, 1999.
- [5] P. L. Huang, Z. Huang, H. Mashimo et al., "Hypertension in mice lacking the gene for endothelial nitric oxide synthase," *Nature*, vol. 377, no. 6546, pp. 239–242, 1995.
- [6] W. Li, S. Mital, C. Ojaimi, A. Csiszar, G. Kaley, and T. H. Hintze, "Premature death and age-related cardiac dysfunction in male eNOS-knockout mice," *Journal of Molecular and Cellular Cardiology*, vol. 37, no. 3, pp. 671–680, 2004.
- [7] R. H. Böger, L. M. Sullivan, E. Schwedhelm et al., "Plasma asymmetric dimethylarginine and incidence of cardiovascular disease and death in the community," *Circulation*, vol. 119, no. 12, pp. 1592–1600, 2009.
- [8] S. J. Nicholls, Z. Wang, R. Koeth et al., "Metabolic profiling of arginine and nitric oxide pathways predicts hemodynamic abnormalities and mortality in patients with cardiogenic shock after acute myocardial infarction," *Circulation*, vol. 116, no. 20, pp. 2315–2324, 2007.
- [9] E. Takimoto, H. C. Champion, M. Li et al., "Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load," *Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1221–1231, 2005.
- [10] L. A. Barouch, R. W. Harrison, M. W. Skaf et al., "Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms," *Nature*, vol. 416, no. 6878, pp. 337–340, 2002.

- [11] L. A. Barouch, T. P. Cappola, R. W. Harrison et al., "Combined loss of neuronal and endothelial nitric oxide synthase causes premature mortality and age-related hypertrophic cardiac remodeling in mice," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 6, pp. 637–644, 2003.
- [12] S. P. Jones, J. J. M. Greer, R. van Haperen, D. J. Duncker, R. de Crom, and D. J. Lefer, "Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 8, pp. 4891–4896, 2003.
- [13] M. Scherrer-Crosbie, R. Ullrich, K. D. Bloch et al., "Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice," *Circulation*, vol. 104, no. 11, pp. 1286–1291, 2001.
- [14] F. Ichinose, K. D. Bloch, J. C. Wu et al., "Pressure overload-induced LV hypertrophy and dysfunction in mice are exacerbated by congenital NOS3 deficiency," *The American Journal of Physiology*, vol. 286, no. 3, pp. H1070–H1075, 2004.
- [15] H. Ruetten, S. Dimmeler, D. Gehring, C. Ihling, and A. M. Zeiher, "Concentric left ventricular remodeling in endothelial nitric oxide synthase knockout mice by chronic pressure overload," *Cardiovascular Research*, vol. 66, no. 3, pp. 444–453, 2005.
- [16] E. S. Buys, M. J. Raher, S. L. Blake et al., "Cardiomyocyte-restricted restoration of nitric oxide synthase 3 attenuates left ventricular remodeling after chronic pressure overload," *The American Journal of Physiology*, vol. 293, no. 1, pp. H620–H627, 2007.
- [17] S. Janssens, P. Pokreisz, L. Schoonjans et al., "Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction," *Circulation Research*, vol. 94, no. 9, pp. 1256–1262, 2004.
- [18] I. N. Mungro, R. Gros, X. You et al., "Cardiomyocyte overexpression of iNOS in mice results in peroxynitrite generation, heart block, and sudden death," *Journal of Clinical Investigation*, vol. 109, no. 6, pp. 735–743, 2002.
- [19] G. A. Haywood, P. S. Tsao, H. E. von der Leyen et al., "Expression of inducible nitric oxide synthase in human heart failure," *Circulation*, vol. 93, no. 6, pp. 1087–1094, 1996.
- [20] F. M. Habib, D. R. Springall, G. J. Davies, C. M. Oakley, M. H. Yacoub, and J. M. Polak, "Tumour necrosis factor and inducible nitric oxide synthase in dilated cardiomyopathy," *The Lancet*, vol. 347, no. 9009, pp. 151–155, 1996.
- [21] P. Zhang, X. Xu, X. Hu, E. D. Van Deel, G. Zhu, and Y. Chen, "Inducible nitric oxide synthase deficiency protects the heart from systolic overload-induced ventricular hypertrophy and congestive heart failure," *Circulation Research*, vol. 100, no. 7, pp. 1089–1098, 2007.
- [22] A. J. Cardounel, H. Cui, A. Samouilov et al., "Evidence for the pathophysiological role of endogenous methylarginines in regulation of endothelial NO production and vascular function," *Journal of Biological Chemistry*, vol. 282, no. 2, pp. 879–887, 2007.
- [23] Q. Feng, L. Xiangru, A. J. Fortin et al., "Elevation of an endogenous inhibitor of nitric oxide synthesis in experimental congestive heart failure," *Cardiovascular Research*, vol. 37, no. 3, pp. 667–675, 1998.
- [24] J. T. Kielstein, S. M. Bode-Böger, G. Klein, S. Graf, H. Haller, and D. Fliser, "Endogenous nitric oxide synthase inhibitors and renal perfusion in patients with heart failure," *European Journal of Clinical Investigation*, vol. 33, no. 5, pp. 370–375, 2003.
- [25] A. Surdacki, M. Nowicki, J. Sandmann et al., "Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension," *Journal of Cardiovascular Pharmacology*, vol. 33, no. 4, pp. 652–658, 1999.
- [26] K. Sydow, C. E. Mondon, and J. P. Cooke, "Insulin resistance: potential role of the endogenous nitric oxide synthase inhibitor ADMA," *Vascular Medicine*, vol. 10, no. 1, pp. S35–S43, 2005.
- [27] R. H. Böger, S. M. Bode-Böger, P. S. Tsao, P. S. Lin, J. R. Chan, and J. P. Cooke, "An endogenous inhibitor of nitric oxide synthase regulates endothelial adhesiveness for monocytes," *Journal of the American College of Cardiology*, vol. 36, no. 7, pp. 2287–2295, 2000.
- [28] H.-B. Xiao, Z.-C. Yang, S.-J. Jia et al., "Effect of asymmetric dimethylarginine on atherogenesis and erythrocyte deformability in apolipoprotein E deficient mice," *Life Sciences*, vol. 81, no. 1, pp. 1–7, 2007.
- [29] Y. Chen, Y. Li, P. Zhang et al., "Dimethylarginine dimethylaminohydrolase and endothelial dysfunction in failing hearts," *The American Journal of Physiology*, vol. 289, no. 5, pp. H2212–H2219, 2005.
- [30] F. Cosentino, S. Patton, L. V. d'Uscio et al., "Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats," *Journal of Clinical Investigation*, vol. 101, no. 7, pp. 1530–1537, 1998.
- [31] U. Landmesser, S. Dikalov, S. R. Price et al., "Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension," *Journal of Clinical Investigation*, vol. 111, no. 8, pp. 1201–1209, 2003.
- [32] Y. Xia, V. L. Dawson, T. M. Dawson, S. H. Snyder, and J. L. Zweier, "Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 13, pp. 6770–6774, 1996.
- [33] M.-H. Zou, C. Shi, and R. A. Cohen, "Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite," *Journal of Clinical Investigation*, vol. 109, no. 6, pp. 817–826, 2002.
- [34] K. J. Baek, B. A. Thiel, S. Lucas, and D. J. Stuehr, "Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme," *Journal of Biological Chemistry*, vol. 268, no. 28, pp. 21120–21129, 1993.
- [35] B. R. Crane, A. S. Arvai, D. K. Ghosh et al., "Structure of nitric oxide synthase oxygenase dimer with pterin and substrate," *Science*, vol. 279, no. 5359, pp. 2121–2126, 1998.
- [36] Y. Xia and J. L. Zweier, "Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 13, pp. 6954–6958, 1997.
- [37] N. M. Olken, Y. Osawa, and M. A. Marletta, "Characterization of the inactivation of nitric oxide synthase by N(G)-methyl-L-arginine: evidence for heme loss," *Biochemistry*, vol. 33, no. 49, pp. 14784–14791, 1994.
- [38] A. J. Cardounel, Y. Xia, and J. L. Zweier, "Endogenous methylarginines modulate superoxide as well as nitric oxide generation from neuronal nitric-oxide synthase: differences in the effects of monomethyl- and dimethylarginines in the presence and absence of tetrahydrobiopterin," *Journal of Biological Chemistry*, vol. 280, no. 9, pp. 7540–7549, 2005.
- [39] F. Scalera, J. Borlak, B. Beckmann et al., "Endogenous nitric oxide synthesis inhibitor asymmetric dimethyl L-arginine

- accelerates endothelial cell senescence," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 10, pp. 1816–1822, 2004.
- [40] J. Toth, A. Racz, P. M. Kaminski, M. S. Wolin, Z. Bagi, and A. Koller, "Asymmetrical dimethylarginine inhibits shear stress-induced nitric oxide release and dilation and elicits superoxide-mediated increase in arteriolar tone," *Hypertension*, vol. 49, no. 3, pp. 563–568, 2007.
- [41] S. M. Wells and A. Holian, "Asymmetric dimethylarginine induces oxidative and nitrosative stress in murine lung epithelial cells," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 36, no. 5, pp. 520–528, 2007.
- [42] T. Ogawa, M. Kimoto, and K. Sasaoka, "Occurrence of a new enzyme catalyzing the direct conversion of N<sup>G</sup>,N<sup>G</sup>-dimethyl-L-arginine to L-citrulline in rats," *Biochemical and Biophysical Research Communications*, vol. 148, no. 2, pp. 671–677, 1987.
- [43] J. M. Leiper, J. Santa Maria, A. Chubb et al., "Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases," *Biochemical Journal*, vol. 343, no. 1, pp. 209–214, 1999.
- [44] C. T. L. Tran, M. F. Fox, P. Vallance, and J. M. Leiper, "Chromosomal localization, gene structure, and expression pattern of DDAH1: comparison with DDAH2 and implications for evolutionary origins," *Genomics*, vol. 68, no. 1, pp. 101–105, 2000.
- [45] F. I. Arrigoni, P. Vallance, S. G. Haworth, and J. M. Leiper, "Metabolism of asymmetric dimethylarginines is regulated in the lung developmentally and with pulmonary hypertension induced by hypobaric hypoxia," *Circulation*, vol. 107, no. 8, pp. 1195–1201, 2003.
- [46] X. Hu, D. Atzler, X. Xu et al., "Dimethylarginine dimethylaminohydrolase-1 is the critical enzyme for degrading the cardiovascular risk factor asymmetrical dimethylarginine," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 7, pp. 1540–1546, 2011.
- [47] H. Dayoub, V. Achan, S. Adimoolam et al., "Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence," *Circulation*, vol. 108, no. 24, pp. 3042–3047, 2003.
- [48] X. Hu, X. Xu, G. Zhu et al., "Vascular endothelial-specific dimethylarginine dimethylaminohydrolase-1-deficient mice reveal that vascular endothelium plays an important role in removing asymmetric dimethylarginine," *Circulation*, vol. 120, no. 22, pp. 2222–2229, 2009.
- [49] J. Leiper, M. Nandi, B. Torondel et al., "Disruption of methylarginine metabolism impairs vascular homeostasis," *Nature Medicine*, vol. 13, no. 2, pp. 198–203, 2007.
- [50] P. Zhang, X. Hu, X. Xu, Y. Chen, and R. J. Bache, "Dimethylarginine dimethylaminohydrolase 1 modulates endothelial cell growth through nitric oxide and Akt," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 4, pp. 890–897, 2011.
- [51] T. Ikegami and Y. Matsuzaki, "Ursodeoxycholic acid: mechanism of action and novel clinical applications," *Hepatology Research*, vol. 38, no. 2, pp. 123–131, 2008.
- [52] P. B. Hylemon, H. Zhou, W. M. Pandak, S. Ren, G. Gil, and P. Dent, "Bile acids as regulatory molecules," *Journal of Lipid Research*, vol. 50, no. 8, pp. 1509–1520, 2009.
- [53] M. Trauner, T. Claudel, P. Fickert, T. Moustafa, and M. Wagner, "Bile acids as regulators of hepatic lipid and glucose metabolism," *Digestive Diseases*, vol. 28, no. 1, pp. 220–224, 2010.
- [54] D. J. Parks, S. G. Blanchard, R. K. Bledsoe et al., "Bile acids: natural ligands for an orphan nuclear receptor," *Science*, vol. 284, no. 5418, pp. 1365–1368, 1999.
- [55] J. Sinisalo, H. Vanhanen, P. Pajunen, H. Vapaatalo, and M. S. Nieminen, "Ursodeoxycholic acid and endothelial-dependent, nitric oxide-independent vasodilatation of forearm resistance arteries in patients with coronary heart disease," *British Journal of Clinical Pharmacology*, vol. 47, no. 6, pp. 661–665, 1999.
- [56] P. R. Maloney, D. J. Parks, C. D. Haffner et al., "Identification of a chemical tool for the orphan nuclear receptor FXR," *Journal of Medicinal Chemistry*, vol. 43, no. 16, pp. 2971–2974, 2000.
- [57] K. Ma, P. K. Saha, L. Chan, and D. D. Moore, "Farnesoid X receptor is essential for normal glucose homeostasis," *Journal of Clinical Investigation*, vol. 116, no. 4, pp. 1102–1109, 2006.
- [58] C. J. Sinal, M. Tohkin, M. Miyata, J. M. Ward, G. Lambert, and F. J. Gonzalez, "Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis," *Cell*, vol. 102, no. 6, pp. 731–744, 2000.
- [59] J. A. Talwalkar and K. D. Lindor, "Primary biliary cirrhosis," *The Lancet*, vol. 362, no. 9377, pp. 53–61, 2003.
- [60] L. Schild, F. Dombrowski, U. Lendeckel, C. Schulz, A. Gardemann, and G. Keilhoff, "Impairment of endothelial nitric oxide synthase causes abnormal fat and glycogen deposition in liver," *Biochimica et Biophysica Acta*, vol. 1782, no. 3, pp. 180–187, 2008.
- [61] L. Schild, I. Jaroskova, U. Lendeckel, G. Wolf, and G. Keilhoff, "Neuronal nitric oxide synthase controls enzyme activity pattern of mitochondria and lipid metabolism," *FASEB Journal*, vol. 20, no. 1, pp. 145–147, 2006.
- [62] U. Özcan, E. Yilmaz, L. Özcan et al., "Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes," *Science*, vol. 313, no. 5790, pp. 1137–1140, 2006.
- [63] S. von Haehling, J. C. Schefold, E. A. Jankowska et al., "Ursodeoxycholic acid in patients with chronic heart failure: a double-blind, randomized, placebo-controlled, crossover trial," *Journal of the American College of Cardiology*, vol. 59, no. 6, pp. 585–592, 2012.
- [64] T. Hu, M. Chouinard, A. L. Cox et al., "Farnesoid X receptor agonist reduces serum asymmetric dimethylarginine levels through hepatic dimethylarginine dimethylaminohydrolase-1 gene regulation," *Journal of Biological Chemistry*, vol. 281, no. 52, pp. 39831–39838, 2006.
- [65] J. Li, A. Wilson, X. Gao et al., "Coordinated regulation of dimethylarginine dimethylaminohydrolase-1 and cationic amino acid transporter-1 by farnesoid X receptor in mouse liver and kidney and its implication in the control of blood levels of asymmetric dimethylarginine," *Journal of Pharmacology and Experimental Therapeutics*, vol. 331, no. 1, pp. 234–243, 2009.
- [66] A. Mencarelli, S. Cipriani, B. Renga et al., "FXR activation improves myocardial fatty acid metabolism in a rodent model of obesity-driven cardiotoxicity," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 23, pp. 94–101, 2013.
- [67] L. Zhang, T. Li, D. Yu, B. M. Forman, and W. Huang, "FXR protects lung from lipopolysaccharide-induced acute injury," *Molecular Endocrinology*, vol. 26, no. 1, pp. 27–36, 2012.

## Research Article

# Baicalin's Therapeutic Time Window of Neuroprotection during Transient Focal Cerebral Ischemia and Its Antioxidative Effects *In Vitro* and *In Vivo*

Fafeng Cheng,<sup>1</sup> Yi Lu,<sup>1</sup> Xianggen Zhong,<sup>1</sup> Wenting Song,<sup>2</sup> Xueqian Wang,<sup>1</sup> Xiaoguang Sun,<sup>1</sup> Jianguo Qin,<sup>3</sup> Shaoying Guo,<sup>4</sup> and Qingguo Wang<sup>1</sup>

<sup>1</sup> College of Basic Medicine, Beijing University of Chinese Medicine, Beijing 100029, China

<sup>2</sup> Xiyuan Hospital of China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>3</sup> Dongfang Hospital, The Second Clinical Medical College of Beijing University of Chinese Medicine, Beijing 100078, China

<sup>4</sup> The Central Hospital of Handan, Handan, Hebei 056001, China

Correspondence should be addressed to Qingguo Wang; wangqg8558@sina.com

Received 26 January 2013; Accepted 11 May 2013

Academic Editor: Hao Xu

Copyright © 2013 Fafeng Cheng et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

We investigated the effects of baicalin on an ischemia-reperfusion-induced brain injury model in rats and its antioxidative activities *in vitro* and *in vivo*. An ischemia-reperfusion injury of the brain via a middle cerebral artery occlusion (MCAO) was induced in rats. Baicalin was injected at different time points (0, 2, 4, and 6 h) after the MCAO was induced. Baicalin can improve neurological function and significantly decrease brain infarction within a time window of 4 h. Moreover, baicalin was able to reduce cell apoptosis and had the strong antioxidative effect of reducing reactive oxygen species production and malondialdehyde generation. In contrast, baicalin interfered with superoxide dismutase and nicotinamide adenine dinucleotide 2'-phosphate oxidase activities. Moreover, baicalin also exhibited strong neuroprotective effects against H<sub>2</sub>O<sub>2</sub>-mediated injury and improved the SOD activity of neurons. Furthermore, baicalin demonstrated good scavenging of hydroxyl radicals, superoxide anions, and DPPH radicals and exerted an additional effect of inhibiting xanthine oxidase. Baicalin showed beneficial effects against MCAO-induced injury within a 4 h time window, and its antioxidative effects both *in vitro* and *in vivo* may partly elucidate its mechanism of action.

## 1. Introduction

Ischemic stroke is a life-threatening disease that is characterized by high morbidity and mortality. Although the pathogenesis of ischemic stroke remains unknown, several studies have suggested that free radicals might be involved in the inflammatory injury and oxidative stress that often accompanies this acute brain disease [1, 2]. Reactive oxygen species can attack proteins, deoxyribonucleic acid, and lipid membranes, thereby disrupting cellular function and integrity, leading to mitochondrial damage [3, 4]. These effects are commonly referred to as "oxidative stress" [5]. Therefore, removing free radicals or preventing their formation can be a potential therapeutic strategy. Several herbs in TCM have been used for thousands of years and were recently found to have antioxidative effects [6, 7]. Baicalin is one of several effective

ingredients that is enriched in the dried root of the *Scutellaria baicalensis* Georgi, which is commonly used in traditional Chinese medicine (Figure 1).

Many studies have been conducted to investigate baicalin's health-promoting effects, in particular, its antibacterial, antiviral, anticancer, anti-inflammatory, and antioxidative effects [7–11]. Recent studies revealed that baicalin can protect against neuronal cell death and enhance neurological function following cerebral ischemia [7, 12, 13]. In addition, Zhang et al. [14] demonstrated that baicalin can pass through the blood-brain barrier and distribute within brain tissue, specifically in the hippocampus, striatum, cortex, and thalamus.

In clinical practice, the timely treatment of stroke patients is not always possible, and the therapeutic time window is often correlated with therapeutic effect. In recent years,

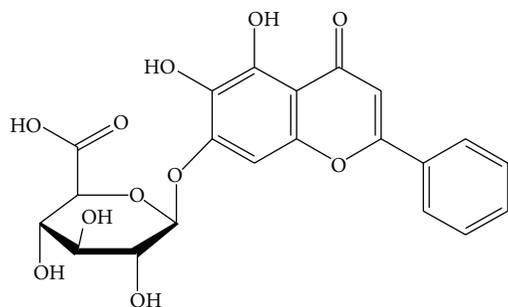


FIGURE 1: Chemical structure of baicalin.

studies that focused on the therapeutic time window have attracted more attention from the committee of the Stroke Therapy Academic Industry Roundtable [15]. Although previous studies [12, 14, 16, 17] have investigated the therapeutic effects of baicalin, studies of its antioxidative activities and cytoprotective effects on primary neurons have been few, and the therapeutic time window of baicalin for cerebral ischemia has not yet been reported.

This study determined baicalin's scavenging activities of hydroxyl radicals, superoxide anions, and DPPH radicals and investigated its regulatory effects on the enzyme activities of such enzymes as xanthine oxidase, NADPH oxidase, and SOD. In this study, we focused on the therapeutic time window of baicalin for MCAO rats. Primary neurons were injured by  $H_2O_2$  to test for neuroprotective effects after baicalin treatment.

## 2. Materials and Methods

**2.1. Animals and Drugs.** Forty-eight healthy, male, C57BL/6 mice (25–30 g), 96 healthy, male, Sprague Dawley (SD) rats (230–250 g), and 10 newborn SD rats (born within 12 h) were purchased from Vital River Laboratories, Beijing, China (number SCXK (Beijing) 2008-0001) and housed in the Central Laboratory, Beijing University of Chinese Medicine, at a room temperature of  $25 \pm 1^\circ C$ , with a relative humidity of 40–60%, automatic day-night rhythm and free access to standard lab chow and tap water. Any procedure involving animals and their care were performed according to the Prevention of Cruelty to Animals Act 1986 and the NIH Guidelines for Care and Use of Laboratory Animals and local laws.

Baicalin (5,6-dihydroxy-7-O- $\beta$ -D-glucopyranosyl-2-penyl-4H-1-benzopyran-4-one) was purchased commercially from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

### 2.2. In Vivo Experiments

**2.2.1. MCAO Model.** Transient focal ischemia was induced by a filament occlusion of the right middle cerebral artery [18]. Briefly, rats were anesthetized with 4% hydrochloride (350 mg/kg, i.p.) and kept under a heating lamp to maintain their core body temperature at  $36.5 \pm 0.5^\circ C$ . The left common carotid artery, internal carotid artery (ICA), and

external carotid artery (ECA) were surgically exposed under a dissecting microscope, and the ECA was coagulated at the bifurcation point. A 0.24 mm diameter nylon filament (tip diameter  $0.32 \pm 0.02$  mm; Beijing Sunbio Biotech, Beijing, China) was inserted into the ICA through the ECA stump and was gently advanced (10 mm) to occlude the origin of the middle cerebral artery. After 1.5 h of MCAO, the filament was removed to restore blood flow (reperfusion) [19]. The rats were then allowed to recover after an incision closure and were housed individually. The MCAO procedure for the mice was similar to that for the rats, with the exception of the use of a nylon filament with a diameter of 0.16 mm and a round tip of  $0.20 \pm 0.01$  mm.

**2.2.2. Groupings of Animals and the Administration of Drugs.** Twenty-four SD rats were used to establish the MCAO model. After elimination of the animals that died or those in which the model was not performed properly, the animals were randomly assigned into two equally sized groups. Baicalin (15 mg/kg) [17] was injected via the tail vein at 0 and 4 h after the onset of ischemia and then twice daily from day 2. The MCAO with vehicle treatment group was injected with the same volume of saline (Figure 2(a)).

For the therapeutic time window experiments, 72 rats were randomly assigned to the sham, MCAO-only, and 0, 2, 4, and 6 h baicalin-treated groups. These animals were injected with 15 mg/kg baicalin (or 0.9 mL/100 g saline in the MCAO-only group). Baicalin treatments were first administered at 0, 2, 4 or 6 h after the onset of ischemia in the different groups, and saline was administered at 0 h in the MCAO-only group. All of the animals in baicalin treatment groups were given a second administration of baicalin 4 h after the first injection, and MCAO-only group received a second administration of saline 4 h after MCAO (Figure 2(c)).

Mice were divided into sham, MCAO-only, and baicalin (15 mg/kg)-treated groups, with 16 animals in each group. All of the mice were subjected to a MCAO; those animals that died or those animals in which the MCAO failed were eliminated from the study. Baicalin (15 mg/kg) or saline was administered via the tail vein at 0 and 4 h after the onset of ischemia. After 24 h, 5 mice from each group were sacrificed, and their brains were prepared for the TUNEL and ROS detection assays. The brain homogenates from the other animals in each group were collected to determine the NADPH oxidase, SOD, and MDA levels.

**2.2.3. Neurological Evaluation.** The neurological function of the surviving rats was evaluated with a scoring system that was previously described by Garcia et al. [20] which consists of 6 tests: spontaneous activity, symmetry in the movement of their 4 limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch. The score obtained from each rat was the sum of the 6 test scores with the maximum score of 18. The neurological evaluation was performed at 24 h after the onset of ischemia for rats in the time window experiments and at 3 other time points (at 24, 48, and 72 h after MCAO) for the other two groups (Figures 2(a) and 2(c)).

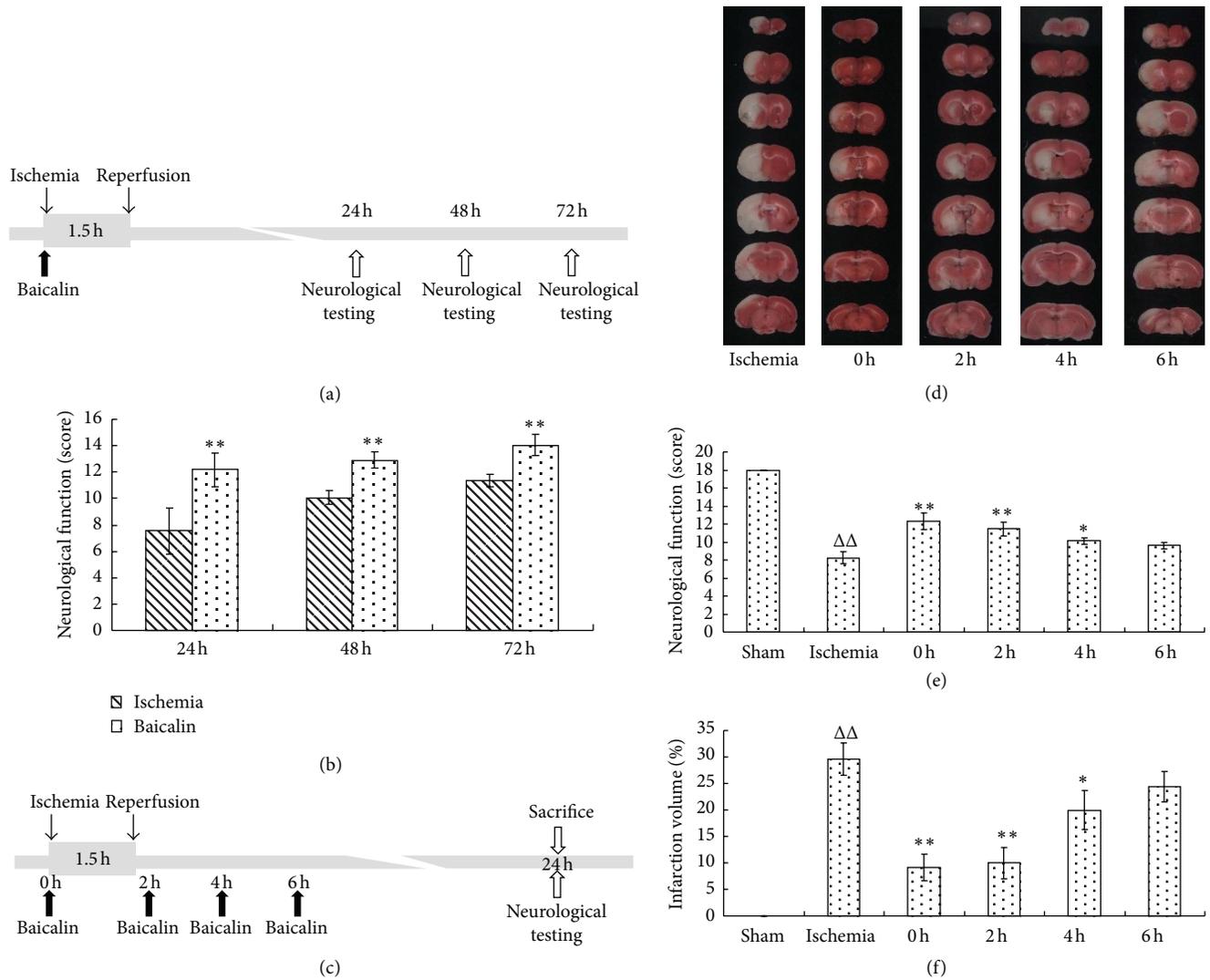


FIGURE 2: Therapeutic efficacy of baicalin in rats undergoing middle cerebral artery occlusion. (a) Flow chart of the determination of neurological function in injured rats at 24, 48, and 72 h after MCAO. (b) The neurological scores were determined in rats with MCAO (1.5 h) at various survival times after the onset of ischemia. Baicalin (15 mg/kg) or saline treatments were administered at 0 and 4 h after the onset of ischemia and after 24 h twice a day. The scores shown are presented as the means  $\pm$  SEM.  $**P < 0.01$ , baicalin group is significantly different from the corresponding control group ( $n = 8$  for each group). (c) A flow chart of the time window experiment of baicalin. (d) Seven coronal brain sections (2 mm thick) were selected for tetrazolium chloride staining. The red stain represents noninjured (normal) tissues; white represents the infarct region. (e) The infarct volume was quantified as a percentage of the total volume, with large infarcts corresponding to a more severe injury. (f) The neurological scores in rats after 1.5 h MCAO and 22.5 h reperfusion. Baicalin (15 mg/kg) treatments were first administered at 0, 2, 4, or 6 h after the onset of ischemia in the different groups, and saline was administered at 0 h in the ischemic group. In addition, 4 h after the first treatment, baicalin was administered again.  $\Delta\Delta P < 0.01$ , ischemic group versus sham;  $**P < 0.01$ ,  $*P < 0.05$ , baicalin-treated groups versus ischemia group ( $n = 10$  for each group).

**2.2.4. Calculation of the Infarct Volume.** After the evaluation for neurological function, the rats were sacrificed and their brains were harvested for TTC staining (Nanjing Green-synthesis Biochemical Co., Ltd., Nanjing, Jiangsu, China). The percentage of the infarct volume from the total brain volume represented the degree of cerebral infarction. Serial coronal sections (2 mm thickness) were prepared and soaked in 2% TTC phosphate buffer at 37°C for 10 minutes in the dark. In this assay, the normal brain tissues were stained red, while the infarct tissues remained unstained (white).

The sections were soaked in 4% paraformaldehyde phosphate buffer for 30 minutes, arranged serially, and then scanned (Tsinghua Unisplendour A688, Xi'an, China). The red and white stained areas were measured using a computer color multimedia image analysis system (Image-Pro Plus6.0, Media Cybernetics, WY, USA). The percentage of infarction was determined by the following equation [18].

$$\% \text{Infarction volume} = \left( \frac{\text{Infarction volume}}{\text{Total volume of slice}} \times 100 \right). \quad (1)$$

**2.2.5. ROS Measurement through DHE Staining.** Brain ROS production was determined using dihydroethidium (DHE) microfluorography [18]. DHE is a cell permeable dye, which can be oxidized into ethidium and other products by superoxide [21]. The animals were sacrificed after 24 h MCAO, and the brains were removed, frozen, and then cryosectioned at a thickness of 20  $\mu\text{M}$ . Sections obtained from the prefrontal cortex were collected and an ROS fluorescence detection kit (Genmed, WY, USA) was used. The DHE solution was superfused on to the brain sections for 60 minutes, and fluorescence intensity was detected using fluorescence microscopy (ZEISS, LSM510 meta, Germany). The fluorescence intensity of 5 different fields in the prefrontal cortex of the ischemic hemisphere was averaged and expressed as relative fluorescence units (RFU) [22].

**2.2.6. TUNEL Detection.** After sampling and cutting the brain sections as previously described, TUNEL staining was performed using a detection kit for programmed cell death (In Situ Cell Death Detection Kit, TMR Red, Roche, USA) according to the manufacturer's instruction. Five areas obtained from each section were examined by fluorescence microscopy (ZEISS, LSM510 meta, Germany) in the prefrontal cortex of the ischemic hemisphere, and the TUNEL-positive cells were quantified [23].

**2.2.7. Measurement of NADPH Oxidase.** The forebrain from the ischemic side was homogenized with saline for the measurement of NADPH oxidase activity. The cytosolic (supernatant) and membrane (pellet) fractions were separated using a Protein Extraction Kit (Transmembrane Protein Extraction Kit, Novagen, Darmstadt, Germany). The membrane fraction was then used for further analysis of NADPH oxidase enzymatic activity [24] and performed according to previously described protocols [24, 25]. Aliquots of the brain homogenate were incubated with NADPH at 37°C, and the NADPH oxidase enzymatic activity was determined every 10 minutes by measuring the reduction of NADPH with a plate reader spectrophotometer (450 nm) and a Radical Detector kit (Genmed, USA). The NADPH oxidase activity was normalized by the amount of protein in each sample, and the increase in absorbance was measured every 10 and 20 minutes. The activity was calculated as  $\text{mU}/(\text{g prot} \times \text{min})$ .

**2.2.8. Detection of SOD and MDA.** The brain was removed 24 h after ischemia, and the cortex was isolated from the lesioned hemisphere and homogenized, and the supernatant was collected [18]. The MDA production reflects the degree of lipid peroxidation injury [26]. The MDA was determined using a kit according to the manufacturer's directions (Nanjing Jiancheng Bioengineering Institute). In addition, the SOD activity was determined using the xanthine oxidase method [26] according to the manufacturer's directions (Nanjing Jiancheng Bioengineering Institute).

### 2.3. In Vitro Experiments

**2.3.1. Protection against  $\text{H}_2\text{O}_2$ -Induced Cell Toxicity in Primary Rat Cortical Neuronal Cultures.** Primary rat cortical

neuronal cultures were prepared from the cerebral cortices of newborn SD rats. Twelve-day-old cultures were used in this study. The cells were seeded in a 96-well flat bottom plate at a density of  $2.0 \times 10^5$  cells/mL and were either incubated with different concentrations of  $\text{H}_2\text{O}_2$  or treated with baicalin at a concentration of 10, 20, 40, 80, and 200  $\mu\text{M}$  in addition to 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . After 16 h of incubation [27], the cell viability and toxicity were determined. The cell viability was assessed by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA), which yields a blue formazan product in living cells, but not in dead cells or their lytic debris. MTT was dissolved in a serum-free glucose solution and added to the culture at a final concentration of 0.5 mg/mL. After 2 h of incubation at 37°C, the medium was carefully removed, and 100  $\mu\text{L}$  DMSO was added to each well. The plates were then read at 580 nm on a spectrophotometer (Bio-Rad, CA, USA) and the results of the neuronal viability was determined as a percentage compared to controls [27]. The LDH release in the medium was measured using a kit from Nanjing Jiancheng Bioengineering Institute and performed according to the manufacturer's directions. The results were expressed as U/L, and the SOD activity in the neurons was also detected. The cells were washed with ice cold PBS, collected in 0.1 M PBS/0.05 mM EDTA buffered solution, and then homogenized using sonication. The homogenates were centrifuged at 10000 r/min for 10 min at 4°C, and the SOD activity in the supernatants was determined using a kit from Nanjing Jiancheng Bioengineering Institute and performed according to the manufacturer's directions.

#### 2.3.2. Detection of Antioxidative Activity of Baicalin in Several Chemical Systems

**Assay of Hydroxyl Radical Scavenging.** The hydroxyl radicals were generated in an  $\text{H}_2\text{O}_2$ - $\text{FeSO}_4$  system by the oxidation of  $\text{FeSO}_4$  and were assayed by a colorimetric change of salicylic acid, according to previously described protocols [28]. In this experiment, the hydroxyl radicals were generated in a reactive solution containing 0.5 mL of 9 mM  $\text{FeSO}_4$ , 0.5 mL of 9 mM salicylic acid-ethanol solution, and the samples were to be tested at different concentrations. Lastly, 5 mL of 9 mM  $\text{H}_2\text{O}_2$  was added to this reactive solution. The mixture was incubated at 37°C for 1 h and the absorbance of the hydroxylated salicylate complex was measured at 510 nm. The inhibition of hydroxyl radical production was calculated as follows: inhibition rate (%) = (the absorbance of the control group – the absorbance of the test group)/the absorbance of the control group  $\times 100\%$ .

**Superoxide Anion Scavenging Effect.** Superoxide anions were generated enzymatically by the xanthine/xanthine oxidase system according to Toda et al. [29]. Briefly, the reaction mixture consisted of 25 mL of 40 mM sodium carbonate buffer (pH 10) containing 0.1 mM EDTA (pH 10.0), 0.06 mL of 10 mM xanthine, 0.03 mL of 0.5% bovine serum albumin, 0.03 mL of 2.5 mM nitroblue tetrazolium, and 0.06 mL of baicalin (dissolved in DMSO). Baicalin was dissolved in 0.2% DMSO to give final concentrations of 400, 300, 100, 80, 40,

20, and 10  $\mu\text{M}$ . The reaction was initiated by the addition of 0.12 mL xanthine oxidase (0.04 units) into each tube, and the absorbance at 560 nm was recorded for 90 s (by the formation of blue formazan). The control experiments were performed by replacing the sample solution with the same volume of 0.2% DMSO.

**DPPH Radical Scavenging Activity.** The radical scavenging activity on DPPH was detected according to previously described protocols [28]. First, 1 mL of 100 mM acetate buffer (pH 5.5), 1.87 mL of ethanol, and 0.1 mL of ethanol solution of 3 mM of DPPH were mixed in a test tube. Then, 0.03 mL baicalin (dissolved in DMSO) was added to the test tube and incubated at 25°C for 20 min. The absorbance at 517 nm (DPPH,  $\epsilon = 8.32 \times 10^3$ ) was recorded. As a control, 0.03 mL of DMSO was added to the control tube. The scavenging activity was calculated from a decrease in the absorbance and expressed as follows: scavenging activity (%) =  $(A - B)/A \times 100\%$ , where “A” represents the absorbance of the control tube and “B” represents the absorbance of the experimental tube.

**Inhibition of Xanthine Oxidase Activity.** The reaction mixture consisted of 2.5 mL of 40 mM sodium carbonate buffer (pH 10) containing 0.04 mL of 0.1 mM EDTA, 0.04 mL of 10 mM xanthine, and 0.04 mL sample solution (dissolved in 0.2% DMSO). The reaction was initiated by the addition of 0.01 mL of xanthine oxidase (0.04 units), and the absorbance at 293 nm was recorded for 90 s. The control experiments were performed as described previously [30].

**Total Antioxidant Capacity.** The total antioxidative capacity was determined using a spectrophotometric assay kit (Nanjing Jiancheng Bioengineering Institute) and performed according to the manufacturer’s instructions. In brief, 30  $\mu\text{L}$  baicalin at different concentrations was added to the reaction buffer containing xanthine, xanthine oxidase, and hydroxylamine. After a 40 min incubation at 37°C, the accumulation of nitrite was quantified by the Griess reaction. The antioxidative capacity is inversely correlated with the concentration of nitrate [31]. These results were normalized according to the manufacturer’s instructions and expressed as U/L.

**2.4. Statistical Analysis.** The data were analyzed using SPSS 16.0 software. A one-way analysis of variance was performed and was followed by a post hoc analysis for significance using the Student-Newman-Keuls multiple comparison test. All of the values are expressed as the mean  $\pm$  SEM. A value of  $P < 0.05$  was considered to be statistically significant.

### 3. Results

**3.1. Baicalin Effects on MCAO-Induced Cerebral Damage.** Simultaneous administration of baicalin (15 mg/kg) with the onset of ischemia could significantly improve the neurological function of rats at 24, 48, and 72 h after MCAO (Figure 2(a)). Rats in both the MCAO-only and baicalin-treated groups showed a trend towards neurological functional recovery (Figure 2(b)). Importantly, animals in the baicalin-treated group showed enhanced neurological function compared with the MCAO-only group at each time

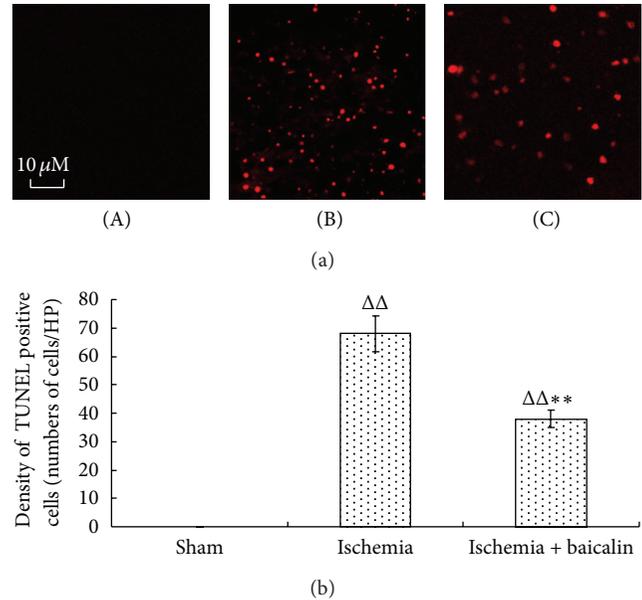


FIGURE 3: Effects of baicalin on TUNEL staining in the prefrontal cortex of MCAO mice. After 24 hr of MCAO, apoptotic cells (a), ( $\times 400$ ) were detected in regions of the prefrontal cortex. The apoptotic cells were labeled with red fluorescence, (A) sham group, (B) ischemia group, and (C) baicalin-treated group. Five animals were selected from each group and 3 sections were selected from each site. Five 400-fold fields of view were randomly selected from each section to quantify the mean of the positive cells. The results are expressed as the mean  $\pm$  SEM (b).  $\Delta\Delta P < 0.01$ , ischemia group versus sham;  $\Delta\Delta^{**} P < 0.01$ , baicalin-treated group versus ischemia group.

point. We chose the 24 h time point after MCAO to assess the neurological function and infarct volume in our time window experiments (Figure 2(c)). We found that administration of baicalin at 0, 2, and 4 h after MCAO can reduce the infarct size by 68.94%, 66.3%, and 32.6%, respectively (Figures 2(d) and 2(e)), and significantly increased the rats’ neurological scores (Figure 2(f)) compared with the MCAO-only group.

No significant differences were observed between the groups treated with baicalin at 6 h after MCAO and the MCAO-only group. Thus, baicalin decreased the infarct volume and improved neurological function in a time window of 4 h. Cellular apoptosis is an important mechanism of neuronal injury after brain ischemia. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used to test the neuroprotective effects of baicalin in MCAO rats [23]. A large number of TUNEL-positive neurons were observed in the prefrontal cortex 24 h after ischemia (Figure 3(a)). In the baicalin treated group (15 mg/kg), the number of TUNEL-positive neurons was reduced by 44% in the prefrontal cortex compared with the MCAO-only group (Figure 3(b)).

**3.2. The Antioxidative Effects of Baicalin in MCAO Mice.** Reactive oxygen species (ROS) production was detected using DHE staining. As shown in Figure 4(a), no fluorescence was detected in the brains obtained from the sham mice (Figure 4(a)). A large number of neuronal cells contributed

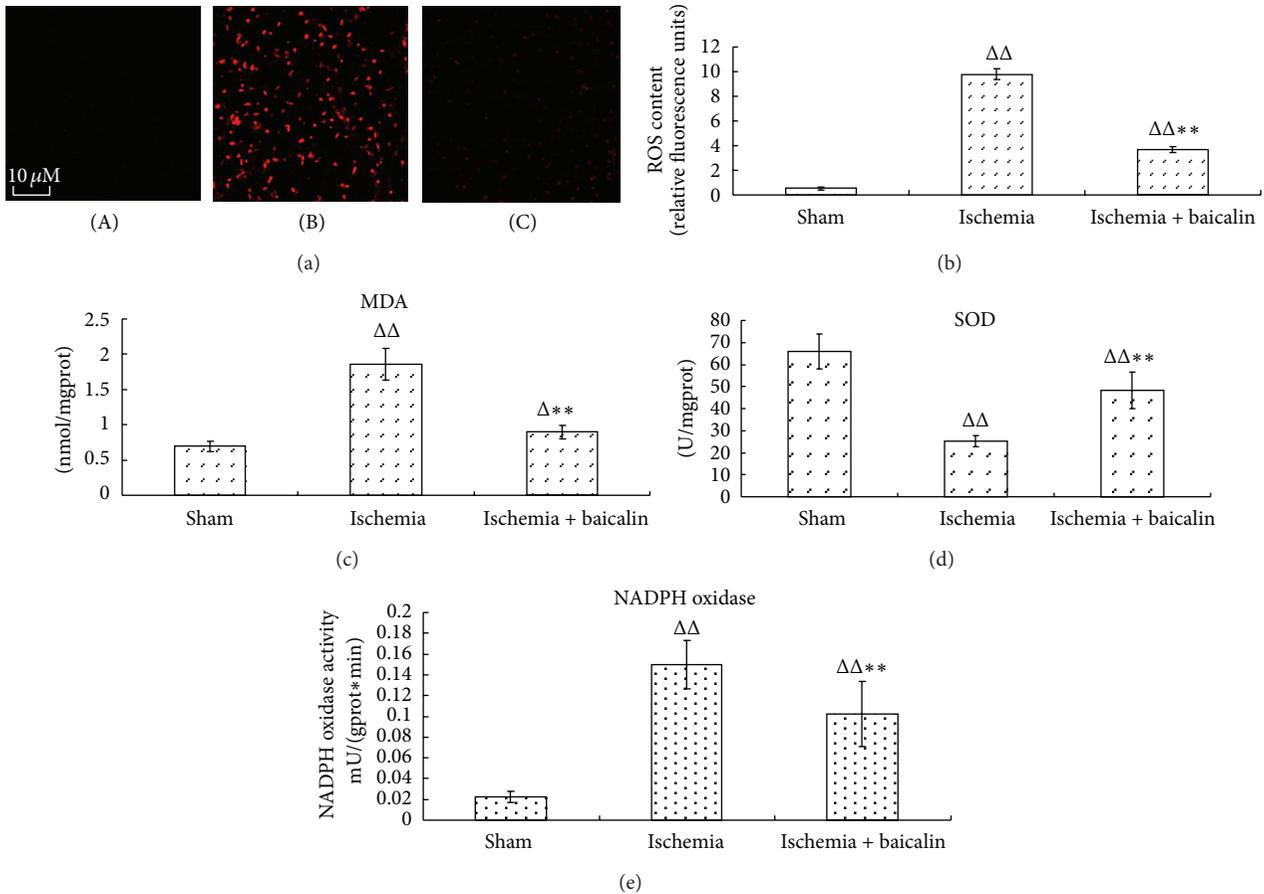


FIGURE 4: Effects of baicalin on the oxidative stress in mice undergoing a middle cerebral artery occlusion. Twenty-four hours after the onset of MCAO, the ROS were detected in regions of the prefrontal cortex using DHE staining. The relative intensity of the red fluorescence represents the ROS content in the prefrontal cortex region of the injured hemisphere in the prefrontal cortex, (A) sham group, (B) ischemia group, and (C) baicalin treatment group ((a), DHE staining  $\times 200$ ). The relative fluorescence intensity in 5 sites obtained from one section was determined by fluorescence microscopy. The mean value of the ROS content was calculated and expressed as the mean  $\pm$  SEM,  $n = 5$  (b). The MDA content, NADPH oxidase activity, and SOD activity are shown in (c), (d), and (e), respectively ( $n = 7$ ).  $\Delta\Delta P < 0.01$ , ischemia group versus sham;  $**P < 0.01$ , baicalin-treated group versus ischemia group.

to the significantly enhanced fluorescence observed in the prefrontal cortex region at 24 h after MCAO. After treatment with baicalin, the number of fluorescent neuronal cells was reduced, and the fluorescence intensity was decreased. A quantitative analysis showed that the fluorescence in the cortex was significantly decreased in the baicalin group compared with the MCAO-only group ( $P < 0.01$ , Figure 4(b)). In another assay, the malondialdehyde (MDA) content in the brain tissue was substantially increased (approximately three times) compared with the sham group 24 h after MCAO, and the MDA content was more than 50% less in the MCAO-only group compared to the baicalin-treated group (Figure 4(c)). These results are consistent with our ROS detection data. The antioxidative effects of baicalin were associated with the regulation of the activity of superoxide dismutase (SOD) and nicotinamide adenine dinucleotide 2'-phosphate (NADPH) oxidase. SOD, an endogenous antioxidant, is constitutively active in normal brain tissues [32]; however, in this study, its activity was significantly decreased 24 h after MCAO, which

is consistent with the result obtained by a previous study [33]. Interestingly, we found that the SOD activity in the baicalin group was approximately twice that of the MCAO-only group (Figure 4(d)). NADPH oxidase plays an important role in ROS generation after cerebral ischemia [34, 35], and its activity is normally low in uninjured animal brain tissues but significantly increases after cerebral ischemia. As shown in Figure 4(e), baicalin significantly inhibited the increase of NADPH oxidase. These results suggest that baicalin has strong antioxidative effects on reducing ROS production and MDA generation, as well as regulates SOD activity and NADPH oxidase activity after MCAO-induced injury.

### 3.3. Neuroprotective Effects on $H_2O_2$ -Induced Neuronal Injury.

After the primary rat cortical neuronal cultures were incubated with different concentrations of  $H_2O_2$  for 16 h, we found that cell viability decreased with increasing  $H_2O_2$  concentrations. Treatment with 300  $\mu M$   $H_2O_2$  resulted in

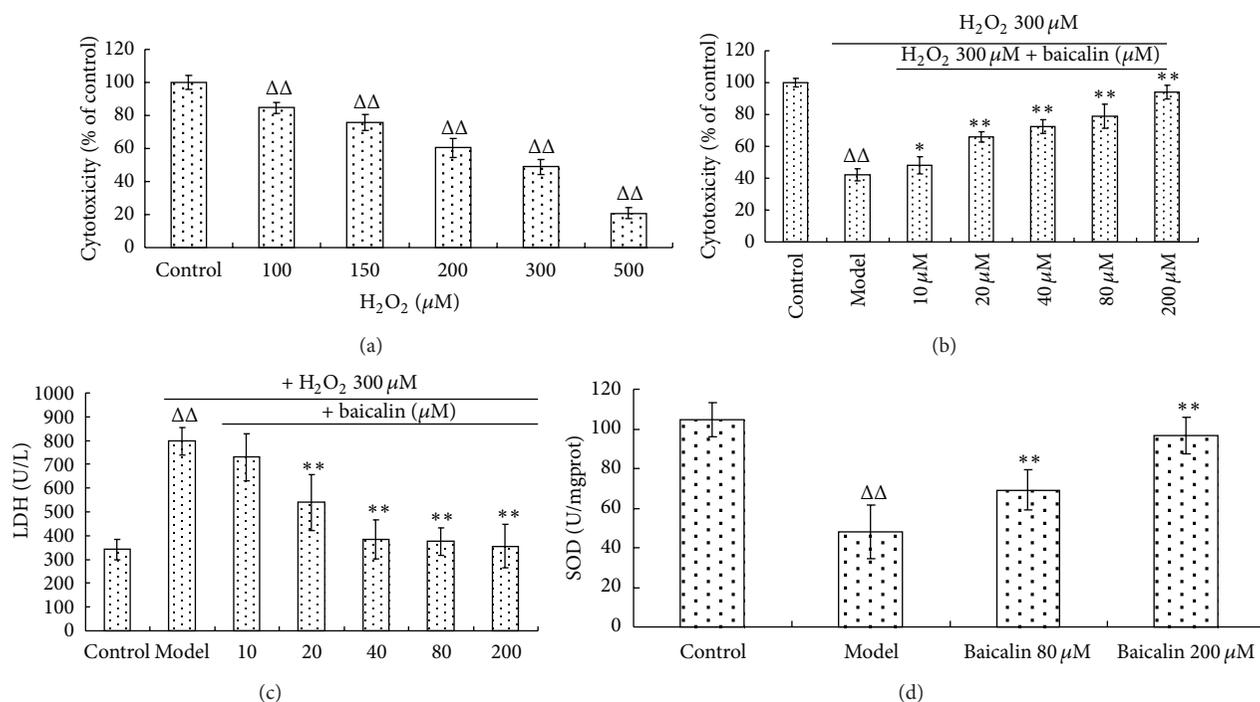


FIGURE 5: Protection effects of baicalin against H<sub>2</sub>O<sub>2</sub>-induced cell toxicity in primary rat cortical neuronal cultures. (a) The cell viability of primary rat cortical neuronal cultures injured by different concentrations of H<sub>2</sub>O<sub>2</sub> after incubation for 16 h. An H<sub>2</sub>O<sub>2</sub> concentration of 300 μM was chosen for the following experiments. (b) The effects of baicalin at concentrations of 10, 20, 40, 80, and 200 μM on the viability of H<sub>2</sub>O<sub>2</sub> (300 μM) injured neurons. (c) Baicalin reduced the levels of LDH in the cell supernatant. (d) Baicalin at 80 and 200 μM increased the SOD activity of neuronal cultures.  $\Delta\Delta P < 0.01$ , H<sub>2</sub>O<sub>2</sub>-treated groups versus control; \*\*P < 0.01, \*P < 0.05, baicalin-treated groups versus H<sub>2</sub>O<sub>2</sub>-only ( $n = 8$  for each group).

only 45% cell survival compared with the untreated group. (Figure 5(a)). This *in vitro* model was used to test the antioxidative effects of baicalin. MTT test results showed that with increasing concentrations of baicalin, there was a corresponding increase in cell survival, demonstrating a dose-dependent relationship between baicalin and cell viability (Figure 5(b)). We found no significant differences in the cell viability between the 200 μM baicalin-treated and normal groups. Accordingly, the LDH leakage in the H<sub>2</sub>O<sub>2</sub>-only group was much higher than in the untreated cells, and this leakage decreased with the addition of baicalin, as shown in Figure 4(c). Baicalin (200 μM) decreased LDH leakage by more than 50% compared with the H<sub>2</sub>O<sub>2</sub>-only group, which is not significantly different from untreated cells (Figure 5(c)). These results indicate that baicalin exhibited strong neuroprotective effects against H<sub>2</sub>O<sub>2</sub>-mediated injury. Furthermore, baicalin can also significantly improve the SOD activity of neurons, the activity of which decreased after H<sub>2</sub>O<sub>2</sub>-induced injury (Figure 5(d)).

**3.4. Oxygen Radical Scavenging and Other Baicalin Antioxidative Activities.** Several tests were performed to investigate the antioxidative activities of baicalin in various signaling systems. First, the antioxidative activity of baicalin was determined by measuring the scavenging of hydroxyl radicals (OH<sup>•</sup>). The hydroxyl radical is the most reactive member of the reactive oxygen species, and it induces severe damage to

DNA, lipids, and proteins. The OH<sup>•</sup> scavenging activity of baicalin is assessed by its competition with salicylic acid for OH<sup>•</sup> radicals in the OH<sup>•</sup> generating/detecting system. In this study, the hydroxyl radical-scavenging effect of baicalin was found to be 43.36% and 98.71% at concentrations of 600 μM and 1000 μM, respectively (Figure 6(a)). The IC<sub>50</sub> value was found to be 390 μM. Thus, baicalin is a good scavenger of hydroxyl radicals. The scavenging activity of baicalin against the enzymatically generated superoxide anions in the xanthine/xanthine oxidase pathway is shown in Figure 6(b). Baicalin reduced superoxide anion formation in a dose-dependent manner and with an IC<sub>50</sub> value of 134.66 μM. Next, the decolorizing activity of DPPH was detected. We found that the DPPH radical scavenging activity increased with increasing concentrations of baicalin (Figure 6(c)). This result indicated that baicalin can quench the DPPH radical with an IC<sub>50</sub> value of 64.92 μM.

The antioxidative activities of some flavonoids (e.g., quercetin and luteolin) are caused by both radical scavenging and the inhibition of enzymatic activity. Xanthine oxidase is known to convert hypoxanthine to xanthine and finally to uric acid. To evaluate the effects of baicalin on xanthine oxidase activity, the formation of uric acid was measured. Baicalin inhibited the oxygen-atom-transfer reaction in a dose-dependent manner (Figure 6(d)). Moreover, the IC<sub>50</sub> value was calculated as 134.66 μM. Finally, the total antioxidative capacity was assessed. Consistent with the above results,

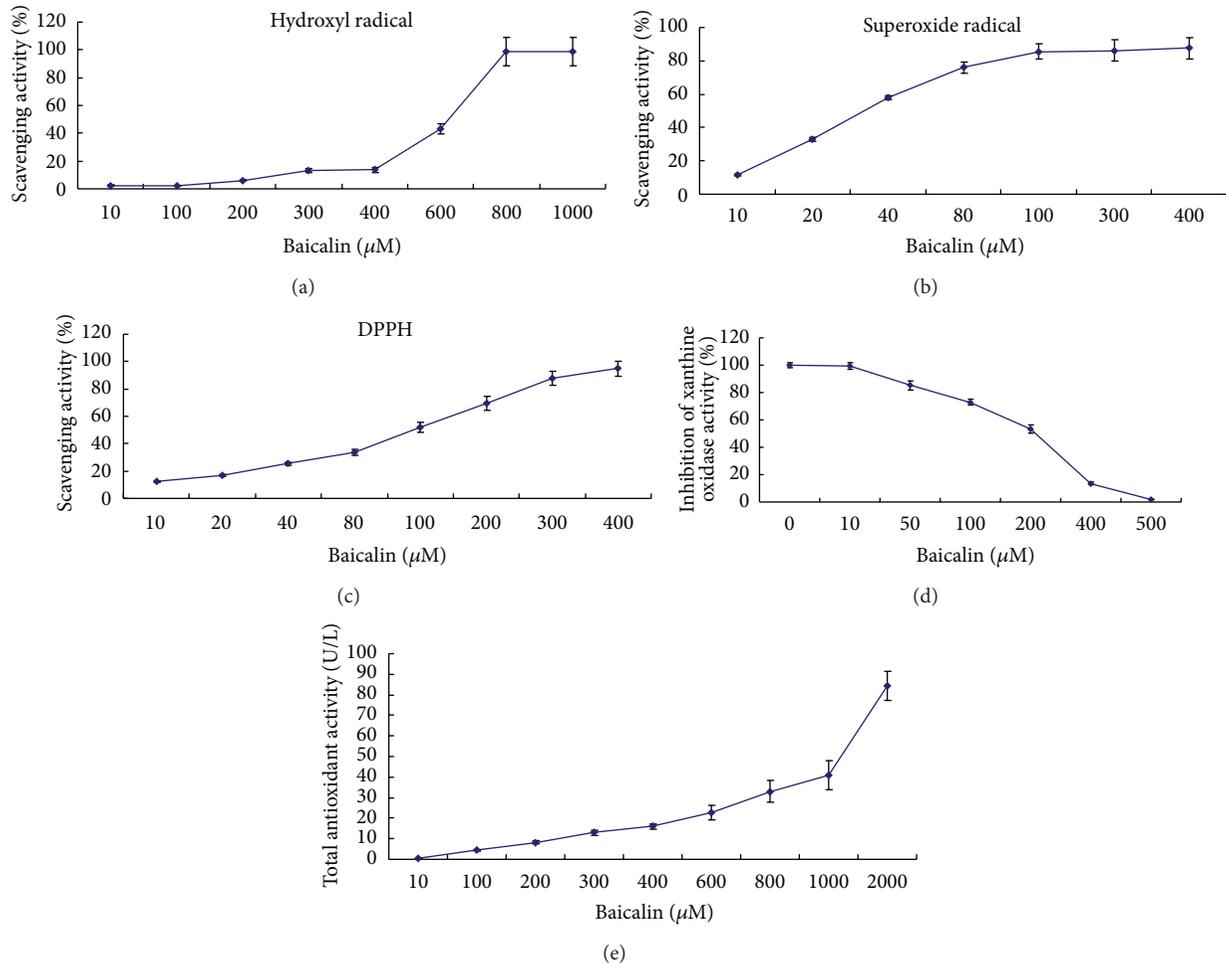


FIGURE 6: Free radical scavenging and antioxidative activity of baicalin. (a) Hydroxyl radical scavenging activity of baicalin. (b) Superoxide anion scavenging effect. (c) DPPH radical scavenging activity. (d) Inhibition of xanthine oxidase activity. (e) Total antioxidative activity. All of the experiments were repeated 3 times.

baicalin showed a strong total antioxidative capacity and exhibited dose-dependent effects (Figure 6(e)).

In conclusion, baicalin showed good scavenging activity on hydroxyl radicals, superoxide anions, and DPPH radicals and exerted the additional effect of inhibiting xanthine oxidase.

#### 4. Discussion

This study defined a therapeutic time window for the neuroprotective effects of baicalin in MCAO-induced ischemic rats. Furthermore, our results provided important insight into the antioxidative effects of baicalin *in vitro* and *in vivo*.

There is accumulating evidence that *Scutellaria baicalensis* Georgi containing various flavonoids including baicalin possess neuroprotective effects against cerebral ischemic injury [16, 36]. Previous studies reported that baicalin exhibited protective effects in a model of focal cerebral ischemia [12, 16]. However, its therapeutic time window was unclear. Abundant evidence has shown that a number of neuroprotective drugs, which had shown efficacy in ischemic animal

models in previous studies, have failed in clinical trials due to inadequate investigation of optimal therapeutic timewindows [18]. Therefore, it is necessary to explore therapeutic time windows for drug treatment in animal models as a part of the evaluation of the neuroprotective effects of these drugs during ischemia. In this study, we confirmed that baicalin significantly improved the neurological score and reduced the infarct volume. Furthermore, the therapeutic time window of baicalin was established at 4 h in MCAO injured rats. Time window is crucial in clinical ischemia treatment. Stroke patients generally get medicine treatment after certain time interval since ischemia onset; so, it is very important to have a certain time window for proposal medicine. For instance, time window of thrombolytic drugs is 3 to 6 h. With a longer time window, more chances will provide to protect patients. In this study, mice experiments showed that the time window of baicalin is 4 h, which indicates a promising clinical use.

Additional lines of evidence from various studies have converged to suggest that oxidative stress, characterized by an overproduction of ROS, contributes to ischemia-induced injury in the following ways: lipid peroxidation; protein

denaturation; inactivation of enzymes; nucleic acid and DNA damage, which results in the release of  $\text{Ca}^{2+}$  from intracellular stores; damage to the cytoskeletal structure; and chemotaxis [1, 2, 37]. To explore a possible mechanism of baicalin on the relief of oxidative stress induced by ischemia-reperfusion injury, we investigated the baicalin-dependent effects on ROS generation, MDA content, SOD activity, and NADPH oxidase activity in MCAO mouse brain tissue homogenates. Our results showed that baicalin significantly reduced ROS production and decreased MDA content, indicating that baicalin attenuates cerebral ischemia-reperfusion-induced oxidation injury *in vivo*.

An  $\text{H}_2\text{O}_2$ -induced primary neuronal injury *in vitro* model was employed to test the antioxidative and neuroprotective effects of baicalin. Cortical neurons are particularly vulnerable to  $\text{H}_2\text{O}_2$  injury because of their relatively low levels of antioxidant enzymes and dependence on mitochondrial respiration [38]. After incubation with  $300\ \mu\text{M}$   $\text{H}_2\text{O}_2$ , the neurons exhibited a decrease in SOD activity, an increase in LDH leakage, and 55% cell death. Treatment with baicalin significantly increased the cell viability, reduced the LDH leakage, and enhanced the SOD activity. These results indicate that baicalin exerts beneficial antioxidative effects.

ROS such as superoxide anions ( $\text{O}^{2-}$ ), peroxy nitrite, and hydroxyl radicals have significant cellular effects that lead to tissue destruction and cell death. These effects are a consequence of high concentrations of ROS that exceed the ability of antioxidant defense mechanisms to counterbalance the damaging effects. ROS are a natural by-product of oxygen metabolism.  $\text{O}^{2-}$  is the primary ROS, and it can transform into other ROS [37]. Moreover,  $\text{O}^{2-}$  is produced in tissues via a number of enzymatic reactions and common cellular sources, including mitochondrial respiration, xanthine oxidase, NADPH oxidase, and nitric oxidase synthetase (NOS) [39–42]. In our study, several tests were performed *in vitro* and *in vivo* to determine the antioxidative mechanisms of baicalin.

SOD can catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, which is important in antioxidant defense *in vivo* [32, 33]. To detect the antioxidative activity, it is important to assess this enzyme [43]. After the ischemia-reperfusion injury, the SOD activity in brain tissue was decreased. Baicalin treatment increased the SOD content, ameliorating the severity of ischemic stroke. The results obtained from the SOD detection in neuronal cultures were consistent with the tests performed *in vivo*. Both sets of results verified the upregulation effects observed with baicalin treatment on SOD activity. Recent evidence indicates that NADPH oxidase plays a key role in cerebral ischemia [34, 35], and some NADPH oxidase regulators have been previously shown to be cerebroprotective [44]. This study demonstrated that baicalin negatively regulates the NADPH oxidase activity that was increased during the cerebral ischemia-reperfusion injury.

To investigate baicalin-dependent effects in various signaling pathways, we measured the scavenging activities of hydroxyl radicals, superoxide anions, and DPPH radicals and the inhibition of xanthine oxidase. Our results showed that baicalin demonstrated beneficial effects on both direct free

radical scavenging activities and the inhibition of xanthine oxidase. We concluded that baicalin has beneficial antioxidative effects through a variety of mechanisms. Baicalin, a flavonoid antioxidant, plays multiple roles including the direct quenching of reactive oxygen species, the inhibition of enzymes involved in the production of the ROS (e.g., xanthine and NADPH oxidases), the increase of enzymatic activity involved in antioxidant defenses (e.g., SOD), (potentially) the chelation of low valent metal ions (i.e.,  $\text{Fe}^{2+}$  or  $\text{Cu}^{2+}$ ) [30, 45].

In summary, baicalin showed beneficial effects against MCAO-induced injury with a therapeutic time window of 4 h after occlusion. Its antioxidative effects both *in vitro* and *in vivo* might partly elucidate its mechanism of action. Further investigation is necessary to characterize the neuroprotective effects of baicalin, which may provide insight into novel therapeutic strategies for ischemia and other neuronal injuries.

## 5. Conclusion

Baicalin has neuroprotective effects in MCAO rats within a therapeutic time window of 4 h. Baicalin showed good antioxidative effects both *in vitro* and *in vivo*. Baicalin can play multiple roles including oxygen radical scavenging and regulation of enzymes.

## Abbreviations

MCAO:	Middle cerebral artery occlusion
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
MDA:	Malondialdehyde
NADPH:	Nicotinamide adenine dinucleotide 2'-phosphate.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Author's Contribution

Fafeng Cheng and Yi Lu equally contributed to this paper. Qingguo Wang obtained funding and supervised this study. Fafeng Cheng conducted the experiments, conceived and designed the study, and wrote the draft of the paper. Yi Lu conducted the animal experiments, conceived and designed this study, and participated in the writing of the paper. Xianggen Zhong and Xueqian Wang helped revise the paper and provided technical support. Wenting Song and Jianguo Qin prepared the neuronal cultures. Xiaoguang Sun and Shaoying Guo helped perform the animal experiments and index detections.

## Acknowledgments

This study was supported by the Science and Technology Major Projects for Major New Drugs (Qingkailing injection for treatment of ischemic stroke, no. 2009ZX09102-136)

and the Special Research Foundation of Young teachers of Beijing University of Chinese Medicine (Qingkailing's effect on endoplasmic reticulum stress based on Yi Bing Tong Zhi).

## References

- [1] F. M. Faraci, "Reactive oxygen species: influence on cerebral vascular tone," *Journal of Applied Physiology*, vol. 100, no. 2, pp. 739–743, 2006.
- [2] M. Lafon-Cazal, S. Pietri, M. Culcasi, and J. Bockaert, "NMDA-dependent superoxide production and neurotoxicity," *Nature*, vol. 364, no. 6437, pp. 535–537, 1993.
- [3] H. Ischiropoulos and J. S. Beckman, "Oxidative stress and nitration in neurodegeneration: cause, effect, or association?" *Journal of Clinical Investigation*, vol. 111, no. 2, pp. 163–169, 2003.
- [4] M. J. M. Ezoulin, J.-E. Ombetta, H. Dutertre-Catella, J.-M. War-net, and F. Massicot, "Antioxidative properties of galantamine on neuronal damage induced by hydrogen peroxide in SK-N-SH cells," *NeuroToxicology*, vol. 29, no. 2, pp. 270–277, 2008.
- [5] K. Hensley, M. L. Maidt, Z. Yu, H. Sang, W. R. Markesbery, and R. A. Floyd, "Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation," *Journal of Neuroscience*, vol. 18, no. 20, pp. 8126–8132, 1998.
- [6] Y. Lu, Q. Wang, M. F. Melzig, and K. Jenett-Siems, "Extracts of *Cynomorium songaricum* protect SK-N-SH human neuroblastoma cells against staurosporine-induced apoptosis potentially through their radical scavenging activity," *Phytotherapy Research*, vol. 23, no. 2, pp. 257–261, 2009.
- [7] S. H. Jung, K. D. Kang, D. Ji et al., "The flavonoid baicalin counteracts ischemic and oxidative insults to retinal cells and lipid peroxidation to brain membranes," *Neurochemistry International*, vol. 53, no. 6–8, pp. 325–337, 2008.
- [8] L.-L. Liu, L.-K. Gong, H. Wang et al., "Baicalin inhibits macrophage activation by lipopolysaccharide and protects mice from endotoxin shock," *Biochemical Pharmacology*, vol. 75, no. 4, pp. 914–922, 2008.
- [9] B.-Q. Li, T. Fu, Y.-D. Yan, N. W. Baylor, F. W. Ruscetti, and H.-F. Kung, "Inhibition of HIV infection by baicalin—a flavonoid compound purified from Chinese herbal medicine," *Cellular and Molecular Biology Research*, vol. 39, no. 2, pp. 119–124, 1993.
- [10] J.-M. Hwang, C.-J. Wang, F.-P. Chou et al., "Protective effect of baicalin on tert-butyl hydroperoxide-induced rat hepatotoxicity," *Archives of Toxicology*, vol. 79, no. 2, pp. 102–109, 2005.
- [11] M. Li-Weber, "New therapeutic aspects of flavones: the anti-cancer properties of Scutellaria and its main active constituents Wogonin, Baicalein and Baicalin," *Cancer Treatment Reviews*, vol. 35, no. 1, pp. 57–68, 2009.
- [12] X.-K. Tu, W.-Z. Yang, S.-S. Shi, C.-H. Wang, and C.-M. Chen, "Neuroprotective effect of baicalin in a rat model of permanent focal cerebral ischemia," *Neurochemical Research*, vol. 34, no. 9, pp. 1626–1634, 2009.
- [13] J. Xu, Y. Murakami, K. Matsumoto et al., "Protective effect of Oren-gedoku-to (Huang-Lian-Jie-Du-Tang) against impairment of learning and memory induced by transient cerebral ischemia in mice," *Journal of Ethnopharmacology*, vol. 73, no. 3, pp. 405–413, 2000.
- [14] L. Zhang, D. Xing, W. Wang, R. Wang, and L. Du, "Kinetic difference of baicalin in rat blood and cerebral nuclei after intravenous administration of *Scutellariae Radix* extract," *Journal of Ethnopharmacology*, vol. 103, no. 1, pp. 120–125, 2006.
- [15] M. Fisher, G. Feuerstein, D. W. Howells et al., "Update of the stroke therapy academic industry roundtable preclinical recommendations," *Stroke*, vol. 40, no. 6, pp. 2244–2250, 2009.
- [16] X. Xue, X.-J. Qu, Y. Yang et al., "Baicalin attenuates focal cerebral ischemic reperfusion injury through inhibition of nuclear factor  $\kappa$ B p65 activation," *Biochemical and Biophysical Research Communications*, vol. 403, no. 3–4, pp. 398–404, 2010.
- [17] Z.-J. Zhang, P. Li, Z. Wang et al., "A comparative study on the individual and combined effects of baicalin and jasmminoidin on focal cerebral ischemia-reperfusion injury," *Brain Research*, vol. 1123, no. 1, pp. 188–195, 2006.
- [18] F. Cheng, X. Zhong, Y. Lu et al., "Refined Qingkailing protects MCAO mice from endoplasmic reticulum stress-induced apoptosis with a broad time window," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 567872, 2012.
- [19] Q. Cai, H. W. Wang, S. Y. Hua, J. Z. Tan, T. Zhou, and C. S. Li, "Neuroprotective efficacy of sodium tanshinone B on hippocampus neuron in a rat model of focal cerebral ischemia," *Chinese Journal of Integrative Medicine*, vol. 18, no. 11, pp. 837–845.
- [20] J. H. Garcia, S. Wagner, K.-F. Liu, X.-J. Hu, and J. P. Mohr, "Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats: statistical validation," *Stroke*, vol. 26, no. 4, pp. 627–635, 1995.
- [21] V. P. Bindokas, J. Jordán, C. C. Lee, and R. J. Miller, "Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine," *Journal of Neuroscience*, vol. 16, no. 4, pp. 1324–1336, 1996.
- [22] H. Girouard, G. Wang, E. F. Gallo et al., "NMDA receptor activation increases free radical production through Nitric Oxide and NOX2," *Journal of Neuroscience*, vol. 29, no. 8, pp. 2545–2552, 2009.
- [23] H. Li, L.-L. Cai, J.-G. Liu et al., "Effect of early intervention with extract of Huannao Yicong Decoction on the pathologic picture of hippocampus and neurocyte apoptosis in APP transgenic mice model of dementia," *Chinese Journal of Integrative Medicine*, vol. 17, no. 6, pp. 430–435, 2011.
- [24] Q. Wang, A. Y. Sun, A. Simonyi et al., "Ethanol preconditioning protects against ischemia/reperfusion-induced brain damage: role of NADPH oxidase-derived ROS," *Free Radical Biology and Medicine*, vol. 43, no. 7, pp. 1048–1060, 2007.
- [25] A. T. Whaley-Connell, E. M. Morris, N. Rehmer et al., "Albumin activation of NAD(P)H oxidase activity is mediated via Rac1 in proximal tubule cells," *American Journal of Nephrology*, vol. 27, no. 1, pp. 15–23, 2007.
- [26] G. Lazzarino, B. Tavazzi, D. Di Pierro, R. Vagnozzi, M. Penco, and B. Giardina, "The relevance of malondialdehyde as a biochemical index of lipid peroxidation of postischemic tissues in the rat and human beings," *Biological Trace Element Research*, vol. 47, no. 1–3, pp. 165–170, 1995.
- [27] M.-H. Ka, E. H. Choi, H.-S. Chun, and K.-G. Lee, "Antioxidative activity of volatile extracts isolated from *Angelica tenuissima* roots, peppermint leaves, pine needles, and sweet flag leaves," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 10, pp. 4124–4129, 2005.
- [28] Z. Gongke, K. Yingzhen, C. Kairong, L. Zhixiao, and W. Yafu, "Hydroxyl radical scavenging activity of  $\beta$ -N-oxalyl-L- $\alpha$ ,  $\beta$ -diaminopropionic acid," *Phytochemistry*, vol. 58, no. 5, pp. 759–762, 2001.
- [29] S. Toda, M. Kumura, and M. Ohnishi, "Effects of phenol-carboxylic acids on superoxide anion and lipid peroxidation

- induced by superoxide anion," *Planta Medica*, vol. 57, no. 1, pp. 8–10, 1991.
- [30] N. Masuoka, T. Isobe, and I. Kubo, "Antioxidants from *Rabdosia japonica*," *Phytotherapy Research*, vol. 20, no. 3, pp. 206–213, 2006.
- [31] R. Li, W.-Q. Wang, H. Zhang et al., "Adiponectin improves endothelial function in hyperlipidemic rats by reducing oxidative/nitrative stress and differential regulation of eNOS/iNOS activity," *American Journal of Physiology*, vol. 293, no. 6, pp. E1703–E1708, 2007.
- [32] J. M. McCord and I. Fridovich, "Superoxide dismutase: the first twenty years (1968–1988)," *Free Radical Biology and Medicine*, vol. 5, no. 5–6, pp. 363–369, 1988.
- [33] H. Kinouchi, H. Kamii, S. Mikawa, C. J. Epstein, T. Yoshimoto, and P. H. Chan, "Role of superoxide dismutase in ischemic brain injury: a study using SOD-1 transgenic mice," *Cellular and Molecular Neurobiology*, vol. 18, no. 6, pp. 609–620, 1998.
- [34] R. H. Fabian, J. R. Perez-Polo, and T. A. Kent, "Perivascular nitric oxide and superoxide in neonatal cerebral hypoxia-ischemia," *American Journal of Physiology*, vol. 295, no. 4, pp. H1809–H1814, 2008.
- [35] A. M. Brennan, S. Won Suh, S. Joon Won et al., "NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation," *Nature Neuroscience*, vol. 12, no. 7, pp. 857–863, 2009.
- [36] Q. B. Zhou, C. Z. Duan, Q. Jia, P. Liu, and L. Y. Li, "Baicalin attenuates focal cerebral ischemic reperfusion injury by inhibition of protease-activated receptor-1 and apoptosis," *Chinese Journal of Integrative Medicine*, 2013.
- [37] K. K. Griendling, D. Sorescu, and M. Ushio-Fukai, "NAD(P)H oxidase: role in cardiovascular biology and disease," *Circulation Research*, vol. 86, no. 5, pp. 494–501, 2000.
- [38] E. R. Whittemore, D. T. Loo, and C. W. Cotman, "Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons," *NeuroReport*, vol. 5, no. 12, pp. 1485–1488, 1994.
- [39] I. Wiswedel, O. Ulbricht, and W. Augustin, "Studies of lipid peroxidation in isolated rat heart mitochondria," *Biomedica Biochimica Acta*, vol. 48, no. 2–3, pp. S73–S76, 1989.
- [40] T. Kober, I. König, M. Weber, and G. Kojda, "Diethyldithiocarbamate inhibits the catalytic activity of xanthine oxidase," *FEBS Letters*, vol. 551, no. 1–3, pp. 99–103, 2003.
- [41] P. J. Pagano, Y. Ito, K. Tornheim, P. M. Gallop, A. I. Tauber, and R. A. Cohen, "An NADPH oxidase superoxide-generating system in the rabbit aorta," *American Journal of Physiology*, vol. 268, no. 6, pp. H2274–H2280, 1995.
- [42] F. Cosentino, S. Patton, L. V. d'Uscio et al., "Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats," *The Journal of Clinical Investigation*, vol. 101, no. 7, pp. 1530–1537, 1998.
- [43] Y. Wang, X. Huang, Q. H. Liang et al., "A strategy for detecting absorbed bioactive compounds for quality control in the water extract of rhubarb by ultra performance liquid chromatography with photodiode array detector," *Chinese Journal of Integrative Medicine*, vol. 18, no. 9, pp. 690–698.
- [44] X.-L. Zhu, L.-Z. Xiong, Q. Wang et al., "Therapeutic time window and mechanism of tetramethylpyrazine on transient focal cerebral ischemia/reperfusion injury in rats," *Neuroscience Letters*, vol. 449, no. 1, pp. 24–27, 2009.
- [45] Z. Gao, K. Huang, X. Yang, and H. Xu, "Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi," *Biochimica et Biophysica Acta*, vol. 1472, no. 3, pp. 643–650, 1999.

## Review Article

# Chinese Herbal Medicine Qi Ju Di Huang Wan for the Treatment of Essential Hypertension: A Systematic Review of Randomized Controlled Trials

Jie Wang,<sup>1</sup> Xingjiang Xiong,<sup>1</sup> Guoyan Yang,<sup>2</sup> Yuqing Zhang,<sup>3</sup> Yongmei Liu,<sup>1</sup> Yun Zhang,<sup>1</sup> Zhenpeng Zhang,<sup>1</sup> Jun Li,<sup>1</sup> and Xiaochen Yang<sup>1</sup>

<sup>1</sup> Department of Cardiology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100053, China

<sup>2</sup> Centre for Evidence-Based Chinese Medicine, Beijing University of Chinese Medicine, Beijing 100029, China

<sup>3</sup> Department of Clinical Epidemiology and Biostatistics, McMaster University, ON, Canada L8S 4L8

Correspondence should be addressed to Xiaochen Yang; [avill1988@126.com](mailto:avill1988@126.com)

Received 18 January 2013; Revised 25 April 2013; Accepted 12 May 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Jie Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Background.** Chinese herbs are potentially effective for hypertension. Qi Ju Di Huang Wan (QJDHW) is a commonly used Chinese herbal medicine as a monotherapy or in combination with other antihypertensive agents for the treatment of essential hypertension (EH). However, there is no critically appraised evidence such as systematic reviews or meta-analyses on the effectiveness and safety of QJDHW for EH. **Methods and Findings.** CENTRAL, PubMed, CBM, CNKI, VIP, and online clinical trial registry websites were searched for published and unpublished randomized controlled trials (RCTs) of QJDHW for essential hypertension up to January 2013 with no language restrictions. A total of 10 randomized trials involving 1024 patients were included. Meta-analysis showed that QJDHW combined with antihypertensive drugs was more effective in lowering blood pressure and improving TCM syndrome for the treatment of essential hypertension than antihypertensive drugs used alone. No trials reported severe adverse events related to QJDHW. **Conclusions.** Our review suggests that QJDHW combined with antihypertensive drugs might be an effective treatment for lowering blood pressure and improving symptoms in patients with essential hypertension. However, the finding should be interpreted with caution because of the poor methodological quality of included trials. There is an urgent need for well-designed, long-term studies to assess the effectiveness of QJDHW in the treatment of essential hypertension.

## 1. Introduction

Hypertension is an increasingly prevalent chronic condition that is associated with serious morbidity and mortality. It is an important risk factor for the development and progression of cardiovascular disease, which is predicted that it will become the leading cause of death and disability worldwide by 2020 [1]. Hypertension is classified as either essential hypertension (EH) or secondary hypertension, and EH accounts for about 90–95% of the cases characterized by high blood pressure with no obvious underlying medical causes [2]. In developing countries, it is a major medical concern that the high rate of undetected and untreated EH [3]. In China, the prevalence of EH is currently 18.8% [4]. The high rates of EH, especially

undiagnosed EH, throughout the oriental countries double the risk of cardiovascular diseases, including coronary heart disease, congestive heart failure, ischemic and hemorrhagic stroke, renal failure, and peripheral arterial disease [5].

There is a growing tendency for people to turn to complementary and alternative medicine (CAM) [6, 7]. In addition, the increasing prevalence of hypertension creates a broad market for alternative therapy to aid the management of blood pressure [8]. Several CAM clinical studies, including a substantial number of randomized controlled trials (RCTs) and systematic reviews, have shown that CAM is effective and safe for the treatment of hypertension [9–12]. It can also improve appetite, intestinal motility, metabolism, and emotional factors such as stress. Furthermore, studies showed

that the application of CAM could enable tailored therapy in clinical practice, including lifestyle modification and individual choice of drugs lowering blood pressure [13, 14].

Qi Ju Di Huang Wan (QJDHW) decoction containing eight commonly used herbs (Chinese wolfberry fruit, chrysanthemum flower, common yam rhizome, tree peony bark, water plantain rhizome, *cornus*, and poria) had been used to relief symptoms like dizziness and vertigo for thousands of years in China. From the perspective of traditional Chinese medicine, it is believed that the mechanism of QJDHW may be related to calming the liver, suppressing liver yang hyperactivity, and nourishing kidney yin. Biochemically, QJDHW showed a good effect in decreasing the concentrations of angiotensin in plasma and myocardium, reducing the endothelin (ET) content and improving kidney blood stream in rats with essential hypertension [15–17].

Currently, it is common to see patients with essential hypertension seek QJDHW used alone or combined with antihypertensive agents as an alternative method. Recent studies also showed that QJDHW could help to control blood pressure [18, 19]. However, the evidence examining the effectiveness of QJDHW for essential hypertension has never been systematically summarized. Thus, we performed this systematic review to critically assess the effectiveness of QJDHW for the treatment of essential hypertension.

## 2. Methods

**2.1. Search Strategy.** We searched the following sources up to January 2013: the Cochrane library, including the Cochrane Central Register of Controlled Trials (CENTRAL, 2012), PubMed, Chinese bases, including Chinese Biomedical Literature Database (CBM), Chinese National Knowledge Infrastructure (CNKI), and Chinese Scientific Journal Database (VIP). In addition, we also searched the databases of clinical trials such as Current Controlled Trials (<http://www.controlled-trials.com/isrctn/>), the National Centre for Complementary and Alternative Medicine (NCCAM) at the National Institutes of Health (NIH) (<http://www.nccam.nih.gov/>), and the Complementary and Alternative Medicine Specialist Library at the NHS National Library for Health (<http://www.library.nhs.uk/cam/>). The searching terms were “Qi Ju Di Huang Wan”, “Yuan Fa Xing Gao Xue Ya (essential hypertension)”, and “Gao Xue Ya (hypertension)”. No language restriction was applied.

**2.2. Inclusion Criteria and Exclusion Criteria.** Our paper was restricted to RCTs that compared QJDHW or modified QJDHW, regardless of the preparation, with conventional antihypertensive drugs. RCTs comparing QJDHW combined antihypertensive drugs with antihypertensive drugs were also included. Quasi-RCTs were not considered. Animal studies, clinical trials including case report, case series traditional reviews were excluded. The main outcome measure was blood pressure (BP). The other outcome measures included the *Scale for TCM Syndrome and Symptom Differentiation* (TCM-SSD), the level of blood lipids (BL), plasma viscosity (PV),

angiotensin II (AngII), endothelin (ET), calcitonin gene-related peptide (CGRP), and safety. For TCM-SSD, the effect was presented as markedly effective, effective, and ineffective. Markedly effective was defined as the main symptoms such as headache, dizziness, palpitations, insomnia, tinnitus, and irritability disappeared or the TCM-SSD scores reduced rate  $\geq 70\%$ ; effective was defined as the main symptoms relieved or 70% > TCM-SSD scores reduced rate  $\geq 30\%$ ; ineffective was defined as the main symptoms that do not change or the TCM-SSD scores reduced rate  $< 30\%$ .

**2.3. Study Selection and Data Extraction.** The titles and abstracts of potentially relevant references were identified through the literature search and reviewed independently by 2 reviewers (G. Yang and Y. Zhang) according to pre-defined criteria. Discrepancies were resolved by consensus with another investigator (J. Wang). The following data were extracted: (1) citations (authors of study, year of publication), (2) methodological information, (3) participants information (sample size, age), (4) detailed information of interventions and controls, (5) outcome measures, and (6) adverse events.

**2.4. Trial Quality Assessment.** We assessed the methodological quality of included RCTs using Cochrane risk of bias tool. It has the following six domains: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome data (attrition bias), incomplete outcome data (attrition bias), and selective reporting (reporting bias). The judgment was given as “high risk”, “unclear risk”, or “low risk”: trials that met all the criteria were categorized as low risk of bias; those that met none of the criteria were categorized as high risk of bias; the others were categorized as an unclear risk of bias if insufficient information was available to make a judgment. Disagreements were resolved by discussion.

**2.5. Data Analysis.** The statistical package (RevMan 5.1.7) provided by Cochrane Collaboration was used for data analyses. Dichotomous data were expressed as risk ratio (RR) and continuous outcomes as weighted mean difference (WMD), with their 95% confidence intervals (CI), respectively. Meta-analysis was performed if the intervention, control, and outcome were the same or similar. The statistical heterogeneity was examined with the  $I^2$ -test, where  $I^2$  values of 50% or more were considered to be an indicator of a substantial heterogeneity. In the absence of significant heterogeneity, we pooled data using a fixed-effect model ( $I^2 < 50\%$ ); otherwise we used random effects model ( $I^2 > 50\%$ ) [20]. To maximize the similarities among studies that would be combined, and data were further stratified where possible into subgroups based on different types of interventions.

## 3. Result

**3.1. Description of Included Trials.** We identified 136 potentially relevant references from the electronic and manual searches. After screening the titles and abstracts, 94 studies

were excluded because of duplicated publication (55 studies), animal studies (4 studies), and noncontrolled clinical trials (35 studies) including case report, case series, and traditional review. Full texts of 42 papers were retrieved, and finally 10 RCTs [21–30] were included. A total of 5 RCTs [21–25] were included in meta-analysis. All the RCTs were conducted in China and published in Chinese. The search for ongoing registered trials identified no trials (Figure 1).

The characteristics of the 10 RCTs are summarized in Table 1. The total number of participants with essential hypertension was 1024. The age of participants varied from 32 to 80 years. Five different diagnostic criteria of hypertension were used in the included trials: one trial [21] used 1999 WHO-ISH guidelines for the management of hypertension (1999 WHO-ISH GMH), one trial [23] used Seventh Report of the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7), one trial [22] used China Guidelines on Prevention and Management of High Blood Pressure-2005 (CGPMHBP-2005), one trial [28] used CGPMHBP-2000, one trial [26] used 1978 WHO-ISH guidelines for the management of hypertension (1999 WHO-ISH GMH), and five trials [24, 25, 27, 29, 30] only demonstrated patients with essential hypertension. Of the 10 trials, three trials [21, 23, 26] reported TCM diagnostic criteria with yindeficiency and excessive yang syndrome and used Guidelines of Clinical Research of New Drugs of Traditional Chinese Medicine (GCRNDTCM); one trial [25] only demonstrated patients with yindeficiency and excessive yang syndrome in TCM, and six trials [22–24, 27–30] have not reported TCM diagnostic criteria.

The interventions included Qi Ju Di Huang Wan in preparation of (modified) decoction and pill. The compositions of different QJDHW preparations are presented in Table 2. Of the 10 trials, 4 trials [21, 26, 27, 30] investigated the preparation of “Qi Ju Di Huang Wan” used alone versus antihypertensive drugs, and the rest 6 trials [22–25, 28, 29] compared the preparation of “Qi Ju Di Huang Wan” plus antihypertensive drugs versus antihypertensive drugs. The treatment duration ranged from 4 to 24 weeks.

**3.2. Methodological Quality of Included Trials.** The methodological quality of the 10 trials was generally low. All of the 10 included trials mentioned the randomized allocation of participants, and only two trials [23, 27] stated the methods of sequence generation of random number table. However, insufficient information was provided to judge whether it was conducted properly or not. Among the 10 trials, allocation concealment and double blind were not mentioned. None of the trials reported a dropout or withdrawae, and none of the trials reported sample size calculation. Since the protocols of all the 10 included trials were not accessible, selective reporting was generally unclear. In addition, no trial reported a followup (Figures 2 and 3).

### 3.3. Effect of the Interventions

**3.3.1. “Qi Ju Di Huang Wan” versus Antihypertensive Drugs (Western Medicine).** Four trials [21, 26, 27, 30] compared

the preparation of “Qi Ju Di Huang Wan” used alone with antihypertensive drugs.

**Blood Pressure.** There was no significant difference between the two groups in systolic blood pressure (WMD: 0.10 [–3.38, 3.58];  $P = 0.96$ ) and diastolic blood pressure (WMD: –0.20 [–2.42, 2.02];  $P = 0.86$ ) after 8 weeks of treatment (see Tables 3 and 4).

**TCM-SSD Scores.** One trial [21] reported the TCM-SSD scores. The result showed a significant difference between QJDHW and antihypertensive drugs ( $P = 0.02$ ).

**3.3.2. “Qi Ju Di Huang Wan” Plus Antihypertensive Drugs versus Antihypertensive Drugs.** Six trials [22–25, 28, 29] compared the combination of modified QJDHW plus antihypertensive drugs with antihypertensive drugs.

**Blood Pressure.** Three trials [22–24] showed that there was a significant difference between treatment and control groups in systolic blood pressure (WMD: –5.52 [–8.96, –2.08];  $P = 0.002$ ) and diastolic blood pressure (WMD: –5.26 [–6.83, –3.70];  $P < 0.00001$ ). The forest plot was shown in the Figure 4.

**TCM-SSD Scores.** Two trials [23, 25] reported the TCM-SSD scores. The meta-analysis showed that the combination group had a beneficial effect on the improvement of TCM syndrome, compared to the antihypertensive drugs used alone (RR: 1.48 [1.20, 1.82];  $P = 0.00003$ ). We could not obtain more details of the TCM-SSD scores. So we just conducted an analysis of dichotomous data between groups (see Table 5).

**3.3.3. Other Outcomes (BL, PV, AngII, ET, and CGRP).** One trial [21] showed that after 8 weeks of treatment, the level of blood lipid (BL) and plasma viscosity (PV) decreased significantly ( $P < 0.05$ ) in QJDHW group compared to captopril group. One trial [23] showed that after 4 weeks of treatment, the level of angiotensin II (Ang II), endothelin (ET) decreased significantly ( $P < 0.01$ ) whereas the level of calcitonin gene-related peptide (CGRP) increased significantly ( $P < 0.05$ ) in QJDH pill plus felodipine (plendil) group compared to felodipine (plendil) group. Furthermore, one trial [25] showed that after 8 weeks of treatment, the level of blood lipid (BL) decreased significantly ( $P < 0.05$ ) in QJDH pill plus verapamil group compared to verapamil group.

**3.3.4. Sensitivity Analysis, Subgroup Analysis, and Publication Bias.** Due to no sufficient number of trials, we failed to conduct sensitivity analysis and subgroup analysis and also failed to perform funnel plot to detect publication bias.

**3.4. Adverse Effect.** Only one trial mentioned the adverse effect in captopril group such as dry cough [21]. Six trials [21–26] reported no side effect in the QJDHW group compared to antihypertensive drugs.

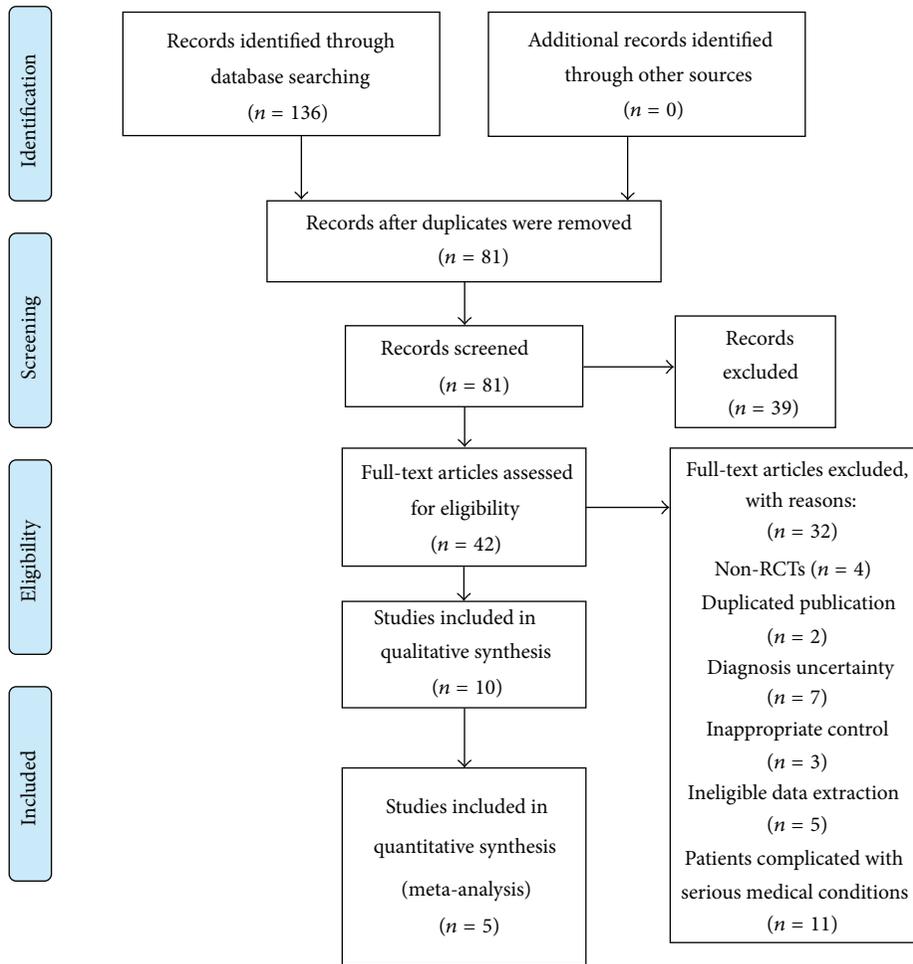


FIGURE 1: Flow Diagram of the literature searching and study selection.

	Zhu 2012	Zhou 2002	Zhong 2006	Yang and cheng 2005	Ou 2002	Liu 2008	Ling et al 2011	Li et al 2012	Du et al 2009	
Random sequence generation (selection bias)	+	+	-	-	?	?	+	?	+	
Allocation concealment (selection bias)	-	-	-	-	-	-	?	-	?	
Blinding of participants and personnel (performance bias)	?	?	?	?	?	?	?	?	?	
Blinding of outcome assessment (detection bias)	?	?	?	?	?	?	?	?	?	
Incomplete outcome data (attrition bias)	+	+	-	-	?	-	+	-	+	
Selective reporting (reporting bias)	-	+	?	+	-	?	+	-	?	
Other bias	-	+	-	?	-	?	+	?	+	

FIGURE 2: Risk of bias summary: review authors' judgments about each risk of bias item for each included study.

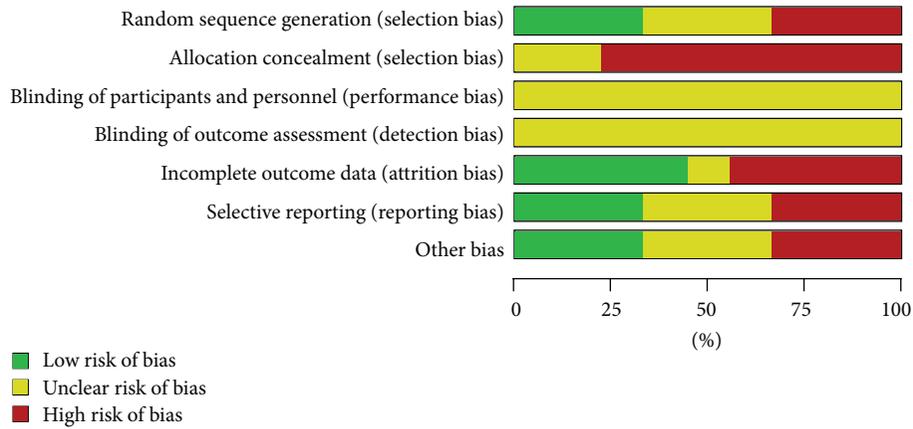
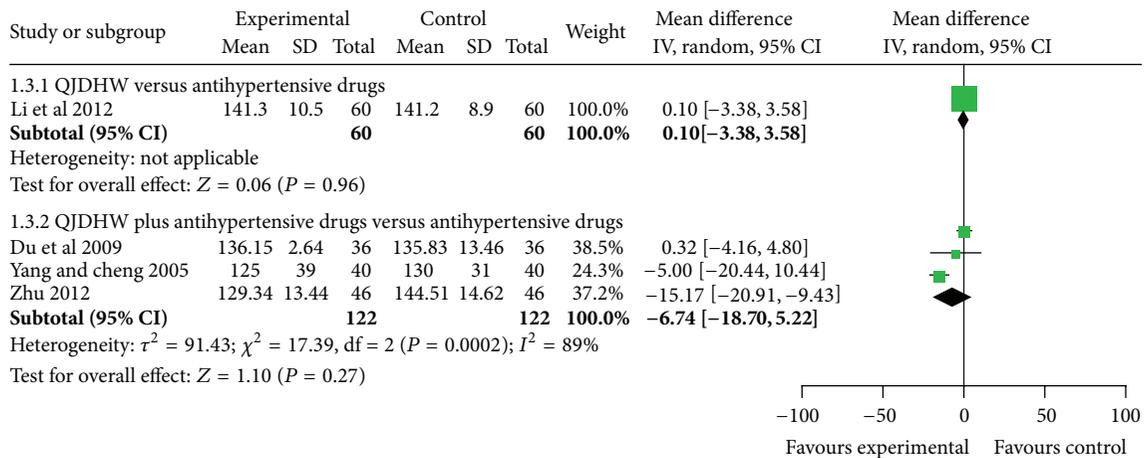
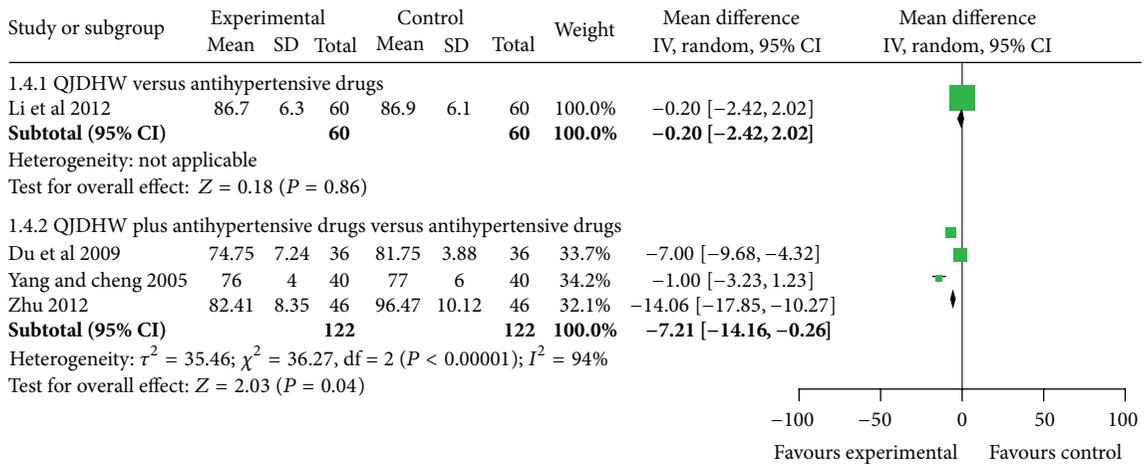


FIGURE 3: Risk of bias graph: review authors’ judgments about each risk of bias item presented as percentages across all included studies.



(a)



(b)

FIGURE 4: The forest plot of comparison of two groups for the outcome of blood pressure: (a) outcome of systolic blood pressure, (b) outcome of diastolic blood pressure.

TABLE 1: Characteristics and methodological quality of included studies.

Study ID	Sample	Diagnosis standard	Intervention	Control	Course (week)	Outcome measure
Li et al., 2012 [21]	120	1999 WHO-ISH GMH; GCRNDTCM	QJDHW	Captopril	8	BP; TCM-SSD; BL; PV; side effect
Zhu, 2012 [22]	92	CGPMHBP-2005	QJDH pill plus nifedipine controlled release tablet	Nifedipine controlled release tablet	8	BP
Du et al., 2009 [23]	120	JNC-7; GCRNDTCM	QJDH pill plus felodipine (plendil)	Felodipine (plendil)	4	BP; TCM-SSD; Ang II, ET, CGRP
Yang and Cheng, 2005 [24]	120	Hypertension diagnostic criteria (unclear)	modified QJDHW plus metoprolol	Metoprolol	8	BP
OuYang, 2002 [25]	102	Hypertension diagnostic criteria (unclear); TCM diagnostic criteria (unclear)	QJDH pill plus verapamil	Verapamil	8	BP; TCM-SSD; BL
Zhang, 1999 [26]	70	1999 WHO-ISH GMH; GCRNDTCM	QJDH pill	Nifedipine	8	BP
Ling, 2011 [27]	60	Hypertension diagnostic criteria (unclear)	QJDH pill	Captopril	4	BP
Liu, 2008 [28]	180	CGPMHBP-2000	QJDH pill plus Western drugs	Western drugs	24	BP
Zhong, 2006 [29]	80	Hypertension diagnostic criteria (unclear)	QJDH pill plus antihypertensive tablets	Antihypertensive tablets	4	BP
Zhou, 2002 [30]	80	Hypertension diagnostic criteria (unclear)	QJDH pill	Nifedipine controlled release tablet	4	BP

Abbreviations: QJDHW: Qi Ju Di Huang Wan; WHO-ISH GMH: WHO-ISH guidelines for the management of hypertension; GCRNDTCM: Guidelines of Clinical Research of New Drugs of Traditional Chinese Medicine; CGPMHBP: China Guidelines on Prevention and Management of High Blood Pressure; JNC-7: Seventh Report of the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure; TCM: traditional Chinese medicine; TCM-SSD: TCM syndrome and symptom differentiation; BP: blood pressure; BL: blood lipid; PV: plasma viscosity; Ang II: angiotensin II; ET: endothelin; CGRP: calcitonin gene-related peptide.

#### 4. Discussion

With the acceptance and popularity of CAM, the potential role of herbal remedies in global health care is being increasingly recognized in the recent years. Currently, more and more systematic reviews (SRs) and meta-analysis have been conducted to assess the efficiency of CAM for EH [31–39]. It has made great contributions to the health and well-being of the people for the unique advantages of CAM in preventing and curing diseases, rehabilitation, and health care. To the best of our knowledge, this is the first systematic review and meta-analysis of RCTs for QJDHW in treating essential hypertension. Our review suggests that QJDHW may be effective for the treatment of hypertension. Based on the findings of meta-analyses of blood pressure and TCM-SSD scores, the preparation of “Qi Ju Di Huang Wan” including pill and decoction used alone or combined with antihypertensive drugs may have some beneficial effects on patients with essential hypertension. This review has the following limitations. Firstly, 5 databases have been searched up to January 2013 including the Cochrane library, PubMed, CBM, CNKI, and VIP. In addition, the databases of clinical trials such as Current Controlled Trials, the National Centre for Complementary and Alternative Medicine have also been

searched without language restriction. However, all included trials were published in Chinese.

Secondly, the methodological quality of most included trials is generally low. Details of randomization were unclear. Concealment of allocation and blinding methods were not described, and reports of dropouts and withdrawals were incomplete. There were two trials (RCTs) [23, 27], stated randomization method through random number table. For the other five trials including Zhu 2012, Du et al. 2009, Yang and Cheng 2005, OuYang 2002, and Zhang 1999 [22–26], they only mentioned that “patients were randomized into two groups”, with no detailed information of randomization generation. All of the included trials did not describe the blinding, we could not judge whether there were performance bias and detection bias because of the awareness of the therapeutic interventions for the subjective outcome measures [40–47]. All the included trials used blood pressure as a primary outcome measure, but half of the included trials evaluated the effectiveness with numerical values. The rest of the trials presented the effect as markedly effective, effective, and ineffective. We have tried to contact authors to get further information either by telephone or email. Unfortunately, no replies and information was got. We recommend that future researchers should follow the basic guidelines for

TABLE 2: Composition of different QJDHW preparations.

Study ID	Preparation	Composition
Li et al., 2012 [21]	Decoction	Chrysanthemum flower 20 g, Chinese wolfberry fruit 15 g, prepared rehmannia root 20 g, <i>Cornus</i> 15 g, common yam rhizome 15 g, poria 15 g, water plantain rhizome 12 g, tree peony bark 10 g, danshen root 15 g, earth worm 20 g, red peony root 12 g, two-toothed achyranthes root 20 g, gambir plant 12 g, and common self-heal fruit spike 15 g. Severe dizziness and tinnitus plus dragon bones 20 g and oyster shell 20 g; vexing heat in the five centers and red tongue plus common anemarrhena rhizome 12 g and Chinese wolfberry root bark 12 g; amnesia and lumbago plus Tortoise plastron 15 g, <i>Eucommia</i> bark 12 g, Chinese taxillus herb 15 g, and deer antler glue 10 g (melted in decoction).
Zhu, 2012 [22]	pill	Chinese patent medicine
Du et al., 2009 [23]	pill	Chinese patent medicine
Yang and Cheng, 2005 [24]	Modified QJDHW	Chrysanthemum flower 20 g, Chinese wolfberry fruit 15 g, prepared rehmannia root 20 g, <i>Cornus</i> 15 g, common yam rhizome 15 g, poria 15 g, water plantain rhizome 12 g, and tree peony bark 10 g.
Ou Yang, 2002 [25]	pill	Chinese patent medicine
Zhang, 1999 [26]	pill	Chinese patent medicine
Ling, 2011 [27]	pill	Chinese patent medicine
Liu, 2008 [28]	pill	Chinese patent medicine
Zhong, 2006 [29]	pill	Chinese patent medicine
Zhou, 2002 [30]	pill	Chinese patent medicine

Abbreviations: QJDHW: Qi Ju Di Huang Wan.

TABLE 3: Analyses of systolic blood pressure.

Trials	WMD [95% CI]	P value
<i>QJDHW versus antihypertensive drugs</i>		
QJDHW versus captopril	1 0.10 [-3.38, 3.58]	0.96
<i>Meta-Analysis</i>	1 0.10 [-3.38, 3.58]	0.96
<i>QJDHW plus antihypertensive drugs versus antihypertensive drugs</i>		
QJDH pill plus nifedipine controlled release tablet versus nifedipine controlled release tablet	1 -15.17 [-20.91, -9.43]	<0.00001
QJDH pill plus felodipine (plendil) versus felodipine (plendil)	1 0.32 [-4.16, 4.80]	0.89
Modified QJDHW plus metoprolol versus metoprolol	1 -5.00 [-20.44, 10.44]	0.53
<i>Meta-Analysis</i>	3 -5.52 [-8.96, -2.08]	0.002

Abbreviations: QJDHW: Qi Ju Di Huang Wan.

TABLE 4: Analyses of diastolic blood pressure.

Trials	WMD [95% CI]	P value
<i>QJDHW versus antihypertensive drugs</i>		
QJDHW versus captopril	1 -0.20 [-2.42, 2.02]	0.86
<i>Meta-Analysis</i>	1 -0.20 [-2.42, 2.02]	0.86
<i>QJDHW plus antihypertensive drugs versus antihypertensive drugs</i>		
QJDH pill plus nifedipine controlled release tablet versus nifedipine controlled release tablet	1 -14.06 [-17.85, -10.27]	<0.00001
QJDH pill plus felodipine (plendil) versus felodipine (plendil)	1 -7.00 [-9.68, -4.32]	<0.00001
Modified QJDHW plus metoprolol versus metoprolol	1 -1.00 [-3.23, 1.23]	0.38
<i>Meta-Analysis</i>	3 -5.26 [-6.83, -3.70]	<0.00001

Abbreviations: QJDHW: Qi Ju Di Huang Wan.

TABLE 5: Analyses of TCM-SSD Scores.

Trials	Intervention (n/N)	Control (n/N)	RR [95% CI]	P value	
<i>QJDHW versus antihypertensive drugs</i>					
QJDHW versus captopril	1	56/60	47/60	1.19 [1.03, 1.38]	0.02
<i>Meta-Analysis</i>	1	56/60	47/60	1.19 [1.03, 1.38]	0.02
<i>QJDHW plus antihypertensive drugs versus antihypertensive drugs</i>					
QJDH pill plus felodipine (plendil) versus felodipine (plendil)	1	31/36	24/36	1.29 [0.99, 1.8]	0.06
QJDH pill plus verapamil versus verapamil	1	31/34	18/34	1.72 [1.23, 2.40]	0.001
<i>Meta-Analysis</i>	2	62/70	42/70	1.48 [1.20, 1.82]	0.0003

Abbreviations: QJDHW: Qi Ju Di Huang Wan.

reporting clinical trials such as the Consolidated Standards of Reporting Trials (CONSORT) statement.

Thirdly, the treatment duration of most included studies was short, varying from 4 to 8 weeks. Only one trial has a long duration of 24 weeks. Since hypertension is a chronic condition, it is a great concern of patients about the effect of long-term treatment. Indeed, none of the included trials reported the mortality rate or the incidence of complications. Moreover, hypertension may exacerbate with or without treatment, especially in China; a major risk factor for hypertension is unbalanced intake of dietary sodium and potassium. Studies have indicated that high dietary sodium intake may change the circadian rhythm of 24-hour blood pressure, which is characterized by a higher nighttime blood pressure. The prevalence of isolated nighttime hypertension, which is defined as a nighttime systolic/diastolic blood pressure more than 120/70 mm Hg and a daytime systolic/diastolic blood pressure less than 135/85 mm Hg, is higher in Chinese than in Europeans [48, 49]. Therefore, a longer follow-up period with serial measurements of outcomes is suggested to determine the long-term effectiveness of QJDHW prescriptions. Thus, RCTs of QJDHW prescriptions with design to measure the followup of outcomes are urgently needed.

Fourthly, among the included trials, there is inadequate reporting on adverse events. None of the five trials reported information on the adverse effect of QJDHW decoctions or pills. Due to the limited information of adverse events, we could not draw definite conclusions on the safety of QJDHW prescriptions. Though most of Chinese herbal medicines and Chinese patent medicines are widely accepted and safely used, the increasing reports of liver and kidney toxicity and other adverse events related to Chinese medicines [50–59] draw much attention to the concern of safety. We recommend that future clinical trials should use QJDHW prescriptions with caution and report adverse events appropriately.

Fifthly, there may be publication bias in this review. We doubted whether all the RCTs have positive effect of QJDHW prescriptions when analyzed by standard statistical techniques using risk ratios or mean differences. For this, extensive searches for unpublished material have been conducted, but no unpublished “negative” studies were found.

In general, comparing to three categories (calcium antagonist, beta blocker, and angiotensin-converting enzyme inhibitors) of antihypertensive drugs such as captopril, nifedipine, felodipine (plendil), metoprolol, and verapamil, the

preparation of QJDHW appears to lower blood pressure and improve the symptoms with less adverse event. The combination of QJDHW and antihypertensive drugs may have significant effectiveness compared to antihypertensive drugs used alone. However, the quality of RCTs included in our review was limited for us to draw definite conclusions about QJDHW. More rigorous RCTs are required to be performed to prove the effectiveness of QJDHW for treating hypertension.

## 5. Conclusions

Our review indicates that QJDHW for treating essential hypertension has some beneficial effects compared to antihypertensive drugs, although the results are of limited value due to the clinical heterogeneity and low methodological quality of the included studies which prevent us from drawing a definitive conclusion for the effectiveness of QJDHW. However, QJDHW preparations are relatively safe, as a traditional Chinese medical therapy for improving symptoms of essential hypertension. Nevertheless, questions that cannot be conclusively answered at present include whether QJDHW prescriptions should be widely recommended and what the most effective preparation of QJDHW is. More well-designed, long-term clinical trials are needed.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Authors' Contribution

Xiaochen Yang, Xingjiang Xiong, and Guoyan Yang contributed equally to this paper.

## Acknowledgments

The current work was partially supported by the National Basic Research Program of China (973 Program, no. 2003CB517103) and the National Natural Science Foundation Project of China (no. 90209011). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the paper.

## References

- [1] World Health Organization, 2008–2013 action plan for the global strategy for the prevention and control of noncommunicable diseases, 2008, <http://www.who.int/nmh/Actionplan-PC-NCD-2008.pdf>.
- [2] O. A. Carretero and S. Oparil, “Essential hypertension. Part I: definition and etiology,” *Circulation*, vol. 101, no. 3, pp. 329–335, 2000.
- [3] D. O. Abegunde, C. D. Mathers, T. Adam, M. Ortegón, and K. Strong, “The burden and costs of chronic diseases in low-income and middle-income countries,” *Lancet*, vol. 370, no. 9603, pp. 1929–1938, 2007.
- [4] J. G. Wang and Y. Li, “Characteristics of hypertension in the Chinese population,” *Current Hypertension Reports*, vol. 14, no. 5, pp. 410–415, 2012.
- [5] S. Stewart and K. Sliwa, “Preventing CVD in resource-poor areas: perspectives from the ‘real-world’,” *Nature Reviews Cardiology*, vol. 6, pp. 489–492, 2009.
- [6] E. Ernst, “Prevalence of use of complementary/alternative medicine: a systematic review,” *Bulletin of the World Health Organization*, vol. 78, no. 2, pp. 252–257, 2000.
- [7] K. J. Chen, K. K. Hui, M. S. Lee, and H. Xu, “The potential benefit of complementary/alternative medicine in cardiovascular diseases,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 125029, 1 page, 2012.
- [8] E. Ernst, K. L. Resch, S. Mills et al., “Complementary medicine—a definition,” *British Journal of General Practice*, vol. 45, pp. 445–506, 1995.
- [9] M. S. Lee, T. Y. Choi, B. C. Shin et al., “Cupping for hypertension: a systematic review,” *Clinical and Experimental Hypertension*, vol. 32, no. 7, pp. 423–425, 2010.
- [10] M. H. Hur, M. S. Lee, C. Kim, and E. Ernst, “Aromatherapy for treatment of hypertension: a systematic review,” *Journal of Evaluation in Clinical Practice*, vol. 18, no. 1, pp. 37–41, 2012.
- [11] J. I. Kim, J. Y. Choi, H. Lee et al., “Moxibustion for hypertension: a systematic review,” *BMC Cardiovasc Disord*, vol. 10, article 33, 2010.
- [12] M. S. Lee, E. N. Lee, J. I. Kim, and E. Ernst, “Tai chi for lowering resting blood pressure in the elderly: a systematic review,” *Journal of Evaluation in Clinical Practice*, vol. 16, no. 4, pp. 818–824, 2010.
- [13] J. Wang, P. Q. Wang, and X. J. Xiong, “Current situation and re-understanding of syndrome and formula syndrome in Chinese medicine,” *Internal Medicine*, vol. 2, no. 3, article 113, 2012.
- [14] H. Xu and K. Chen, “Integrative medicine: the experience from China,” *Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 3–7, 2008.
- [15] Z. J. Yue, Z. D. Zou, D. H. Li et al., “Effect of reinforcing kidney on blood pressure and kidney blood stream in SHR,” *Zhong Guo Shi Yan Fang Ji Xue Za Zhi*, vol. 15, no. 1, pp. 42–44, 2009.
- [16] F. F. Duo, Z. D. Zou, W. J. Wang et al., “Effect of Qi Ju Di Huang Wan on injured vascular endothelial cell ultrastructure induced by Ang II,” *Zhong Guo Yi Yao Dao Kan*, vol. 10, 2010.
- [17] Z. J. Yue, Z. D. Zou, D. H. Li et al., “Effect of reinforcing kidney on blood pressure and kidney function in SHR,” *Zhong Guo Shi Yan Fang Ji Xue Za Zhi*, vol. 15, no. 8, pp. 63–65, 2009.
- [18] J. Wang and X. J. Xiong, “Outcome measures of Chinese herbal medicine for hypertension: an overview of systematic reviews,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 697237, 7 pages, 2012.
- [19] X. J. Xiong, X. C. Yang, Y. M. Liu et al., “Chinese herbal formulas for treating hypertension in traditional Chinese medicine: perspective of modern science,” *Hypertension Research*, 2013.
- [20] J. P. T. Higgins and S. Green, *Corchrane Reviewers’ Handbook 5.1.0*, Version 5.1.0, Review Manager (RevMan) [Computer program], 2011.
- [21] W. Li, H. G. Chen, and W. You, “Clinical effect of Qi Ju Di Huang decoction on 60 patients with essential hypertension,” *Zhong Guo Min Jian Liao Fa Za Zhi*, vol. 20, no. 2, pp. 37–38, 2012.
- [22] C. Q. Zhu, “The effect of Qi Ju Di Huang Wan on elderly essential hypertension combined with nifedipine controlled release tablet,” *Yao Wu Yu Lin Chuang Za Zhi*, vol. 2, no. 2, pp. 119–121, 2012.
- [23] B. J. Du, Z. F. Huang, and X. C. Kong, “Effects of Qi Ju Di Huang Wan combined with felodipine on angiotensin, endothelin and CGRP on patients with essential hypertension,” *Zhong Yi Yao Xin Xi Za Zhi*, vol. 26, no. 4, pp. 53–54, 2009.
- [24] S. Yang and Y. P. Cheng, “The effect of Qi Ju Di Huang Wan on essential hypertension combined with metoprolol,” *Lin Chuang Hui Cui Za Zhi*, vol. 20, no. 17, pp. 1005–1006, 2005.
- [25] Y. P. OuYang, “Clinical effect of Qi Ju Di Huang Wan combined with verapamil on 34 patients with elderly essential hypertension,” *Hu Nan Zhong Yi Yao Dao Bao*, vol. 8, no. 1, pp. 15–16, 2002.
- [26] Q. Z. Zhang, “Clinical effect of Qi Ju Di Huang Wan on patients with deficiency of yin of essential hypertension,” *Shaanxi Zhong Yi Yao*, vol. 7, pp. 987–990, 1999.
- [27] C. D. Ling, “Clinical effect of Qi Ju Di Huang Wan combined with Ni Fuda on 30 patients with essential hypertension,” *Shi Yong Zhong Yi Yao*, vol. 7, pp. 325–329, 2011.
- [28] Y. L. Liu, “The impact of Chinese medicine against male sexual dysfunction due to hypertension,” *He Nan Zhong Yi Yao Xue Bao*, vol. 16, pp. 1256–1259, 2008.
- [29] Y. Zhong, “Clinical effect of Qi Ju Di Huang Wan on 50 patients with essential hypertension,” *Zhong Yi Za Zhi*, vol. 17, pp. 856–860, 2006.
- [30] Y. F. Zhou, “Clinical effect of Qi Ju Di Huang Wan on 80 patients with deficiency of yin of essential hypertension,” *Shaanxi Zhong Yi Yao*, vol. 8, pp. 532–539, 2002.
- [31] J. Wang, B. Feng, X. C. Yang et al., “Tianma gouteng yin as adjunctive treatment for essential hypertension: a systematic review of randomized controlled trials,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 706125, 18 pages, 2013.
- [32] X. J. Xiong, X. C. Yang, B. Feng et al., “Zhen gan xi feng decoction, a traditional Chinese herbal formula, for the treatment of essential hypertension: a systematic review of randomized controlled trials,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 982380, 9 pages, 2013.
- [33] J. Wang, X. C. Yang, B. Feng et al., “Is Yangxue Qingnao Granule combined with antihypertensive drugs, a new integrative medicine therapy, more effective than antihypertensive therapy alone in treating essential hypertension?” *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 540613, 8 pages, 2013.
- [34] X. J. Xiong, X. C. Yang, W. Liu et al., “Banxia baizhu tianma decoction for essential hypertension: a systematic review of randomized controlled trials,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 271462, 10 pages, 2012.

- [35] J. Wang, K. W. Yao, X. C. Yang et al., "Chinese patent medicine liu wei di huang wan combined with antihypertensive drugs, a new integrative medicine therapy, for the treatment of essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 714805, 7 pages, 2012.
- [36] J. Wang and X. J. Xiong, "Current situation and perspectives of clinical study in integrative medicine in China," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.
- [37] A. Ferreira, "Integrative medicine for hypertension: the earlier the better for treating who and what are not yet ill," *Hypertension Research*, 2013.
- [38] M. S. Lee, H. J. Lim, M. S. Lee, and H. S. Jang, "Perceptions, knowledge and misuse of an oriental herbal drug: a survey of 608 Korean female nursing college students," *Complementary Therapies in Clinical Practice*, vol. 11, no. 3, pp. 200–204, 2005.
- [39] M. S. Ju, S. Lee, I. Bae et al., "Effects of aroma massage on home blood pressure, ambulatory blood pressure, and sleep quality in middle-aged women with hypertension," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 403251, 7 pages, 2013.
- [40] K. F. Schulz, L. Chalmers, R. J. Hayes, and D. G. Altman, "Empirical evidence of bias: dimensions of methodological quality associated with estimates of treatment effects in controlled trials," *Journal of the American Medical Association*, vol. 273, no. 5, pp. 408–412, 1995.
- [41] J. Hu, J. Zhang, W. Zhao, Y. Zhang, L. Zhang, and H. Shang, "Cochrane systematic reviews of Chinese herbal medicines: an overview," *PLoS ONE*, vol. 6, no. 12, Article ID e28696, 2011.
- [42] L. Liu, "The clinical trial barriers," *Nature*, vol. 480, no. 7378, article S100, 2011.
- [43] Y. Tu, "The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine," *Nature Medicine*, vol. 17, no. 10, pp. 1217–1220, 2011.
- [44] W. Chen, C. E. D. Lim, H. J. Kang, and J. Liu, "Chinese herbal medicines for the treatment of type A H1N1 influenza: a systematic review of randomized controlled trials," *PLoS ONE*, vol. 6, no. 12, Article ID e28093, 2011.
- [45] M. Y. Liu and K. J. Chen, "Convergence: the tradition and the modern," *Chinese Journal of Integrative Medicine*, vol. 18, no. 3, pp. 164–165, 2012.
- [46] Z. Junhua, S. Hongcai, G. Xiumei et al., "Methodology and reporting quality of systematic review/meta-analysis of traditional Chinese medicine," *Journal of Alternative and Complementary Medicine*, vol. 13, no. 8, pp. 797–805, 2007.
- [47] J. Wang and X. J. Xiong, "Control strategy on hypertension in Chinese medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 284847, 6 pages, 2012.
- [48] R. C. Hermida, M. H. Smolensky, D. E. Ayala et al., "2013 ambulatory blood pressure monitoring recommendations for the diagnosis of adult hypertension, assessment of cardiovascular and other hypertension-associated risk, and attainment of therapeutic goals," *Chronobiology International*, vol. 30, no. 3, pp. 355–410, 2013.
- [49] B. Wizner, D. G. Dechering, L. Thijs et al., "Short-term and long-term repeatability of the morning blood pressure in older patients with isolated systolic hypertension," *Journal of Hypertension*, vol. 26, no. 7, pp. 1328–1335, 2008.
- [50] X. J. Xiong, F. Y. Chu, H. X. Li, and Q. Y. He, "Clinical application of the TCM classic formulae for treating chronic bronchitis," *Journal of Traditional Chinese Medicine*, vol. 31, no. 1, pp. 69–72, 2011.
- [51] J. Wang, R. van der Heijden, S. Spruit et al., "Quality and safety of Chinese herbal medicines guided by a systems biology perspective," *Journal of Ethnopharmacology*, vol. 126, no. 1, pp. 31–41, 2009.
- [52] K. Chan, "Some aspects of toxic contaminants in herbal medicines," *Chemosphere*, vol. 52, no. 9, pp. 1361–1371, 2003.
- [53] D. Melchart, K. Linde, S. Hager, D. Shaw, and R. Bauer, "Liver enzyme elevations in patients treated with traditional Chinese medicine," *Journal of the American Medical Association*, vol. 282, no. 1, pp. 28–29, 1999.
- [54] L. G. Miller, "Herbal medicinals: selected clinical considerations focusing on known or potential drug-herb interactions," *Archives of Internal Medicine*, vol. 158, no. 20, pp. 2200–2211, 1998.
- [55] A. Tachjian, V. Maria, and A. Jahangir, "Use of herbal products and potential interactions in patients with cardiovascular diseases," *Journal of the American College of Cardiology*, vol. 55, no. 6, pp. 515–525, 2010.
- [56] Z. Y. Shen and X. Chen, "Analysis on 99 cases of adverse reactions of Chinese patent drugs," *African Journal of Microbiology Research*, vol. 6, no. 8, pp. 1742–1746, 2012.
- [57] H. Xu and K. J. Chen, "Herb-drug interaction: an emerging issue of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 3, pp. 195–196, 2010.
- [58] P. Windrum, D. R. Hull, and T. C. M. Morris, "Herb-drug interactions," *Lancet*, vol. 355, no. 9208, pp. 1019–1020, 2000.
- [59] A. Fugh-Berman, "Herb-drug interactions," *Lancet*, vol. 355, no. 9198, pp. 134–138, 2000.

## Research Article

# Effective Components of *Panax quinquefolius* and *Corydalis tuber* Protect Myocardium through Attenuating Oxidative Stress and Endoplasmic Reticulum Stress

Mei Xue,<sup>1</sup> Meilin Liu,<sup>2</sup> Xinyuan Zhu,<sup>2</sup> Lin Yang,<sup>1</sup> Yu Miao,<sup>1</sup> Dazhuo Shi,<sup>1</sup> and Huijun Yin<sup>1</sup>

<sup>1</sup> Cardiovascular Center, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>2</sup> Department of Geriatric, Peking University First Hospital, Beijing 100034, China

Correspondence should be addressed to Huijun Yin; [huijunyin@yahoo.com.cn](mailto:huijunyin@yahoo.com.cn)

Received 8 March 2013; Revised 3 June 2013; Accepted 10 June 2013

Academic Editor: Hao Xu

Copyright © 2013 Mei Xue et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Both oxidative stress and endoplasmic reticulum stress (ERS) have been implicated in carcinogenesis and neurological diseases, while there are few reports about the mechanisms of them in the progression of acute myocardial infarction (AMI). This study examined oxidative stress and ERS in a rat model of AMI and evaluated their role in therapy by metoprolol and effective components of *Panax quinquefolius* and *Corydalis tuber* (EPC). In the present study a rat model of AMI was established by ligation of the left anterior descending coronary artery. After oral administration of metoprolol or low-to-high doses of EPC for 2 weeks, serum malondialdehyde (MDA), superoxide dismutase (SOD), and 8-iso-prostaglandin F<sub>2</sub>α (8-iso-PGF<sub>2</sub>α) were detected using enzyme-linked immunosorbent assay (ELISA). Quantitative real-time PCR and Western blotting were used to examine mRNA and protein expressions of the hallmarks of ERS-glucose-regulated protein-78 (GRP78) and CCAAT/enhancer-binding protein homologous protein (CHOP). We confirmed that both metoprolol and moderate-to-high dose of EPC decreased 8-iso-PGF<sub>2</sub>α serum level and downregulated the mRNA and protein expressions of GRP78 and CHOP in myocardium, while EPC also increased SOD serum level. These results indicated that metoprolol and EPC protect the myocardium by attenuating oxidative stress and ERS induced by myocardial infarction, highlighting the ERS pathways as potential therapeutic targets for AMI.

## 1. Introduction

Acute myocardial infarction (AMI) is a severe stress condition that causes extensive biochemical changes, which is associated with increasing production of reactive oxygen species (ROS) [1]. The imbalance between ROS production and antioxidant defenses leads to the condition known as oxidative stress. Detrimental effects of ROS are clearly demonstrated by the findings that in transgenic mice in which an antioxidant protein, superoxide dismutase (SOD), is overexpressed, infarct size is markedly reduced [2, 3]. There is a growing body of evidence which indicates that oxidative stress plays an important role in the initiation and progression of myocardial infarction (MI) [4–7].

The endoplasmic reticulum (ER) is a multifunctional intracellular organelle responsible for the synthesis and folding of proteins as well as calcium storage and signaling.

Various stimuli, such as ischemia, hypoxia, oxidative stress, and inflammatory factors, have been suggested to triggering ER dysfunction, which are designated as ER stress (ERS) [8, 9]. Cells alleviate ERS through the unfolded protein response (UPR). The upregulation of ER chaperones, such as the glucose-regulated protein-78 (GRP78), contributes to the repair of unfolded proteins. However, if stress is sustained, the UPR causes cell death by transcriptional induction of CCAAT/enhancer-binding protein homologous protein (CHOP), the caspase-12 dependent pathway, and activation of the c-Jun NH<sub>2</sub>-terminal kinase 1 (JNK1) dependent pathway [10]. Recently, Mitra et al. [11] reported that GRP78, as an ER-resident protein, assisting in protein folding and the most important upstream regulator of the UPR, was exclusively upregulated during MI. Exclusive upregulation of CHOP in MI hearts and nuclear translocation of CHOP in the hypoxic cardiomyocytes signifies induction of ERS-mediated

apoptosis (Figure 1) [11]. Further, some data suggest that oxidative stress and ERS reinforce each other in thymic lymphomagenesis and sporadic amyotrophic lateral sclerosis [12–14], while there are very few reports about the mechanisms of them in the progression of MI.

The extracts of *Panax quinquefolius* and *Corydalis tuber* (EPC), composed of *Panax quinquefolius* saponins and tetrahydropalmatine mainly, showed good effects for the treatment of ischemic cardiovascular diseases in clinic. *Panax quinquefolius* saponins and tetrahydropalmatine have been shown to have protective effects against oxidative stress [15–17]. Recent study demonstrated that *Panax quinquefolius* saponins can also reduce myocardial hypoxia-reoxygenation injury by inhibiting excessive ERS [18]. So we hypothesized that oxidative stress and ERS play important roles in the pathogenesis of MI. And this study was therefore undertaken to investigate whether EPC can protect myocardium against MI by suppressing oxidative stress and excessive ERS, the key proteins—GRP78 and CHOP.

## 2. Materials and Methods

**2.1. EPC Preparation.** EPC was provided by Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. The main components were shown in Table 1, measured by high performance liquid chromatogram (HPLC) method.

**2.2. Animals and Experimental Protocol.** A total of 100 male Wistar rats weighing  $180 \pm 20$  g were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Certificate no. SCXK Beijing 2005-0013). The protocol was approved by the animal care and ethics committee of the China Academy of Chinese Medical Sciences. Sham group comprised 10 randomly selected rats, and the remainder was randomly divided into 5 groups, namely, control group, metoprolol group, low-dose EPC group, moderate-dose EPC group, and high-dose EPC group, with 18 rats in each group. The left anterior descending (LAD) coronary artery was ligated in the 5 groups to establish MI model according to Olivetti's methods as described before [19, 20]. The rats were anesthetized by intraperitoneal injection of urethane solution (20%) at a dose of 0.6 mL/kg. The rats in sham group did not undergo ligation. Of the surviving rats, metoprolol (AstraZeneca Pharmaceutical Co., Ltd., batch no.: 1012055), EPC were administered to metoprolol group (9 mg/kg), low-dose EPC group (0.54 g/kg), moderate-dose EPC group (1.08 g/kg), and high-dose EPC group (2.16 g/kg) by gastrogavage, respectively, once every 24 h for two weeks, and an equal volume of normal saline was given to sham group and control group [21]. One hour after the last administration, the blood samples were collected from the abdominal aorta of rats and kept in a red tube biochemical procoagulant at room temperature for 60 min. The serum was separated by low-speed centrifugation and then was stored at  $-80^{\circ}\text{C}$  for use. The myocardial tissues below the ligation were stored in liquid nitrogen for Western blotting analysis.

**2.3. Enzyme-Linked Immunosorbent Assay.** The serum levels of malondialdehyde (MDA), SOD, and 8-iso-prostaglandin

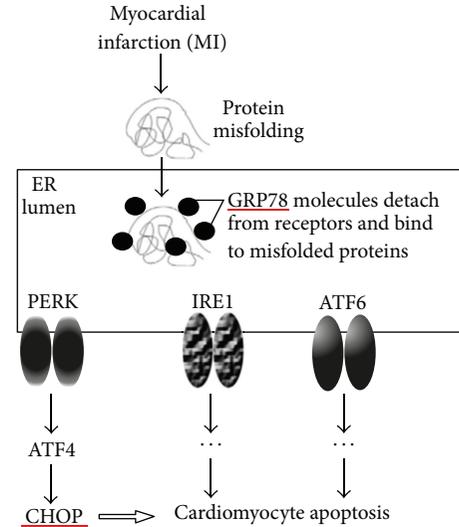


FIGURE 1: ERS during MI [11].

TABLE 1: Quality evaluation of EPC.

Major constituent	Content (%)
Ginsenoside Rg1	0.11
Ginsenoside Re	1.88
Ginsenoside Rb1	5.30
Tetrahydropalmatine	0.07

F2 $\alpha$  (8-iso-PGF2 $\alpha$ ) were detected using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The ELISA kits were provided by Sino-American Biotechnology Co., Ltd. (Wuhan, China). A Multiskan type 3 microplate reader (Thermo Scientific) was used for detection.

**2.4. Quantitative Real-Time Polymerase Chain Reaction (PCR).** Total mRNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The mRNA was reverse transcribed to cDNA using M-MLV reverse transcriptase PCR Kit (TaKaRa). The primer sets for GRP78 (forward 5'-CCTGGTTCTGCTTGATGTGT-3' and reverse 5'-TCGTTACCTTCGTAGACCTT-3'), CHOP (forward 5'-CCAGGAAACGAAGAGGAAGA-3' and reverse 5'-GGT-GCTTGTGACCTCTGCT-3'), and glyceraldehydes phosphate dehydrogenase (GAPDH) (forward 5'-CAACTCCCT-CAAGATTGTCAGCAA-3' and reverse 5'-GGCATGGAC-TGTGGTCATGA-3') were synthesized by Shanghai Sangon Biotech Co., Ltd. PCR amplification of GRP78, CHOP, and GAPDH cDNAs was performed with 1.5  $\mu\text{L}$  cDNA in the same parameters. The reverse transcription PCR and analysis were performed using the ABI PRISM 7500 sequence detection system. Reactions were run for optimal cycles with predenaturalization at  $94^{\circ}\text{C}$  for 15 min; denaturation, annealing, and extension at  $94^{\circ}\text{C}$  for 15 s,  $60^{\circ}\text{C}$  for 34 s,  $72^{\circ}\text{C}$  for 15 s and repeated for 40 cycles; and lastly extension at  $72^{\circ}\text{C}$  for 10 min. The housekeeping gene GAPDH was used for

internal control. The  $2^{-\Delta\Delta CT}$  method [22] was used to analyze the relative changes in gene expression.

**2.5. Western Blotting.** The myocardium tissues were homogenized and lysed in lysis buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were then incubated with the primary antibody against GRP78 (Abcam, USA) and CHOP (Cell Signaling Technology, USA) at 4°C overnight, and then the membrane was incubated with appropriate secondary antibody. After washing, membranes were exposed to X-ray film. The staining was quantified by scanning the films and the band density was determined with Image-Pro Plus software.

**2.6. Statistical Analysis.** All data from at least 9 (ELISA results) or 5 (real-time Quantitative PCR and Western blotting analysis) independent experiments were expressed as means  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) was carried out for the comparison of means. All statistical analyses were performed with SPSS version 11.0, and *P* values of less than 0.05 were considered to be statistically significant.

### 3. Results

**3.1. General Condition.** All the survived rats underwent operation exhibited normal physical appearance and behavior during the gavage period of different drugs. The survival outcome after LAD ligation is presented in Table 2.

**3.2. Expressions of MDA, SOD, and 8-Iso-PGF2 $\alpha$  in Serum.** The serum concentrations of MDA, SOD, and 8-iso-PGF2 $\alpha$  are shown in Figure 2. The serum MDA and 8-iso-PGF2 $\alpha$  levels in control group were significantly increased, while the serum SOD level decreased, compared to sham group (*P* < 0.05). Moderate-to-high dose EPC increased SOD, decreased 8-iso-PGF2 $\alpha$ , and metoprolol also decreased 8-iso-PGF2 $\alpha$ , when, respectively, compared with control group (*P* < 0.05).

**3.3. EPC Reduces GRP78 and CHOP mRNA Expressions in Infarcted Myocardium.** Alterations in mRNA expression of GRP78 and CHOP in infarcted myocardium were detected by quantitative real-time PCR. Compared with sham group, the gene expression of GRP78 and CHOP increased after experimental AMI (*P* < 0.05). Metoprolol and moderate-to-high dose EPC significantly reduced the mRNA expression of GRP78 and CHOP when compared to that of control group (*P* < 0.05). The results are shown in Figure 3.

**3.4. EPC Decreases GRP78 and CHOP Protein Expressions in Infarcted Myocardium.** Alterations in protein expression of GRP78 and CHOP in infarcted myocardium were detected by Western blotting. As seen in Figure 4, the protein expression of GRP78 and CHOP increased after experimental AMI (*P* < 0.05). Compared with control group, metoprolol and

TABLE 2: The outcome after LAD ligation.

Group	<i>N</i>	Dead rats ( <i>n</i> )	Surviving rats ( <i>n</i> )
Sham	10	0	10
Control	18	9	9
Metoprolol	18	6	12
Low EPC	18	9	9
Moderate EPC	18	7	11
High EPC	18	8	10

moderate-to-high dose EPC significantly decreased the protein expression of GRP78 and CHOP (*P* < 0.05).

### 4. Discussion

In the setting of AMI, ROS has been indicated playing a significant role in tissue necrosis and ischemia-reperfusion injury [23, 24]. Several pathways exist to protect against damage induced by ROS, with those best characterized in the heart being the superoxide dismutase. Overexpression of SOD has been shown to reduce infarct size in mice, which supports the contention that SOD is a major defense mechanism against ROS and a critical determinant in the tolerance of the heart to oxidative stress [25]. One method to quantify oxidative injury is to measure lipid peroxidation. MDA, one of the end-products of lipid peroxidation driven by ROS, can contribute significantly to the oxidative damage of proteins as it occurs under conditions of oxidative stress in age-related diseases and ischemic heart disease [26, 27]. Quantification of 8-iso-PGF2 $\alpha$  derived from the nonenzymatic oxidation of arachidonic acid provides an accurate assessment of oxidative stress both *in vitro* and *in vivo* [28, 29], which was also identified as an independent and cumulative risk marker of coronary heart disease [30]. In the present study, the expressions of MDA and 8-iso-PGF2 $\alpha$  in control group were increased compared to sham group, while the expression of SOD decreased, which indicates that MI conditions induce oxidative stress.

Perturbations of ER homeostasis affect protein folding and cause ERS. MI conditions induce accumulation of unfolding or misfolding proteins within the ER. ER can sense the stress and then respond to it through translational attenuation, upregulation of the genes for ER chaperones and related proteins, and degradation of unfolded proteins by a quality-control system [31]. GRP78, belonging to the heat shock protein 70 group and widely used as a marker for ERS, plays an important role in many cellular processes, which can contribute to the repair of unfolded proteins [32]. One important component of the ERS-mediated apoptosis pathway is CHOP, which encourages ROS production by depleting the cell of glutathione [31]. The results showed that both the gene and protein expressions of GRP78 and CHOP in control group were increased compared to sham group, indicating that MI conditions also induce ERS. Therefore, MI conditions induce both excessive ERS and oxidative stress.

Beta-blockers have been used extensively in the last 40 years after AMI as part of primary therapy and in secondary

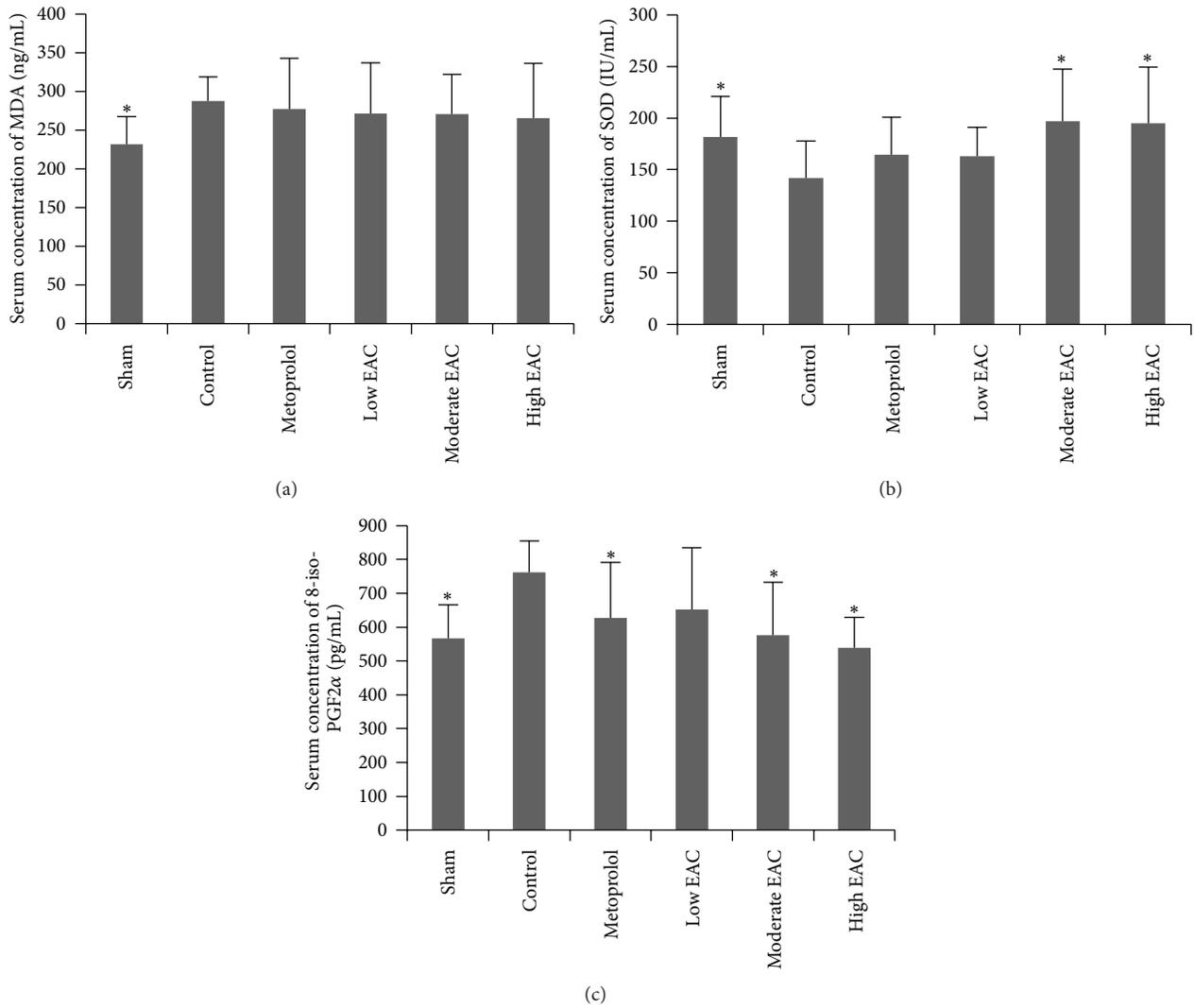


FIGURE 2: Serum concentration of MDA (a), SOD (b), and 8-iso-PGF2α (c). The error bars denote SD (\* $P < 0.05$  compared with control group).

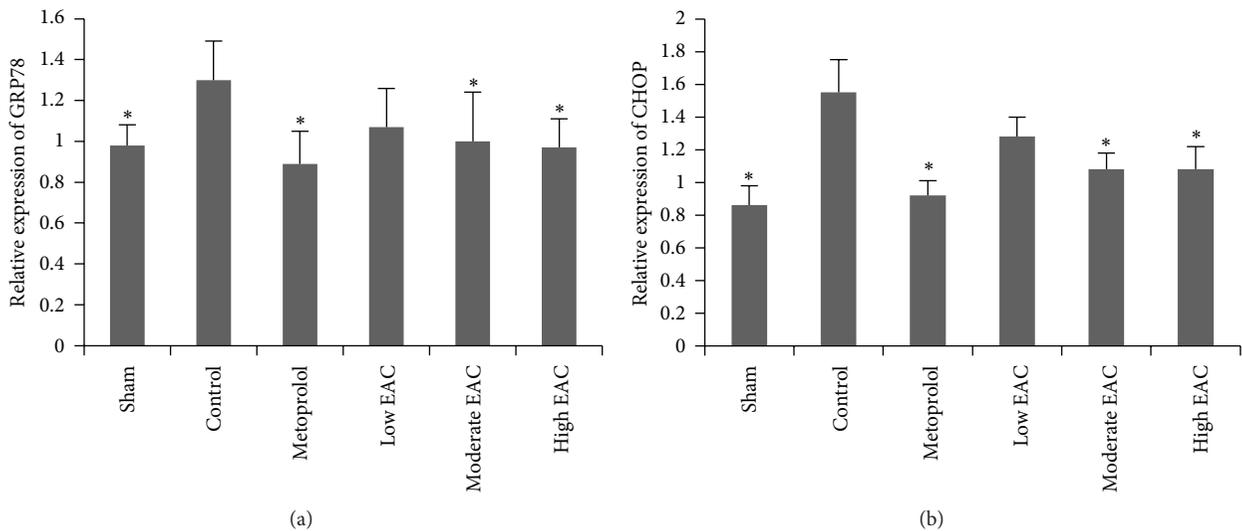


FIGURE 3: Expressions of GRP78 and CHOP mRNA in infarcted myocardium. The gene expressions of GRP78 and CHOP were determined by quantitative real-time PCR. GAPDH was used as a control reference. The error bars denote SD (\* $P < 0.05$  compared with control group;  $n = 6$ ).

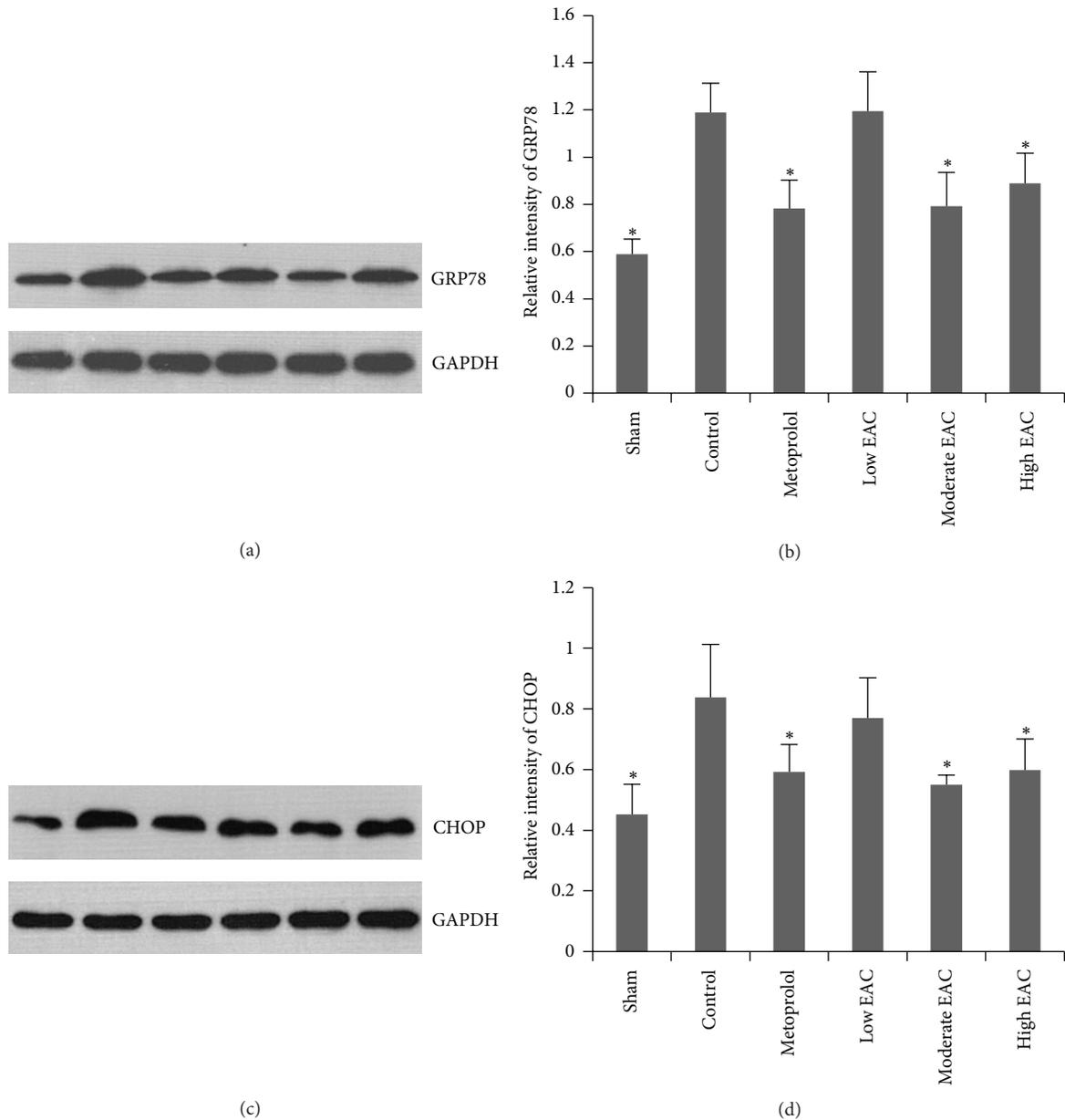


FIGURE 4: Expressions of GRP78 and CHOP protein in infarcted myocardium. The expressions of GRP78 and CHOP protein in infarcted myocardium were performed by Western blotting ((a) and (c)). Quantification of protein expressions were shown in (b) and (d). The error bars denote SD (\* $P < 0.05$  compared with control group;  $n = 6$ ).

prevention. Metoprolol, a Beta-blocker, as a cornerstone in the therapy of the postinfarct heart, has an important effect on decreasing mortality in patients after AMI [33]. George et al. reported that metoprolol can significantly improve cardiac function, result in normalized ERS marker, and reduce DNA damage in a coronary embolization model of heart failure [34]. The aforesaid results showed that metoprolol downregulated the expressions of GRP78 and CHOP in myocardium subjected to MI, protecting the myocardium by attenuating ERS. Metoprolol also decreased 8-iso-PGF $2\alpha$  serum level so as to suppress oxidative stress invoked by MI. Therefore, metoprolol protect myocardium by suppressing excessive ERS and oxidative stress.

EPC, the extracts of *Panax quinquefolius* and *Corydalis tuber*, has been used for the treatment of ischemic cardiovascular diseases for years in clinic. *Panax quinquefolius* saponins and tetrahydropalmatine are the main components of EPC determined by HPLC method. Previous animal experiments and clinical trials have shown that *Panax quinquefolius* saponins have antioxidant effects, and its protective effects may be mostly attributed to scavenging H $_2$ O $_2$  and hydroxyl radicals, enhancing the activities of superoxide dismutase and catalase, suppressing ROS-induced Jun N-terminal kinase activation [35–37]. Tetrahydropalmatine has been shown to have a protective effect against oxidative stress, which significantly reduced intracellular ROS formation and

enhanced the production of intracellular antioxidants—SOD. Wang et al. reported that *Panax quinquefolius* saponins suppressed hypoxia-reoxygenation-induced excessive ERS, as evidenced by reduced caspase 12 activation and decreased GRP78, calreticulin, and CHOP [38]. Our findings presented here confirm and extend findings of the aforesaid works. EPC exhibited significant protective effects against oxidative stress injury in myocardium after MI by increasing SOD and decreasing 8-iso-PGF $2\alpha$ . Moderate-to-high dose EPC significantly decreased the mRNA and protein expressions of GRP78 and CHOP when compared with control group, indicating that EPC could alleviate injury of myocardium subjected to MI by suppressing excessive ERS. Based on our study, ERS and oxidative stress are potential therapeutic targets for human AMI. The beneficial effects of metoprolol on MI are mediated, at least in part, through the prevention of oxidative stress and ERS induced damage. EPC is an effective compound for treatment of MI by suppressing excessive ERS and oxidative stress, which provides experimental evidence for the clinical application of EPC.

## 5. Conclusions

Metoprolol and EPC protect the myocardium by attenuating oxidative stress and ERS in MI rats, highlighting the ERS pathways as potential therapeutic targets for MI. Further mechanistic study will be necessary to elucidate these interactions fully.

## Acknowledgments

This study was supported by National Science and Technology Major Project (Grant no. 2009ZX09103-441) and Chinese National Natural Scientific Fund (Grants nos. 81030063 and 81102722).

## References

- [1] M. D. Bagatini, C. C. Martins, V. Battisti et al., "Oxidative stress versus antioxidant defenses in patients with acute myocardial infarction," *Heart and Vessels*, vol. 26, no. 1, pp. 55–63, 2011.
- [2] M. Hori and K. Nishida, "Oxidative stress and left ventricular remodeling after myocardial infarction," *Cardiovascular Research*, vol. 81, no. 3, pp. 457–464, 2009.
- [3] Z. Chen, B. Siu, Y.-S. Ho et al., "Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice," *Journal of Molecular and Cellular Cardiology*, vol. 30, no. 11, pp. 2281–2289, 1998.
- [4] E. D. van Deel, Z. Lu, X. Xu et al., "Extracellular superoxide dismutase protects the heart against oxidative stress and hypertrophy after myocardial infarction," *Free Radical Biology and Medicine*, vol. 44, no. 7, pp. 1305–1313, 2008.
- [5] M. T. Gökdemir, H. Kaya, O. Söğüt, Z. Kaya, L. Albayrak, and A. Taşkın, "The role of oxidative stress and inflammation in the early evaluation of acute non-ST-elevation myocardial infarction: an observational study," *Anatolian Journal of Cardiology*, vol. 13, no. 2, pp. 131–136, 2013.
- [6] M. Garelnabi, V. Gupta, V. Mallika, and J. Bhattacharjee, "Platelets oxidative stress in Indian patients with ischemic heart disease," *Journal of Clinical Laboratory Analysis*, vol. 24, no. 1, pp. 49–54, 2010.
- [7] S. Aksoy, N. Cam, U. Gurkan et al., "Oxidative stress and severity of coronary artery disease in young smokers with acute myocardial infarction," *Cardiology Journal*, vol. 19, no. 4, pp. 381–386, 2012.
- [8] D. Ron, "Translational control in the endoplasmic reticulum stress response," *Journal of Clinical Investigation*, vol. 110, no. 10, pp. 1383–1388, 2002.
- [9] C. Xu, B. Bailly-Maitre, and J. C. Reed, "Endoplasmic reticulum stress: cell life and death decisions," *Journal of Clinical Investigation*, vol. 115, no. 10, pp. 2656–2664, 2005.
- [10] W. Xin, X. Li, X. Lu, K. Niu, and J. Cai, "Involvement of endoplasmic reticulum stress-associated apoptosis in a heart failure model induced by chronic myocardial ischemia," *International Journal of Molecular Medicine*, vol. 27, no. 4, pp. 503–509, 2011.
- [11] A. Mitra, T. Basak, K. Datta, S. Naskar, S. Sengupta, and S. Sarkar, "Role of  $\alpha$ -crystallin B as a regulatory switch in modulating cardiomyocyte apoptosis by mitochondria or endoplasmic reticulum during cardiac hypertrophy and myocardial infarction," *Cell Death and Disease*, vol. 4, no. 4, article e582, 2013.
- [12] M. Yan, J. Shen, M. D. Person et al., "Endoplasmic reticulum stress and unfolded protein response in Atm-deficient thymocytes and thymic lymphoma cells are attributable to oxidative stress," *Neoplasia*, vol. 10, no. 2, pp. 160–167, 2008.
- [13] C. M. Haynes, E. A. Titus, and A. A. Cooper, "Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death," *Molecular Cell*, vol. 15, no. 5, pp. 767–776, 2004.
- [14] E. V. Ilieva, V. Ayala, M. Jové et al., "Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis," *Brain*, vol. 130, no. 12, pp. 3111–3123, 2007.
- [15] K. T. Kim, K. M. Yoo, J. W. Lee, S. H. Eom, I. K. Hwang, and C. Y. Lee, "Protective effect of steamed American ginseng (*Panax quinquefolius* L.) on V79-4 cells induced by oxidative stress," *Journal of Ethnopharmacology*, vol. 111, no. 3, pp. 443–450, 2007.
- [16] J.-T. Xie, Z.-H. Shao, T. L. Vanden Hoek et al., "Antioxidant effects of ginsenoside Re in cardiomyocytes," *European Journal of Pharmacology*, vol. 532, no. 3, pp. 201–207, 2006.
- [17] J. Li, Z. H. Shao, J. T. Xie et al., "The effects of ginsenoside Rb1 on JNK in oxidative injury in cardiomyocytes," *Archives of Pharmacological Research*, vol. 35, no. 7, pp. 1259–1267, 2012.
- [18] C. Wang, Y.-Z. Li, X.-R. Wang, Z.-R. Lu, D.-Z. Shi, and X.-H. Liu, "Panax quinquefolium saponins reduce myocardial hypoxia-reoxygenation injury by inhibiting excessive endoplasmic reticulum stress," *Shock*, vol. 37, no. 2, pp. 228–233, 2012.
- [19] M. Xue, H. Yin, L. Zhang et al., "Dynamic expression of the main related indicators of thrombosis, inflammatory reaction and tissue damage in a rat model of myocardial infarction," *Molecular Medicine Reports*, vol. 4, no. 4, pp. 693–696, 2011.
- [20] Y. Guo, H.-J. Yin, D.-Z. Shi, and K.-J. Chen, "Effects of tribuli saponins on left ventricular remodeling after acute myocardial infarction in rats with hyperlipidemia," *Chinese Journal of Integrative Medicine*, vol. 11, no. 2, pp. 142–146, 2005.
- [21] Q. Chen, *Methodological Study of Chinese Herbs Pharmacology*, People's Medical Publishing House, Beijing, China, 1996.
- [22] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.
- [23] T. Yoshida, N. Maulik, R. M. Engelman, Y.-S. Ho, and D. K. Das, "Targeted disruption of the mouse sod I gene makes the hearts vulnerable to ischemic reperfusion injury," *Circulation Research*, vol. 86, no. 3, pp. 264–269, 2000.

- [24] G. K. Asimakis, S. Lick, and C. Patterson, "Postischemic recovery of contractile function is impaired in SOD2+/- but not SOD1+/- mouse hearts," *Circulation*, vol. 105, no. 8, pp. 981-986, 2002.
- [25] E. P. Chen, H. B. Bittner, R. D. Davis, R. J. Folz, and P. Van Trigt, "Extracellular superoxide dismutase transgene overexpression preserves postischemic myocardial function in isolated murine hearts," *Circulation*, vol. 94, supplement 9, pp. II412-II417, 1996.
- [26] M. Garelnabi, V. Gupta, V. Mallika, and J. Bhattacharjee, "Platelets oxidative stress in Indian patients with ischemic heart disease," *Journal of Clinical Laboratory Analysis*, vol. 24, no. 1, pp. 49-54, 2010.
- [27] H. H. F. Refsgaard, L. Tsai, and E. R. Stadtman, "Modifications of proteins by polyunsaturated fatty acid peroxidation products," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 2, pp. 611-616, 2000.
- [28] S. Tacconelli, M. L. Capone, and P. Patrignani, "Measurement of 8-iso-prostaglandin F2alpha in biological fluids as a measure of lipid peroxidation," *Methods in Molecular Biology*, vol. 644, pp. 165-178, 2010.
- [29] G. L. Milne, S. C. Sanchez, E. S. Musiek, and J. D. Morrow, "Quantification of F2-isoprostanes as a biomarker of oxidative stress," *Nature Protocols*, vol. 2, no. 1, pp. 221-226, 2007.
- [30] E. Schwedhelm, A. Bartling, H. Lenzen et al., "Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: a matched case-control study," *Circulation*, vol. 109, no. 7, pp. 843-848, 2004.
- [31] S. Oyadomari and M. Mori, "Roles of CHOP/GADD153 in endoplasmic reticulum stress," *Cell Death and Differentiation*, vol. 11, no. 4, pp. 381-389, 2004.
- [32] K. F. Ferri and G. Kroemer, "Organelle-specific initiation of cell death pathways," *Nature Cell Biology*, vol. 3, no. 11, pp. E255-263, 2001.
- [33] W. Koenig, H. Lowel, M. Lewis, and A. Hormann, "Long-term survival after myocardial infarction: relationship with thrombolysis and discharge medication. Results of the Augsburg myocardial infarction follow-up study 1985 to 1993," *European Heart Journal*, vol. 17, no. 8, pp. 1199-1206, 1996.
- [34] I. George, H. N. Sabbah, K. Xu, N. Wang, and J. Wang, "beta-Adrenergic receptor blockade reduces endoplasmic reticulum stress and normalizes calcium handling in a coronary embolization model of heart failure in canines," *Cardiovascular Research*, vol. 91, no. 3, pp. 447-455, 2011.
- [35] K. T. Kim, K. M. Yoo, J. W. Lee, S. H. Eom, I. K. Hwang, and C. Y. Lee, "Protective effect of steamed American ginseng (*Panax quinquefolius* L.) on V79-4 cells induced by oxidative stress," *Journal of Ethnopharmacology*, vol. 111, no. 3, pp. 443-450, 2007.
- [36] J.-T. Xie, Z.-H. Shao, T. L. Vanden Hoek et al., "Antioxidant effects of ginsenoside Re in cardiomyocytes," *European Journal of Pharmacology*, vol. 532, no. 3, pp. 201-207, 2006.
- [37] J. Li, Z. H. Shao, J. T. Xie et al., "The effects of ginsenoside Rb1 on JNK in oxidative injury in cardiomyocytes," *Archives of Pharmacological Research*, vol. 35, no. 7, pp. 1259-1267, 2012.
- [38] C. Wang, Y.-Z. Li, X.-R. Wang, Z.-R. Lu, D.-Z. Shi, and X.-H. Liu, "Panax quinquefolium saponins reduce myocardial hypoxia-reoxygenation injury by inhibiting excessive endoplasmic reticulum stress," *Shock*, vol. 37, no. 2, pp. 228-233, 2012.

## Review Article

# Systematic Review of Compound Danshen Dropping Pill: A Chinese Patent Medicine for Acute Myocardial Infarction

Jing Luo,<sup>1</sup> Hao Xu,<sup>2</sup> and Keji Chen<sup>2</sup>

<sup>1</sup> Graduate School, Beijing University of Chinese Medicine, Beijing 100029, China

<sup>2</sup> Cardiovascular Diseases Center, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

Correspondence should be addressed to Hao Xu; xuhaotcm@hotmail.com and Keji Chen; keji\_chen@yahoo.com

Received 26 February 2013; Accepted 23 May 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Jing Luo et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Objective.** This paper systematically evaluated the efficacy and safety of compound Danshen dropping pill (CDDP) in patients with acute myocardial infarction (AMI). **Methods.** Randomized controlled trials (RCTs), comparing CDDP with no intervention, placebo, or conventional western medicine, were retrieved. Data extraction and analyses were conducted in accordance with the Cochrane standards. We assessed risk of bias for each included study and evaluated the strength of evidence on prespecified outcomes. **Results.** Seven RCTs enrolling 1215 patients were included. CDDP was associated with statistically significant reductions in the risk of cardiac death and heart failure compared with no intervention based on conventional therapy for AMI. In addition, CDDP was associated with improvement of quality of life and impaired left ventricular ejection fraction. Nevertheless, the safety of CDDP was unproven for the limited data. The quality of evidence for each outcome in the main comparison (CDDP versus no intervention) was “low” or “moderate.” **Conclusion.** CDDP showed some potential benefits for AMI patients, such as the reductions of cardiac death and heart failure. However, the overall quality of evidence was poor, and the safety of CDDP for AMI patients was not confirmed. More evidence from high quality RCTs is warranted to support the use of CDDP for AMI patients.

## 1. Introduction

Acute myocardial infarction (AMI) is a serious type of coronary heart disease (CHD) and a major cause of death worldwide with an estimated annual incidence rate of seven million people [1]. As a result of coronary artery thrombotic occlusion from plaques rupture or erosion, AMI usually leads to death if complicated by severe heart failure, malignant ventricular arrhythmia, or cardiac rupture [1, 2]. Despite the application of percutaneous coronary intervention (PCI) and conventional western medicine, AMI patients remain at certain risk of in-hospital death and complications as well as recurrent acute cardiovascular events [2–4]. With more and more clinicians successfully applied traditional Chinese medicine (TCM) in CHD prevention and treatment based on conventional therapy, the effects of TCM for CHD have drawn more and more attention [5–8].

Compound Danshen dropping pill (CDDP, also known as the “Dantonic Pill”), a Chinese oral patent medicine,

has been widely used for cardiovascular diseases, including AMI, in China and some Asia countries. The phase II clinical trial of CDDP to treat chronic stable angina (<http://clinicaltrials.gov/>, NCT00797953) had been completed in the United States in 2010. Moreover, this drug has been approved by the Australian Therapeutic Goods Administration for use and is widely available in Australia [9]. CDDP consists of three compositions, namely, *Radix Salviae Miltiorrhizae*, *Radix Notoginseng*, and *Borneolum Syntheticum*. These compositions and their pharmacological actions [10–15] are listed in Table 1 with common, pinyin, and Latin names. Previous pharmacologic studies and randomized clinical trials have indicated the potential benefit of CDDP for patients with AMI [16–21]. Recent systematic reviews [22–24] also revealed potential benefits of CDDP for angina pectoris. The efficacy and safety of CDDP for AMI, however, have not been systematically evaluated. The aim of this study was to assess the efficacy and safety of CDDP on the treatment of AMI patients.

TABLE 1: Compositions of compound Danshen dropping pill.

Common name	Pinyin name	Latin name	Pharma. actions
Danshen root	Danshen	<i>Radix Salviae Miltiorrhizae</i>	Dilates coronary vessels and antimyocardial ischemia inhibit platelet aggregation and thrombosis, decrease cholesterol and endothelial damage, scavenge free radicals, antilipid peroxidative, and antiatherosclerosis, and reduce myocardial ischemia-reperfusion injury, anti-inflammatory [10, 11].
Sanchi root	Sanqi	<i>Radix Notoginseng</i>	Dilates blood vessel increases blood platelet number to promote hemostasis, inhibits platelet aggregation and thrombosis, and reduces viscosity of whole blood, decreases the heart rate and myocardial ischemia-reperfusion injury, inhibits proliferation of vascular smooth muscle cell, decreases cholesterol and antiatherosclerosis, antioxidation [12, 13].
Borneol	Bingpian	<i>Borneolum Syntheticum</i>	Analgesia and sedation boost other drugs' bioavailability, anti-inflammatory, and decreases the heart rate and myocardial oxygen consumption [14, 15].

## 2. Methods

**2.1. Inclusion and Exclusion Criteria.** Randomized controlled trials (RCTs) comparing CDDP with no intervention, placebo, or conventional western medicine were sought regardless of their publication status. Participants of any gender, age, or ethnic origin with AMI meeting with one of the past or current definitions of AMI [25–29] were included. Those without description of diagnostic criteria but stated patients with definite AMI were also considered. Quasi-randomized trials and animal experiments were excluded. Trials with CDDP as adjunctive therapy or with duration less than four weeks were also excluded.

Primary outcomes consisted of all-cause mortality, cardiac mortality, recurrent myocardial infarction (RMI), and revascularization, including PCI and coronary artery bypass graft (CABG). Secondary outcomes included heart failure, readmission, left ventricular ejection fraction (LVEF), recurrent angina, adverse events and health-related quality of life measured by a validated tool.

### 2.2. Source of Literature and Search Strategy

**2.2.1. Electronic Searches.** We searched the following databases up to October 2012 for the identification of RCTs both published and unpublished: Pubmed, The Cochrane Library, Chinese Biomedical Database (CBM), Chinese VIP Information (VIP), China National Knowledge Infrastructure (CNKI), Wanfang Databases, China Proceedings of Conference Full-text Database (CPCD), Chinese Doctoral Dissertations Full-text Database (CDFD), and Chinese Master's Theses Full-text Database (CMFD). Search strategy in Table 2 was used in The Cochrane Library and adapted appropriately for other databases.

In addition, we searched databases of ongoing trials: ClinicalTrials.gov (<http://clinicaltrials.gov/>) and Current Controlled Trials (<http://www.controlled-trials.com/>).

**2.2.2. Additional Searches.** We also searched the reference lists of studies included in this systematic review and of other relevant reviews to identify missing relevant articles.

TABLE 2: Search strategy for the Cochrane library.

Strategy
No. 1 Danshen pill
No. 2 salvia pill
No. 3 compound Danshen
No. 4 compound salvia
No. 5 composite Danshen
No. 6 composite salvia
No. 7 Dantonic Pill
No. 8 CDDP
No. 9 CSDP
No. 10 FFDS
No. 11 myocardial infarction [MeSH]
No. 12 coronary disease [MeSH]
No. 13 coronary artery disease [MeSH]
No. 14 acute coronary syndrome [MeSH]
No. 15 myocardial infarct*
No. 16 AMI
No. 17 MI
No. 18 acute coronary syndrome
No. 19 (1, 2, 3, 4, 5, 6, 7, 8, 9, or 10)
No. 20 (11, 12, 13, 14, 15, 16, 17, or 18)
No. 21 (19 and 20)

**2.3. Study Identification and Data Extraction.** Two authors (Jing Luo, Hao Xu) independently screened the titles and abstracts of references for potentially relevant RCTs. Full texts of potentially eligible articles were retrieved for further identification according to the inclusion and exclusion criteria. Any disagreement was resolved by consensus.

Two authors (Jing Luo, Hao Xu) independently extracted data using a preset data extraction form. Characteristics of RCTs including methods, participants, interventions, comparisons, and outcomes were extracted. We obtained missing information from the original authors whenever possible and resolved any disagreement through discussion or consulting the third author (Keji Chen).

**2.4. Assessment of Risk of Bias and Quality of Evidence.** Two authors (Jing Luo, Hao Xu) independently assessed the methodological quality of each of the included studies using the Cochrane “risk of bias” criteria [30], which covers the following items: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias. Disagreements were resolved by consensus. For each item, a low risk was considered when we judged a “Yes,” conversely, a “No” for a high risk, and otherwise for an unclear risk.

We also evaluated the quality of evidence of each outcome using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach [31], as recommended by the Cochrane Collaboration. Patient important outcomes in the main comparison were judged across five factors: limitations in study design and execution, inconsistency of results, indirectness of evidence, imprecision, and publication bias. Accordingly, we graded the quality of evidence in this review as very low, low, moderate, or high.

**2.5. Data Analysis.** We used RevMan 5.1 software for data analyses. Studies were stratified by the different types of comparisons. We performed intention-to-treat analysis (ITT) for dichotomous data and presented outcome data as risk ratio (RR) with corresponding 95% confidence interval (CI). We calculated mean difference (MD) with its 95% CI for continuous outcomes. Fixed effect model was used to analyze data with low heterogeneity ( $I^2 \leq 50\%$ ); random effects model was applied if heterogeneity is significant ( $50\% < I^2 < 75\%$ ). Results were not pooled for data with high heterogeneity ( $I^2 \geq 75\%$ ) [32], in which case we explored potential causes of heterogeneity by conducting subgroup analyses based on the characteristics of intervention (dosage, duration) and the types of conventional therapy (PCI versus thrombolysis). We also performed sensitivity analyses on studies with lower methodological quality, in order to investigate whether the inclusion of such studies altered the conclusion of the meta-analysis. Possible publication bias was checked using funnel plots when the number of included studies of any particular outcome is greater than eight.

### 3. Results

**3.1. Study Identification.** A total of 564 references were found according to search strategy, of which 261 were excluded for duplicates among databases. After screening the abstract, we excluded 231 articles. 72 potentially eligible studies were retrieved for further identification, of which 65 were excluded because they did not meet the prespecified inclusion criteria described in the methods. At last, seven eligible RCTs [19–21, 33–36] were included. No ongoing trial was found. Please refer to Figure 1 for a more detailed illustration of the data screening process.

**3.2. Description of Included Studies.** The characteristics of the included seven studies [19–21, 33–36] are summarized in Table 3. Each of the studies was conducted in China. One

postgraduate dissertation [35] was unpublished in 2010, and the others were published from 2006 to 2011. One study [19] was of multicenter design, but the others were of single centre trials.

The number of participants in the individual study ranged from 45 to 500, with a total of 1215 in this review (583 in intervention groups and 632 in control groups). There were 863 males and 352 females included in the review, with mean age, where given [19, 20, 34–36], ranging from 52 to 66 years. All of the participants were diagnosed with AMI by different diagnostic criteria: two studies [20, 21] used the WHO diagnostic criteria; one study [33] used ACC/AHA diagnostic criteria; four studies [19, 34–36] without specified diagnostic criteria but mentioned “patients with AMI were eligible to include.” Two studies [19, 35] only included patients with ST-elevation myocardial infarction (STEMI), one study excluded AMI without Q wave [21], and the others did not introduce the types of AMI (four studies) [20, 33, 34, 36].

All participants in the intervention groups were treated with CDDP, 10 pills three times a day (tid) orally based on conventional therapy since the day of diagnosis [19, 20, 33–36]. Only one study [21] began the CDDP treatment four to five weeks later after diagnosis and changed the dosage from 10 pills tid to five pills tid after 60 days of treatment. The duration of treatment was mainly as same as the length of follow up, ranging from four weeks to 12 months. One study [21] was designed as three groups with two comparisons including CDDP versus no intervention and CDDP versus propranolol. Six studies consisted of two groups (one study [36] compared CDDP with placebo and the others [19, 20, 33–35] focused on CDDP compared with no intervention). In total, there were three comparisons in the review: (1) CDDP plus conventional therapy versus conventional therapy (six studies) [19–21, 33–35]; (2) CDDP plus conventional therapy versus placebo plus conventional therapy (one study) [36]; (3) CDDP plus conventional therapy versus propranolol plus conventional therapy (one study) [21].

Five studies [19–21, 35, 36] reported mortality including all-cause mortality (four studies) [19–21, 35] and cardiac mortality (three studies) [21, 35, 36]. Two studies provided numerical information on RMI [21, 36], but the data could not be pooled for the different comparisons. Four studies [19–21, 36] reported heart failure. Three studies [19, 20, 36] provided the number of patients having recurrent angina. Besides the incidence of readmission and adverse events (narrative introduction), one study [36] also assessed the QOL by questionnaire score, and the questionnaire was designed referring to Treatment of Mild Hypertension Study (TOMHS) and Medical Outcomes Study 36-Item Short-Form Health Survey (SF-36). Five studies [19, 21, 33, 34, 36] assessed the LVEF with the aim of evaluating the heart function. None of the included studies mentioned revascularization.

#### 3.3. Quality of Included Studies

**3.3.1. Risk of Bias in Included Studies.** Risk of bias summaries for each outcome in the included RCTs at the study level are presented in Figures 2–10. No study was felt to have a low

TABLE 3: Characteristics of included studies.

ID	Sample size (I/C)	Age (y, I/C)	Diagnostic criteria of AMI	Type of AMI	Intervention	Control	Duration of treatment	Follow up	Outcomes	Baseline report
Li et al. 2011 [19]	500 (252/248)	60.10 ± 9.60/ 56.70 ± 7.80	Not specified	STEMI	CDDP 10 pills tid + CT (the same as control)	CT (western medicines + PCI)	30 days	30 days	All-cause mortality, shock, arrhythmia, LVEF%, HF, angina, myocardial enzyme.	Yes
Lin 2011 [33]	90 (46/44)	43–75/36–72	ACC/AHA 2004	Unclear	CDDP 10 pills tid + CT (the same as control)	CT (western medicines)	6 weeks	6 weeks	LVEF%, WBC, CRP, Chinese symptoms.	Yes (narrative only)
Ma 2010 [35]	163 (78/85)	62.55 ± 11.95/ 66.02 ± 11.40	Not specified	STEMI	CDDP 10 pills tid + CT (the same as control)	CT (western medicines or plus PCI/thrombolysis)	1 month	3 months	All-cause mortality, IL-6, cardiac mortality, hs-CRP, MACEs, MMP-9, TNF- $\alpha$ .	Yes
Li et al. 2010 [36]	63 (42/21)	58.40 ± 11.60	Not specified	Unclear	CDDP 10 pills tid + CT (the same as control)	Placebo + CT (western Medicines + thrombolysis).	4 months	4 months	Cardiac mortality, LVEF%, readmission, QOL, RMI, HF, angina, adverse events.	Yes (narrative only)
Guo et al. 2010 [20]	136 (76/60)	55.60 ± 12.5/ 51.80 ± 13.60	WHO	Unclear	CDDP 10 pills tid + CT (the same as control)	CT (western medicines + thrombolysis)	4 weeks	4 weeks	All-cause mortality, shock, HF, recanalization, angina, myocardial enzyme.	Yes (narrative only)
Xu and Wang 2007 [21]	218 (66/72/80)	36–75/37–78 /32–79	WHO	With Q-wave	CDDP 10 pills tid dl-60, then 5 pills tid + CT (the same as control)	Propranolol 10–15 mg tid + CT (no detail); CT (no detail)	12 months	12 months	All-cause mortality, RMI, cardiac mortality, HF, arrhythmia, LVEF%.	Yes (narrative only)
Mei et al. 2006 [34]	45 (23/22)	56.11 ± 11.13	Not specified	Unclear	CDDP 10 pills tid + CT (the same as control)	CT (western medicines)	6 months	6 months	LVEF%, SV, CO	Unclear

Notes: CT: conventional therapy; HF: heart failure; AHA: American heart Association; ACC: American College of Cardiology; CRP: C-reactive protein; hs-CRP: high sensitive C-reactive protein; WBC: white blood cell; MACEs: major adverse cardiac events; WHO: World Health Organization; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; MMP-9: matrix metalloproteinase-9; IL-6: interleukin-6; SV: stroke volume; CO: cardiac output; tid: three times a day.

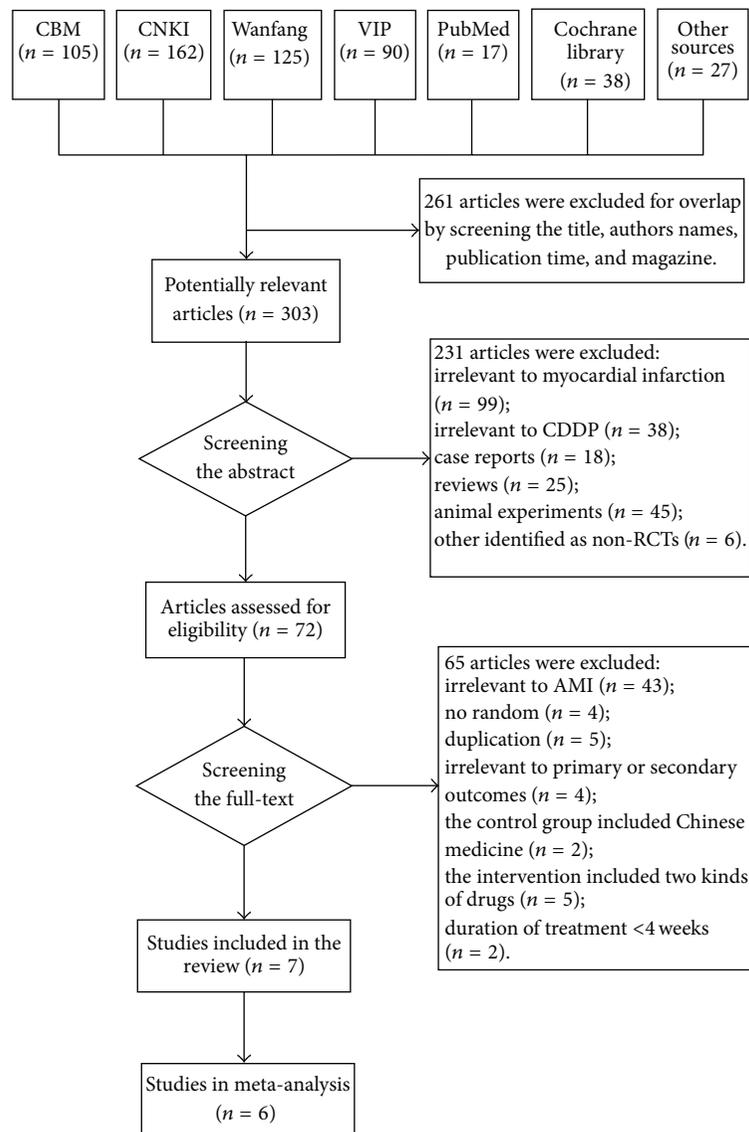


FIGURE 1: Flow chart of study search and identification.

risk of bias. Of the seven studies, one [35] introduced the random sequence being generated from a random number table, and the others just mentioned “patients were randomly allocated” without the method of randomization. Only one study [19] reported allocation concealment. None of the studies described blinding of participants and personnel although one study [36] used placebo. All of the studies did not report blinding of outcome assessment. Neither withdrawals nor losses to follow up were reported in the studies. One study [19] had incomplete outcome data. Five studies [20, 21, 33, 35, 36] reported the comparability of the baseline among groups, but four of them did not provide baseline data [20, 21, 33, 36]. The multicenter study [19], with other similar baselines, reported that the rate of diabetes patients in the intervention group was higher than the control group. In addition, no study mentioned prior sample size estimation or ITT analysis for any outcome.

After we contacted with the original authors by telephone and email, only one author [19] told us that there was no blinding of participants or personnel in their study, and the randomization was designed by public health statistics teaching and research section of Tianjin Medical University; he did not know any other details. In fact, due to a number of unsuccessful contacts and some unclear or unavailable replies, most of our questions were not resolved.

**3.3.2. Quality of Evidence in Included Studies.** The quality of evidence for each outcome in the main comparison (CDDP versus no intervention) was ranged from “low” to “moderate” (Table 4). Quality assessment of the evidence in accordance with the GRADE approach showed some limitations of the study design and execution, inconsistency, indirectness, and imprecision. Due to the low number of included studies for each outcome, we could not create funnel plots to detect

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Guo et al. 2010	?	?	?	+	?	+	?
Li et al. 2011	?	+	-	+	?	-	?
Ma 2010	+	?	?	+	?	+	?
Xu and Wang 2007	?	?	?	+	?	+	?

+ Low risk  
 - High risk  
 ? Unclear

FIGURE 2: Risk of bias summary—all-cause mortality.

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Li et al. 2010	?	?	?	?	?	+	?
Ma 2010	+	?	?	?	?	+	?
Xu and Wang 2007	?	?	?	?	?	+	?

+ Low risk  
 - High risk  
 ? Unclear

FIGURE 3: Risk of bias summary—cardiac mortality.

publication bias. For each outcome, there were one or two serious limitations among the five factors. For example, because of the serious risk of bias and imprecision for all-cause mortality in the main comparison, we downgraded the quality rating by two levels, thus the quality of evidence for this outcome was low. The quality of evidence was moderate for cardiac mortality and heart failure, low for all-cause mortality, RMI, recurrent angina, and LVEF.

3.4. Effect of Interventions (Table 5 to Table 7)

3.4.1. All-Cause Mortality (Table 5). Four studies [19–21, 35] reported all-cause mortality in two different comparisons. Meta-analysis of the four studies showed no statistically significant difference in the risk of all-cause death between CDDP and no intervention (RR 0.65; 95%CI 0.37 to 1.14;  $n = 945$ ). Sensitive analysis, excluding the lower quality study [19], found that CDDP was associated with a statistically significant reduction in the risk of all-cause death compared with no intervention without heterogeneity (RR 0.51; 95%CI 0.27 to 0.98; three studies,  $n = 445$ ;  $I^2 = 0\%$ ) [20, 21, 35]. A single study reported that there was no statistical difference in reducing all-cause mortality between CDDP and propranolol on the basis of conventional therapy (RR 0.65; 95%CI 0.16 to 2.63;  $n = 138$ ) [21]. The associated risk of bias is presented

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Li et al. 2010	?	?	?	?	?	+	?
Xu and Wang 2007	?	?	?	?	?	+	?

+ Low risk  
 - High risk  
 ? Unclear

FIGURE 4: Risk of bias summary—recurrent myocardial infarction.

TABLE 4: GRADE analysis: summary of findings for the main comparison.

Compound Danshen dropping pill versus no intervention for acute myocardial infarction						
Patient or population: patients with acute myocardial infarction						
Settings: inpatients and outpatients						
Intervention: compound Danshen dropping pill (CDDP)						
Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	None of Participants (studies)	Quality of the evidence (GRADE)	Comments
	Assumed risk	Corresponding risk				
Control						
CDDP						
<i>All-cause mortality</i> Follow-up: 4 weeks–12 months	<i>Study population</i>					
	63 per 1000	41 per 1000 (23–72)	RR 0.65 (0.37–1.14)	945 (4 studies)	⊕⊕ ⊕ ⊕ low <sup>1,2</sup>	
	104 per 1000	68 per 1000 (38–119)				
		<i>Moderate</i>				
<i>Cardiac mortality</i> Follow-up: 3–12 months	<i>Study population</i>					
	127 per 1000	55 per 1000 (25–121)	RR 0.43 (0.2–0.95)	309 (2 studies)	⊕⊕ ⊕ ⊕ moderate <sup>3,4</sup>	
	127 per 1000	55 per 1000 (25–121)				
		<i>Moderate</i>				
<i>Recurrent myocardial infarction</i> Follow-up: mean 12 months	<i>Study population</i>					
	100 per 1000	30 per 1000 (7–138)	RR 0.30 (0.07–1.38)	146 (1 study)	⊕⊕ ⊕ ⊕ low <sup>5</sup>	
	100 per 1000	30 per 1000 (7–138)				
		<i>Moderate</i>				
<i>Heart failure</i> Follow-up: 4 weeks–12 months	<i>Study population</i>					
	85 per 1000	35 per 1000 (19–64)	RR 0.41 (0.22–0.75)	782 (3 studies)	⊕⊕ ⊕ ⊕ moderate <sup>1</sup>	
	150 per 1000	62 per 1000 (33–112)				
		<i>Moderate</i>				

TABLE 4: Continued.

Compound Danshen dropping pill versus no intervention for acute myocardial infarction					
<i>Study population</i>					
<i>Recurrent angina</i>					
Clinical diagnosis based on patients complaint	211 per 1000	70 per 1000 (21–217)	RR 0.33 (0.1–1.03)	636 (2 studies)	⊕⊕⊕⊕ low <sup>1,6,7</sup>
Follow-up: 4 weeks–30 days		<i>Moderate</i>			
	201 per 1000	66 per 1000 (20–207)			
<i>Left ventricular ejection fraction</i>					
Measured with echocardiogram. Scale from 30% to 75%. Duration of treatment: 4–6 weeks	The mean left ventricular ejection fraction in the control groups was 50.48% <sup>9</sup>	The mean left ventricular ejection fraction in the intervention groups was 5.71% higher (4.38%–7.04% higher)		590 (2 studies)	⊕⊕⊕⊕ low <sup>1,8</sup>
<i>Left ventricular ejection fraction</i>					
Measured with echocardiogram. Scale from: 30%–75%. Duration of treatment: 6–12 months	The mean left ventricular ejection fraction in the control groups was 49.71% <sup>9</sup>	The mean left ventricular ejection fraction in the intervention groups was 3.82% higher (2.46%–5.19% higher)		191 (2 studies)	⊕⊕⊕⊕ low <sup>3,8</sup>

\*The basis for the assumed risk (e.g., the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95%CI).

CI: confidence interval; RR: risk ratio; GRADE Working Group grades of evidence:

High quality: further research is very unlikely to change our confidence in the estimate of effect.

Moderate quality: further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.

Low quality: further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.

Very low quality: we are very uncertain about the estimate.

<sup>1</sup>One study had selective reporting. For the other studies, the overall risk of bias was felt to be unclear.

<sup>2</sup>95%CI includes possibility of both benefits and harms, and the sample size was not the optimal information size. After sensitive analysis excluding the lower quality study, the result suggested benefit, but the sample size was still small.

<sup>3</sup>The overall risk of bias of the studies was unclear. The sample size was not the optimal information size.

<sup>4</sup>95%CI included only benefit, so we were cautious about downgrading the imprecision although the sample size was less than the optimal information size.

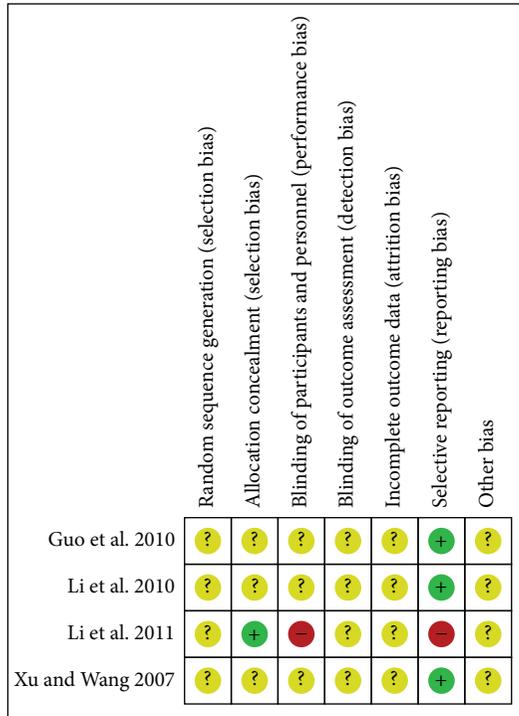
<sup>5</sup>Unclear risk of bias and only 146 patients enrolled. 95%CI included possibility of both benefits and harms.

<sup>6</sup>The heterogeneity ( $I^2 = 61\%$ ) can be explained by the major differences of conventional therapy and sample size between the two studies, and this outcome is not so important affect the decision-making; therefore, we did not downgrade for this factor.

<sup>7</sup>95%CI suggested benefit as well as no benefit.

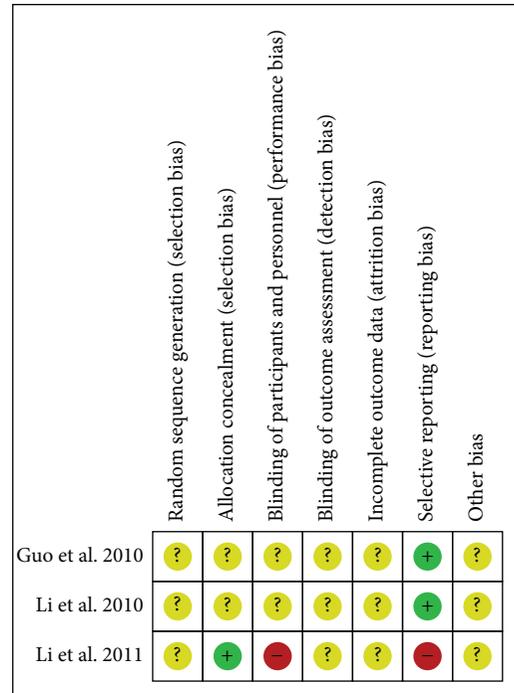
<sup>8</sup>This is an indirect outcome for AMI patients.

<sup>9</sup>Final measurements at the end of the study.



+ Low risk  
 - High risk  
 ? Unclear

FIGURE 5: Risk of bias summary—heart failure.



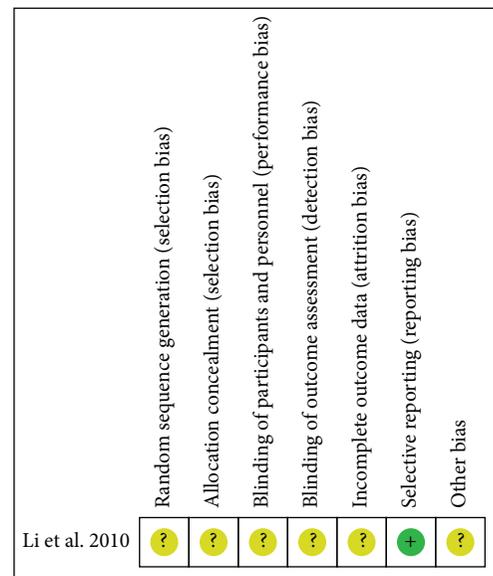
+ Low risk  
 - High risk  
 ? Unclear

FIGURE 6: Risk of bias summary—recurrent angina.

in Figure 2. The quality of evidence in the main comparison (CDDP versus no intervention) was low (Table 4).

3.4.2. *Cardiac Mortality (Table 5)*. Three studies [21, 35, 36] assessed cardiac mortality in three different comparisons. Meta-analysis of two studies [21, 35] showed that CDDP was associated with a statistically significant reduction in the risk of cardiac death compared with no intervention without heterogeneity (RR 0.43; 95%CI 0.20 to 0.95;  $n = 309$ ;  $I^2 = 0\%$ ). Compared with placebo on the basis of conventional therapy, CDDP had no statistically significant advantage in reducing cardiac mortality (RR 0.50; 95%CI 0.03 to 7.60; one study,  $n = 63$ ) [36]. A single study reported a similar result between CDDP and propranolol (RR 0.81; 95%CI 0.17 to 3.76;  $n = 138$ ) [21]. Figure 3 presents the associated risk of bias. The quality of evidence in the main comparison (CDDP versus no intervention) was moderate (Table 4).

3.4.3. *Recurrent Myocardial Infarction (Table 5)*. Two studies [21, 36] reported RMI in three different comparisons. None of the comparisons, however, presented a statistically significant difference in the risk of RMI: CDDP versus no intervention (RR 0.30; 95%CI 0.07 to 1.38; one study,  $n = 146$ ) [21]; CDDP versus placebo (RR 0.50; 95%CI 0.11 to 2.27; one study,  $n = 63$ ) [36]; CDDP versus propranolol (RR 0.73; 95%CI 0.13 to 4.22; one study,  $n = 138$ ) [21]. The associated risk of



+ Low risk  
 - High risk  
 ? Unclear

FIGURE 7: Risk of bias summary—readmission.

TABLE 5: Analyses of primary outcomes.

Outcomes (comparisons)	Treatment ( <i>n/N</i> )	Control ( <i>n/N</i> )	Weight (%)	RR	95% CI
<i>(1) All-cause mortality</i>					
<i>(1.1) CDDP + conventional therapy versus conventional therapy</i>					
Guo et al. 2010 [20]	5/76	5/60	19.10	0.79	[0.24, 2.60]
Li et al. 2011 [19]	6/252	4/248	13.80	1.48	[0.42, 5.17]
Ma 2010 [35]	5/78	11/85	36.10	0.50	[0.18, 1.36]
Xu and Wang 2007 [21]	3/66	10/80	31.00	0.36	[0.10, 1.27]
<b>Total (FEM, <math>I^2 = 0\%</math>)</b>			<b>100.00</b>	<b>0.65</b>	<b>[0.37, 1.14]</b>
<i>Sensitive analysis</i>					
Guo et al. 2010 [20]	5/76	5/60	22.20	0.79	[0.24, 2.60]
Ma 2010 [35]	5/78	11/85	41.80	0.50	[0.18, 1.36]
Xu and Wang 2007 [21]	3/66	10/80	35.90	0.36	[0.10, 1.27]
<b>Total (FEM, <math>I^2 = 0\%</math>)</b>			<b>100.00</b>	<b>0.51</b>	<b>[0.27, 0.98]</b>
<i>(1.2) CDDP + conventional therapy versus propranolol + conventional therapy</i>					
Xu and Wang 2007 [21]	3/66	5/72	100.00	0.65	[0.16, 2.63]
<i>(2) Cardiac mortality</i>					
<i>(2.1) CDDP + conventional therapy versus conventional therapy</i>					
Ma 2010 [35]	5/78	11/85	53.80	0.50	[0.18, 1.36]
Xu and Wang 2007 [21]	3/66	10/80	46.20	0.36	[0.10, 1.27]
<b>Total (FEM, <math>I^2 = 0\%</math>)</b>			<b>100.00</b>	<b>0.43</b>	<b>[0.20, 0.95]</b>
<i>(2.2) CDDP + conventional therapy versus placebo + conventional therapy</i>					
Li et al. 2010 [36]	1/42	1/21	100.00	0.50	[0.03, 7.60]
<i>(2.3) CDDP + conventional therapy versus propranolol + conventional therapy</i>					
Xu and Wang 2007 [21]	3/66	4/72	100.00	0.81	[0.17, 3.76]
<i>(3) Recurrent myocardial infarction</i>					
<i>(3.1) CDDP + conventional therapy versus conventional therapy</i>					
Xu and Wang 2007 [21]	2/66	8/80	100.00	0.30	[0.07, 1.38]
<i>(3.2) CDDP + conventional therapy versus placebo + conventional therapy</i>					
Li et al. 2010 [36]	3/42	3/21	100.00	0.50	[0.11, 2.27]
<i>(3.3) CDDP + conventional therapy versus propranolol + conventional therapy</i>					
Xu and Wang 2007 [21]	2/66	3/72	100.00	0.73	[0.13, 4.22]

bias is presented in Figure 4. The quality of evidence in the main comparison (CDDP versus no intervention) was low (Table 4).

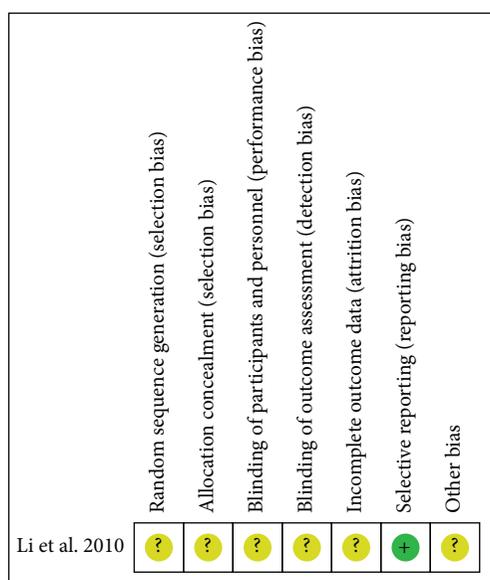
**3.4.4. Heart Failure (Table 6).** Four studies [19–21, 36] reported heart failure in three different comparisons. Meta-analysis of three studies [19–21] found that CDDP was associated with a statistically significant reduction in the risk of heart failure compared with no intervention with no heterogeneity (RR 0.41; 95%CI 0.22 to 0.75;  $n = 782$ ;  $I^2 = 0\%$ ). Sensitive analysis, excluding the lower quality study [19], got a similar conclusion (RR 0.30; 95%CI 0.14 to 0.65; two studies,  $n = 282$ ;  $I^2 = 0\%$ ) [20, 21]. Compared with propranolol on the basis of conventional therapy, CDDP still presented a statistical difference in reducing heart failure (RR 0.26; 95%CI 0.07 to 0.99; one study,  $n = 138$ ) [21]. Nevertheless, compared with placebo on the basis of conventional therapy, CDDP showed no effect in the reduction of heart failure (RR 0.63; 95%CI 0.19 to 2.09; one study,  $n = 63$ ) [36]. The associated risk of bias is presented in Figure 5. And the

quality of evidence in the main comparison (CDDP versus no intervention) was moderate (Table 4).

**3.4.5. Recurrent Angina (Table 6).** Three studies [19, 20, 36] assessed the number of patients having recurrent angina in two different comparisons. While meta-analysis of two studies showed that CDDP was associated with a statistically significant reduction in the risk of recurrent angina compared with no intervention; the heterogeneity was significant (RR 0.43; 95%CI 0.29 to 0.64;  $n = 636$ ;  $I^2 = 61\%$ ) [19, 20]. We, hence, examined the data and looked over the papers carefully. We found that besides the types of conventional therapy, the sample sizes between the two studies were also of big differences. Furthermore, one study was high risk of bias [19]. Random effects model, therefore, was used and got a different result without statistical difference (RR 0.33; 95%CI 0.10 to 1.03;  $n = 636$ ). Compared with placebo on the basis of conventional therapy, CDDP still showed no effect in the reduction of recurrent angina (RR 0.55; 95%CI 0.29 to 1.02; one study,  $n = 63$ ) [36]. Figure 6 presents the associated

TABLE 6: Analyses of secondary outcomes.

Outcomes (comparisons)	Treatment (n/N)	Control (n/N)	Weight (%)	RR	95% CI
<i>(1) Heart failure</i>					
<i>(1.1) CDDP + conventional therapy versus conventional therapy</i>					
Xu and Wang 2007 [21]	3/66	12/80	32.40	0.30	[0.09, 1.03]
Guo et al. 2010 [20]	5/76	13/60	43.40	0.30	[0.11, 0.80]
Li et al. 2011 [19]	6/252	8/248	24.10	0.74	[0.26, 2.10]
<b>Total (FEM, I<sup>2</sup> = 0%)</b>			<b>100.00</b>	<b>0.41</b>	<b>[0.22, 0.75]</b>
Sensitive analysis					
Xu and Wang 2007 [21]	3/66	12/80	57.30	0.30	[0.09, 1.03]
Guo et al. 2010 [20]	5/76	13/60	42.70	0.30	[0.11, 0.80]
<b>Total (FEM, I<sup>2</sup> = 0%)</b>			<b>100.00</b>	<b>0.30</b>	<b>[0.14, 0.65]</b>
<i>(1.2) CDDP + conventional therapy versus placebo + conventional therapy</i>					
Li et al. 2010 [36]	5/42	4/21	100.00	0.63	[0.19, 2.09]
<i>(1.3) CDDP + conventional therapy versus propranolol + conventional therapy</i>					
<b>Xu and Wang 2007 [21]</b>	<b>3/66</b>	<b>11/72</b>	<b>100.00</b>	<b>0.26</b>	<b>[0.07, 0.99]</b>
<i>(2) Recurrent angina</i>					
<i>(2.1) CDDP + conventional therapy versus conventional therapy</i>					
Guo 2010 [20]	2/76	11/60	33.40	0.14	[0.03, 0.62]
Li et al. 2011 [19]	27/252	54/248	66.60	0.49	[0.32, 0.75]
<b>Total (REM, I<sup>2</sup> = 61%)</b>			<b>100.00</b>	<b>0.33</b>	<b>[0.10, 1.03]</b>
<i>(2.2) CDDP + conventional therapy versus placebo + conventional therapy</i>					
Li et al. 2010 [36]	12/42	11/21	100.00	0.55	[0.29, 1.02]
<i>(3) Readmission</i>					
<i>CDDP + conventional therapy versus placebo + conventional therapy</i>					
Li et al. 2010 [36]	3/42	4/21	100.00	0.38	[0.09, 1.52]



+ Low risk  
 - High risk  
 ? Unclear

FIGURE 8: Risk of bias summary—QOL.

risk of bias. The quality of evidence in the main comparison (CDDP versus no intervention) was low (Table 4).

3.4.6. *Readmission (Table 6)*. Only one study reported readmission in the comparison of CDDP plus conventional therapy versus placebo plus conventional therapy (RR 0.38; 95%CI 0.09 to 1.52; n = 63) [36]. The associated risk of bias is presented in Figure 7.

3.4.7. *Quality of Life (Table 7)*. One study assessed QOL by questionnaire score. The questionnaire was designed referring to TOMHS and SF-36. Compared with placebo group on the basis of conventional therapy, patients in the group treated with CDDP had higher scores (MD 12.60; 95%CI 3.23 to 21.97; n = 63) [36]. The associated risk of bias is presented in Figure 8.

3.4.8. *Left Ventricular Ejection Fraction (Table 7)*. Five studies [19, 21, 33, 34, 36] assessed LVEF in three different comparisons. Meta-analysis (random effects model) of four studies [19, 21, 33, 34] found that CDDP was associated with a statistically significant increase in LVEF compared with no intervention (MD 4.79%; 95%CI 3.31 to 6.28; n = 781). For the significant heterogeneity (I<sup>2</sup> = 51%) among

TABLE 7: Analyses of secondary outcomes.

Outcomes (comparisons)	Treatment			Control			Weight (%)	MD	95% CI
	Mean	SD	N	Mean	SD	N			
<i>(4) LVEF%</i>									
<i>(4.1) CDDP + conventional therapy versus conventional therapy</i>									
Mei et al. 2006 [34]	60.80	7.20	23	59.20	6.80	22	10.50	1.60	[-2.49, 5.69]
Xu and Wang 2007 [21]	51.20	4.30	66	47.10	4.60	80	34.60	4.10	[2.65, 5.55]
Li et al. 2011 [19]	57.10	8.70	252	51.90	9.90	248	31.70	5.20	[3.57, 6.83]
Lin 2011 [33]	54.50	6.80	46	47.80	3.90	44	23.30	6.70	[4.42, 8.98]
<b>Total (REM, I<sup>2</sup> = 51%)</b>							<b>100.00</b>	<b>4.79</b>	<b>[3.31, 6.28]</b>
<i>Subgroup analysis (according to duration of treatment)</i>									
<i>(4.1.1) 30 days–6 weeks</i>									
Li et al. 2011 [19]	57.10	8.70	252	51.90	9.90	248	33.90	5.20	[3.57, 6.83]
Lin 2011 [33]	54.50	6.80	46	47.80	3.90	44	17.40	6.70	[4.42, 8.98]
<b>Subtotal (FEM, I<sup>2</sup> = 9%)</b>							<b>51.30</b>	<b>5.71</b>	<b>[4.38, 7.04]</b>
<i>(4.1.2) 6 months–12 months</i>									
Mei et al. 2006 [34]	60.80	7.20	23	59.20	6.80	22	5.40	1.60	[-2.49, 5.69]
Xu and Wang 2007 [21]	51.20	4.30	66	47.10	4.60	80	43.30	4.10	[2.65, 5.55]
<b>Subtotal (FEM, I<sup>2</sup> = 22%)</b>							<b>48.70</b>	<b>3.82</b>	<b>[2.46, 5.19]</b>
<i>(4.2) CDDP + conventional therapy versus placebo + conventional therapy</i>									
Li et al. 2010 [36]	55.69	9.34	42	50.21	7.83	21	100.00	5.48	[1.10, 9.86]
<i>(4.3) CDDP + conventional therapy versus propranolol + conventional therapy</i>									
Xu and Wang 2007 [21]	51.20	4.30	66	49.60	5.00	72	100.00	1.60	[0.05, 3.15]
<i>(5) Quality of life (score)</i>									
<i>CDDP + conventional therapy versus placebo + conventional therapy</i>									
Li et al. 2010 [36]	110.28	19.33	42	97.68	17.13	21	100.00	12.60	[3.23, 21.97]

the studies, we examined the data and looked over the papers carefully. We found that there was a significant difference in the duration of treatment among the studies. Therefore, we conducted a subgroup analysis according to the duration of treatment. In the subgroup analysis of patients with 30 days to six weeks treatment [19, 33] versus six months to 12 months treatment [21, 34], the test effect still had statistical significant but without significant heterogeneity: MD 5.71% (95%CI 4.38 to 7.04; two studies,  $n = 590$ ;  $I^2 = 9\%$ ) for 30 days to six weeks treatment versus MD 3.82% (95%CI 2.46 to 5.19; two studies,  $n = 191$ ;  $I^2 = 22\%$ ) for six months to 12 months treatment. Compared with placebo on the basis of conventional therapy, CDDP also presented a statistical difference in the increase of LVEF (MD 5.48%; 95%CI 1.10 to 9.86; one study,  $n = 63$ ) [36]. In addition, a single study reported a similar result between CDDP and propranolol (MD 1.60%; 95%CI 0.05 to 3.15;  $n = 138$ ) [21]. Figure 9 presents the associated risk of bias. The quality of evidence in the main comparison (CDDP versus no intervention) was low (Table 4).

**3.4.9. Adverse Events.** One of the seven studies reported adverse events [36]. The authors described that there were mild adverse events in the CDDP group such as blushing (1/63 patient), abdominal distention (2/63 patients), dizziness, and distention of head (2/63 patients). However, all of the adverse events remitted spontaneously. There were no significant differences between the two groups in blood glucose, hepatic

function, and renal function after treatment. The associated risk of bias is presented in Figure 10.

#### 4. Discussion

Seven RCTs including 1215 participants were included in this review. CDDP presented statistically significant benefit on the incidence of cardiac death and heart failure as compared with no intervention based on conventional therapy for AMI. Compared with propranolol, CDDP showed the similar effect on heart failure. In addition, the benefit of CDDP on LVEF was statistically significant both in short-term (30 days to six weeks) and long-term (six months to 12 months) treatment compared with no intervention, placebo, or propranolol. CDDP was also associated with a statistically significant improvement in QOL compared with placebo on the basis of conventional therapy. However, it was not associated with a statistically significant effect on RMI, readmission, or recurrent angina. Unfortunately, no data was available to assess the effect of CDDP on revascularization.

The discrepancy between the effect on all-cause mortality before and after sensitive analysis might be related to the lower quality study [19]. Although CDDP was found to be beneficial for the reduction of all-cause mortality after sensitive analysis, the effect still need to be demonstrated due to the low quality of the evidence.

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Li et al. 2010	?	?	?	?	?	+	?
Li et al. 2011	?	+	-	?	?	-	?
Lin 2011	?	?	?	?	?	+	?
Mei et al. 2006	?	?	?	?	?	+	?
Xu and Wang 2007	?	?	?	?	?	+	?

- + Low risk
- High risk
- ? Unclear

FIGURE 9: Risk of bias summary—LVEF.

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Li et al. 2010	?	?	?	?	?	+	?

- + Low risk
- High risk
- ? Unclear

FIGURE 10: Risk of bias summary—adverse events.

When we mention TCM, often natural products with fewer side effects come to mind. In fact, systematic reviews [22–24] do indicate fewer mild side effects of CDDP for angina pectoris. A latest parallel double blind randomized placebo-controlled trial also showed no significant adverse effects of CDDP for hypercholesterolemia patients [9]. However, in this review, only one study with small simple size described mild adverse events of CDDP with spontaneous remission. Due to the insufficient data, it is too early to evaluate the safety of CDDP for AMI patients at present. We, therefore, suggest detailed description of adverse events in the future studies of CDDP.

We have to consider a number of limitations in this review before recommending the conclusion to clinical practitioners. (1) We might miss some unpublished relevant studies since we only searched unpublished studies from CPCD, CDFD, and CMFD. What is more, we could not create a funnel plot to check for possible publication bias for each outcome due to the low number of included studies. Publication bias might exist in our results. (2) None of the included studies was assessed to be at low risk of bias. The main reasons are as follows: firstly, the method of random sequence generation was unclear in most of the studies, and only one study reported allocation concealment; most of the studies might have selection bias; secondly, no study described double blind method as well as the blinding of outcome assessment; both selection bias and detection bias might exist in the conclusion; thirdly, neither withdrawals nor losses to follow up was reported in each study; this could lead to a high risk of attrition bias; fourthly, one study [19] had selective reporting on cardiac mortality and RMI which should be reported in accordance with its study plan; this could induce reporting bias. In addition, all of the included studies did not mention ITT analysis, which might lead to some other bias. (3) Most of the durations of follow up were short; the reliability and validity of some outcomes such as mortality could be influenced. (4) The small number of included studies and the different comparisons among the studies precluded us from conducting subgroup analyses to explore effect modifiers such as duration of intervention and type of conventional therapy. (5) For some outcomes, only single study provided data and most of the studies did not meet the calculated optimal information size. This might influence the precision of results, which could downgrade the quality of evidence. (6) We assessed the quality of evidence for each outcome according to the GRADE approach with caution. However, the overall quality of evidence in the main comparison was poor, which can weaken the strength of recommendation.

Although this systematic review suggests some benefits of CDDP for AMI patients, the recommendation of findings was limited due to the poor quality studies. Therefore, rigorously designed clinical trials are warranted to further demonstrate the effectiveness and safety of CDDP for AMI. Moreover, we suggest that researchers of RCTs provide complete, clear, and transparent information on their methodologies and findings in the future. This is important for readers or reviewers to assess and use RCTs accurately. Thus, we expect that more RCTs of TCM will be appropriately designed, conducted, and

reported according to the CONSORT statement [37] or the CONSORT statement for herbal interventions [38].

## 5. Conclusion

This systematic review found the following potential benefits from CDDP added to conversional therapy in AMI patients: reduction of cardiac death and heart failure, improvement of QOL and LVEF. However, the benefits should be considered due to the poor quality of evidence. In addition, the safety of CDDP has not been confirmed for the deficiency of available studies. More high quality evidence from high quality RCTs is needed to support the clinical use of CDDP for AMI patients.

## Conflict of Interests

The authors declare no conflict of interests.

## Authors' Contribution

J. Luo developed the search strategy and data extraction form, searched and identified trials, extracted data and analyzed data, and drafted the paper. H. Xu conceived and designed the study, helped with development of the search strategy, identified trials and extracted data, verified data analyses, and revised the paper. K. J. Chen conceived and designed the study, provided methodological perspectives, and revised the paper.

## Acknowledgments

The current work was supported by the National Key Basic Research Program of China (no. 2006CB504803) and the Twelve Five-year Plan of China (nos. 2013BAI02B01 and 2013BAI13B01). The authors thank Jun Xia, researcher of Cochrane Schizophrenia Group, for her kind help in the writing of this review and Stephanie Sampson, researcher of Cochrane Schizophrenia Group, and Yaolong Chen, researcher of Chinese GRADE Center, for advice on the quality assessment in the review.

## References

- [1] H. D. White and D. P. Chew, "Acute myocardial infarction," *The Lancet*, vol. 372, no. 9638, pp. 570–584, 2008.
- [2] P. Libby, "Current concepts of the pathogenesis of the acute coronary syndromes," *Circulation*, vol. 104, no. 3, pp. 365–372, 2001.
- [3] X.-J. Ma, H.-J. Yin, and K.-J. Chen, "Appraisal of the prognosis in patients with acute myocardial infarction treated with primary percutaneous coronary intervention," *Chinese Journal of Integrative Medicine*, vol. 15, no. 3, pp. 236–240, 2009.
- [4] K. J. Chen, K. K. Hui, M. S. Lee, and H. Xu, "The potential benefit of complementary/alternative medicine in cardiovascular diseases," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 125029, 1 page, 2012.
- [5] G. Dobos and I. Tao, "The model of Western integrative medicine: the role of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 11–20, 2011.
- [6] A. S. Ferreira and A. J. Lopes, "Chinese medicine pattern differentiation and its implications for clinical practice," *Chinese Journal of Integrative Medicine*, vol. 17, no. 11, pp. 818–823, 2011.
- [7] H. Xu and K.-J. Chen, "Integrating traditional medicine with biomedicine towards a patient-centered healthcare system," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 83–84, 2011.
- [8] K.-J. Chen and A.-P. Lu, "Situation of integrative medicine in China: results from a National Survey in 2004," *Chinese Journal of Integrative Medicine*, vol. 12, no. 3, pp. 161–165, 2006.
- [9] K. A. O'Brien, S. H. Ling, E. Abbas et al., "A Chinese herbal preparation containing radix salviae miltiorrhizae, radix notoginseng and borneolum syntheticum reduces circulating adhesion molecules," *Evidence-based Complementary and Alternative Medicine*, vol. 2011, Article ID 790784, 6 pages, 2011.
- [10] X.-Y. Ji, B. K.-H. Tan, and Y.-Z. Zhu, "Salvia miltiorrhiza and ischemic diseases," *Acta Pharmacologica Sinica*, vol. 21, no. 12, pp. 1089–1094, 2000.
- [11] C. D. Wing-Shing, C. M. Koon, C. F. Ng et al., "The roots of Salvia miltiorrhiza (Danshen) and Pueraria lobata (Gegen) inhibit atherogenic events: a study of the combination effects of the 2-herb formula," *Journal of Ethnopharmacology*, vol. 143, no. 3, pp. 859–866, 2012.
- [12] J. Leng, C. M. Fu, and F. Wan, "Research progress on chemical compositions and pharmacological effects of panaxatriol saponins," *West China Journal of Pharmaceutical Sciences*, vol. 26, no. 1, pp. 83–86, 2011.
- [13] Z. G. Yang, A. Q. Chen, and S. D. Yu, "Research progress of pharmacological actions of Panax notoginseng," *Shanghai Journal of Traditional Chinese Medicine*, vol. 39, no. 4, pp. 59–62, 2005.
- [14] S. R. Wu, G. Cheng, and Y. Feng, "Progress in studies on pharmacology of borneol," *Chinese Traditional and Herbal Drugs*, vol. 32, no. 12, pp. 90–92, 2001.
- [15] Y. Liu, B. L. Zhang, and L. M. Hu, "General view on pharmacological research of borneol," *Tianjin Journal of Traditional Chinese Medicine*, vol. 20, no. 4, pp. 85–87, 2003.
- [16] Y. Lu and M. J. Jia, "Pharmacological and clinical research of Compound Danshen Dropping Pill in coronary heart disease," *Chinese Heart Journal*, vol. 12, no. 5, pp. 418–419, 2000.
- [17] J. Sun, S. H. Huang, B. K.-H. Tan et al., "Effects of purified herbal extract of Salvia miltiorrhiza on ischemic rat myocardium after acute myocardial infarction," *Life Sciences*, vol. 76, no. 24, pp. 2849–2860, 2005.
- [18] X. Ji, B. K.-H. Tan, Y. C. Zhu, W. Linz, and Y. Z. Zhu, "Comparison of cardioprotective effects using ramipril and DanShen for the treatment of acute myocardial infarction in rats," *Life Sciences*, vol. 73, no. 11, pp. 1413–1426, 2003.
- [19] G. P. Li, X. T. Zheng, H. Z. Wang et al., "Multicenter investigation of Compound Danshen Dripping Pills on short-term clinical events in patient with ST elevation myocardial infarction undergoing primary PCI (MICD-STEMIPCI)," *Chinese Journal of Interventional Cardiology*, vol. 19, no. 1, pp. 24–28, 2011.
- [20] H. Y. Guo, S. F. Lin, and X. Z. Kang, "Effects of Compound Danshen Dripping Pills on reperfusion injury after intravenous thrombolytic therapy in acute myocardial infarction," *Journal of Chinese Physician*, vol. 38, no. 7, pp. 49–50, 2010.
- [21] H. Xu and J. Wang, "Effects of Compound Danshen Dripping Pill on survival rate of patients with acute myocardial infarction," *Proceeding of Clinical Medicine*, vol. 16, no. 6, pp. 429–430, 2007.

- [22] Y. M. Guo, C. Zhang, Q. L. Cha et al., "Compound Salvia Droplet Pill for treatment of coronary heart disease: a systematic review," *Journal of Shanghai University of Traditional Chinese Medicine*, vol. 26, no. 3, pp. 24–31, 2012.
- [23] J. Zhang, M.-Z. Zhang, and L. Wang, "The systematic review of compound Danshen dripping pills in treatment of coronary heart disease," *Chinese Journal of New Drugs*, vol. 18, no. 6, pp. 465–468, 2009.
- [24] W. Chen, L. M. Qin, Z. H. Liu, and Z. R. Xu, "The systematic review of Compound Danshen Dropping Pills in treatment of stable angina pectoris," *Journal of Shandong University of Traditional Chinese Medicine*, vol. 36, no. 4, pp. 287–291, 2012.
- [25] Chinese Society of Cardiology of Chinese Medical Association and Editorial Board of Chinese Journal of Cardiology, "Guideline for the diagnosis and treatment of patients with acute myocardial infarction," *Chinese Journal of Cardiology*, vol. 29, no. 12, pp. 9–24, 2001.
- [26] E. Braunwald, E. M. Antman, J. W. Beasley et al., "ACC/AHA guidelines for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction: executive summary and recommendations: a report of the American College of Cardiology/American Heart Association task force on practice guidelines (committee on the management of patients with unstable angina)," *Circulation*, vol. 102, no. 10, pp. 1193–1209, 2000.
- [27] Chinese Society of Cardiology of Chinese Medical Association and Editorial Board of Chinese Journal of Cardiology, "Guideline for diagnosis and treatment of patients with unstable angina and non-ST-segment elevation myocardial infarction," *Chinese Journal of Cardiology*, vol. 35, no. 4, pp. 295–304, 2007.
- [28] Chinese Society of Cardiology of Chinese Medical Association and Editorial Board of Chinese Journal of Cardiology, "Recommendation of the application of universal definition of myocardial infarction in China," *Chinese Journal of Cardiology*, vol. 36, no. 10, pp. 867–869, 2008.
- [29] Chinese Society of Cardiology of Chinese Medical Association and Editorial Board of Chinese Journal of Cardiology, "Guideline for diagnosis and treatment of patients with ST-elevation myocardial infarction," *Chinese Journal of Cardiology*, vol. 38, no. 8, pp. 675–687, 2010.
- [30] J. P. T. Higgins and S. Green, *Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0*, The Cochrane Collaboration, 2011, <http://handbook.cochrane.org/>.
- [31] J. Brozek, A. Oxman, and H. Schünemann, "GRADEpro. [Computer program]. Version 3.2 for Windows," 2008, <http://ims.cochrane.org/revman/other-resources/gradepro>.
- [32] J. P. T. Higgins, S. G. Thompson, J. J. Deeks, and D. G. Altman, "Measuring inconsistency in meta-analyses," *British Medical Journal*, vol. 327, no. 7414, pp. 557–560, 2003.
- [33] X. D. Lin, "Clinical trial of Compound Danshen Dripping Pill on ventricular remodeling after acute myocardial infarction," *World Chinese Medicine*, vol. 6, no. 2, pp. 111–112, 2011.
- [34] F. G. Mei, Y. Q. Zhang, Z. L. Wang, and G. Zhao, "Effects of Compound Danshen Dripping Pill on BNP and left ventricular function in patients with acute myocardial infarction," *Heilongjiang Journal of Traditional Chinese Medicine*, vol. 6, pp. 4–6, 2006.
- [35] Y. H. Ma, *Relationship Between Serum Level of Tumor Necrosis Factor-Alpha, Matrix Metalloproteinase-9 and Interleukin-6 and Clinical Prognosis and the Effects of Compound Danshen Dripping Pill on STEMI*, Tianjin Medical University, Tianjin, China, 2010.
- [36] X. F. Li, F. Liu, T. L. Jiang, Z. J. Wang, and Z. H. Wu, "Efficacy of compound Danshen drop pill 42 patients with early acute myocardial infarction," *Chinese Journal of New Drugs*, vol. 19, no. 18, pp. 1699–1702, 2010.
- [37] K. F. Schulz, D. G. Altman, and D. Moher, "CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials," *Annals of Internal Medicine*, vol. 152, no. 11, pp. 726–732, 2010.
- [38] J. J. Gagnier, H. Boon, P. Rochon, D. Moher, J. Barnes, and C. Bombardier, "Reporting randomized, controlled trials of herbal interventions: an elaborated CONSORT statement," *Annals of Internal Medicine*, vol. 144, no. 5, pp. 364–367, 2006.

## Research Article

# The Effect of Ouhyul Herbal Acupuncture Point Injections on Shoulder Pain after Stroke

Yu-Ri Seo,<sup>1</sup> Woo-Sang Jung,<sup>2</sup> Seong-Uk Park,<sup>1</sup> Sang-Kwan Moon,<sup>1</sup>  
Jung-Mi Park,<sup>1</sup> and Joo-Young Park<sup>1</sup>

<sup>1</sup> Department of Cardiovascular & Neurologic Diseases, College of Korean Medicine, Kyung-Hee University, Seoul, Republic of Korea

<sup>2</sup> Department of Cardiovascular and Neurologic Diseases, Hospital of Oriental Medicine, Kyung Hee University, 1 Hoegi, Seoul 130-702, Republic of Korea

Correspondence should be addressed to Woo-Sang Jung; [wsjung@khu.ac.kr](mailto:wsjung@khu.ac.kr)

Received 20 October 2012; Revised 20 May 2013; Accepted 21 May 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Yu-Ri Seo et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

An effective and safe remedy for shoulder pain is needed as shoulder pain is a common complication of stroke and restricts recovery of patients. This study was carried out to evaluate the effect of Ouhyul herbal acupuncture point injection (O-API) on shoulder pain in patients with stroke. Twenty-four participants with shoulder pain after stroke were recruited and randomized to the O-API and control groups. Treatment was conducted for 2 weeks three times per week. We evaluated the effects of treatment with a numerical rating scale (NRS), painless passive range of motion (PROM) of external shoulder rotation, and the Fugl-Meyer Motor Assessment (FMMA) at baseline, each week, and 1 week after the final treatment. All measures were similar between the O-API and control groups at baseline. The O-API group showed significant improvement on the NRS compared with that in the control group after 2 weeks of treatment, and the treatment effect was maintained until the follow-up period. PROM decreased significantly in both groups, but the reduction was maintained only in the O-API group. No significant difference was observed on the FMMA between the two groups. O-API resulted in significant improvement in shoulder pain after stroke, and its effect was maintained after termination of treatment without any severe side effects.

## 1. Introduction

Shoulder pain is one of the most common stroke complications (9–84%) [1, 2]. Various controversial theories about the mechanisms generating shoulder pain after stroke have been proposed, but the most plausible theories attribute the cause to subluxation, which occurs easily after stroke because of the structural characteristics of the shoulder, spasticity, and contracture that originate as a change in muscle balance after an upper motor neuron disorder. Rotator cuff abnormalities and flaccid muscles near the glenohumeral joint are prone to trauma [3].

Poststroke shoulder pain is generally treated with non-steroidal anti-inflammatory drugs, steroid injections, positioning and handling, shoulder strapping, or electrical stimulation, but these are imperfect methods for treating shoulder pain after stroke because of their side effects, insufficient effect duration, and statistically insignificant effects [4].

Patients with stroke and severe shoulder pain are apt to withdraw from rehabilitation programs [5], stay longer in the hospital [6], and complain of a poor quality of life [7]; thus, an effective remedy for reducing shoulder pain after stroke is strongly needed to promote rehabilitation treatment. Ouhyul herbal acupuncture point injection (O-API) is a treatment used in Korean medical hospitals, in which a herbal drug-like plant extract is injected at acupuncture points.

Therefore, in this study, we evaluated the effect of O-API on shoulder pain after stroke by measuring pain relief, functional improvement in the shoulder joint, and the increase in shoulder range of motion.

## 2. Materials and Methods

**2.1. Study Design.** This was a prospective, randomized, double-blind, and controlled clinical trial conducted from

September 2010 to February 2012 at the Kyung-Hee Korean Medical Center, Korea.

Participants were randomly allocated to the O-API or the normal saline- (NS-) API groups. They were randomized off-site using a blocked stratified procedure that consisted of a block size of four with one stratum and two groups with different baseline NRS scores ( $>5$  and  $\leq 5$ ). The randomization was conducted by an independent physician who was not involved in the inclusion or exclusion process, treatment, or assessment procedures. We use the result of the study about the effect of Bee venom acupuncture point injection on the shoulder pain after stroke as a significant level of more than 95% and power of 80%, and we decided to recruit 30 patients for the study.

Participants were screened for eligibility using a chart review and interview on the first visit and randomly assigned to the groups. They were treated by the O-API or the NS-API three times per week during the 2-week treatment. Effectiveness was assessed every week, and participants received at least four treatments. Participants attended a follow-up session 1 week after the final treatment and were assessed.

**2.2. Study Participants.** The study participants were recruited by referral from Korean medical doctors at Kyung-Hee Korean Medical Center. Patients were recruited through the advertisement.

Participants were informed about the study protocol and allowed to be included in this study after their written consent. This study protocol was approved by the Institutional Review Board of Kyung-Hee Korean Medical Center (KOMC IRB 2010-10).

Inclusion criteria were (1) shoulder pain with a NRS  $> 2$ , (2) alert mental status and ability to answer the survey, (3) no treatment plans that would affect shoulder pain during the study, and (4) consent to join the study.

Exclusion criteria were (1) patients with any infection, abscess, and those who were unable to receive invasive treatment due to unstable vital signs, (2) a history of fracture or trauma at the shoulder prior to stroke onset, and (3) patients who complained of shoulder pain as a result of fracture or trauma after stroke.

And we recruited patients without division into acute or subacute.

**2.3. Intervention.** The O-API was prepared at Kyung-Hee Korean Medical Center and the NS-API was produced at JW Pharmaceutical, a Korean drug manufacturing company. O-API consists of eight herbal medicines, including 20 g *Gardeniae Fructus*, 8 g *Corydalis Tuber*, 8 g *olibanum*, 8 g *myrrha*, 6 g *Persicae Semen*, 6 g *Paeoniae Radix Rubra*, 6 g *Salviae Miltiorrhizae Radix*, and 4 g *Sappan Lignum*. The herbs were placed in 660 mL distilled water and refluxed for 2 hours, and the liquefied steam was collected in a flask. The mixture was brought up to 200 mL with distilled water, and 1.8 g NaCl was added. The solution was filtered and subdivided into 20 mL sterilized bottles, closed with an aluminum cap, and sterilized in an autoclave. There are studies about herbal acupuncture point injection that herbal

acupuncture point injection has an effect on headache, cervical spondylosis, and knee osteoarthritis.

We followed the STRICTA guidelines. Korean API is based on the modern innovation of traditional acupuncture and strengthens and sustains the effects of acupuncture. In this study, five needles were inserted per participant per session and unilateral LI 15, TE 14, GB 21, SI 11, and SI 12 were used as acupuncture points. Needles were applied to the subcutaneous tissue at the acupoints. No muscle twitch response was observed, but participants felt a needling sensation as the fluid was injected. After injecting 0.1 cc at each point, the 30-gauge needle was pulled out immediately. These processes were also adjusted to normal saline group. Participants received two treatment sessions and were observed at one follow-up session. Each session was provided after a 1-week interval, and participants received three treatments per session. Participants could receive any other treatment but they should not change treatments likely to affect their shoulder pain from 1 week before the start of our study to the follow-up session. The practitioner in this study had 6 years of education and 2 years of clinical experience working at Kyung-Hee Korean Medical Center.

A placebo effect may be associated with pain control, but the NS injection only had sodium chloride as the control.

**2.4. Outcome Measures.** Participants were evaluated by the numerical rating scale (NRS), painless passive range of motion (PROM) of external shoulder rotation, and the Fugl-Meyer Motor Assessment (FMMA) and the McGill Pain Questionnaire-short form (MPQ-SF) at each visit by the same examiner blinded to group allocation. The NRS score revealed subjective pain intensity level from 0 (no pain) to 10 (the most intense pain imaginable).

The MPQ-SF is a multidescriptive measure of pain that consists of 22 pain questions. It reveals the nature of shoulder pain.

PROM is the painless passive range of motion of shoulder external rotation. Participants were placed in a supine position with 45° abduction of the shoulder joint and 90° flexion of the elbow joint with the forearm pronated. The examiner externally rotated the shoulder until the subjects felt pain and measured the angle between the line connecting the olecranon to the styloid process of the ulna and the horizontal plane. PROM is an objective pain level measure. We assessed PROM with goniometer after treatment in each session. The normal range of PROM is 0–90 degrees. And PROM decreases as symptoms are improved.

FMMA is a scale that shows motor function of the upper extremities and consists of assessment items segmented for several extremity regions. Unlike other assessment scales, it reveals shoulder function.

Detailed information on these measurement items and relevant scores are listed in the Appendix (see Supplementary Material available online at <http://dx.doi.org/10.1155/2013/504686>).

**2.5. Statistical Analysis.** The analysis was based on a per-protocol basis. Between-group differences in demographic

TABLE 1: Baseline demographic and clinical characteristics of the participants.

Characteristic	O-API	NS-API	P value
Age (years)	63.8 ± 10.8	67.4 ± 7.8	0.416
Sex (male : female)	6 : 7	4 : 7	0.697
Duration of disease (day)	56.5 ± 29.7	53.5 ± 26.7	1.000
Number of treatment times	5.7 ± 0.7	5.6 ± 0.8	0.858
The other treatment for shoulder pain after stroke (no.)	3	1	0.596
Western medicine	1	1	1.000
Herbal medicine	0	0	1.000
Physical treatment	2	0	1.000
Acupuncture and moxibustion	0	0	1.000
Stroke type (infarction : hemorrhage)	11 : 2	5 : 6	0.082
Stroke recurrent (no.)	1	1	1.000
Underlying disease (no.)			
Hypertension	8	11	0.041
Diabetes	4	3	1.000
Dislipidemia	7	2	0.105
Heart disease	1	2	0.576

and baseline characters were tested with the chi-square test for categorical variables and the Mann-Whitney  $U$ -test for continuous variables. Although this trial's sample size was small, we confirmed that our results were normally distributed using the Kolmogorov-Smirnov test. We used the paired  $t$ -test for within-group comparisons and the independent  $t$ -test for between-group comparisons and to check for changes in each outcome measure. All results are expressed as mean  $\pm$  standard deviation. SPSS 12.0 for Windows was used for the analyses (SPSS, Inc., Chicago, IL, USA). A  $P < 0.05$  was considered significant.

### 3. Results

**3.1. Baseline Participant Characteristics.** Twenty-nine participants were included. Three participants in the O-API group and two in the NS-API group withdrew or were lost to followup at 5 days after the endpoint of the study. Thirteen participants in the O-API group and 11 in the NS-API group completed the interventions and provided complete data at the followup. Therefore, 24 participants were included in the analysis. Table 1 shows the baseline characteristics of the study population. No differences were observed in the baseline characteristics between the groups.

The participants usually described their pain as "heavy, splitting, fearful, or hot-burning" on the MPQ-SF.

**3.2. Changes in NRS Scores.** NRS scores were not significantly different between the groups at baseline. A significant decrease in NRS score was observed in the O-API group after treatment but not in the NS-API group ( $P = 0.267$ ). The significant difference in the NRS score in the O-API group ( $P = 0.000$ ) was maintained at the 1-week followup, whereas NRS score in the NS-API group showed no decrease compared with that at baseline.

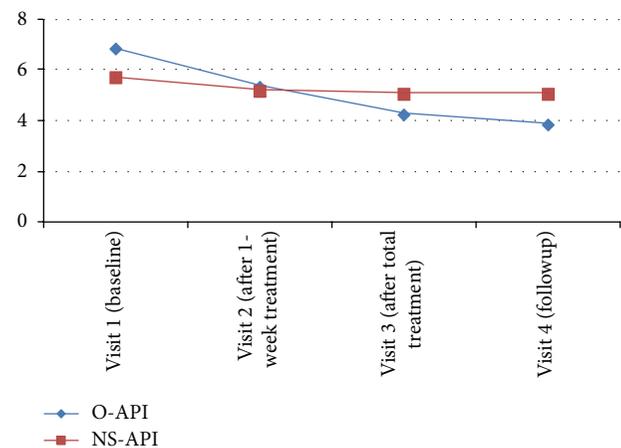


FIGURE 1: Numerical rating scale (NRS) scores in each group during the trial.

The O-API group demonstrated a significant change according to the independent  $t$ -test compared with that in the NS-API group (Figure 1).

**3.3. Changes in PROM Scores.** The PROM score in the O-API group was similar ( $48.85 \pm 21.03$ ) to that in the NS-API group ( $33.55 \pm 19.43$ ) prior to treatment. Significant decreases in PROM scores were observed compared with those at baseline in both groups. PROM score decreased after treatment in the O-API group ( $12.23 \pm 10.96$ ) but was not different from that in the NS-API group ( $5.36 \pm 7.66$ ).

However, the decrease in PROM in the O-API group from baseline to the 1-week follow-up period was significant, whereas that in the NS-API group was not (Figure 2).

**3.4. Changes in FMMA Scores.** Baseline FMMA scores were not different between the two groups. FMMA scores in both

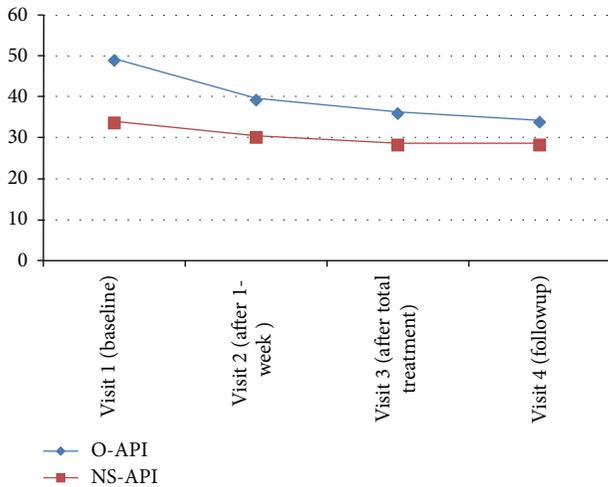


FIGURE 2: Passive range of motion (PROM) of external shoulder rotation in each group.

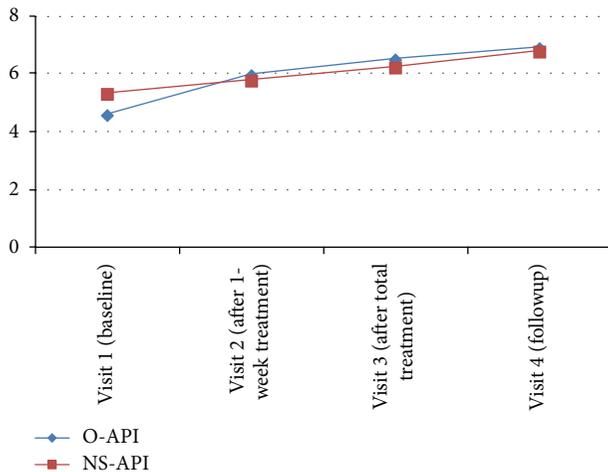


FIGURE 3: Fugl-Meyer Motor Assessment (FMMA) in each group.

groups increased significantly after treatment, and the increases were significantly different between the two groups. The O-API group ( $1.92 \pm 1.19$ ) demonstrated greater improvement than that in the NS-API group ( $0.91 \pm 1.04$ ) ( $P = 0.039$ ).

The increases on the FMMA in the two groups from baseline to the 1-week follow-up period were significantly sustained (Figure 3).

**3.5. Adverse Events.** One of the 16 O-API participants complained of pantalagia during the study period. Among 13 subjects, one in the NS-API group had transient local site pain and one complained of fatigue.

No serious adverse events were reported.

We assessed the adverse effect with the subjective and objective symptoms that participants complain. And we considered of them in a mild level when patients do not need the treatment, a moderate level when patients need

the treatment without dropout, and a severe level when patients need treatment with drop out.

## 4. Discussion

This study was a well-designed randomized clinical trial to evaluate the effects of O-API to treat shoulder pain after stroke. As a result, we confirmed that O-API not only reduced shoulder pain after stroke but also rehabilitated functional disorders of a hemiplegic extremity based on the results of the NRS and FMMA.

The O-API group showed significant improvement on then NRS compared with that in the NS-API group and the improvement was maintained until the follow-up period (1 week after the end of the study).

FMMA and PROM showed significant improvements in both groups at the final treatment session. A significant effect was detected in the PROM scale of the O-API group compared with that in the control group, indicating that both O-API and NS-API had an effect on shoulder range of motion and function of painful shoulders but that O-API showed more improvement in functional recovery than that of NS-API.

When we checked the scale at 1 week after the end of the study, the PROM effect in the O-API group was maintained at the followup but both treatments had a similar significant effect on the FMMA. This result indicates that O-API has a continuous effect on PROM compared with that of NS-API but that both NS-API and O-API resulted in continuous improvement in shoulder joint function.

O-API had a better effect on subjective pain sensation than that of NS-API and its effects were maintained. O-API and NS-API both improved limits of shoulder range of motion, but O-API had a more lasting effect. O-API has more significant improvement than that of NS-API in shoulder joint function but only during the treatment period.

We chose O-API as an alternative treatment because of its pain control effect [8], but no experimental studies are available about how O-API improves shoulder pain after stroke. Some clinical trials have shown that O-API has a significant analgesic effect on somatic pain such that from whiplash injury [9] and low back pain [10]. Several studies have reported the effects of O-API in rats. For example, acupuncture point injection of olibanum (one of the O-API components) has an analgesic effect by changing the amount of serotonin [11]. Cho et al. reported that when a Gardeniae Fructus aqueous extract is injected into the sprained ankle of rats, it improves stepping force of the ankle-sprained limb and decreases paw edema produced by ankle sprain. These analgesic effects on ankle sprain pain can be explained due to the regulation of nitric oxide by suppressing inducible nitric oxide synthase and cyclooxygenase-2 (COX-2) protein expression [12].

Furthermore, a report about the analgesic effect of an acetic acid *Persicaria Semen* extract on mice exposed to a hot plate has been published [13].

In addition, studies about the oral administration of each herbal medicine in the *Ouhyul* herbal acupuncture mixture reveal a pain-controlling effect. A *Salvia miltiorrhiza* extract

decreases tumor necrosis factor- $\alpha$  and COX-2 mRNA *in vitro* [14]. Paeoniae Radix improved pain in an *in vivo* experiment that used the stretching and Randall-Selitto methods [15].

Some limitations of this study should be mentioned. The follow-up duration was too short, and it was unclear how to treat the shoulder pain with O-API after stroke. Experimental studies about which herbs in O-API are responsible for the shoulder pain effects are needed. Regardless of these limitations, this trial was important because it evaluated shoulder joint function and the pain and duration of the effect unlike previous O-API studies.

## 5. Conclusion

The results showed that the O-API was effective for shoulder pain after stroke without critical side effects. Sustainability of the treatment effect was also demonstrated in this trial; thus, we conclude that O-API is a suitable therapy for shoulder pain. A larger, longer term follow-up study is needed to confirm the results.

## Acknowledgments

The authors would like to thank all the professors and coworkers in the department for their help. And they do not have a direct financial support from any other companies or organizations and there was no conflict of interests in this study.

## References

- [1] I. Lindgren, A.-C. Jönsson, B. Norrving, and A. Lindgren, "Shoulder pain after stroke: a prospective population-based study," *Stroke*, vol. 38, no. 2, pp. 343–348, 2007.
- [2] D. C. Poulin, A. Barsauskas, B. Berenbaum et al., "Painful shoulder in the hemiplegic and unilateral neglect," *Archives of Physical Medicine and Rehabilitation*, vol. 71, no. 9, pp. 673–676, 1990.
- [3] M. Murie-Fernández, M. Carmona Iragui, V. Gnanakumar, M. Meyer, N. Foley, and R. Teasell, "Painful hemiplegic shoulder in stroke patients: causes and management," *Neurologia*, vol. 27, no. 4, pp. 234–244, 2012.
- [4] C. I. M. Price, "Shoulder pain after stroke: a research challenge," *Age and Ageing*, vol. 31, no. 3, pp. 36–38, 2002.
- [5] R. M. Braun, F. West, V. Mooney, V. L. Nickel, B. Roper, and C. Caldwell, "Surgical treatment of the painful shoulder contracture in the stroke patient," *Journal of Bone and Joint Surgery A*, vol. 53, no. 7, pp. 1307–1312, 1971.
- [6] C. W. Roy, M. R. Sands, L. D. Hill, A. Harrison, and S. Marshall, "The effect of shoulder pain on outcome of acute hemiplegia," *Clinical Rehabilitation*, vol. 9, no. 1, pp. 21–27, 1995.
- [7] M. Widar, G. Ahlström, and A.-C. Ek, "Health-related quality of life in persons with long-term pain after a stroke," *Journal of Clinical Nursing*, vol. 13, no. 4, pp. 497–505, 2004.
- [8] J.-M. Park, S.-U. Park, W.-S. Jung, and S.-K. Moon, "Carthami-Semen acupuncture point injection for chronic daily headache: a pilot, randomised, double-blind, controlled trial," *Complementary Therapies in Medicine*, vol. 19, no. 1, pp. S19–S25, 2011.
- [9] S. Hyun, S. Beom-Ryong, S. Min-Seop, and Y. Tae-Han, "Effects of jungsongouhyul herbal acupuncture(JSO) multi-treatment for whiplash injury by traffic accident," *Journal of Korean Institute of Herbal-Acupuncture*, vol. 8, pp. 59–65, 2005.
- [10] L. Sung-hwan, K. Min-wan, L. Hyun, and L. So-yol, "Effectiveness of Bee-venom acupuncture and ouhyul herbal acupuncture in herniation of nucleus pulposus-comparison with acupuncture therapy only," *The Journal of Korean Acupuncture & Moxibustion Society*, vol. 24, pp. 197–205, 2007.
- [11] H. Lee and S. Lim, "Effects of olibanum aqua-acupuncture on analgesia and the contents of serotonin in the several regions of brain in rats," *The Journal of Korean Oriental Medicine*, vol. 14, pp. 246–261, 1997.
- [12] K. S.-T. Cho, M.-S. Park, S. - Kim, Y.-T. Park, K.-J. Kim, and K.-S. Sohn, "In-Cheul. Effect of Frutuscardeniae herbal acupuncture on the rat model of ankle sprain pain," *The Korean Society of Meridian & Acupoint*, vol. 22, pp. 57–74, 2005.
- [13] D. H. Kim, K. S. Lee, and B. K. Song, "A study on the analgesic and anticoagulative effects of pericae semen and carthamiflos of aqua-acupuncture," *The Society of Oriental Obstetrics and Gynecology*, vol. 13, pp. 60–73, 2000.
- [14] K. Hee-Eun, M. Sang-Yeon, and K. Jang-Hyun, "In vitro study of anti-inflammatory and analgesic effects of salvia miltiorrhiza (SM) extracts using luciferase reporter gene assay," *The Journal of Korean Oriental Medicine*, vol. 29, pp. 88–99, 2008.
- [15] H. Nam-Doo, K. Chong-Woo, and S. Hyun-Dae, "A study on the analgesic and anti-convulsional effect of paeoniae radix," *The Korean Society of Pharmacognosy*, vol. 10, pp. 119–124, 1979.

## Review Article

# Cardiovascular Effects of Salvianolic Acid B

**Jie Wang, Xingjiang Xiong, and Bo Feng**

*Department of Cardiology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Xicheng District, Beijing 100053, China*

Correspondence should be addressed to Bo Feng; [fengbo263@163.com](mailto:fengbo263@163.com)

Received 17 January 2013; Accepted 11 May 2013

Academic Editor: Keji Chen

Copyright © 2013 Jie Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Salvianolic acid B (SAB, Sal B) is the representative component of phenolic acids derived from the dried root and rhizome of *Salvia miltiorrhiza* Bge (Labiatae) which has been used widely and successfully in Asian countries for clinical therapy of various vascular disturbance-related diseases for hundreds of years. However, its exact cardioprotective components and the underlying mechanism for therapeutic basis are still poorly understood. This paper discussed and elucidated the underlying biological mechanisms and pharmacology of Sal B and their potential cardioprotective effects.

## 1. Introduction

Salvia is the *Salvia miltiorrhiza*'s dried roots and rhizomes which belongs to plants of Labiatae *Lagurus* grass species (see Figure 1). The herb is some bitter and slightly cold in flavor and enters heart and liver meridian. Salvia is a commonly used herbal medicine for “invigorating” the blood and reducing blood clotting in eastern countries, particularly in China. Currently, it is widely used for the treatment of cardiovascular diseases (CVDs) and cerebrovascular diseases [1–3] and is gaining more and more popularity both in eastern and western countries, including the United States, European countries, and so forth. Furthermore, it can exert protecting effect on liver [4–6], kidneys [7–9], and lungs [10, 11], especially improving ischemia- /reperfusion- (I-/R-) induced injury. According to the theory of traditional Chinese medicine (TCM), it has the effect of promoting blood circulation to clear blood stasis, regulating menstruation and relieving analgesia, clearing heart fire and calming the nerves. Modern pharmacology studies have shown that Salvia has many pharmacological effects, such as increasing coronary blood flow, reducing excitability and conductivity of myocardial, protecting against myocardial ischemic/reperfusion injury, improving microcirculation, antiplatelet aggregation and thrombosis, protecting and improving the kidney function, and reducing blood viscosity as well as antibacterial, anti-inflammatory and antioxidant protection against brain tissue I/R injury.

Salvia has been used for various diseases related to blood stasis syndrome in China for thousands of years, and now, it is widely used for CVDs [12]. During the past 60 years, much significant progress has been made from theory, experiments to clinic fields based on the inherit, and innovation of thoughts in TCM to clarify the treatment principle and method of Salvia and Salvia preparations, which has already got consensus and increasing popularity in medical community in China. Currently, a growing number of medical researchers have focused on the chemical constituents of Salvia. Main chemical constituents of *Salvia miltiorrhiza* roots extract are classified into two major categories: water-soluble compounds (WSC) and lipophilic diterpenoid quinines (LDQ) [13]. According to the pharmacological structure of phenolic acid compounds, we can further divide the WSC and LDQ into single phenolic acids (protocatechuic aldehyde, protocatechuic acid, caffeic acid, and 3,4-dihydroxyphenyl lactic acid) and polyphenolic acids (rosmarinic acid, lithospermic acid, salvianolic acid A, salvianolic acid B, and other salvianolic acids). The major LDQs are tanshinone I (TsI), tanshinone IIA (TsIIA), tanshinone IIB (TsIIB), and other tanshinones [14]. In recent years, studies both in vivo and in vitro have confirmed that salvianolic acid can regulate the signal transduction pathways of vascular endothelial cells, vascular smooth muscle cells, and cardiac cells to prevent and treat cardiovascular damage [15]. Currently, preparations derived from Salvia are widely used in clinical treatment with symptoms or diagnosis of coronary



(a) Portion above ground



(b) Roots for pharmaceutical use

FIGURE 1: Morphology of *Salvia miltiorrhiza*.

heart disease, chest tightness, and angina embolism. The most commonly used formulations of *Salvia* are injections, dipping pills, and so on, including, Danshen injection, the *Salvia* infusion injection, the salvianolate injection (lyophilized), Xiang Dan injection, Danxiang Guanxin injection, Danhong injection (infusion injection), and Danshen dripping pill (DSP). Currently, with increasing studies on *Salvia miltiorrhiza* including randomized controlled trials (RCTs) and systematic reviews (SRs), DSP which is composed of *Salvia miltiorrhiza* (Danshen) is apparently more effective than ISDN (isosorbide dinitrate) in treating angina pectoris [16]. As we know, the main constituents of *Salvia* are water-soluble components, such as 3,4-dihydroxyphenyl lactic acid (also called danshensu), salvianolic acid A, salvianolic acid B, and so on. Salvianolic acid B is the main constituent of *Salvia* phenolic acid and the most active constituent of water-soluble salvianolic acid substances. *Salvia* phenolic acids could elevate the ability of antioxidation, affect the blood lipid metabolism, and inhibit the generation atherosclerosis, which basically represents the traditional role of the activating blood circulation and dissolving stasis of *Salvia* in

TCM [17]. Salvianolic acid B, also known as satanic acid B or lithospermic acid B, is a condensate of three molecules danshennol and one molecule of caffeic acid (see Figure 2). It is a pale yellow amorphous powder in character. In this paper, the pharmacology of salvianolic acid B in the treatment for CVDs was reviewed.

## 2. Cardiovascular Pharmacology

**2.1. Antioxidant Effect.** The oxidative stress of organism can produce a large number of reactive oxygen species (ROS), which can lead to ischemic cardiomyopathy through direct or indirect way. So the antioxidative stress is an important part of protecting ischemic myocardium. Salvianolic acid B is a new generation of the natural antioxidants, which typically presents in the form of a metal salt, especially, magnesium salts. This compound has a plurality of phenolic hydroxyl group, so it has strong antioxidant activity. There are at least six experiments demonstrate the antioxidant role of salvianolic acid B. It can influence  $Ca^{2+}$  aggregation and endothelial cell NO release of hypoxia/reoxygenation-induced cell. When acid B concentration is 2.5, 5, and 10 mg/l, cell viability and superoxide dismutase (SOD) activity are enhanced, and the formation of malondialdehyde (MDA) in human umbilical vein endothelial cells (ECV304) is inhibited. Hypoxia/reoxygenation stimulation can increase the expression of human umbilical vein endothelial intracellular  $Ca^{2+}$  concentration, NO release, and eNOS mRNA, but reduce the expression of iNOS mRNA. SalB can alleviate damage of the hypoxia/reoxygenation stimulation to ECV304 cell, increase the release of NO which is closely related to alleviation of cell damage [18]. SalB inhibits HG-induced oxidative stress and reduces the generation of ROS and 8-hydroxy-2-deoxyguanosine (8-OHDG) and mitochondrial depolarization and apoptosis in a dose-dependent manner. It can downregulate the expression of Bax and AIF nuclear translocation and cytochrome c release mediated by HG, but upregulate the expression of Bcl-2 induced by HG. Besides, SalB attenuated HG-induced caspase of the enzyme 3, 9 and minimize PARP cleavage of Schwann cells (SCs). SalB antagonist oxidative stress, mitochondrial activation pathway, and apoptosis of SCs are induced by high glucose [19]. SalB inhibits angiotensin II or  $H_2O_2$  and TNF- $\alpha$ -induced gelatinolytic activity in human aortic smooth muscle cells (HASMCs) in a concentration-dependent manner because salvianolic acid B scavenged  $H_2O_2$  in a dose-dependent manner in test tube [20]. One research showed that both SalB and EGb 761 were able to scavenge  $O_2^+$  and OH, inhibit lipid peroxidation of microsomes, and protect SH-SY5Y cells against  $H_2O_2$ -induced oxidative damage. SalB exerts more antioxidant efficiency than EGb 761 [21]. Both salvianolic acid B and danshensu exhibit higher scavenging activities against free hydroxyl radicals (HO), superoxide anion radicals ( $O_2^-$ ), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals, and 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals and weaker iron chelating and hydrogen peroxide ( $H_2O_2$ ) scavenging activities than vitamin C [22]. Antioxidant effect of salvianolic acid B in vitro is shown in Table 1.

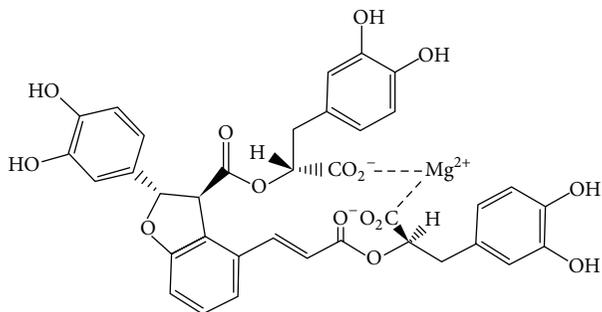


FIGURE 2: Salvianolic acid B-Major water-soluble compounds derived of *Salvia miltiorrhiza*.

TABLE 1: Antioxidant effect of salvianolic acid B in vitro.

Cells/tissues	Effects	Reference
ECV304	Activity of SOD, release of NO, aggregation of $Ca^{2+}$ .	Luo et al., 2002 [18]
SCs	Generation of ROS, generation of 8-OHDG, expression of Bax, AIF nuclear translocation, cytochrome C release, caspase of the enzyme 3, 9, cleavage of PARP, expression of Bcl-2.	Sun et al., 2012 [19]
HASMCs	Activity of gelatinolytic, scavenge $H_2O_2$ .	Zhang and Wang, 2006 [20]
SH-SY5Y	Scavenge $O_2^+$ and OH, reduce oxidative damage.	Liu et al., 2006 [21]

Note: HEK293T cell: human embryonic kidney cells; HO-1: heme oxygenase-1; ROS: reactive oxygen species; Nrf2: nuclear factor 2-related factor 2; SOD: superoxide dismutase; MDA: malondialdehyde; ECV304: human umbilical vein endothelial cells; TMP: tetramethylpyrazine; HASMCs: human aortic smooth muscle cells; 8-OHDG: 8-hydroxy-2-deoxyguanosine; SCs: Schwann cells; MMP-2: matrix metalloproteinase-2; PARP: poly ADP-ribose polymerase.

**2.2. Antiplatelet Aggregation, Anticoagulant, and Antithrombotic Effect.** Platelet plays a key role in platelet thrombosis. Many thrombotic diseases have hyperthyroidism characteristics of aggregation of platelet releasing, so inhibiting of platelet aggregation is of great significance for the prevention of CVDs. Previous studies have shown that salvianolic acid B can inhibit platelet aggregation and adhesion, and the progression is related to integrin  $\alpha 2\beta 1$ , but the specific mechanism of action is still unclear. Salvianolic acid B controlled more than 20 kinds of protein expression, such as 70 kDa heat shock protein, forest domain protein CLP36 copine I, peroxiredoxin-2, coronin-1 B, and cytoplasmic dynein intermediate chain 2C. The experiments predict and verify that integrin  $\alpha 2\beta 1$  may be the target of salvianolic acid B. Integrin protein signaling cascade network includes regulation intracellular levels of  $Ca^{2+}$  and cytoskeleton-related proteins such as coronin-1B and cytoskeleton structure of platelets [23]. SalB inhibits platelet aggregation and activation in patients and stabilizes plaque by reducing MMP-9 and

improve prognosis [24]. Other studies about salvianolic acid B on eNOS activity of platelet endothelial cell platelet concluded that certain dose concentration range (<10 mg/l) of salvianolate can significantly increase eNOS activity and promote the production of L-citrulline and the release of NO, which can inhibit  $Ca^{2+}$  transmembrane transport of platelet to inhibit platelet aggregation and thrombosis [25–27]. SAB and tetramethylpyrazine (TMP) could inhibit shear-induced platelet aggregation (SIPA) with a dose-dependent manner in SD rats. Magnesium lithospermate B (MLB) inhibits the aggregation and 5-HT release in rabbit platelets and attenuates intracellular calcium concentration by inhibiting the rise of  $[Ca^{2+}]_i$  in thrombin stimulated platelets but decreases the  $[Ca^{2+}]_i$  in resting platelets [28]. SAB inhibited static platelet adhesion to a synthetic peptide specific for the collagen receptor  $\alpha 2\beta 1$  and binding of an antibody against  $\alpha 2\beta 1$  to platelets and inhibited the interaction of soluble  $\alpha 2\beta 1$  to immobilized collagen in a solid phase [29]. Antiplatelet aggregation, anticoagulant, and antithrombotic effect of salvianolic acid B in vitro and in vivo is shown in Table 2.

**2.3. Promoting Cardiac Angiogenesis.** Coronary revascularization surgery has resolved the problem of epicardial vascular occlusion, but no-reflow, reperfusion injury, restenosis, stent thrombosis, and other clinical tricky problems still remained as a pressing issue. The pathomechanism is directly related to the formation of collateral circulation and coronary microcirculation and endothelial cell injury. Therefore, it is of particularly importance to promote formation of collateral circulation and angiogenesis in myocardial ischemic area currently. Salvianolic acid B and Danshen crude extract can promote cell growth and differentiation. SalB can upregulate matrix metalloproteinase 2 (MMP-2) gene and upregulate vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor R2 (VEGF-R2) genes [30]. Promoting cardiac angiogenesis effect of salvianolic acid B in vitro is shown in Table 3.

**2.4. Protecting Myocardial Cells from Apoptosis.** Apoptosis is an important mechanism of acute myocardial ischemia and reperfusion myocardial cell death. Excessive accumulation of ROS leads to oxidative stress. The progression can induce cell death process after regulating series of Intracellular signaling pathways [31], such as PI3K/Akt pathway, TAB1-P38 apoptosis signaling pathway, and caspase-3 apoptotic pathway. In these pathways, the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-13-kinase (PI3K)/Akt pathway play a major role in cell growth, survival, differentiation, and apoptosis [32]. Studies showed that PI3K inhibitor (LY294002) prevents ERK pathway activation induced by hydrogen peroxide and protects cells from apoptosis, and SalB could inhibit  $H_2O_2$ -induced cell apoptosis mainly through the PI3K/Akt pathway (ERK upstream) [33]. Salvianolic acid B can significantly reduce the myocardial infarct size and blood lactate dehydrogenase level of model rat with acute myocardial infarction. Further studies showed that SalB can enhance cell activity

TABLE 2: Anti-platelet aggregation, anticoagulant, and antithrombotic effect of salvianolic acid B in vitro and in vivo.

Type	Cells/tissues	Effects	Reference
In vitro research	Human platelet endothelial cell	Activity of eNOS, L-arginine to L-citrulline, release of NO.	Radomski et al., 1990 [25, 26]
	Platelet	Collagen receptor $\alpha 2\beta 1$ .	Wu et al., 2008 [29]
Type	Organ/animals	Effects	Reference
In vivo research	Rats blood	Induce SIPA. 20 kinds of protein expression, $\alpha 2\beta 1$ integrin protein,	Li et al., 2004 [28]
	Rats blood	levels of intracellular $Ca^{2+}$ , cytoskeleton-related proteins, cytoskeleton platelets structure.	Ma et al., 2011 [23]

Note: eNOS: endothelial nitric oxide synthase; L-arginine: left-handed arginine; SIPA: shear-induced platelet aggregation.

TABLE 3: Promoting cardiac angiogenesis effect of salvianolic acid B in vitro.

Cells/tissues	Effects	Reference
HUVEs	Expression of MMP-2 gene, expression of VEGF, VEGF gene, VEGF receptor 2 genes.	Lay et al., 2003 [30]

Note: VEGF: vascular endothelial growth factor receptor; MMP-2: matrix metalloproteinase-2; HUVEC: human umbilical vein endothelial cells.

and reduce the number of sub-G1 and apoptotic nuclei of ischemic cell model in order to show its antiapoptotic effects. The specific mechanism is as follows: salvianolic acid B specifically inhibits phosphorylation of p38 mediated by TAB1 (TGF- $\beta$ -activated protein kinase 1 binding protein 1) by interfering with the interaction of TAB1 and P38 [34]. One research showed that the concentration of acid B is higher in the acute myocardial infarction rats model compared with nonischemic myocardial area, indicating that salvianolic acid B can improve cardiac function and myocardial tissue structure. Biochemical analysis showed salvianolic acid B can regulate the expression of 36 kinds of proteins in rats with AMI, which is composed of the mesh part of the diagram of cell's apoptosis and metabolism. Salvianolic acid B can also inhibit polymerase 1 pathway, improve the integrity of the mitochondria and nuclei in heart tissue of acute myocardial infarction, and protect myocardial cells from apoptosis [35]. The experiment proved that treatment of 50  $\mu$ M LAB can significantly reduce death. LAB significantly reduced phosphorylation of p38 and JNK induced by cytokine, which is in accordance with  $\beta$ -cells decrease in cleaved caspase-3 activity by a significant activation expression of of Nrf2-HO-1 (the heme oxygenase 1) and SIRT-1. LAB also has a protective effect on cytokine-induced caspase-3 apoptotic pathway [31].

Hunger for three hours can lead to myocardial cells induced autophagy, which is an important reason for myocardial cell's damage. Salvianolic acid B can protect of starving cells and inhibit of apoptosis process by blocking early stages of autophagic flux, respectively [36]. One research showed PC12 cells pretreated with SalB (10 nmol/L, 100 nmol/L, 1 mol/L) manifested relatively low proportion of apoptosis (15.7%, 13.5%, 11.8%). The mechanism is that Par-4 is involved in the protective effect of SalB against A-beta-induced damage while salB can largely prevent the increase in Par-4 expression of the A-beta-induced PC12 cells. Magnesium lithospermate B exhibits direct superoxide radicals scavenging and xanthine oxidase inhibitory activity [37]. The conclusion can be verified by experiments of protecting HL-60 cells from superoxide radicals-induced apoptosis in the xanthine oxidase reaction [38]. SM treatment is able to induce the highest frequency of apoptosis in cholesterol-fed balloon-injury rabbits, upregulate the expression of p53 and the frequency of TUNEL-positive cells [39]. SMND-309, is a new derivate of salvianolic acid B. It can prevent the elevation in ST segment level and the increase in serum creatine kinase-MB, lactate dehydrogenase, alanine aminotransferase and cardiac troponin T content, increase the activities of superoxide dismutase, catalase and glutathione peroxidase, decrease the content of malondialdehyde in myocardium, reduce the myocardium necrosis scores and the number of apoptosis cardiocytes, upregulated the expression of anti-apoptotic protein, Bcl-2; and downregulate the expression of proapoptotic protein, Bax [40]. Protecting myocardial cells from apoptosis of salvianolic acid B in vivo and in vitro is shown in Table 4.

### 2.5. Inhibiting Ischemia and Hypoxia of Myocardial Injury.

Myocardial ischemia and hypoxia diseases such as coronary heart disease threaten human health severely. Both physicians and researchers have made great effort in looking for effective drug of anti-ischemic hypoxic/hypoxia. Salvianolic acid B could antagonize voltage-dependent  $Ca^{2+}$  channels and therefore synergistically reduce cardiac ischemic injury with the antioxidant effects [41]. Other researches also studied the protective effect of salvianolic acid B on NO. The research confirmed for the first time that salvianolic acid B and tanshinone IIA promote left-handed arginine (L-arginine) uptake by enhancing expression of catalase (CAT) and increasing phosphorylation of eNOS through AMPK-PI3 K-Akt signaling pathway. Results showed that NO is a key factor for salvianolic acid B to reverse myocardial ischemia and hypoxia damage [42]. In the early stages of LPS-induced neonatal rat cardiomyocytes injury, TLR4-NF $\kappa$ B-TNF $\alpha$  signaling pathway which is not directly related to this process with HSP70 is activated quickly. Mechanism of salvianolic acid B protection of the ischemic myocardium is related to suppressing TLR4-NF $\kappa$ B-TNF $\alpha$  signaling pathway in dose-dependent manner [43]. SalB exerts cardioprotective effect on large MI mediated by reversing upregulation of leptin, endothelin pathways and oxidative stress, and recovering the normal expressions of SERCA2a and PLB in myocardium [44]. MLB may protect the heart from ischemic/reperfused

TABLE 4: Protecting myocardial cells from apoptosis of salvianolic acid B in vivo and in vitro.

Type	Cells/tissues	Effects	Reference
In vitro research	rCMECs	PI3K/Akt pathway, ERK upstream.	Blanc et al., 2003 [32]
	H9C2	TAB1-P38 apoptosis signaling pathway, cell activity, reduce sub-G1, inhibit p38 phosphorylation, interaction of TAB1 and P38.	Du et al., 2010 [34]
	INS-1	Phosphorylation of p38, phosphorylation of JNK, expression of Nrf2-HO-1, expression of SIRT-1, caspase-3 apoptotic pathway.	Han et al., 2011 [36]
	PC12	Expression of Par-4, superoxide radicals scavenging, xanthine oxidase inhibitory activity.	Tang and Zhang, 2002 [37]
	HL-60	Superoxide radicals-induced apoptosis, xanthine/xanthine oxidase reactions.	Liu et al., 2009 [38]
	Rabbit neointimal cell	Expression of p53, frequency of TUNEL-positive cells.	Hung et al., 2001 [39]
Type	Organ/animals	Effects	Reference
In vivo research	Rat heart	Myocardial infarct size, blood lactate dehydrogenase.	Du et al., 2010 [34]
	Rat heart	Cardiac function, myocardial tissue structure, expression of 36 kinds of proteins, ADP-ribose polymerase-1 pathway, integrity of mitochondria, integrity of nuclei.	Xu et al., 2011 [35]
	Rat heart	ST segment level, serum creatine kinase-MB, lactate dehydrogenase, alanine aminotransferase, cardiac troponin T content, activities of superoxide dismutase, activities of catalase, activities of glutathione peroxidase, expression of anti-apoptotic protein, expression of Bcl-2, expression of proapoptotic protein, expression of Bax.	Yang et al., 2010 [40]

Note: Nrf2: nuclear factor 2-related factor 2; TAB1: TGF- $\beta$ -activated protein kinase 1 binding protein 1; rCMECs: rat cerebral microvascular endothelial cells; APD: action potential duration; PI3K: phosphatidylinositol-13-kinase; ERK: extracellular-signal-regulated kinase.

injury by decreasing apoptosis through the inhibition activity of JNK3 [45]. Magnesium lithospermate B can induce eNOS expression in the endothelial cells of BAs and improve endothelial dysfunction. MLB inhibits ET-1 production in SAH animals via an NO-dependent mechanism [46]. Inhibiting ischemia and hypoxia of myocardial injury of salvianolic acid B in vitro is shown in Table 5.

**2.6. Endothelial Cell Protection.** Under normal circumstances, vascular endothelial secretion of vasoactive substances, which regulate vasomotor to protect the vessel wall from the infiltration of inflammatory cells, could inhibit thrombosis and vascular smooth proliferation of muscle cell. Many factors can cause vascular endothelial injury

and dysfunction. It is the the first stage of atherosclerosis. The endothelial cell protection role of salvianolic acid B is essential for the occurrence and development of atherosclerosis. Studies revealed that when the concentration of SME is 50 mg/mL and 100 mg/mL and concentrations of salvianolic acid B were 1, 2.5, 5, 10, 20 mg/mL, the expression of the VCAM-1 was lower, and the expression of ICAM-1 was also significantly reduced in a dose-dependent manner. SME may exert endothelial cell protection role by downregulating VCAM-1 and ICAM-1 in dose-dependent manner [47]. Salvianolic acid B can reduce the endothelial dependent vasodilation decline of Otsuka Long-Evans Tokushima Fatty (OLETF) rat, but increase the level of serum nitrite and lower serum AGEs concentration. The mechanism is related to Akt phosphorylation as well as

TABLE 5: Inhibiting ischemia and hypoxia of myocardial injury of salvianolic acid B in vitro.

Type	Cells/tissues	Effects	Reference
In vivo research	Guinea pig heart	Anti-voltage-dependent Ca <sup>2+</sup> channels.	Wang et al., 2006 [41]
	Rat heart	Upregulation of leptin, upregulation of oxidative stress, endothelin pathways, expressions of SERCA2a, expressions of PLB.	He et al., 2008 [44]
	SAH rat	Induce eNOS expression, improve endothelial dysfunction, ET-1 production.	Chang et al., 2011 [46]
Type	Organ/animals	Effects	Reference
In vitro research	HUVEs	Promote L-arginine uptake, expression of catalase (CAT), phosphorylation of eNOS, AMPK-PI3K-Akt signaling pathway, NO.	Pan et al., 2011 [42]
	Neonatal rat cardiomyocytes	TLR4-NFκB-TNFα signaling pathway.	Wang et al., 2011 [43]

Note: SAH: subarachnoid hemorrhage; TNF-α: tumor necrosis factor α; HUVEC: shuman umbilical vein endothelial cells; NF-κB: nuclear factor κB; TLR: toll-like receptor; eNOS: endothelial nitric oxide synthase.

reducing the O bit N-acetylglucosamine amine of eNOS. The mechanism is also related to increasing expression of 3-phosphoinositide kinase/Akt signaling pathway-dependent Nrf-2 as well as reducing the oxidative stress caused by hyperglycemia and apoptosis of vascular endothelial cell [48]. Salvianolic acid B exerts protective effect on vascular endothelial cells by inhibiting TNF-α-induced PAI-1 (plasminogen activator inhibitor type 1) mRNA production and protein secretion [49]. SalB induces the expression of GRP78 by activating ATF6 and the PERK-eIF2a-ATF4 pathway and protects human endothelial cells from oxidative stress-induced cellular damage [50]. Endothelial cell protection of salvianolic acid B in vivo and in vitro is shown in Table 6.

**2.7. Improving Hemorheology.** Pharmacological studies have shown that the change of blood flow state is one of the important causes of thrombosis. And fibrinogen plays an important role in platelet aggregation, and so reducing fibrinogen can reduce thrombosis in a certain sense. Salvianolic acid B and paeonol compounds can significantly decrease the fibrinogen and malondialdehyde levels in a dose-dependent manner, increase high-density lipoprotein levels, improve the rabbit blood viscosity and plasma viscosity, decrease NO/ET proportion, and decrease lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) levels in a dose-dependent manner. It is proved that salvianolic acid B can improve blood hemorheology, reduce oxidative damage, improve the vascular endothelial cell function, and prevent the development of coronary artery disease [51]. SalB increased the fibrinolytic and anticoagulant potential of cultured HUVECs by upregulating the expression of t-PA and TM and by downregulating the expression of PAI-1 [52]. Both DLA and SAB can inhibit venular thrombosis induced by photochemical reaction (PR) thrombosis in rat mesentery and delay thrombus-initiation

time [53]. Improving hemorheology of salvianolic acid B in vivo is shown in Table 7.

**2.8. Acting on Ion Channel Function.** Many ion channels are closely related to cardiovascular disease. It is unclear whether and how MLB affects the cardiac ion channels. The occurrence of cardiovascular disease may be limited to not only a single ion channel, but to multiorganization, multicell network level of ion channel interactions. Recently, many researchers focus on ion channels and regulatory proteins associated. Whether the ion channel leads to cardiovascular disease by causing arrhythmogenic is not yet formed as a conclusion. There are at least two experiments about SalB acting on the BK<sub>Ca</sub> channel. One experiment confirmed MLB can make arterial vasodilating through the activation of BK<sub>Ca</sub> channel (big-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels) of smooth muscle cell and the increase of endothelial NO release [54]. Another one verified that salvianolic acid B could activate the opening of the BK<sub>Ca</sub> channels of the porcine coronary artery smooth muscle cells. Cumulative application of salvianolic acid B (30–300 μM) caused an L-NNA- (100 μM) insensitive potentiation of the outward BK<sub>Ca</sub> (iberiotoxin-sensitive Ca<sup>2+</sup>-activated K<sup>+</sup>) current amplitude. Salvianolic acid B (300 μM) caused an ODQ-sensitive enhancement of the outward BK<sub>Ca</sub> current amplitude [55]. An experiment stimulated SH-SY5Y neuroblastoma cells in tumor cells with different concentrations of ouabain or MLB, using Fluo4-AM (fluorescent dye) measurements to measure Ca<sup>+</sup> level of cells. It is confirmed that elevation of ouabain and MLB can cause increase of intracellular Ca<sup>2+</sup> levels, which may be related to inhibition activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase enzyme [56]. MLB reversibly inhibited L-type Ca<sup>2+</sup> current (I<sub>Ca,L</sub>) on single ventricular myocytes of adult guinea pigs. The inhibition was use dependent and voltage dependent and the voltage-dependent Ca<sup>2+</sup> antagonistic effect of MLB works

TABLE 6: Endothelial cell protection of salvianolic acid B in vivo and in vitro.

Type	Cells/tissues	Effects	Reference
In vitro research	HAECs	Expression of VCAM-1, expression of ICAM-1, Akt phosphorylation,	Chen et al., 2001 [47]
	HUVECs and HAECs	O bit N-acetylglucosamine amine of eNOS, 3-phosphoinositide kinase/Akt signaling pathway, expression of Nrf-2	Kim et al., 2010 [48]
	HUVECs	Expression of PAI-1 Mrna.	Zhou et al., 2005 [49]
	HUVECs	Expression of GRP78, ATF6, and the PERK-eIF2a-ATF4 pathway.	Wu et al., 2009 [50]
Type	Organ/animals	Effects	Reference
In vivo research	OLETF Diabetic rat	Endothelial dependent vasodilation, levels of serum nitrite, serum AGEs concentration, reduce the oxidative stress.	Kim et al., 2010 [48]

Note: HUVEC: human umbilical vein endothelial cells; HAEC: human aortic endothelial cells; OLETF: Otsuka Long-Evans Tokushima Fatty; PAI-1: plasminogen activator inhibitor type 1; eNOS: endothelial nitric oxide synthase; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule 1.

TABLE 7: Improving hemorheology of salvianolic acid B in vitro and in vivo.

Type	Cells/tissues	Effects	Reference
In vitro research	HUVECs	Expression of t-PA and TM, expression of PAI-1.	Wang et al., 2009 [53]
Type	Organ/animals	Effects	Reference
In vivo research	Rabbits heart	Decrease fibrinogen and level, decrease malondialdehyde level, increase high-density lipoprotein level, improve rabbit blood viscosity, improve rabbit plasma viscosity, decrease NO/ET proportion, decrease LDH, decrease CPK.	Shi et al., 2007 [52]

Note: CPK: creatine phosphokinase; LDH: lactate dehydrogenase.

in concert with its antioxidant action for attenuating heart ischemic injury. When the concentration of MLB is up to 300 AM, there is no significant effect on the fast-inactivating  $\text{Na}^+$  current ( $I_{\text{Na}}$ ), but on delaying rectifier  $\text{K}^+$  current ( $I_{\text{K}}$ ) and inward rectifier  $\text{K}^+$  current [57]. The vasorelaxant effects of salvianolic acid B were produced by inhibition of  $\text{Ca}^{2+}$  influx in the vascular smooth muscle cells. The opening of  $\text{K}^+$  channels had a minor contribution to their effects [58]. Acting on ion channel function of salvianolic acid B in vitro is shown in Table 8.

**2.9. Anti-Inflammatory Effect.** Various researches demonstrated that inflammatory response was involved in the process of myocardial infarction (MI), endothelium injury, atherosclerosis, and cardiovascular hypertrophy [59, 60], which have been mostly introduced in the former paragraphs. Adhesion and migration of white blood cells in the vessel wall is an early manifestation of atherosclerosis formation. The use of antioxidants to inhibit the expression of adhesion molecules can prolong the progression of atherosclerosis. Salvianolic acid B is considered to be promising powerful

antioxidants. One research study mechanism of salvianolic acid B and *Salvia hydroalcoholic extract* (SME) to TNF- $\alpha$  induced HAECs. When concentration of salvianolic acid B is 0.48 times, it can significantly inhibit nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) activity of TNF- $\alpha$ -induced HAECs. It confirmed the exact anti-inflammatory effect of salvianolic acid B [61]. Shih Chung Chen have proved that salvianolic acid B significantly inhibits the phosphorylation of JAK2 (tyrosine 1007/1008) and STAT1 (Tyr701 and serine 727 (Ser727)) induced by IFN- $\gamma$ . The specific mechanism may be that salvianolic acid B inhibits STAT1 downstream target chemoattractant factor IP-10, MIG, I-TAC induced by IFN- $\gamma$  and inhibits the secretion of promoter activity of IP-10 and IP-10 protein. Salvianolic acid B can also reduce the adhesion role of monocyte to endothelial cells when endothelial cells stimulated with IFN- $\gamma$  were used as experimental object. Salvianolic acid B also increases PIAS1 and SOCS1 expression. This may contribute to its inhibition of JAK-STAT1 signaling pathway [62]. SalB significantly reduced the production of NO, TNF- $\alpha$ , IL-1b, and ROS induced by LPS treatment in rat primary microglia in a dose-dependent manner [63]. The activation

TABLE 8: Acting on ion channel function of salvianolic acid B in vitro.

Type	Cells/tissues	Effects	Reference
In vitro Research	SH-SY5Y	Suppress $\text{Na}^+ - \text{K}^+$ -ATP enzyme, increase intracellular $\text{Ca}^{2+}$ levels.	Chen et al., 2010 [56]
	Porcine CASMs	Activate $\text{BK}_{\text{Ca}}$ channels, L-NNA insensitive, $\text{BK}_{\text{Ca}}$ current amplitude, inhibited L-type $\text{Ca}^{2+}$ current, delay rectifier $I_{\text{K}}$ , inward rectifier $I_{\text{K}}$ .	Lam et al., 2006 [55]
	VSMCs	Activation of $\text{BK}_{\text{Ca}}$ channel, increase NO release.	Zhang et al., 2010 [54]
Type	Organ/animals	Effects	Reference
In vivo research	Rat coronary artery	Inhibition of $\text{Ca}^{2+}$ influx.	Lam et al., 2006 [58]

Note: VSMC: vascular smooth muscle cells;  $\text{BK}_{\text{Ca}}$ : iberiotoxin-sensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current;  $I_{\text{Ca,L}}$ : L-type  $\text{Ca}^{2+}$  current;  $I_{\text{Na}}$ :  $\text{Na}^+$  current;  $I_{\text{K}}$ :  $\text{K}^+$  current.

of T lymphocytes contributes to the inflammatory processes of atherosclerotic diseases. MLB inhibits IL-2, IL-4, TNF- $\alpha$ , and interferon-gamma production; reduces the expressions of T cell activation markers CD 25 and CD 69; down-regulates activator protein-1 (AP-1), nuclear factor kappa B (NF- $\kappa$ B), and octamer binding transcription factor (Oct-1) DNA-binding activity, and also inhibits c-Jun N-terminal kinase (JNK),  $\text{I}\kappa\text{B}\alpha$  degradation, nuclear translocation of p65 and p50, and decreased  $\text{I}\kappa\text{B}\alpha$  kinase (IKK) activity through suppressing JNK-AP-1, IKK- $\text{I}\kappa\text{B}\alpha$ -NF- $\kappa$ B, and Oct-1 signaling pathways [64]. SalB suppresses the expression of proinflammatory cytokines TNF- $\alpha$ , IL-1, and enhance and the expression of anti-inflammatory cytokines IL-10 and TGF- $\beta$ 1. All of these findings extended the protective role of SalB in the model of TBI [65]. SalB treatment also suppressed the pathway of ERK1/2, JNK, and p38 mitogen-activated protein kinase. It can also attenuate the increase in prostaglandin E2 production and NADPH oxidase activity in LPS-treated HASMCs [66]. SalB and LSS treatment inhibit TNF- $\alpha$ -induced NF- $\kappa$ B activation evidenced by  $\text{I}\kappa\text{B}\alpha$  degradation and p65 nuclear translocation in HAECs. SalB has a combination effect with LSS to reduce the expression of three adhesion molecules (VCAM-1, ICAM-1, and E-selectin), leading to reduced monocyte adhesion to HAECs [67]. Anti-inflammatory protection role of Salvianolic acid B in vitro is shown in Table 9.

**2.10. Preventing Cell Migration, Proliferation, and Intimal Hyperplasia.** Proliferation of vascular smooth muscle cells (VSMC) and migration of platelet-derived growth factor (PDGF) play an important role in the development of atherosclerosis and restenosis. One in vitro research studied the therapeutic potential of neointimal formation of salvianolic acid B to carotid artery injury rat and the PDGF signaling pathway which stimulates the proliferation of vascular smooth muscle cell and migration. It is demonstrated that SalB directly scavenges reactive oxygen species in the system of xanthine oxidase and reduces the generation of reactive oxygen species in the PDGF-BB-induced vascular smooth

muscle cells. In rat carotid artery balloon-injury model, SalB plays an important role in preventing the formation process of neointimal mediated by injury and prevents proliferation and migration of vascular smooth muscle cell in vitro mediated by PDGF-BB. In view of this, it is believe that salvianolic acid B has prospects in the prevention of atherosclerosis and postangioplasty restenosis [68]. SDF-1 $\alpha$  significantly promotes growth and migration of A10 cells, while SalB can significantly reverse the impact of costimulation group. Similarly, SalB significantly downregulated the upregulation Raf-1, MEK, and ERK1/2 phosphorylation of ERK1/2, FAK, and phosphorylated FAK stimulated by CXCR4 SDF-1 $\alpha$  and increased activity of NF- $\kappa$ B promoter. In addition, SalB is also effective in reducing intimal hyperplasia induced by balloon angioplasty. In short, SalB can prevent cell proliferation, migration, and subsequent neointimal hyperplasia. This pharmacological mechanism can be explained by theory of inhibiting receptor expression levels of the CXCR4 and expression of downstream molecular SDF-1 $\alpha$ /CXCR4 [69]. SalB could inhibit high glucose-induced human mesangial cells proliferation and extracellular matrix production in a dose-dependent manner through modulating the cell-cycle progress and MMP-2 and MMP-9 activities via suppressing NF- $\kappa$ B activation [70]. Preventing cell migration, proliferation, and intimal hyperplasia salvianolic acid B in vitro is shown in Table 10.

**2.11. Antiatherosclerosis.** Atherosclerosis is characterized by the lipid calming on affected artery intima, complex carbohydrates accumulate, and middle arterial disease. Coronary atherosclerosis is of great harm, which could lead to the stenosis or obstruction of blood vessels. Currently, it is demonstrated that SalB can act on Nrf2-ARE signaling pathway and p38-MAPK signaling pathway to prevent the occurrence of atherosclerotic disease. SalB can also activate NAD(P)H quinone oxidoreductase-1 (NQO1) by pathway of nuclear factor erythroid 2-related factor-2 antioxidant responsive element (Nrf2-ARE), thereby inhibiting the vascular injury and vascular smooth muscle cell proliferation

TABLE 9: Anti-inflammatory effect of salvianolic acid B in vitro.

Type	Cells/tissues	Effects	Reference
In vitro research	HAECs	Activity of NF- $\kappa$ B.	Sun et al., 2011 [61]
	Endothelial cells	JAK-STAT1 signaling pathway, inhibit phosphorylation of JAK2, inhibit phosphorylation of STAT1, inhibit IP-10, MIG, and I-TAC, reduce adhesion role of monocyte to endothelial cells, increase expression of PIAS1, increase expression of SOCS1.	Chen et al., 2006 [66]
	Rat primary microglia	Production of IL-1b and ROS, production of NO, TNF- $\alpha$ , inhibit IL-2, IL-4, TNF- $\alpha$ .	Cheng et al., 2012 [64]
	Human peripheral T lymphocyte	Expressions of CD 25 and CD 69, down-regulate AP-1, NF-Kb, down-regulate Oct-1DNA-binding activity, inhibit c-JNK, I $\kappa$ B $\alpha$ degradation, inhibit nuclear translocation of p65 and p50, IKK activity, suppress JNK-AP-1, IKK-I $\kappa$ B $\alpha$ -NF- $\kappa$ B and Oct-1 signaling pathways, phosphorylation of ERK1/2.	Chen et al., 2011 [65]
	HASMCs	Phosphorylation of JNK, pathway of ERK1/2, c-JNK, and p38 MAPK, prostaglandin E2 production, NADPH oxidase activity.	Chen et al., 2006 [66]
	HAECs	NF- $\kappa$ B activation, VCAM-1, ICAM-1, and E-selectin.	Xie et al., 2010 [67]
Type	Organ/animals	Effects	Reference
In vivo research	Mice brain	Expression of TNF- $\alpha$ , expression of IL-1, expression of IL-10 and TGF- $\beta$ 1.	Chen et al., 2006 [66]

Note: PAI-1: plasminogen activator inhibitor type 1; NF- $\kappa$ B: nuclear factor  $\kappa$ B; HAEC: human aortic endothelial cells; p38 MAPK: p38 mitogen-activated protein kinase.

and migration. It might be the potential molecular target of salvianolic acid B against atherosclerosis [71]. Anti-atherosclerotic of salvianolic acid B also has relation with inhibition of H-monDC mature. The oxidation of low-density lipoprotein (ox-LDL) can promote the mature of H-monDC, stimulate cells expression of CD40, CD86, CD1a, HLA-DR and IL-12, IL-10, production of TNF- $\alpha$  and upregulate signaling pathway. SalB can suppress the above process and activate PPAR $\gamma$  nuclear translocation in order to reduce the ox-LDL-induced upregulation of TLR4 and primary reactive protein 88 myeloid differentiation, and also inhibit downstream p38-MAPK signaling cascade pathway [72]. Salvianolic acid B can antagonize lipid uptake process of Scavenger receptor mediated by CD36 and reduce low density lipoprotein (mLDL) uptake in a dose-dependent manner in phorbol-12-myristate-13-acetate (PMA)-stimulated THP-1 and RAW 264.7 cells, thus preventing of atherosclerotic disease [73]. SalB significantly attenuate upregulations of both MMPs and the LPS-induced cell migration as well as downregulation of the extracellular-signal-regulated kinase1/2 (ERK1/2) and c-Jun NH2-terminal kinase (JNK) [74]. Antiatherosclerosis of Salvianolic acid B in vitro is shown in Table 11.

*2.12. Inhibiting Left Ventricular Remodeling.* Acute myocardial infarction may lead to left ventricular remodeling, and then cause congestive heart failure. Therefore, it is necessary to study treatment strategies of inhibiting left ventricular remodeling. Salvianolic acid B could selectively inhibit the activity of MMP-9 in a rat model of myocardial infarction. Salvianolic acid B can also effectively increase the thickness of the left ventricular wall in the myocardial infarction rats to improve the contraction of the heart, and reduce cardiac fibrosis. Previous experiments confirmed the exact role of anti-cell fibrosis of salvianolic acid B, but the specific mechanism of action was still unclear. There are a variety of hypotheses [75]. Salvianolic acid B inhibits the synthesis of type I collagen of non-TGF- $\beta$ 1 stimulated human hepatic stellate cell line (LX-2), the anti-fiber of mechanism is related to direct inhibiting p38 signaling pathway and cross effect of the Smad to ERK signaling pathway. Cardiac fibroblasts play a key role in cardiac function. As we all know, MMP-9 greatly influence the occurrence and development of cardiac remodeling [76]. One study about the catalytic MMP-9 CD (domain of MMP-9) and neonatal cardiac fibroblasts showed 200 nm MMP-9 CD can stimulate cardiac fibroblast

TABLE 10: Preventing cell migration, proliferation, and intimal hyperplasia salvianolic acid B in vitro.

Cells/tissues	Effects	Reference
rVSMCs	PDGF signaling pathway, scavenge ROS, system of xanthine oxidase, generation of ROS, process of neointimal, prevent proliferation, prevent migration.	Hur et al., 2008 [68]
A10	Expression of SDF-1 $\alpha$ /CXCR4, regulate Raf-1 and MEK, regulate ERK1/2 and phosphorylation ERK1/2, regulate FAK and phosphorylated FAK, activity of NF- $\kappa$ B promoter.	Pan et al., 2012 [69]
Human mesangial cells	Modulate the cell-cycle progress, activity of MMP-2, activity of MMP-9, suppress NF- $\kappa$ B activation.	Luo et al., 2008 [70]

Note: ROS: reactive oxygen species; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; PDGF: platelet-derived growth factor; A10 cells: vascular smooth muscle cells.

TABLE 11: Antiatherosclerosis of salvianolic acid B in vitro.

Cells/tissues	Effects	Reference
VSMCs	Nrf2-ARE signaling pathway, activation of NQO1.	Hur et al., 2010 [71]
H-monDC	Suppress activation of PPAR $\gamma$ nuclear translocation, expression of CD40, CD86, CD1a, and HLA-DR, expression of IL-12 and IL-10, production of TNF- $\alpha$ , regulation of TLR4, myeloid differentiation of primary reactive protein 88, p38-MAPK signaling pathway.	Sun et al., 2011 [72]
Macrophage	Reduce mLDL uptake, antagonize CD36.	Bao et al., 2012 [73]
HASMCs	MMPs protein synthesis, downregulate ERK1/2, downregulate c-JNK.	Lin et al., 2007 [74]

Note: H-monDC: human monocyte-derived dendritic cells; Nrf2: nuclear factor 2-related factor 2; NQO1: NAD(P)H quinone oxidoreductase-1; PPAR: poly ADP-ribose polymerase; ARE: antioxidant responsive element; VSMC: vascular smooth muscle cells; IL: interleukin; TLR: toll like receptor; TNF: tumor necrosis factor.

migration; increase collagen synthesis; upregulate secretion of ICAM, TNF- $\alpha$ , IL-6 and VCAM-1; and downregulate the expression of VEGF. This is closely related to cell proliferation [77]. SalB can inhibit A-beta aggregation and fibril formation and the cellular toxicity of aged A-beta towards PC12

cells [78]. Antimyocardia fibrosis, inhibiting left ventricular remodeling of salvianolic acid B in vivo and in vitro is shown in Table 12.

**2.13. Antiarrhythmic.** It is generally believed that the anti-arrhythmic and local anesthetics drugs mainly act on voltage-gated Na<sup>+</sup> channels. A new view showed Na<sup>+</sup> channel agonist has the positive inotropic effect. Salvianolic acid B is regarded as a new kind of Na<sup>+</sup> channel agonist. It can slow down the inactivation of Na<sup>+</sup> channel and increase action potential duration (APD). One research proved dmLSB has no apparent influence to currents of K<sup>+</sup> channels or Ca<sup>+</sup> channel; it only selectively affects Na<sup>+</sup> current ( $I_{NA}$ ). dmLSB slows down  $I_{NA}$  kinetics inactivation by increasing the proportion of material that cannot cause persistent sodium electricity loss of live. dmLSB only prolongs APD and then affects EAD. It is different from other Na<sup>+</sup> channel agonists which impact EAD and cause arrhythmia. Therefore, the clinical use of dmLSB is more safer and more promising [79]. Salvianolic acid B has the same molecular mechanism of inhibition of Na<sup>+</sup>-K<sup>+</sup>-ATP enzyme activity with cardiac glycosides. And MLB has lower cytotoxic effect than ouabain, so it will become great potential substitutes for cardiac glycosides with a wide range of clinical trials. Anti-arrhythmic effect of salvianolic acid B in vitro is shown in Table 13.

### 3. Discussion and Perspective

Currently, the high incidence of cardiovascular diseases (CVDs) worldwide potentially threaten human health [80–84]. The prevalence of CVDs is incessantly increasing and it is still the most common cause of death. History of application of herbal medicine as representative of complementary and alternative medicines in China has been lasting for thousands of years. Traditional Chinese medicine (TCM) has also formed a particular way which other therapeutics cannot match with it on diagnosis and treatment of the disease. And a variety of practices including Chinese herb and formulas, acupuncture, moxibustion, cupping, qigong, Tai Chi, diet, and exercise therapy were originated in China [85–87]. Nowadays, Chinese scholars combine traditional Chinese medicine with modern medicine perfectly carrying forward integrative mode. It takes the advantage of theory and practice of Chinese and modern medicine and exerts dual effect to improve clinical therapy efficacy. Chinese scholars have made great achievement on reducing the mortality and improving the quality of life by using patterns of integrative mode on diagnosis and treatment of cardiovascular, cerebrovascular disease and the tumor disease. And study on the blood stasis syndrome (BSS) and promoting blood circulation and removing blood stasis (PBCRBS) is the most active field of research of integration of traditional and western medicine in China [88, 89]. Scholars studying herbs of accelerating blood circulation (ABC) have made remarkable achievements in recent years [90, 91].

Many Chinese herbal medicine have function of accelerating blood circulation, clearing blood stasis, and dredging the meridians, such as Danshen, chuanxiong, chishao, and

TABLE 12: Inhibiting left ventricular remodeling of salvianolic acid B in vivo and in vitro.

Type	Cells/tissues	Effects	Reference
In vitro research	LX-2	p38 signaling pathway, cross effect from the Smad to ERK signaling pathway, synthesis of type I collagen, MMP-9 CD,	Lv and Xu, 2012 [76]
	HL-60	cardiac fibroblast migration, collagen synthesis, secretion of cytokine (ICAM, TNF- $\alpha$ , IL-6, and sVCAM-1), expression of VEGF.	Jiang et al., 2010 [77]
	PC12	A-beta aggregation, fibril formation, cellular toxicity of aged A-beta.	Tang and Zhang, 2011 [78]
Type	Organ/animals	Effects	Reference
In vivo research	Rat heart	Activity of MMP-9, increase the thickness of the left ventricular wall, improve the contraction of the heart, reduce cardiac fibrosis.	Wang et al., 2011 [75]

Note: MMP-9 CD: catalytic domain of MMP-9; LX-2: stellate cell lines; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule 1; IL: interleukin; TNF: tumor necrosis factor; MMP-9 CD: catalytic domain of MMP-9; ERK: extracellular-signal-regulated kinase; VEGF: vascular endothelial growth factor receptor.

TABLE 13: Anti-arrhythmic of salvianolic acid B in vitro.

Cells/tissues	Effects	Reference
Rat ventricular myocytes	$I_{NA}$ kinetics inactivation, prolong APD, then affect EAD, increase APD.	Yoon et al., 2004 [79]

Note: APD: action potential duration; EAD: early after depolarization.

honghua. Herbal medicines are great treasure that nature gifts to human and have made great contribution to human health [92–95]. Conclusive evidence can be found in the prevention and treatment of cardiovascular disease whether from traditional medicine or modern pharmacology research perspective. *Salvia* is the most widely used traditional Chinese medicine in the field of cardiovascular and cerebrovascular diseases. Currently, with increasing popularity of complementary and alternative medicine among CVDs patients, constituents of Chinese herb formulas are the key research areas [96, 97]. Many researches demonstrated that Chinese herbs can definitely regulate whole body by acting on multilevel and multitargets. Among them, salvianolic acid B is a water-soluble antioxidant from *Salvia* extract. It plays significant role of antioxidant effect; antiplatelet aggregation, anticoagulant, and antithrombotic effect; promoting cardiac angiogenesis; antiatherosclerosis; protecting myocardial cells from apoptosis; inhibiting left ventricular remodeling; inhibiting ischemia and hypoxia of myocardial injur; and protection of endothelial cell. SalB also has the protection effect of anti-arrhythmic, improving hemorheology;

acting on ion channel function anti-inflammatory protection, and preventing cell migration, proliferation, and intimal hyperplasia. Though SalB has so many effects on preventing and treatment of CVDs, there are also some problems we need to arise to develop both efficacious and pharmaceutical medicines. On current, Research about role of anti-inflammatory and effect of protecting myocardial cells from apoptosis were performed more frequently than other studies. Nevertheless there is only a few studies published about the promoting cardiac angiogenesis and anti-arrhythmic effect. Also there is deficiency of in vivo research on effect of antioxidant; anti-arrhythmic; antiatherosclerosis, promoting cardiac angiogenesis and preventing cell migration, proliferation, and intimal hyperplasia. So, further systematic in vivo researches are warranted to explore and verify the potential effect to provide precise guidance for clinical use and new drug discovery. Furthermore, there is also no randomized controlled trials (RCTs) and systematic reviews (SRs) about SalB. So, it is imperative to conduct multicentered, large-sized samples and randomized and arid controlled trials to reasonably evaluate the efficacy and safety of Chinese herb and formulas for CVDs. As we know, active ingredients with potential protecting and treating CVDs are material basis of Chinese herb and formulas [98, 99]. However there are so many active ingredients in Chinese herb, so large quantity of active ingredients should be identified, extracted, and purified. Correspondingly, more research should be designed and complemented to explain the mechanism of each agent. All the above problems seriously limit the research and progress on CVDs treatment and should be solved as soon as possible in future researches.

## Abbreviations

8-OHDG:	8-Hydroxy-2-deoxyguanosine	NF- $\kappa$ B:	Nuclear factor $\kappa$ B
A10 cells:	Vascular smooth muscle cells	NO:	Nitric oxide
ABC:	Accelerating blood circulation	NQO1:	NAD(P)H quinine oxidoreductase-1
AIF:	Apoptosis inducing factor	Nrf2:	Nuclear factor 2-related factor 2
APD:	Action potential duration	O <sup>2-</sup> :	Superoxide anion radicals
ARE:	Antioxidant responsive element	OLET:	Otsuka Long-Evans Tokushima Fatty
ATF4:	Activating transcription factor 4	ox-LDL:	Oxidation of low-density lipoprotein
BA:	Basilar artery	p38 MAPK:	p38 mitogen-activated protein kinase
BK <sub>Ca</sub> :	Iberiotoxin-sensitive Ca <sup>2+</sup> -activated K <sup>+</sup> current	PAI-1:	Plasminogen activator inhibitor type 1
BSS:	Blood stasis syndrome	PARP:	Poly (ADP-ribose) polymerase
CPK:	Creatine phosphokinase	PBCRBS:	Promoting blood circulation and removing blood stasis
CVDs:	Cardiovascular diseases	PDGF:	Platelet-derived growth factor
DLA:	3,4-Dihydroxy-phenyl lactic acid	PERK:	Pancreatic ER kinase (PKR)-like ER kinase
dmLSB:	Dimethyl lithospermate B	PI3K:	Phosphatidylinositol-13-kinase
DPPH:	1,1-Diphenyl-2-picryl-hydrazyl	PLB:	Phospholamban
DSP:	Danshen dripping pill	PMA:	Phorbol-12-myristate-13-acetate
EAD:	Early after depolarization	PR:	Photochemical reaction
ECV304:	Human umbilical vein endothelial cells	rCMEC:	Rat cerebral microvascular endothelial cells
Egb 761:	Extract ginkgo biloba 761	RCTs:	Randomized controlled trials
eIF2a:	Eukaryotic translation initiation factor 2a	ROS:	Reactive oxygen species
eNOS:	Endothelial nitric oxide synthase	SAB:	Salvianolic acid B
ERK:	Extracellular-signal-regulated kinase	SAH:	Subarachnoid hemorrhage
ET-1:	Endothelin-1	Sal B:	Salvianolic acid B
GRP78:	Glucose-regulated protein 78	SAPK:	Stress-activated protein kinase
H <sub>2</sub> O <sub>2</sub> :	Hydrogen peroxide	SCs:	Schwann cells
HAEC:	Human aortic endothelial cells	SERCA2a:	Sarco/endoplasmic reticulum ATPase 2a
HASMCs:	Human aortic smooth muscle cells	SIPA:	Shear-induced platelet aggregation
HEK293T cell:	Human embryonic kidney cells	SM:	Salvia miltiorrhiza
HG:	High glucose	SME:	Salvia hydroalcoholic extract
H-monDC:	Human monocyte-derived dendritic cells	SOD:	Superoxide dismutase
HO:	The heme oxygenase	SRs:	Systematic reviews
HUVECs:	Human umbilical vein endothelial cells	TAB1:	TGF- $\beta$ -activated protein kinase 1 binding protein 1
I/R:	Ischemia and reperfusion	TBI:	Traumatic brain injury
I <sub>Ca,L</sub> :	L-type Ca <sup>2+</sup> current	TLR:	Toll like receptor
ICAM-1:	Intercellular adhesion molecule 1	TM:	Thrombomodulin
I <sub>K</sub> :	K <sup>+</sup> Current	TMP:	Tetramethylpyrazine
IL:	Interleukin	TNF:	Tumor necrosis factor
IL-1b:	Interleukin-1b	t-PA:	Tissue-type plasminogen activator
I <sub>Na</sub> :	Na <sup>+</sup> current	TsI:	Tanshinone I
iNOS:	Induced nitric oxide synthase	TsIIA:	Tanshinone IIA
JAK:	Januskinase	TsIIB:	Tanshinone IIB
JNK:	c-Jun N-terminal kinase	TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
L-arginine:	Left-handed arginine	VCAM-1:	Vascular cell adhesion molecule-1
LDH:	Lactate dehydrogenase	VEGF:	Vascular endothelial growth factor receptor
LDQ:	Lipophilic diterpenoid quinines	VSMC:	Vascular smooth muscle cells
LPS:	Lipopolysaccharide	WSC:	Water-soluble compounds.
LV:	Left ventricular		
LX-2:	Stellate cell lines		
MDA:	Malondialdehyde		
MI:	Myocardial infarction		
MLB:	Magnesium lithospermate B		
MMP-2:	Matrix metalloproteinase-2		
MMP-9 CD:	Catalytic domain of MMP-9		
MMP-9:	Matrix metalloproteinase-9		
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate		

## Conflict of Interests

All authors manifest that there is no conflict of interests.

## Author's Contribution

J. Wang and X. Xiong contributed equally to this paper.

## Acknowledgment

The current work was partially supported by the National Basic Research Program of China (973 Program, no. 2003-CB517103) and the National Natural Science Foundation Project of China (no. 90209011).

## References

- [1] L. Zhou, Z. Zuo, and M. S. S. Chow, "Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use," *Journal of Clinical Pharmacology*, vol. 45, no. 12, pp. 1345–1359, 2005.
- [2] B. Wu, M. Liu, and S. Zhang, "Dan shen agents for acute ischaemic stroke," *Cochrane Database of Systematic Reviews*, vol. 18, no. 2, Article ID CD004295, 2007.
- [3] C. Wang, X. Zhao, S. Mao, Y. Wang, X. Cui, and Y. Pu, "Management of SAH with traditional Chinese medicine in China," *Neurological Research*, vol. 28, no. 4, pp. 436–444, 2006.
- [4] M. Oda, H. Yokomori, and J. Y. Han, "Regulatory mechanisms of hepatic microcirculatory hemodynamics: hepatic arterial system," *Clinical Hemorheology and Microcirculation*, vol. 34, no. 1-2, pp. 11–26, 2006.
- [5] Y. Horie, J. Y. Han, S. Mori et al., "Herbal cardiogenic pills prevent gut ischemia/reperfusion-induced hepatic microvascular dysfunction in rats fed ethanol chronically," *World Journal of Gastroenterology*, vol. 11, no. 4, pp. 511–515, 2005.
- [6] H. C. Xing, L. J. Li, K. J. Xu et al., "Effects of *Salvia miltiorrhiza* on intestinal microflora in rats with ischemia/reperfusion liver injury," *Hepatobiliary and Pancreatic Diseases International*, vol. 4, no. 2, pp. 274–280, 2005.
- [7] C. G. Chen and Y. P. Wang, "Magnesium lithospermate B ameliorates renal cortical microperfusion in rats," *Acta Pharmacologica Sinica*, vol. 27, no. 2, pp. 217–222, 2006.
- [8] S. C. Hoffmann, R. L. Kampen, S. Amur et al., "Molecular and immunohistochemical characterization of the onset and resolution of human renal allograft ischemia-reperfusion injury," *Transplantation*, vol. 74, no. 7, pp. 916–923, 2002.
- [9] Y. Bando, Y. Tsukamoto, T. Katayama et al., "ORP150/HSP12A protects renal tubular epithelium from ischemia-induced cell death," *FASEB Journal*, vol. 18, no. 12, pp. 1401–1403, 2004.
- [10] Y. Chen, Y. Ruan, L. Li et al., "Effects of *Salvia miltiorrhiza* extracts on rat hypoxic pulmonary hypertension, heme oxygenase-1 and nitric oxide synthase," *Chinese Medical Journal*, vol. 116, no. 5, pp. 757–760, 2003.
- [11] J. Reignier, H. Sellak, R. Lemoine et al., "Prevention of ischemia-reperfusion lung injury by sulfated Lewis pentasaccharide," *Journal of Applied Physiology*, vol. 82, no. 4, pp. 1058–1063, 1997.
- [12] L. J. Feldman, D. Himbert, J. M. Juliard et al., "Reperfusion syndrome: relationship of coronary blood flow reserve to left ventricular function and infarct size," *Journal of the American College of Cardiology*, vol. 35, no. 5, pp. 1162–1169, 2000.
- [13] P. Hu, G. A. Luo, Z. Z. Zhao, and Z. H. Jiang, "Quantitative determination of four diterpenoids in radix *Salviae miltiorrhizae* using LC-MS-MS," *Chemical and Pharmaceutical Bulletin*, vol. 53, no. 6, pp. 705–709, 2005.
- [14] J. Y. Han, J. Y. Fan, Y. Horie et al., "Ameliorating effects of compounds derived from *Salvia miltiorrhiza* root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion," *Pharmacology and Therapeutics*, vol. 117, no. 2, pp. 280–295, 2008.
- [15] J. H. C. Ho and C. Y. Hong, "Salvianolic acids: small compounds with multiple mechanisms for cardiovascular protection," *Journal of Biomedical Science*, vol. 18, no. 1, article 30, 2011.
- [16] Y. Jia, F. Huang, S. Zhang, and S. W. Leung, "Is danshen (*Salvia miltiorrhiza*) dripping pill more effective than isosorbide dinitrate in treating angina pectoris? A systematic review of randomized controlled trials," *International Journal of Cardiology*, vol. 157, no. 3, pp. 330–340, 2012.
- [17] Z. X. Shi and G. Li, "Comparative analysis on the major constituents in radix *Salvia miltiorrhizae* injectable preparations," *China Pharmacy*, vol. 20, no. 3, pp. 207–209, 2009.
- [18] W. B. Luo, L. Dong, and Y. P. Wang, "Effect of magnesium lithospermate B on calcium and nitric oxide in endothelial cells upon hypoxia/reoxygenation," *Acta Pharmacologica Sinica*, vol. 23, no. 10, pp. 930–936, 2002.
- [19] L. Q. Sun, J. Zhao, and T. T. Zhang, "Protective effects of salvianolic acid B on schwann cells apoptosis induced by high glucose," *Neurochemical Research*, vol. 37, no. 5, pp. 996–1010, 2012.
- [20] H. S. Zhang and S. Q. Wang, "Salvianolic acid B from *Salvia miltiorrhiza* inhibits tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-induced MMP-2 upregulation in human aortic smooth muscle cells via suppression of NAD(P)H oxidase-derived reactive oxygen species," *Journal of Molecular and Cellular Cardiology*, vol. 41, no. 1, pp. 138–148, 2006.
- [21] C. S. Liu, Y. Cheng, J. F. Hu, W. Zhang, N. H. Chen, and J. T. Zhang, "Comparison of antioxidant activities between salvianolic acid B and *Ginkgo biloba* extract (EGb 761)," *Acta Pharmacologica Sinica*, vol. 27, no. 9, pp. 1137–1145, 2006.
- [22] G. R. Zhao, H. M. Zhang, T. X. Ye et al., "Characterization of the radical scavenging and antioxidant activities of danshensu and salvianolic acid B," *Food and Chemical Toxicology*, vol. 46, no. 1, pp. 73–81, 2008.
- [23] C. Ma, Y. Yao, Q. X. Yue et al., "Differential proteomic analysis of platelets suggested possible signal cascades network in platelets treated with salvianolic acid B," *PLoS ONE*, vol. 6, no. 2, Article ID e14692, 2011.
- [24] X. Y. Mu, "Influence of salvianolate to platelet aggregation and MMP-9 of patients with unstable angina," *Chinese Medical Herald*, vol. 24, pp. 59–60, 2009.
- [25] M. W. Radomski, R. M. J. Palmer, and S. Moncada, "An L-arginine nitric oxide pathway present in human platelets regulates aggregation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 13, pp. 5193–5197, 1990.
- [26] M. W. Radomski, R. M. J. Palmer, and S. Moncada, "Characterization of the L-arginine: nitric oxide pathway in human platelets," *British Journal of Pharmacology*, vol. 101, no. 2, pp. 325–328, 1990.
- [27] J. E. Freedman, J. Loscalzo, M. R. Barnard, C. Alpert, J. F. Keaney, and A. D. Michelson, "Nitric oxide released from activated platelets inhibits platelet recruitment," *The Journal of Clinical Investigation*, vol. 100, no. 2, pp. 350–356, 1997.
- [28] M. Li, C. Zhao, R. N. S. Wong, S. Goto, Z. Wang, and F. Liao, "Inhibition of shear-induced platelet aggregation in rat by tetramethylpyrazine and salvianolic acid B," *Clinical Hemorheology and Microcirculation*, vol. 31, no. 2, pp. 97–103, 2004.
- [29] Y. P. Wu, X. M. Zhao, S. D. Pan et al., "Salvianolic Acid B inhibits platelet adhesion under conditions of flow by a mechanism involving the collagen receptor  $\alpha 2\beta 1$ ," *Thrombosis Research*, vol. 123, no. 2, pp. 298–305, 2008.

- [30] I. S. Lay, J. H. Chiu, M. S. Shiao, W. Y. Lui, and C. W. Wu, "Crude extract of *Salvia miltiorrhiza* and salvianolic acid B enhance in vitro angiogenesis in murine SVR endothelial cell line," *Planta Medica*, vol. 69, no. 1, pp. 26–32, 2003.
- [31] B. W. Lee, S. W. Chun, S. H. Kim et al., "Lithospermic acid B protects beta-cells from cytokine-induced apoptosis by alleviating apoptotic pathways and activating anti-apoptotic pathways of Nrf2-HO-1 and Sirt1," *Toxicology and Applied Pharmacology*, vol. 252, no. 1, pp. 47–54, 2011.
- [32] A. Blanc, N. R. Pandey, and A. K. Srivastava, "Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H<sub>2</sub>O<sub>2</sub> in vascular smooth muscle cells: potential involvement in vascular disease," *International journal of molecular medicine*, vol. 11, no. 2, pp. 229–234, 2003.
- [33] C. L. Liu, L. X. Xie, M. Li, S. S. K. Durairajan, S. Goto, and J. D. Huang, "Salvianolic acid B inhibits hydrogen peroxide-induced endothelial cell apoptosis through regulating PI3K/Akt signaling," *PLoS ONE*, vol. 2, no. 12, Article ID e1321, 2007.
- [34] C. S. Du, R. F. Yang, S. W. Song, and Y. P. Wang, "Magnesium lithospermate B protects cardiomyocytes from ischemic injury via inhibition of TAB1-p38 apoptosis signaling," *Frontiers in Pharmacology*, vol. 1, p. 111, 2010.
- [35] L. L. Xu, Y. P. Deng, and L. X. Feng, "Cardio protection of salvianolic acid B through inhibition of apoptosis network," *PLoS ONE*, vol. 6, no. 9, Article ID e24036, 2011.
- [36] X. Han, J. X. Liu, and X. Z. Li, "Salvianolic acid B inhibits autophagy and protects starving cardiac myocytes," *Acta Pharmacologica Sinica*, vol. 32, no. 1, pp. 38–44, 2011.
- [37] M. Tang and J. Zhang, "Prostate apoptosis response-4 involved in the protective effect of salvianolic acid B against amyloid  $\beta$  peptide-induced damage in PC12 cells," *Japanese Journal of Pharmacology*, vol. 88, no. 4, pp. 422–427, 2002.
- [38] X. Liu, R. Chen, Y. Shang, B. Jiao, and C. Huang, "Superoxide radicals scavenging and xanthine oxidase inhibitory activity of magnesium lithospermate B from *Salvia miltiorrhiza*," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 24, no. 3, pp. 663–668, 2009.
- [39] H. H. Hung, Y. L. Chen, S. J. Lin et al., "A salvianolic acid B-rich fraction of *Salvia miltiorrhiza* induces neointimal cell apoptosis in rabbit angioplasty model," *Histology and Histopathology*, vol. 16, no. 1, pp. 175–183, 2001.
- [40] J. Yang, G. Zhang, J. Tian et al., "Cardioprotective effect of SMND-309, a novel derivative of salvianolic acid B on acute myocardial infarction in rats," *Basic and Clinical Pharmacology and Toxicology*, vol. 106, no. 4, pp. 317–323, 2010.
- [41] W. Wang, G. Y. Hu, and Y. P. Wang, "Selective modulation of L-type calcium current by magnesium lithospermate B in guinea-pig ventricular myocytes," *Life Sciences*, vol. 78, no. 26, pp. 2989–2997, 2006.
- [42] C. Pan, L. Lou, Y. Huo et al., "Salvianolic acid B and Tanshinone IIA attenuate myocardial ischemia injury in mice by no production through multiple pathways," *Therapeutic Advances in Cardiovascular Disease*, vol. 5, no. 2, pp. 99–111, 2011.
- [43] J. Wang, Y. Zhang, and L. L. Guo, "Salvianolic acid B inhibits the TLR4-NF $\kappa$ B-TNF $\alpha$  pathway and attenuates neonatal rat cardiomyocyte injury induced by lipopolysaccharide," *Chinese Journal of Integrative Medicine*, vol. 17, no. 10, pp. 775–779, 2011.
- [44] H. He, M. Shi, X. Zeng et al., "Cardioprotective effect of salvianolic acid B on large myocardial infarction mediated by reversing upregulation of leptin, endothelin pathways, and abnormal expression of SERCA2a, phospholamban in rats," *Journal of Ethnopharmacology*, vol. 118, no. 1, pp. 35–45, 2008.
- [45] L. M. Yang, Y. L. Xiao, and J. H. Ou-Yang, "Inhibition of magnesium lithospermate B on the c-Jun N-terminal kinase 3 mRNA expression in cardiomyocytes encountered ischemia/reperfusion injury," *Acta Pharmaceutica Sinica*, vol. 38, no. 7, pp. 487–491, 2003.
- [46] C. Z. Chang, S. C. Wu, and A. L. Kwan, "Magnesium lithospermate B alleviates the production of endothelin-1 through an NO-dependent mechanism and reduces experimental vasospasm in rats," *Acta Neurochirurgica*, vol. 153, no. 11, pp. 2211–2217, 2011.
- [47] Y. H. Chen, S. J. Lin, H. H. Ku et al., "Salvianolic acid B attenuates VCAM-1 and ICAM-1 expression in TNF- $\alpha$ -treated human aortic endothelial cells," *Journal of Cellular Biochemistry*, vol. 82, no. 3, pp. 512–521, 2001.
- [48] S. H. Kim, S. H. Kim, M. Choi et al., "Natural therapeutic magnesium lithospermate B potently protects the endothelium from hyperglycaemia-induced dysfunction," *Cardiovascular Research*, vol. 87, no. 4, pp. 713–722, 2010.
- [49] Z. Zhou, Y. Liu, A. D. Miao, and S. Q. Wang, "Salvianolic acid B attenuates plasminogen activator inhibitor type I production in TNF- $\alpha$  treated human umbilical vein endothelial cells," *Journal of Cellular Biochemistry*, vol. 96, no. 1, pp. 109–116, 2005.
- [50] H. L. Wu, Y. H. Li, Y. H. Lin et al., "Salvianolic acid B protects human endothelial cells from oxidative stress damage: a possible protective role of glucose-regulated protein 78 induction," *Cardiovascular Research*, vol. 81, no. 1, pp. 148–158, 2009.
- [51] Q. Yang, S. Wang, Y. Xie et al., "Effect of Salvianolic acid b and paeonol on blood lipid metabolism and hemorrhheology in myocardial ischemia rabbits induced by pituitrin," *International Journal of Molecular Sciences*, vol. 11, no. 10, pp. 3696–3704, 2010.
- [52] C. S. Shi, H. C. Huang, H. L. Wu et al., "Salvianolic acid B modulates hemostasis properties of human umbilical vein endothelial cells," *Thrombosis Research*, vol. 119, no. 6, pp. 769–775, 2007.
- [53] F. Wang, Y. Y. Liu, L. Y. Liu et al., "The attenuation effect of 3,4-dihydroxy-phenyl lactic acid and salvianolic acid B on venular thrombosis induced in rat mesentery by photochemical reaction," *Clinical Hemorheology and Microcirculation*, vol. 42, no. 1, pp. 7–18, 2009.
- [54] H. F. Zhang, X. Q. Chen, G. Y. Hu, and Y. P. Wang, "Magnesium lithospermate B dilates mesenteric arteries by activating BK Ca currents and contracts arteries by inhibiting KV currents," *Acta Pharmacologica Sinica*, vol. 31, no. 6, pp. 665–670, 2010.
- [55] F. F. Y. Lam, S. W. Seto, Y. W. Kwan, J. H. K. Yeung, and P. Chan, "Activation of the iberiotoxin-sensitive BKCa channels by salvianolic acid B of the porcine coronary artery smooth muscle cells," *European Journal of Pharmacology*, vol. 546, no. 1–3, pp. 28–35, 2006.
- [56] Y. C. Chen, T. R. Jinn, T. Y. Chung, F. Y. Li, R. J. Fan, and J. Tc Tzen, "Magnesium lithospermate B extracted from *Salvia miltiorrhiza* elevates intracellular Ca<sup>2+</sup> level in SH-SY5Y cells," *Acta Pharmacologica Sinica*, vol. 31, no. 8, pp. 923–929, 2010.
- [57] W. Wang, G. Y. Hu, and Y. P. Wang, "Selective modulation of L-type calcium current by magnesium lithospermate B in guinea-pig ventricular myocytes," *Life Sciences*, vol. 78, no. 26, pp. 2989–2997, 2006.
- [58] F. F. Y. Lam, J. H. K. Yeung, Y. W. Kwan, K. M. Chan, and P. M. Y. Or, "Salvianolic acid B, an aqueous component of danshen (*Salvia miltiorrhiza*), relaxes rat coronary artery by inhibition of calcium channels," *European Journal of Pharmacology*, vol. 553, no. 1–3, pp. 240–245, 2006.

- [59] S. I. Jang, H. J. Kim, Y. J. Kim, S. I. Jeong, and Y. O. You, "Tanshinone IIA inhibits LPS-induced NF- $\kappa$ B activation in RAW 264.7 cells: possible involvement of the NIK-IKK, ERK1/2, p38 and JNK pathways," *European Journal of Pharmacology*, vol. 542, no. 1-3, pp. 1-7, 2006.
- [60] S. I. Jang, S. I. Jeong, K. J. Kim et al., "Tanshinone IIA from salvia miltiorrhiza inhibits inducible nitric oxide synthase expression and production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in activated RAW 264.7 cells," *Planta Medica*, vol. 69, no. 11, pp. 1057-1059, 2003.
- [61] A. J. Sun, H. Y. Liu, and S. J. Wang, "Salvianolic acid B suppresses maturation of human monocyte-derived dendritic cells by activating PPAR $\gamma$ ," *British Journal of Pharmacology*, vol. 164, no. 8, pp. 2042-2053, 2011.
- [62] C. S. Chung, Y. L. Lin, and B. Huang, "Salvianolic acid B suppresses IFN- $\gamma$ -induced JAK/STAT1 activation in endothelial cells," *Thrombosis Research*, vol. 128, no. 6, pp. 560-564, 2011.
- [63] S. X. Wang, L. M. Hu, X. M. Gao, H. Guo, and G. W. Fan, "Anti-inflammatory activity of salvianolic acid B in microglia contributes to its neuroprotective effect," *Neurochemical Research*, vol. 35, no. 7, pp. 1029-1037, 2010.
- [64] C. C. Cheng, S. P. Yang, and W. S. Lin, "Magnesium lithospermate B mediates anti-inflammation targeting activator protein-1 and nuclear factor- $\kappa$  B signaling pathways in human peripheral T lymphocytes," *International Immunopharmacology*, vol. 13, no. 3, pp. 354-361, 2012.
- [65] T. Chen, W. Liu, X. Chao et al., "Salvianolic acid B attenuates brain damage and inflammation after traumatic brain injury in mice," *Brain Research Bulletin*, vol. 84, no. 2, pp. 163-168, 2011.
- [66] Y. L. Chen, C. S. Hu, F. Y. Lin et al., "Salvianolic acid B attenuates cyclooxygenase-2 expression in vitro in LPS-treated human aortic smooth muscle cells and in vivo in the apolipoprotein-E-deficient mouse aorta," *Journal of Cellular Biochemistry*, vol. 98, no. 3, pp. 618-631, 2006.
- [67] L. X. Xie, S. S. K. Durairajan, J. H. Lu et al., "The effect of salvianolic acid B combined with laminar shear stress on TNF- $\alpha$ -stimulated adhesion molecule expression in human aortic endothelial cells," *Clinical Hemorheology and Microcirculation*, vol. 44, no. 4, pp. 245-258, 2010.
- [68] K. Y. Hur, H. J. Seo, E. S. Kang et al., "Therapeutic effect of magnesium lithospermate B on neointimal formation after balloon-induced vascular injury," *European Journal of Pharmacology*, vol. 586, no. 1-3, pp. 226-233, 2008.
- [69] C. H. Pan, C. W. Chen, and S. Ming-Jyh, "Salvianolic acid B inhibits SDF-1 $\alpha$ -stimulated cell proliferation and migration of vascular smooth muscle cells by suppressing CXCR4 receptor," *Vascular Pharmacology*, vol. 56, no. 1, pp. 98-105, 2012.
- [70] P. Luo, Z. Tan, Z. Zhang, H. Li, and Z. Mo, "Inhibitory effects of salvianolic acid B on the high glucose-induced mesangial proliferation via NF- $\kappa$ B-dependent pathway," *Biological and Pharmaceutical Bulletin*, vol. 31, no. 7, pp. 1381-1386, 2008.
- [71] K. Y. Hur, S. H. Kim, M. A. Choi et al., "Protective effects of magnesium lithospermate B against diabetic atherosclerosis via Nrf2-ARE-NQO1 transcriptional pathway," *Atherosclerosis*, vol. 211, no. 1, pp. 69-76, 2010.
- [72] A. J. Sun, H. Y. Liu, and S. J. Wang, "Salvianolic acid B suppresses maturation of human monocyte-derived dendritic cells by activating PPAR $\gamma$ ," *British Journal of Pharmacology*, vol. 164, no. 8, pp. 2042-2053, 2011.
- [73] Y. Bao, L. Wang, and Y. N. Xu, "Salvianolic acid B inhibits macrophage uptake of modified low density lipoprotein (mLDL) in a scavenger receptor CD36-dependent manner," *Atherosclerosis*, vol. 223, no. 1, pp. 152-159, 2012.
- [74] S. J. Lin, I. T. Lee, Y. H. Chen et al., "Salvianolic acid B attenuates MMP-2 and MMP-9 expression in vivo in apolipoprotein-E-deficient mouse aorta and in vitro in LPS-treated human aortic smooth muscle cells," *Journal of Cellular Biochemistry*, vol. 100, no. 2, pp. 372-384, 2007.
- [75] Y. H. Wang, F. Xu, and J. Chen, "Matrix metalloproteinase-9 induces cardiac fibroblast migration, collagen and cytokine secretion: inhibition by salvianolic acid B from *Salvia miltiorrhiza*," *Phytomedicine*, vol. 19, no. 1, pp. 13-19, 2011.
- [76] Z. Lv and L. Xu, "Salvianolic Acid B inhibits ERK and p38 MAPK signaling in TGF- $\beta$ 1-stimulated human hepatic stellate cell Line (LX-2) via distinct pathways," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 960128, 11 pages, 2012.
- [77] B. Jiang, J. Chen, L. Xu et al., "Salvianolic acid B functioned as a competitive inhibitor of matrix metalloproteinase-9 and efficiently prevented cardiac remodeling," *BMC Pharmacology*, vol. 10, article 10, 2010.
- [78] M. K. Tang and J. T. Zhang, "Salvianolic acid B inhibits fibril formation and neurotoxicity of amyloid beta-protein in vitro," *Acta Pharmacologica Sinica*, vol. 22, no. 4, pp. 380-384, 2001.
- [79] J. Y. Yoon, S. H. Ahn, H. Oh et al., "A novel Na<sup>+</sup> channel agonist, dimethyl lithospermate B, slows Na<sup>+</sup> current inactivation and increases action potential duration in isolated rat ventricular myocytes," *British Journal of Pharmacology*, vol. 143, no. 6, pp. 765-773, 2004.
- [80] H. Xu and K. J. Chen, "Making evidence-based decisions in the clinical practice of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 6, pp. 483-485, 2010.
- [81] K. J. Chen, "Clinical service of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 14, no. 3, pp. 163-164, 2008.
- [82] J. Wang and X. J. Xiong, "Current situation and perspectives of clinical study in integrative medicine in China," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.
- [83] J. Wang and X. J. Xiong, "Control strategy on hypertension in Chinese medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 284847, 6 pages, 2012.
- [84] J. Wang and X. J. Xiong, "Outcome measures of Chinese herbal medicine for hypertension: an overview of systematic reviews," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 697237, 7 pages, 2012.
- [85] M. Y. Liu and K. J. Chen, "Convergence: the tradition and the modern," *Chinese Journal of Integrative Medicine*, vol. 18, no. 3, pp. 164-165, 2012.
- [86] J. Wang, P. Q. Wang, and X. J. Xiong, "Current situation and re-understanding of syndrome and formula syndrome in Chinese medicine," *Internal Medicine*, vol. 2, no. 3, Article ID 100013, 2012.
- [87] N. Robinson, "Integrative medicine—traditional Chinese medicine, a model?" *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 21-25, 2011.
- [88] Y. Liu, H. J. Yin, D. Z. Shi, and K. J. Chen, "Chinese herb and formulas for promoting blood circulation and removing blood stasis and antiplatelet therapies," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 184503, 2012.
- [89] H. Xu and K. J. Chen, "Integrating traditional medicine with biomedicine towards a patient-centered healthcare system," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 83-84, 2011.

- [90] K. J. Chen, D. Z. Shi, H. Xu et al., "XS0601 reduces the incidence of restenosis: a prospective study of 335 patients undergoing percutaneous coronary intervention in China," *Chinese Medical Journal*, vol. 119, no. 1, pp. 6–13, 2006.
- [91] H. Xu and K. Chen, "Integrative medicine: the experience from China," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 3–7, 2008.
- [92] K. J. Chen, "Where are we going?" *Chinese Journal of Integrative Medicine*, vol. 16, no. 2, pp. 100–101, 2010.
- [93] X. J. Xiong, F. Y. Chu, H. X. Li, and Q. Y. He, "Clinical application of the TCM classic formulae for treating chronic bronchitis," *Journal of Traditional Chinese Medicine*, vol. 31, no. 1, pp. 69–72, 2011.
- [94] H. Xu and K. J. Chen, "Complementary and alternative medicine: is it possible to be mainstream," *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 403–404, 2012.
- [95] C. Keji and X. Hao, "The integration of traditional Chinese medicine and western medicine," *European Review*, vol. 11, no. 2, pp. 225–235, 2003.
- [96] X. J. Xiong, F. Y. Chu, H. X. Li, and Q. Y. He, "Clinical application of the TCM classic formulae for treating chronic bronchitis," *Journal of Traditional Chinese Medicine*, vol. 31, no. 1, pp. 69–72, 2011.
- [97] X. J. Xiong and J. Wang, "Discussion of related problems in herbal prescription science based on objective indications of herbs," *Journal of Chinese Integrative Medicine*, vol. 8, no. 1, pp. 20–24, 2010.
- [98] X. J. Xiong and J. Wang, "Experience of diagnosis and treatment of exogenous high-grade fever," *Journal of Chinese Integrative Medicine*, vol. 9, no. 6, pp. 681–687, 2011.
- [99] J. Wang and X. J. Xiong, "Explaining syndromes of decoction for removing blood stasis in chest," *Zhongguo Zhong Yao Za Zhi*, vol. 36, no. 21, pp. 3026–3031, 2011.

## Research Article

# Inhibition of NADPH Oxidase Mediates Protective Effect of Cardiogenic Pills against Rat Heart Ischemia/Reperfusion Injury

Xiao-Yuan Yang,<sup>1,2</sup> Na Zhao,<sup>1</sup> Yu-Ying Liu,<sup>1</sup> Bai-He Hu,<sup>1</sup> Kai Sun,<sup>1</sup> Xin Chang,<sup>1</sup>  
Xiao-Hong Wei,<sup>1</sup> Jing-Yu Fan,<sup>1</sup> and Jing-Yan Han<sup>1,2,3,4</sup>

<sup>1</sup> Tansy Microcirculation Research Center, Peking University Health Science Center, Beijing 100191, China

<sup>2</sup> Department of Integration of Traditional Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, 38 Xueyuan Road, Beijing 100191, China

<sup>3</sup> Key Laboratory of Microcirculation, State Administration of Traditional Chinese Medicine, Beijing 100191, China

<sup>4</sup> Key Laboratory of Stasis and Phlegm, State Administration of Traditional Chinese Medicine, Beijing 100191, China

Correspondence should be addressed to Jing-Yan Han; hanjingyan@bjmu.edu.cn

Received 24 February 2013; Revised 14 May 2013; Accepted 22 May 2013

Academic Editor: Hao Xu

Copyright © 2013 Xiao-Yuan Yang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Cardiogenic pill (CP) is a compound Chinese medicine currently used in China for treatment of ischemic angina pectoris. Our previous results indicated that a single dosing of CP pretreatment at 0.8 g/kg attenuates ischemia/reperfusion- (I/R-) induced myocardial injury and cardiac microcirculatory disturbance. The present study aimed to investigate the effect of CP at low dosage in a multiple dosing manner and to uncover the mechanism of antioxidative activity of CP. Male Sprague-Dawley rats were subjected to left anterior descending artery occlusion for 30 min followed by 60 min reperfusion. CP was administered daily by gavage for six days at 0.1, 0.4, and 0.8 g/kg/day before I/R. Results showed that multiple dosing of CP at three doses significantly reduced I/R-induced myocardial injury, microcirculatory disturbance, and oxidative stress. CP dramatically inhibited I/R-induced nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase subunit gp91<sup>phox</sup> expression and p67<sup>phox</sup> and p47<sup>phox</sup> translocation from cytosol to cell membrane. Translocation of cytosolic subunits to membrane is required for the activation of NADPH oxidase. These data suggested that multiple dosing of CP at doses ranging from 0.1 to 0.8 g/kg/day reduced I/R-induced rat myocardial injury and microcirculatory disturbance, which was mediated by inhibition of NADPH oxidase activation.

## 1. Introduction

Coronary artery disease is a leading cause of death all over the world. Although percutaneous coronary intervention (PCI) is currently used in clinic to restore myocardial perfusion, ischemia/reperfusion- (I/R-) induced injury may lead to cardiac electrical dysfunction and permanent contractile disability. Myocardial injury induced by I/R accounts for the majority of death induced by various forms of cardiovascular diseases [1]. Thus, better understanding the complications of I/R is essential for developing effective treatment to prevent I/R insults [2].

Reactive oxygen species (ROS) plays a crucial role in I/R-induced myocardial damage. Excessive ROS produced by I/R results in increased infarct size and various deleterious events [3]. For instance, ROS directly reacts with membrane

lipid, protein, and DNA and eventually causes the damage to cell structure and function [4]. Moreover, massive ROS release leads to activation of transcription factors, such as nuclear factor  $\kappa$ B (NF- $\kappa$ B), which in turn result in augmented expression of adhesion molecules and leukocyte infiltration [5]. Inflammation response then concurs to increased extent of tissue injury during I/R. Besides enhancement of inflammation, ROS causes mitochondrial depolarization and prolonged opening of mitochondrial permeability transition pore, triggering cell apoptosis [6]. Given to the central role of ROS in I/R injury, alleviating the oxidative stress is considered to be a potential option in limiting I/R-induced myocardial injury.

Nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase serves as one of the ROS sources in cardiomyocytes, vascular smooth muscle cells, endothelial

cells, and fibroblasts [7–10]. Once activated by cytosolic regulatory subunits, NADPH oxidase catalyzes electron transfer from NADPH onto molecular  $O_2$  to generate  $O_2^{\cdot-}$  [11]. Emerging evidence suggests the involvement of NADPH oxidase in I/R injury. Fukui et al. reported that the expression of NADPH oxidase 2 (NOX2), the catalytic subunit of the phagocyte NADPH oxidase, also known as gp91<sup>phox</sup>, is elevated in rat myocardium after myocardial infarction [12]. Similar results are observed in human myocardium in a subsequent study [13]. Pathogenic roles of NADPH oxidase-derived ROS are also verified in human I/R injury in vivo [14]. NOX2<sup>-/-</sup> animals with inhibited NADPH oxidase activity exhibit reduced cardiomyocyte apoptosis and improved ventricular function and survival rate after myocardial infarction [15, 16]. Collectively, these results indicate NADPH oxidase as a potential target in treatment of ischemic heart diseases and that pharmacological inhibition of NADPH oxidase might be an efficient strategy to prevent I/R-induced myocardial injury.

Cardiotonic pill (CP), consisting of *Salvia miltiorrhiza* (SM), *Panax notoginseng* (PN), and *Borneol*, is a widely used traditional Chinese medicine (TCM) in China for treating ischemic angina pectoris, which has been scheduled to undergo phase III clinical trials for prevention and treatment of ischemic cardiovascular diseases by the US Food and Drug Administration in 2013. Our previous results showed that a single administration of CP at 0.8 g/kg prevents myocardial damage and apoptosis in rats after 60 min of reperfusion [17], while post-treatment with CP represses I/R-induced myocardial fibrosis in rats [18]. Consistent with the results of CP, accumulating evidence demonstrates the beneficial roles of the components of CP for heart. For instance, SM [19] and its major ingredient, 3,4-dihydroxy-phenyl lactic acid (DLA) [20] and salvianolic acid (SAB) [21], are well established to exhibit antioxidant activity. PN, another ingredient of CP, is reported to attenuate ischemic injury by inhibiting inflammatory response [22]. In spite of this advance, the cardioprotective mechanism of CP is not fully understood at present.

In the present study, we intended to gain further insight into the underlying mechanism of cardioprotection of CP against I/R injury, with particularly focusing on the likely involvement of NADPH oxidase.

## 2. Materials and Methods

**2.1. Animals.** Male Sprague-Dawley (SD) rats weighing 240–260 g were obtained from the Animal Center of Peking University Health Science Center (Certificate no. SCXK (Jing) 2006-0008). Rats were housed in a humidity of 40% ± 5% and a temperature of 22°C ± 2°C under a 12/12 h light/dark cycle. Rats were free to access water and food, while fasted for 12 h before the surgery. All surgical procedures performed on animals were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010-001), according to the guidelines of the Peking University Health Science Center Animal Research Committee.

**2.2. Agents.** CP was purchased from Tasly Pharmaceutical Co., Ltd. (Tianjin, China). An analysis by high performance liquid chromatography was carried out for quality control of CP, with one pill containing 9 mg of SM, 1.76 mg of PN, 0.5 mg of *Borneol*, and 13.74 mg of polyethylene glycol. triphenyl tetrazolium chloride (TTC) was obtained from AMRESCO Co., Ltd. (Solon, Ohio, USA) and dissolved in phosphate buffer at a concentration of 0.375%.

**2.3. Animal Model.** SD rats were randomly divided into five groups: Sham, I/R, CP 0.1 + I/R, CP 0.4 + I/R, and CP 0.8 + I/R. Rats were anesthetized by pentobarbital sodium (60 mg/kg, i.p.). Respiration of rats was maintained by a positive-pressure respirator (ALC-V8, Shanghai, China) through an intratracheal cannula. The heart was exposed by a midsternal thoracotomy, and a 5-0 silk ligature was placed around the left anterior descending coronary artery (LAD) [23]. Location of the ligature was 1-2 mm under the boundary of pulmonary conus and left auricle. The LAD was occluded for 30 min to induce myocardial ischemia and then released for reperfusion for 60 min. In Sham group, the rats underwent all the surgical procedures, except for tightening the ligature. Heart tissues were removed at a level 4 mm above the apex after 60 min of reperfusion for determination of the parameters concerned.

**2.4. Drug Administration.** CP was dissolved in saline. The animals in the groups pretreated with CP received the drug daily by gavage starting from 6 days before I/R at the dose of 0.1 g/kg/day (CP 0.1 + I/R), 0.4 g/kg/day (CP 0.4 + I/R), or 0.8 g/kg/day (CP 0.8 + I/R), which were 1.2-, 4.9-, and 9.9-fold, respectively, the equivalent effective dose used in clinic. In Sham and I/R group, rats received the same amount of saline in the same way. One and a half hour after the last administration of CP or saline on day 6, animals were anesthetized and subjected to surgical procedure.

**2.5. TTC Staining.** At the end of reperfusion, hearts were removed and sliced transversely into five sections (1 mm thick) starting from the ligation site. The slices were incubated in 0.375% TTC at 37°C for 15 min to delineate the infarction area. Slices stained with TTC were analyzed by Image-Pro Plus 5.0 software (Media Cybernetic, Maryland, USA), and infarction was expressed as a percentage of left ventricle area.

**2.6. Myocardial Blood Flow.** Myocardial blood flow (MBF) was measured by a Laser-Doppler Perfusion Imager (PeriScan PIM3, Perimed, Stockholm, Sweden) equipped with a computer. After left thoracotomy, MBF was recorded at baseline (before ischemia), 30 min after ischemia, and 5, 10, 30, and 60 min of reperfusion, respectively. Acquired MBF images were analyzed by LDPIwin 3.1 software (Perimed, Stockholm, Sweden). MBF results were expressed as percentage of the baseline.

**2.7. Red Blood Cell Velocity in and Diameter of Coronary Venules.** Coronary venule was observed by an upright microscope (BX51WI, Olympus, Tokyo, Japan) connecting

with a high-speed video camera (Photron Fastcam-ultimate APX, Tokyo, Japan) under epi-illumination. The red blood cell (RBC) velocity in the venule was recorded at a rate of 1000 frames/s before ischemia (baseline), 30 min after ischemia, and 30, 60 min of reperfusion, respectively. The stored images were replayed at a rate of 25 frames/s. The venule diameter and RBC velocity were calculated using Image-Pro Plus 5.0 software, as described previously [24].

**2.8. FITC-Albumin Leakage from Coronary Venules.** At the end of reperfusion, 50 mg/kg of FITC-conjugated albumin (Sigma-Aldrich, St Louis, USA) was injected into the right femoral vein via intravenous catheter. After FITC-albumin injection, venular images were acquired via an upright fluorescence microscope (DM-LFS, Leica, Mannheim, Germany) equipped with a SIT camera (EB-CCD Camera C7190, Hamamatsu, Shizuoka, Japan) under excitation wavelength of 455 nm. The fluorescence intensities of FITC-albumin inside the venules (Iv) and extravascular interstitial area (Ii) were analyzed with Image-Pro Plus 5.0 software. Albumin leakage from coronary venules was expressed by the ratio of Ii/Iv.

**2.9. Histological and Immunohistochemistry Evaluation of Cardiac Tissues.** Hearts were excised at 60 min of the reperfusion, fixed in 4% paraformaldehyde for 48 h and further prepared for paraffin sectioning. Paraffin sections were stained with hematoxylin and eosin (HE). For immunohistochemistry, the paraffin-embedded sections (5  $\mu$ m) were rehydrated and treated with 0.01 M sodium citrate for antigen retrieval. After blocked with bovine serum albumin, sections were incubated overnight at 4°C in a humidified box with specific antibodies against Capase-3 (1:100, Abcam, Massachusetts, USA), Bcl-2 (1:800, Chemicon International, California, USA), Bax (1:200, NOVUS, Colorado, USA), NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) (1:400, NOVUS, Colorado, USA), and P65 subunit of NF- $\kappa$ B (1:700, NOVUS, Colorado, USA). After washed three times by phosphate-buffered saline (PBS), sections were incubated with a Horse Radish Peroxidase (HRP) conjugated secondary antibody and revealed using the diaminobenzidine (DAB) substrate kit. For each section, five fields were selected from the surrounding infarction areas of the left ventricle and captured at a magnification  $\times$ 200 by a conventional microscope (Digital Sight DS-5M-U1, Nikon, Tokyo, Japan) connected with a digital camera (SZ-40, Olympus, Tokyo, Japan). Mean density was determined by Image-Pro Plus 5.0 software.

**2.10. Detection of Cardiomyocyte Apoptosis.** At the end of reperfusion, rat hearts were excised and prepared for paraffin sections as described above. Section was stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) by using a cell death detection kit (Roche, Basel, Switzerland), according to the manufacturer's protocol. Five visual fields were captured from the surrounding infarction areas of the left ventricle by a laser confocal microscope (Axiovert 200M, Carl Zeiss, Jena, Germany). The number of total nuclei and the TUNEL-positive nuclei in each field were counted and analyzed with Image-Pro Plus 5.0 software.

**2.11. Myocardial Ultrastructure.** Hearts were fixed by infusion with a mixture of 5% paraformaldehyde and 2% glutaraldehyde for 40 min after reperfusion. Then, cardiac tissue was excised from the ischemic region of left ventricle and trimmed into a block 1 mm<sup>3</sup>. The tissue block was then fixed overnight in 3% glutaraldehyde at 4°C and postfixed by 1% osmium tetroxide for 2 h. Tissues were prepared as routing for ultrathin sectioning. Sections were stained with uranyl acetate and lead citrate, then observed using a transmission electron microscope (JEM 1230, JEOL, Tokyo, Japan).

**2.12. Western Blot.** Myocardial tissues were collected from the ischemic areas of the left ventricle and then homogenized. Whole protein was extracted using a protein extraction kit (Applygen Technologies, Beijing, China) and mixed with 5x electrophoresis sample buffer. Cytosol proteins and membrane proteins were separated by Nucl-Cyto-Mem Preparation Kit (Applygen Technologies, Beijing, China), according to the manufacturer's protocol. After separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites were blocked with 5% skimmed milk in Tris-buffered saline Tween (TBS-T). Then, PVDF membranes with target membrane proteins were incubated overnight at 4°C with primary antibodies against gp91<sup>phox</sup> (1:2000, Abcam, Massachusetts, USA), p67<sup>phox</sup> (1:800, Abcam, Massachusetts, USA), p47<sup>phox</sup> (1:200, Santa Cruz Biotechnology, California, USA), p40<sup>phox</sup> (1:200, Santa Cruz Biotechnology, California, USA), and GAPDH (1:3000, Cell Signaling Technology, Vermont, USA). The PVDF membranes with target cytosolic proteins were incubated overnight at 4°C with primary antibodies against p67<sup>phox</sup> (1:800, Abcam, Massachusetts, USA), p47<sup>phox</sup> (1:200, Santa Cruz Biotechnology, California, USA), p40<sup>phox</sup> (1:200, Santa Cruz Biotechnology, California, USA), and  $\beta$ -tubulin (1:3000, Cell Signaling Technology, Vermont, USA). After washing three times by TBS-T, PVDF membranes were then incubated with HRP-conjugated secondary antibody (1:5000, Cell Signaling Technology, Vermont, USA) for 1 h at room temperature. Antibody bindings were revealed using Enhanced Chemiluminescence (ECL) Detection Kit (APPLYGEN, Beijing, China). For quantification, band intensity was assessed via Bio-Rad Quantity One software (Bio-Rad, California, USA).

**2.13. Determination of Malondialdehyde, Superoxide Dismutase, Catalase, and Glutathione Level.** Rats were sacrificed after 60 min of reperfusion; infarcted myocardial tissues were dissected from left ventricle. Immediately after snap frozen in liquid nitrogen, tissues were stored at -80°C till use. Whole proteins were extracted using a protein extraction kit (Applygen, Beijing, China), and protein concentration was determined with BCA protein assay kit (Applygen, Beijing, China), according to manufacturer's instruction. The level of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) were determined by using MDA Detection Kit (Nanjing Jiancheng Institute of

Biotechnology, Nanjing, China), SOD Assay (R&D, Minnesota, USA), CAT ELISA Kit (R&D, Minnesota, USA), GSH ELISA Kit (R&D, Minnesota, USA), respectively. All tests were performed twice according to manufacturer's instruction. Plates were analyzed on MULTISKAN MK3 enzyme microplate reader (Thermo Fisher Scientific Inc., Illinois, USA).

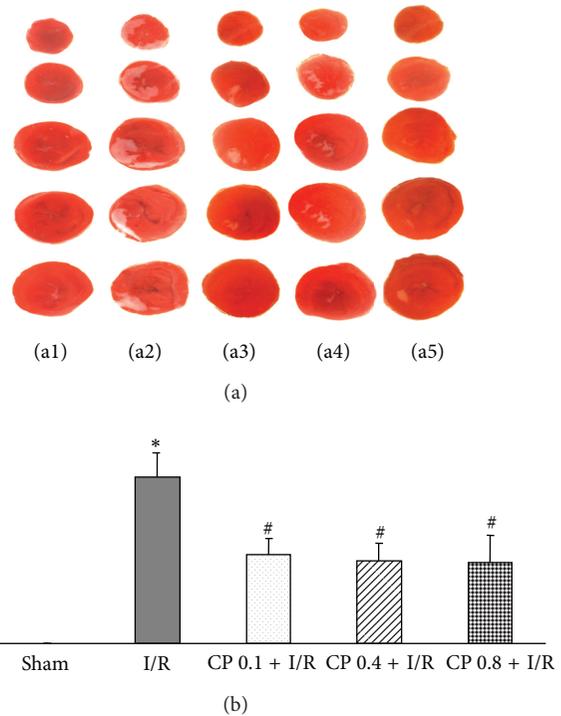
**2.14. Expression of CD18 and CD11b on Neutrophils.** Ten milliliters of blood were taken from the abdominal aorta of rats after 60 min of reperfusion. Blood was anticoagulated with 3.8% sodium citrate at a ratio of 9:1 (v/v). Fifty microliters of blood were then incubated with FITC-labeled anti-CD18 antibody (5  $\mu\text{g}/\text{mL}$ ) (BD, Franklin Lakes, NJ, USA) and FITC-labeled anti-CD11b antibody (5  $\mu\text{g}/\text{mL}$ ) (BD, Franklin Lakes, NJ, USA) for 20 min at room temperature in the dark. The erythrocytes were lysed with hemolysin (BD, New Jersey, USA), according to the manufacturer's instruction. After washing twice by PBS, mean fluorescence intensity was measured using a flow cytometer (FACSCalibur, BD Company, New Jersey, USA). Five thousand neutrophils were sorted and analyzed for each sample as reported previously [17].

**2.15. Peroxide in Neutrophils.** During reperfusion, a hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) sensitive fluorescent probe dihydrorhodamine 123 (DHR) dissolved in 3 mL saline was infused into femoral vein via intravenous catheter (2  $\mu\text{mol}/\text{kg}$ ). At the end of reperfusion, blood was collected from the abdominal aorta of rats and anticoagulated with heparin. Fluorescence intensity of DHR was determined using a flow cytometer (FACS Calibur, BD Company, New Jersey, USA) at excitation light of 510 nm and emission wavelength of 534 nm.

**2.16. Statistical Analysis.** All data were expressed as mean  $\pm$  SEM statistical analysis adopted SPSS 11.5 statistical package and was performed using one-way ANOVA followed by Newman-Keuls test or using two-way ANOVA followed by Bonferroni for multiple comparisons. A *P* value less than 0.05 was considered to be statistically significant.

### 3. Results

**3.1. CP Administration Diminishes I/R-Induced Infarct Size.** Rat hearts were subjected to 30 min of left descending artery occlusion followed by 60 min of reperfusion. Infarct size of various groups was detected to evaluate the cardioprotective role of CP. Representative heart slices stained by TTC to delineate infarct size are shown in Figure 1(a). Apparently, no infarct was observed in myocardial tissue slices from Sham group. However, myocardium sections from I/R group exhibited obvious infarct areas, which were protected by administration of CP at the three doses tested. Quantitative analysis of the infarct size further confirmed that the hearts from CP treated rats showed significantly smaller infarct size compared to those from I/R group, suggesting that CP administration exerted beneficial effects on I/R-challenged myocardium (Figure 1(b)).



**FIGURE 1: CP pretreatment reduces I/R-induced infarct size.** (a) Representative images of myocardial tissue slices stained with TTC in Sham group (a1), I/R group (a2), CP 0.1 + I/R group (a3), CP 0.4 + I/R group (a4), and CP 0.8 + I/R group (a5). White territory represents infarct area. (b) Quantitative results of infarct size in each group. Sham: Sham group; I/R: ischemia/Reperfusion group; CP 0.1 + I/R: CP pretreatment at 0.1 g/kg/day for 6 days plus I/R operation; CP 0.4 + I/R: CP pretreatment at 0.4 g/kg/day for 6 days plus I/R operation; CP 0.8 + I/R: CP pretreatment at 0.8 g/kg/day for 6 days plus I/R operation. The treatment of animals in each group is detailed in Section 2. Results are presented as mean  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$  versus Sham group, #  $P < 0.05$  versus I/R group.

**3.2. CP Administration Inhibits I/R-Induced Myocardial Apoptosis.** To investigate the effect of pretreatment with CP on myocardial apoptosis upon I/R, TUNEL staining and immunohistochemistry of apoptosis-related protein were conducted on myocardium from different groups 60 min after reperfusion. Figure 2(a) illustrates the representative images of left ventricular myocardium stained with TUNEL in surrounding infarct areas from various groups. Only few TUNEL-positive cells were observed in Sham group. In contrast, numerous TUNEL-positive cells were detected in I/R group, which were remarkably reduced in CP pretreated myocardium. Consistent with confocal survey, statistical results also indicated that the hearts from CP pretreated rats showed a significant decrease in the number of TUNEL-positive cells compared to those from I/R group after I/R (Figure 2(e)).

Apoptosis related proteins play a pivotal role in mediating myocardium apoptosis, thus expression of Caspase-3, Bax, and Bcl-2 was assessed by immunohistochemistry in the present study. Figure 2(b) shows the representative immunohistochemistry images of Caspase-3 in all groups.

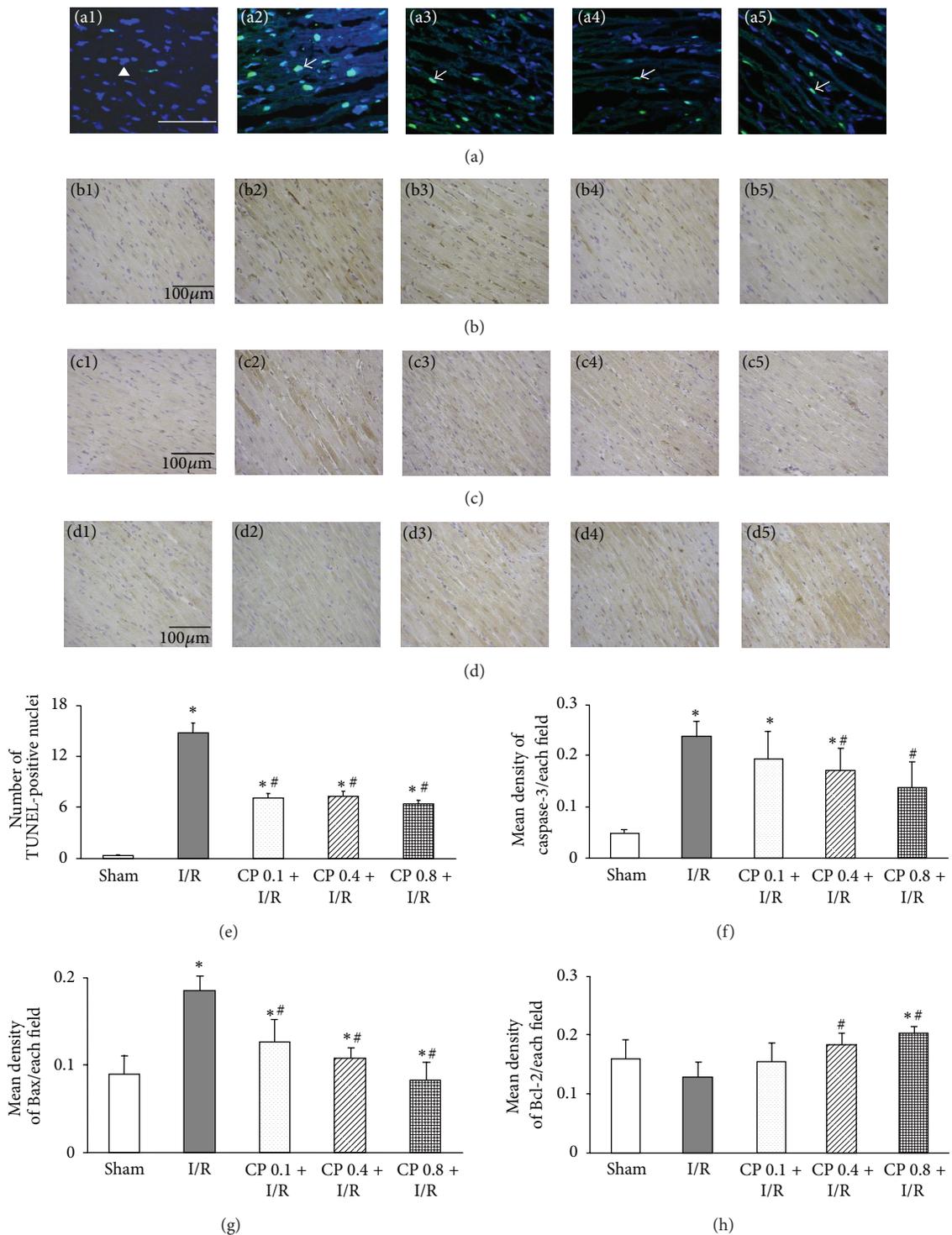


FIGURE 2: The effects of CP pretreatment on I/R-induced apoptosis and the expression of apoptosis related proteins. (a) Representative images of TUNEL stained myocardial sections in various groups. Apoptotic nuclei are indicated by TUNEL staining (green), and total nuclei are identified by Hoechst staining (blue). Arrowheads indicate normal nuclei. Arrows indicate TUNEL positive cells. Bar = 50  $\mu\text{m}$ . (b) Representative photographs of immunohistochemistry staining for Caspase-3 in different groups. (c) Representative images of myocardial slices stained for Bax in different groups. (d) Representative slices of immunohistochemistry staining for Bcl-2 protein in various groups. Immunohistochemistry positive staining cells are shown in brown color. Bar = 100  $\mu\text{m}$ . (e) to (h) Shown are statistical analysis of TUNEL positive nuclei, Caspase-3 expression, Bax expression, and Bcl-2 expression, respectively, in five different groups. 1, 2, 3, 4, and 5 which denotes Sham group, I/R group, CP 0.1 + I/R group, CP 0.4 + I/R group, and CP 0.8 + I/R group, respectively. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$  versus Sham group, #  $P < 0.05$  versus I/R group.

Only a few cells exhibited Caspase-3 positive staining in Sham group, whereas overexpression of Caspase-3 was detected in I/R group. Corresponding statistical results demonstrated that this increased expression of Caspase-3 was dramatically depressed by pretreatment with CP at 0.4 g/kg/day or 0.8 g/kg/day, but not at 0.1 g/kg/day (Figure 2(f)). Similar to the results of Caspase-3, representative pictures (Figure 2(c)) and quantitative analysis (Figure 2(g)) of Bax showed that administration of CP at all three doses markedly reversed upregulated level of Bax induced by I/R. On the contrary, expression of Bcl-2 was decreased significantly after I/R challenge, which was restored by pretreatment with CP at 0.4 g/kg/day or 0.8 g/kg/day, but not at 0.1 g/kg/day (Figures 2(d) and 2(h)). These results suggested that CP exerted its antiapoptosis role by reducing the expression of proapoptotic protein Caspase-3 and Bax and promoting the expression of anti-apoptotic protein Bcl-2 as compared to rats in I/R group.

**3.3. CP Administration Restores RBC Velocity in Coronary Venules after I/R.** Coronary venules and RBC movement inside the coronary venules of a beating heart were clearly recorded by a high-speed video camera (Figure 3(a)). Figure 3(b) illustrates the time course of changes in RBC velocity in coronary venules during I/R in various groups. No obvious difference was detected at baseline among groups. However, marked decrease in RBC velocity in I/R group was observed from the end of ischemia till 60 min of reperfusion, compared with Sham group. Pretreatment with CP significantly restored the decline in RBC velocity induced by I/R.

Statistical analysis in Figure 3(c) showed that diameters of coronary venules at baseline were comparable among the five groups, and no significant alteration in the coronary venule diameters of all groups was detected throughout the whole observation period.

**3.4. CP Administration Prevents Albumin Leakage from Coronary Venules after I/R.** Representative fluorescence images of coronary venules in Figure 4(a) showed that no evident FITC-labeled albumin leakage was detected in Sham group, whereas, obvious leakage was visible in the rats from I/R group, which was markedly reversed by CP pretreatment at all three doses. Likewise, quantified results in Figure 4(b) have shown that I/R group displayed significantly up-regulated albumin leakage from coronary venules as compared to Sham group, while pretreatment with CP remarkably prevented albumin leakage from coronary venules induced by I/R.

**3.5. CP Administration Preserves Myocardial Blood Flow after I/R.** Recovery of RBC velocity in coronary venules and microvascular endothelium barrier suggested that CP pretreatment might improve myocardial coronary perfusion upon I/R. To verify this presumption, MBF was assessed by using a Laser Doppler Perfusion Imager. Acquired representative pictures of MBF at different time points of I/R in all groups are illustrated in Figure 5(a). As shown in Figure 5(b), the time courses of quantitative evaluation of MBF demonstrated that MBF fell dramatically after 30 min of ischemia compared with Sham group; however, this decrease in MBF

during I/R was remarkably recovered by pretreatment with CP at 0.4 g/kg/day and 0.8 g/kg/day. Lower dose (0.1 g/kg/day) of CP showed no effect on MBF after I/R.

**3.6. CP Administration Retains Myocardium Structure after I/R.** Micrographs of HE stained myocardial sections in various groups are presented in Figure 6(a), revealing that distinct morphological injury, such as myocardial fiber disarrangement and disruption, myocardial tissue edema, and leukocyte infiltration, occurred in infarction areas of myocardial tissues from rats exposed to I/R. Noticeably, pretreatment with CP significantly preserved myocardium structure after I/R (Figure 6(a)).

The representative ultrastructure micrographs of myocardium are displayed in Figure 6. Figure 6(b) has shown the representative images of capillary from infarct region of myocardial tissues. Capillary and its surrounding tissues in Sham group displayed normal ultrastructural feature, whereas I/R challenge evoked capillary endothelium damage, caveolae augment; and perivascular edema. CP pretreatment well preserved capillary ultrastructure of myocardium after I/R.

Figure 6(c) shows the representative photographs of myocardial cells from infarct region of myocardial tissues. In Sham group, myocardial cells retained regularly arranged myofilaments and mitochondria with densely packed cristae. Exposure to I/R led to a dramatic myocardium ultrastructure alterations, such as disrupted myofibrils and disordered mitochondrial cristae. This myocardium injury induced by I/R was markedly alleviated by pretreatment with CP, particularly at dose of 0.8 g/kg/day.

**3.7. CP Administration Inhibits Oxidative Stress Induced by I/R.** To investigate oxidative stress, peroxide in blood was detected by using a  $H_2O_2$  sensitive fluorescent probe DHR. The levels of peroxide indicated by fluorescence intensity are illustrated in Figure 7(a). I/R provoked a pronounced increase in peroxide production after 60 min of reperfusion, as compared to Sham group. Pretreatment with CP at dose of 0.4 g/kg/day and 0.8 g/kg/day markedly attenuated I/R-induced peroxide enhancement.

Furthermore, myocardium MDA, an indicator of cellular lipid peroxidation, was also measured in different groups. As shown in Figure 7(b), the level of MDA in I/R group was significantly elevated compared with that in Sham group. The increased production of MDA induced by I/R was dramatically blunted by pretreatment with CP at the three doses examined.

**3.8. CP Administration Inhibits NF- $\kappa$ B Activation and Neutrophil Adhesion Induced by I/R.** As an oxidant-sensitive transcription factor, NF- $\kappa$ B activation plays a central role in the regulation of inflammatory response, via the augmented expression of cytokines and adhesion molecules. Activation of NF- $\kappa$ B was assessed by detecting the expression of nuclear factor  $\kappa$ B inhibitor  $\alpha$  ( $I\kappa B\alpha$ ) and P65 subunit of NF- $\kappa$ B. Figures 8(a) and 8(b) display, respectively, the representative immunohistochemistry images of  $I\kappa B\alpha$  and P65 around the

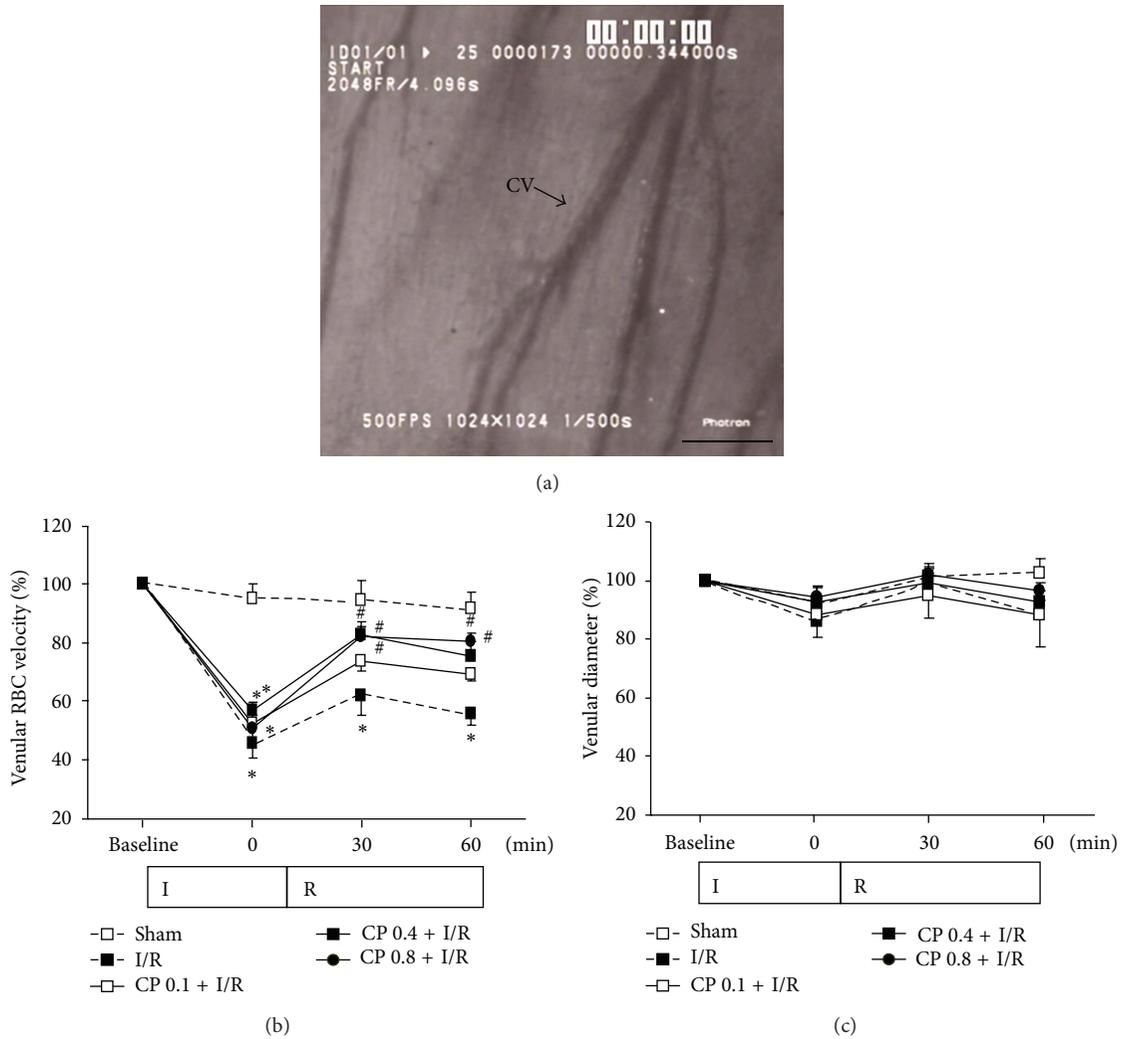


FIGURE 3: Effect of CP pretreatment on RBC velocity and venular diameter. (a) Representative image of cardiac coronary microcirculation. Bar = 100  $\mu$ m. (b) Time course of coronary venular RBC velocity in various groups. Results of RBC velocity are expressed as a percentage of baseline. (c) Quantitative results of coronary venular diameter. Results are presented as mean  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$  versus Sham group, #  $P < 0.05$  versus I/R group.

infarct areas of left ventricular myocardium. As shown in Figure 8(c), I/R provoked a significant  $\text{I}\kappa\text{B}\alpha$  degradation after 60 min of reperfusion as compared to Sham group, and administration of CP 6 days before I/R obviously prevented  $\text{I}\kappa\text{B}\alpha$  degradation evoked by I/R. Supporting this result, the expression of P65 was pronouncedly upregulated by I/R compared to that in Sham group, and elevation in P65 expression was remarkably inhibited by pretreatment of CP (Figure 8(d)).

The interaction between vascular endothelium and neutrophils is a central feature of the inflammatory response [25]. Integrins CD11 and CD18 on neutrophils are engaged in mediating neutrophil firm adhesion to endothelium and transmigration [26]. The expression of CD18 on neutrophils in blood stream was detected by flow cytometry, and the result is shown in Figure 8(e). The amount of CD18 was enhanced after I/R, which was significantly inhibited by

pretreatment with CP. However, no evident difference was detected in the expression of another adhesion molecule CD11b among the five groups at 60 min of reperfusion (Figure 8(f)).

**3.9. CP Administration Enhances Expression of Antioxidant Enzymes after I/R.** Endogenous antioxidant enzymes, such as SOD, CAT, glutathione peroxidase (GSH-PX), and its substrate GSH, work together to reduce free radical to water and minimize ROS-induced injury. Thus, the levels of antioxidants from myocardial tissue were tested as shown in Figure 9. There was no significant difference among the five groups at 60 min of reperfusion in the expression of SOD (Figure 9(a)) and CAT (Figure 9(b)). However, the level of myocardium GSH, a potent reductant, was dramatically reduced by exposure to I/R, which was significantly

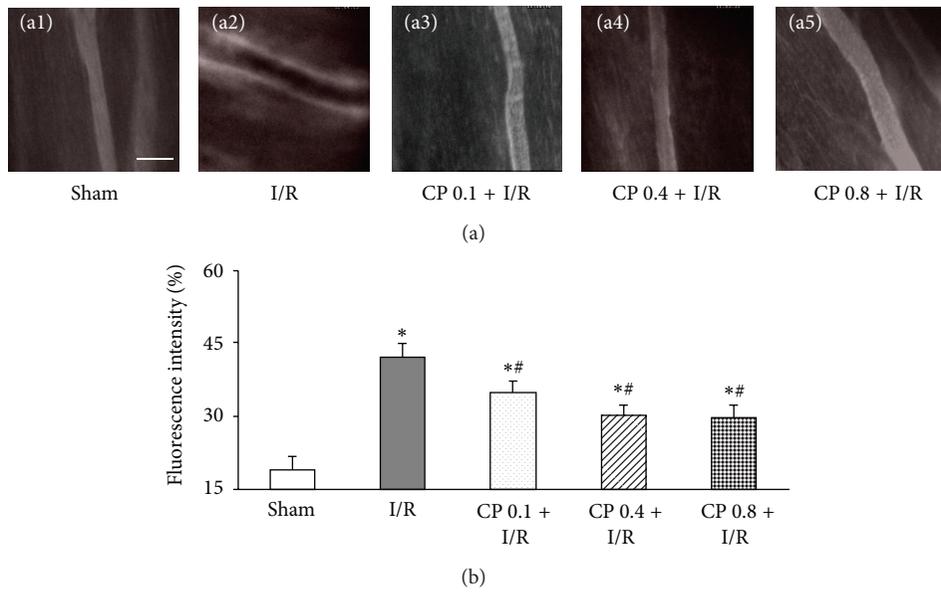


FIGURE 4: CP pretreatment prevents coronary venular albumin leakage. (a) Representative images of albumin leakage from coronary venules in Sham group (a1), I/R group (a2), CP 0.1 + I/R group (a3), CP 0.4 + I/R group (a4), and CP 0.8 + I/R group (a5). Bar = 100  $\mu$ m. (b) Statistical results of albumin leakage expressed as ratio of fluorescence intensity in interstice to that in venular lumen. Results are presented as mean  $\pm$  SEM ( $n = 6$ ). \* $P < 0.05$  versus Sham group, # $P < 0.05$  versus I/R group.

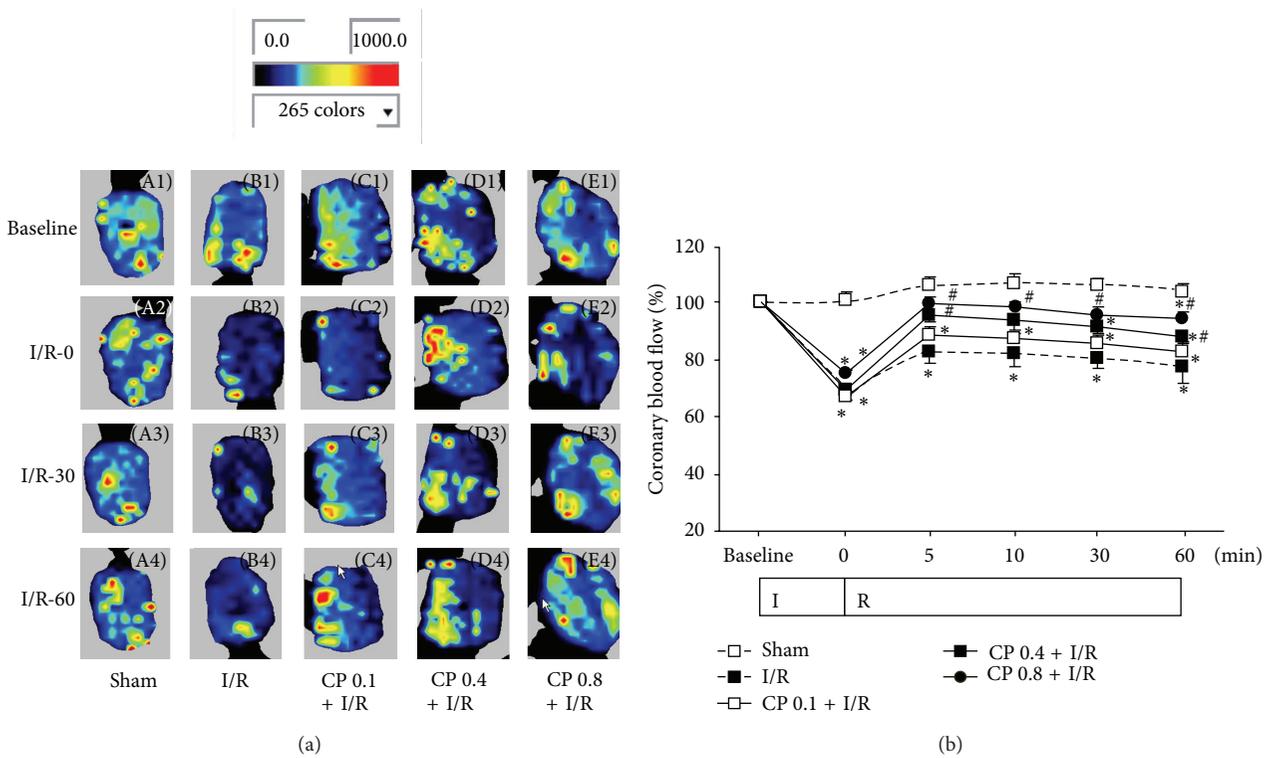


FIGURE 5: CP pretreatment improves MBF. (a) Representative MBF images acquired by Laser Doppler Perfusion Imager in Sham group (A), I/R group (B), CP 0.1 + I/R group (C), CP 0.4 + I/R group (D), and CP 0.8 + I/R group (E). For each group, images at baseline (1) and 0 min (2), 30 min (3), and 90 min (4) of reperfusion (4) are shown, respectively. Color scale illustrates myocardial blood flow from dark blue (low flow) to red (high flow). (b) Time course of quantitative results of MBF in various groups. MBF is expressed as a percentage of baseline MBF. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). \* $P < 0.05$  versus Sham group, # $P < 0.05$  versus I/R group.

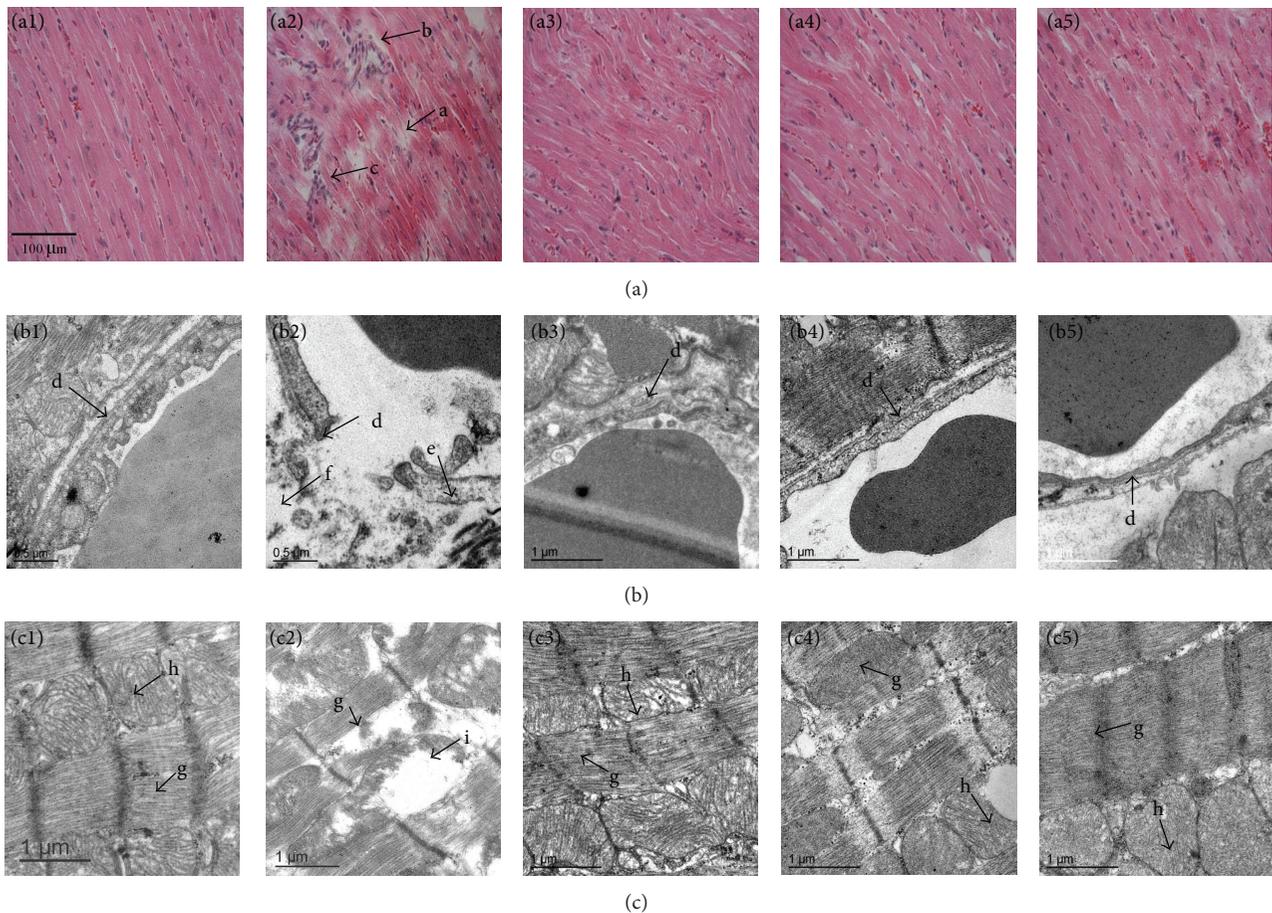


FIGURE 6: CP pretreatment diminishes I/R-induced alteration in myocardial tissue morphology. (a) Representative images of myocardial sections stained with HE. a: Disrupted myocardial fiber. b: Interstitial edema. c: Inflammatory cell infiltration. Bar = 100  $\mu\text{m}$ . (b) Representative electron micrographs of myocardial capillary from various groups. d: Vascular endothelium. e: Caveolae. f: Interstitial edema. (c) Representative electron micrographs of myocardial fiber in different groups. g: Myofilament. h: Mitochondria. i: Corrupted mitochondria. Results are presented for Sham group (1), I/R group (2), CP 0.1 + I/R group (3), CP 0.4 + I/R group (4), and CP 0.8 + I/R group (5).

restored by pretreatment with CP at the three doses tested (Figure 9(c)).

**3.10. CP Administration Prevents NADPH Oxidase Activation after I/R.** In the resting condition, gp91<sup>phox</sup> together with p22<sup>phox</sup> are located in intracellular vesicle membrane [11]. Activation of NADPH oxidase requires interactions with cytosolic organizer and modulator subunits, including p67<sup>phox</sup>, p47<sup>phox</sup>, and p40<sup>phox</sup> to form an enzyme complex. Once activated, vesicles bearing NOX2 enzyme complex translocate towards and fuse with the plasma membrane [11] and transfer single electron from cytoplasmic NADPH across the plasma membrane to extracellular molecular oxygen to generate superoxide.

To examine the activation of NADPH oxidase, expression of gp91<sup>phox</sup> on the membrane and translocations of organizer subunits p67<sup>phox</sup>, p47<sup>phox</sup>, and p40<sup>phox</sup> from cytoplasm to cell membrane were detected. The representative western blot bands and statistical results are shown in Figure 10. Expression of gp91<sup>phox</sup>, p67<sup>phox</sup>, and p47<sup>phox</sup> on cell membrane

was significantly increased after challenge by I/R compared to Sham operation, while this increase was significantly inhibited by pretreatment of CP at all three doses (Figures 10(a) and 10(b)). Correspondently, the expression of p67<sup>phox</sup> and p47<sup>phox</sup> in cytoplasm decreased remarkably after I/R, which was significantly blunted by pretreatment with CP (Figures 10(c) and 10(d)). On the other hand, no evident alteration in p40<sup>phox</sup> expression was observed either on cell membrane or in cytoplasm, implying that p40<sup>phox</sup>, as a modulator, was not involved in the I/R-induced activation of NADPH oxidase.

#### 4. Discussion

In the present study, we confirm that pretreatment with CP for six days at minimal effective dose 0.1 g/kg/day significantly prevented I/R-induced myocardial infarct and apoptosis, cardiac microcirculatory dysfunction, morphological alterations, and oxidative stress. In addition, CP pretreatment dramatically inhibited I/R-induced NADPH oxidase subunits

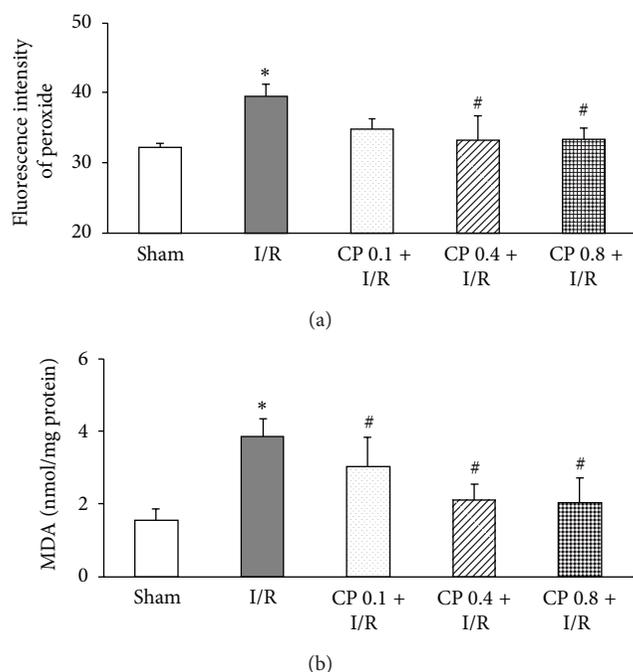


FIGURE 7: CP pretreatment alleviates I/R-induced oxidative stress. (a) Peroxide levels in peripheral blood are presented as fluorescence intensity of peroxide sensitive probe DHR in different groups. (b) MDA level of myocardial tissue in various groups. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). \* $P < 0.05$  versus Sham group, # $P < 0.05$  versus I/R group.

gp91<sup>phox</sup> expression and p67<sup>phox</sup> and p47<sup>phox</sup> translocation from cytoplasm to cell membrane. Thus, this study demonstrates, for the first time, that the antioxidative activity of CP is, at least in part, dependent on its ability to inhibit NADPH oxidase activation.

Although intensive investigations have been carried out in the last decades on protecting cardiomyocytes from I/R injury, no effective therapeutic approach has been applied in clinic. One possible reason is the lack of therapeutic interventions that could simultaneously target multiple pathological events during I/R. CP is proved to be a safe and effective TCM and consisted of diverse components which are reported to relieve a variety of deleterious consequences induced by I/R, including ROS, inflammation, and apoptosis. In 2013, CP has been scheduled to undergo phase III clinical trials for prevention and treatment of ischemic cardiovascular diseases by the US Food and Drug Administration and has thus attracted increasing interest of researchers to explore the protective mechanism of CP and reveal its new clinical indications.

CP is currently used in clinic at the dose of 0.081 g/kg/day in a multiple dosing manner. In the present study, we mimicked the multiple administration drug delivery method in clinic by giving CP for 6 days through gavage. Results show that CP was effective at 0.1, 0.4, and 0.8 g/kg/day, which were 1.2-, 4.9-, and 9.9-fold, respectively, the equivalent effective dose used in human. This result appears inconsistent with

our previous results showing that a single administration of CP 90 min before I/R only at 0.8 g/kg/day, but not lower doses, attenuates cardiac microcirculation disturbance and myocardium injury after 60 min of reperfusion [17]. This discrepancy results from the difference in CP administration protocol, with a multiple dosing in the present study while a single dosing in our previous work, suggesting that CP can be applied in a manner of either a multiple dosing at low dosage, as commonly used in clinic, or of a single dosing, if necessary, at a high dosage.

Intense inflammatory reaction during I/R has been implicated to extend myocardial injury [27]. One of the earliest inflammatory responses involves neutrophil adhesion to capillary endothelium mediated by integrins CD 18, CD 11a, and CD11b [25]. The trapped neutrophils plug the capillary and release autacoids to induce vasoconstriction and platelet aggregation [28]. In the present study, I/R caused an increase in CD18 expression on neutrophils, reduced coronary RBC velocity and myocardial blood flow, which were significantly protected by CP pretreatment, confirming the potential of CP as an anti-inflammatory agent.

Inflammatory responses are associated with increased expression of a range of cytokines and adhesion molecules, majority of which is regulated by NF- $\kappa$ B. NF- $\kappa$ B could be activated by cytokines themselves and free radicals as well [29]. In our experiment, we found that CP pretreatment represses the degradation of I $\kappa$ B $\alpha$ , and inhibits the activation of NF- $\kappa$ B. The available results suggest that this anti-inflammatory effect of CP is, at least in part, attributable to its antioxidant potential.

I/R leads to markedly accelerated ROS production, resulting in deleterious alterations in cell structure and function. Increasing investigation has been carried out to reduce massive ROS in I/R injury. In the present study, CP pretreatment suppressed I/R-induced formation of ROS in both myocardial tissue and peripheral blood. The antioxidant activity of CP mostly, if not all, attributes to its ingredients extracted from SM. Several studies support the cardiac protective effects and oxidant scavenging activity of SM [30]. The water-soluble compounds of SM extract are mainly polyphenols, which possess aromatic ring(s) bearing one or more hydroxyl moieties [31]. Polyphenols are reported to be effective ROS scavengers due to the presence of multiple phenolic hydroxyl groups [32], which accept electron from ROS to form stable phenoxyl radicals and, in turn, interrupt the cascade of oxidation reactions in cell [33].

Oxidative stress after I/R is the result of imbalance between the massive ROS and limited antioxidant defenses. Endogenous enzymatic and nonenzymatic antioxidants include SOD, CAT, GSH-PX, and its cofactor GSH [34]. The results on the effects of CP on myocardial antioxidant expression indicated that the expression of SOD and catalase were not altered by CP administration, while an increased level of GSH was observed after 90 min of reperfusion in CP pretreated group compared with I/R group. This result suggests that, besides direct reaction with ROS by polyphenols, enhanced GSH may be another reason for the reduced oxidative stress by CP pretreatment. The ingredient(s) in CP responsible for this endpoint is unknown

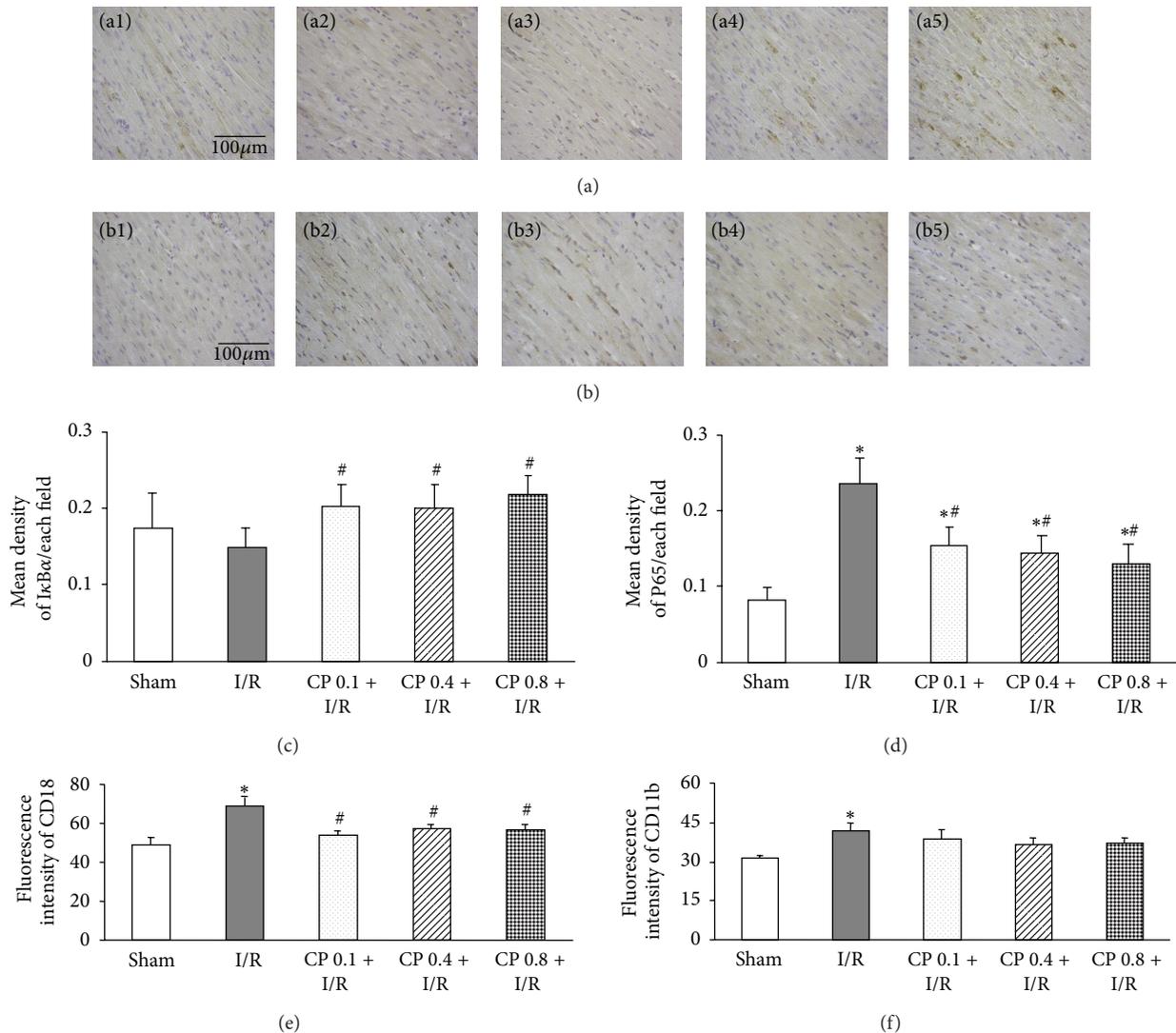


FIGURE 8: Effects of CP pretreatment on NF- $\kappa$ B activation and adhesion molecule expression after I/R. (a) Representative images of immunohistochemistry staining for I $\kappa$ B $\alpha$ . I $\kappa$ B $\alpha$  positive cells are revealed by DAB (brown). Bar = 100  $\mu$ m. (b) Representative photographs of myocardial sections stained for P65 in different groups. P65 proteins are shown in brown color. Bar = 100  $\mu$ m. (c) Statistical results of I $\kappa$ B $\alpha$  expression in five groups. (d) Quantitative analysis of P65 expression in various groups. 1, 2, 3, 4, and 5 denote Sham, I/R, CP 0.1 + I/R, CP 0.4 + I/R, and CP 0.8 + I/R group, respectively. (e) and (f) The expression of adhesion molecules CD18 and CD11b in rat neutrophils, respectively. The expression of adhesion molecules is presented as fluorescence intensity of FITC. Results are presented as mean  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$  versus Sham group, #  $P < 0.05$  versus I/R group.

at present, and the mechanism details behind this effect remain to be clarified.

For many years, researchers have focused on ROS detoxification, either using supplemented antioxidants in a non-targeted and nonspecific way like Vitamin E [35] or drugs that increase endogenous antioxidants such as GSH and SOD mimetics [36, 37]. Although much of the published results support that antioxidants protect cardiomyocytes against the damage induced by I/R, the results of several large randomized clinical trials regarding ROS scavengers in cardiovascular disease have been disappointing [38]. Thus, seeking for an approach able to directly block the enzymes involved in ROS synthesis become an attractive alternative to

inhibit ROS production. NADPH oxidase is a main source of superoxide ( $O_2^{\cdot -}$ ) in most of cardiovascular diseases, which is central to the regulation of other ROS formation [39]. Massive amount of  $O_2^{\cdot -}$  produced by NADPH oxidase activates other enzymes such as xanthine oxidase (XO) and endothelial NO synthase (eNOS) to produce free radicals [40, 41]. Several cell types within heart contribute to NADPH oxidase-derived ROS production, among which vascular endothelial cells and inflammatory cells are well appreciated to produce ROS by activation of NADPH oxidase in response to I/R challenge. Regarding the role of NADPH oxidase in cardiomyocytes in I/R injury, the data so far available are controversial. Evidence in vitro showed that gene transfer of a dominant

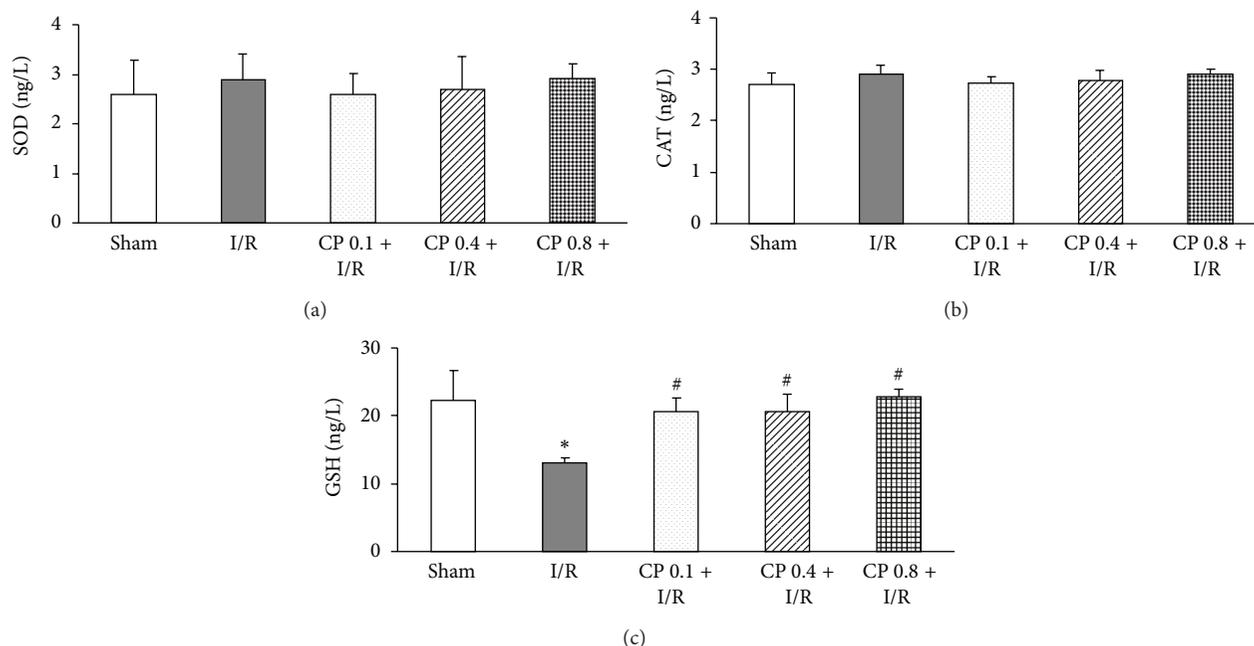


FIGURE 9: The effect of CP pretreatment on the expression of antioxidative enzymes after I/R. (a) Myocardial SOD expression in different groups. (b) The level of myocardial CAT in various groups. (c) GSH expression of myocardial tissues in five groups. Data are presented as mean  $\pm$  SEM ( $n = 6$ ). \* $P < 0.05$  versus Sham group, # $P < 0.05$  versus I/R group.

negative racl gene product (NI7racl) inhibits excessive ROS production in ventricular myocytes, endothelial and vascular muscle cells subjected to reoxygenation injury [42], while racl activation has been recognized to be the first episode of NADPH oxidase activation [39]. Moreover, elevated NOX2 expression was observed in human cardiomyocytes after acute myocardial infarction [13]. On the other hand, both NOX2<sup>-/-</sup> and p47<sup>phox</sup><sup>-/-</sup> mice were not found to exhibit attenuated I/R-induced injury [43, 44]. In the current study, an enhanced activation of NADPH oxidase was revealed in cardiac tissue after I/R. We at present cannot discriminate the cell type that exhibited the activated NADPH oxidase or the relative contribution of each cell type to this outcome. Nonetheless, CP pretreatment attenuated the activation of NADPH oxidase in cardiac tissue, indicating that NADPH oxidase is one of the targets that CP acts to protect heart from I/R damage.

Investigations have been conducted with attempt to find specific agents to inhibit the NADPH oxidase activity. For instance, peptides such as Gp91ds-tat and PR39 were shown to possess decoy p47<sup>phox</sup> binding sites, which prevent interaction between p47<sup>phox</sup> and NOX proteins, in turn, suppress NADPH oxidase activation [45, 46]. However, peptide drugs are limited to parenteral administration. Chemical inhibitors such as apocynin and aminoethyl benzenesulfonyl-fluoride (AEBSF) were reported to block NADPH oxidase assembly and result in a marked impairment of NADPH activation

[47, 48]. However, apocynin and AEBSF have not been thoroughly defined in humans as to their specificity, efficiency, and toxicity. In the present study, we presented evidences showing the inhibitory effect of CP on NADPH oxidase activation by reducing gp91<sup>phox</sup> expression and repressing translocations of p67<sup>phox</sup> and p47<sup>phox</sup> to cell membrane, indicating CP as a potential regime to abate the activation of NADPH oxidase during I/R.

The NADPH oxidase inhibition effect has been previously reported for the components of CP. To this end, salvianolic acid A and salvianolic acid B were shown to attenuate ROS formation and reduce the expression of gp91<sup>phox</sup> and p47<sup>phox</sup> in membrane fraction in hepatic fibrosis model [49]. Tanshinone II A was reported to negatively regulate upstream pathway of NADPH oxidase [50]. Similar inhibitory effects were also observed for other SM components such as rosmarinic acid [51], caffeic acid, and protocatechuic acid [52]. Ginsenoside Rb1, a major component of PN, was found to suppress oxidative damage via inhibition of NADPH oxidase subunit p47<sup>phox</sup> in renal interstitial fibrosis rats [53]. The finding of the present study is in agreement with the reports above. More importantly, the results of the present study argue the superiority of CP as antioxidant over others in that it acts on multiple targets, including inhibition of NADPH oxidase, elevation of GSH, and reaction with ROS via phenolic hydroxyl groups, which collectively and efficiently reduce the oxidative stress injury to cardiac tissue after I/R.

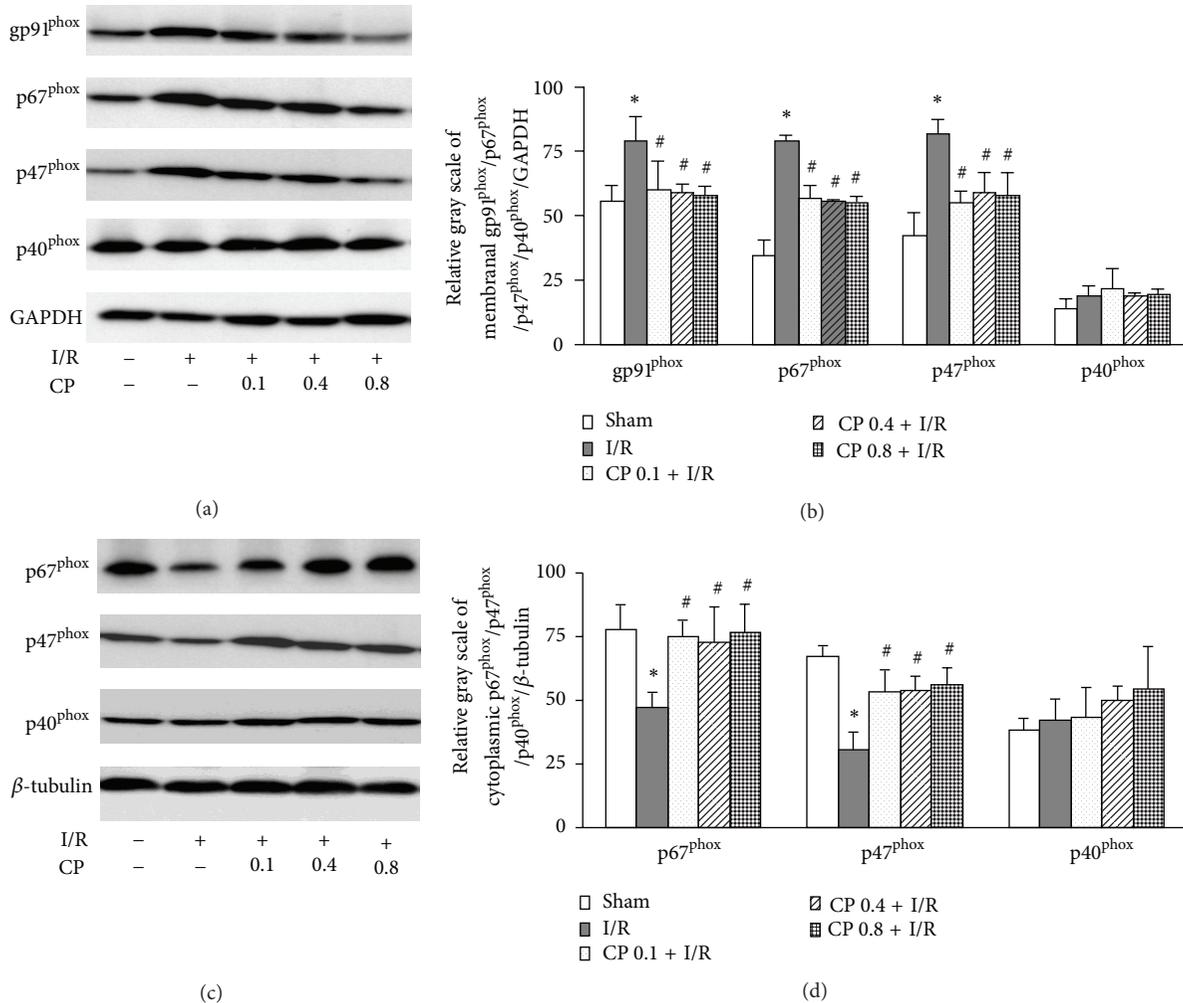


FIGURE 10: CP pretreatment inhibits NADPH oxidase activation induced by I/R. (a) Representative Western blot bands of gp91<sup>phox</sup>, p67<sup>phox</sup>, p47<sup>phox</sup> and p40<sup>phox</sup> on cell membrane. (b) Quantification of cell membrane expression of gp91<sup>phox</sup>, p67<sup>phox</sup>, p47<sup>phox</sup> and p40<sup>phox</sup>. All membrane protein intensities were normalized to GAPDH. (c) Representative Western blot bands of NADPH oxidase organizer subunits p67<sup>phox</sup> and p47<sup>phox</sup> and modulator p40<sup>phox</sup> in cytoplasm. (d) Statistical results of cytoplasm expression of p67<sup>phox</sup>, p47<sup>phox</sup> and p40<sup>phox</sup>. All band intensities were calculated based on the results from 3 independent experiments. All cytosolic protein intensities were normalized to  $\beta$ -tubulin. Results are presented as mean  $\pm$  SEM ( $n = 3$ ). \*  $P < 0.05$  versus Sham group, #  $P < 0.05$  versus I/R group.

This result provides an example showing that a compound medicine may be some time preferable to a single one to cope with an insult.

## 5. Conclusions

In summary, we reported that treatment with CP at doses ranging from 0.1 to 0.8 g/kg/day for 6 days effectively prevents rat heart from I/R-induced impacts, including infarct, apoptosis, microcirculation disturbance, oxidative stress, and inflammatory responses. Besides, CP pretreatment significantly repressed I/R-induced NADPH oxidase subunits gp91<sup>phox</sup> expression and p67<sup>phox</sup> and p47<sup>phox</sup> translocation from cytoplasm to cell membrane. The cardioprotective effects of CP, at least partly, attribute to its antioxidative activity mediated by inhibition of NADPH oxidase activation.

## Conflict of Interests

The authors declare no conflict of interests.

## Authors' Contribution

Xiao-Yuan Yang and Na Zhao equally contributed to this work.

## Acknowledgments

This work was supported by the Production of New Medicine Program of Ministry of Science and Technology of China (2008ZX09401) and the National Natural Science Foundation of China (812736370) for Jing-Yan Han.

## References

- [1] American Heart Association, *Learn and Live. Know the Facts, Get the Stats*, American Heart Association, Dallas, Tex, USA, 2006.
- [2] S. K. Powers, Z. Murlasits, M. Wu, and A. N. Kavazis, "Ischemia-reperfusion-induced cardiac injury: a brief review," *Medicine and Science in Sports and Exercise*, vol. 39, no. 9, pp. 1529–1536, 2007.
- [3] G. Ambrosio, L. C. Becker, G. M. Hutchins, H. F. Weisman, and M. L. Weisfeldt, "Reduction in experimental infarct size by recombinant human superoxide dismutase: insights into the pathophysiology of reperfusion injury," *Circulation*, vol. 74, no. 6, pp. 1424–1433, 1986.
- [4] K. L. Hamilton, "Antioxidants and cardioprotection," *Medicine and Science in Sports and Exercise*, vol. 39, no. 9, pp. 1544–1553, 2007.
- [5] P. Pagliaro, F. Moro, F. Tullio, M. Perrelli, and C. Penna, "Cardioprotective pathways during reperfusion: focus on redox signaling and other modalities of cell signaling," *Antioxidants and Redox Signaling*, vol. 14, no. 5, pp. 833–850, 2011.
- [6] K. A. Webster, "Mitochondrial membrane permeabilization and cell death during myocardial infarction: roles of calcium and reactive oxygen species," *Future Cardiology*, vol. 8, no. 6, pp. 863–884, 2012.
- [7] L. Xiao, D. R. Pimentel, J. Wang, K. Singh, W. S. Colucci, and D. B. Sawyer, "Role of reactive oxygen species and NAD(P)H oxidase in  $\alpha$ 1-adrenoceptor signaling in adult rat cardiac myocytes," *The American Journal of Physiology*, vol. 282, no. 4, pp. C926–C934, 2002.
- [8] K. K. Griendling, C. A. Minieri, J. D. Ollerenshaw, and R. W. Alexander, "Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells," *Circulation Research*, vol. 74, no. 6, pp. 1141–1148, 1994.
- [9] A. Görlach, R. P. Brandes, K. Nguyen, M. Amidi, F. Dehghani, and R. Busse, "A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall," *Circulation Research*, vol. 87, no. 1, pp. 26–32, 2000.
- [10] S. Johar, A. C. Cave, A. Narayanapanicker, D. J. Grieve, and A. M. Shah, "Aldosterone mediates angiotensin II-induced interstitial cardiac fibrosis via a Nox2-containing NADPH oxidase," *FASEB Journal*, vol. 20, no. 9, pp. 1546–1548, 2006.
- [11] K. Bedard and K. H. Krause, "The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology," *Physiological Reviews*, vol. 87, no. 1, pp. 245–313, 2007.
- [12] T. Fukui, M. Yoshiyama, A. Hanatani, T. Omura, J. Yoshikawa, and Y. Abe, "Expression of p22-phox and gp91-phox, essential components of NADPH oxidase, increases after myocardial infarction," *Biochemical and Biophysical Research Communications*, vol. 281, no. 5, pp. 1200–1206, 2001.
- [13] P. A. J. Krijnen, C. Meischl, C. E. Hack et al., "Increased Nox2 expression in human cardiomyocytes after acute myocardial infarction," *Journal of Clinical Pathology*, vol. 56, no. 3, pp. 194–199, 2003.
- [14] S. P. Loukogeorgakis, M. J. van den Berg, R. Sofat et al., "Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans," *Circulation*, vol. 121, no. 21, pp. 2310–2316, 2010.
- [15] Y. H. Looi, D. J. Grieve, A. Siva et al., "Involvement of Nox2 NADPH oxidase in adverse cardiac remodeling after myocardial infarction," *Hypertension*, vol. 51, no. 2, pp. 319–325, 2008.
- [16] F. Qin, M. Simeone, and R. Patel, "Inhibition of NADPH oxidase reduces myocardial oxidative stress and apoptosis and improves cardiac function in heart failure after myocardial infarction," *Free Radical Biology and Medicine*, vol. 43, no. 2, pp. 271–281, 2007.
- [17] N. Zhao, Y. Liu, F. Wang et al., "Cardiotonic pills, a compound Chinese medicine, protects ischemia-reperfusion-induced microcirculatory disturbance and myocardial damage in rats," *The American Journal of Physiology*, vol. 298, no. 4, pp. H1166–H1176, 2010.
- [18] X. H. Wei, Y. Y. Liu, Q. Li et al., "Treatment with cardiotonic pills after ischemia-reperfusion ameliorates myocardial fibrosis in rats," *Microcirculation*, vol. 20, no. 1, pp. 17–29, 2013.
- [19] Z. Qiao, J. Ma, and H. Liu, "Evaluation of the antioxidant potential of *Salvia miltiorrhiza* ethanol extract in a rat model of ischemia-reperfusion injury," *Molecules*, vol. 16, no. 12, pp. 10002–10012, 2011.
- [20] J. Han, Y. Horie, J. Fan et al., "Potential of 3,4-dihydroxyphenyl lactic acid for ameliorating ischemia-reperfusion-induced microvascular disturbance in rat mesentery," *The American Journal of Physiology*, vol. 296, no. 1, pp. G36–G44, 2009.
- [21] G. Zhao, H. Zhang, T. Ye et al., "Characterization of the radical scavenging and antioxidant activities of danshensu and salvianolic acid B," *Food and Chemical Toxicology*, vol. 46, no. 1, pp. 73–81, 2008.
- [22] H. Y. Son, H. S. Han, H. W. Jung, and Y. Park, "Panax notoginseng attenuates the infarct volume in rat ischemic brain and the inflammatory response of microglia," *Journal of Pharmacological Sciences*, vol. 109, no. 3, pp. 368–379, 2009.
- [23] H. Fan, L. Yang, F. Fu et al., "Cardioprotective effects of salvianolic acid a on myocardial ischemia-reperfusion injury in vivo and in vitro," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 508938, 9 pages, 2012.
- [24] Y. Liu, J. Yang, K. Sun, C. Wang, J. Han, and F. Liao, "Determination of erythrocyte flow velocity by dynamic grey scale measurement using off-line image analysis," *Clinical Hemorheology and Microcirculation*, vol. 43, no. 3, pp. 265–267, 2009.
- [25] J. Vinten-Johansen, "Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury," *Cardiovascular Research*, vol. 61, no. 3, pp. 481–497, 2004.
- [26] H. Zhou, J. Liao, J. Aloor et al., "CD11b/CD18 (Mac-1) is a novel surface receptor for extracellular double-stranded RNA to mediate cellular inflammatory responses," *Journal of Immunology*, vol. 190, no. 1, pp. 115–125, 2013.
- [27] M. L. Entman and C. W. Smith, "Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease," *Cardiovascular Research*, vol. 28, no. 9, pp. 1301–1311, 1994.
- [28] N. G. Frangogiannis, C. W. Smith, and M. L. Entman, "The inflammatory response in myocardial infarction," *Cardiovascular Research*, vol. 53, no. 1, pp. 31–47, 2002.
- [29] A. Rahman, J. Kefer, M. Bando, W. D. Niles, and A. B. Malik, "E-selectin expression in human endothelial cells by TNF- $\alpha$ -induced oxidant generation and NF- $\kappa$ B activation," *The American Journal of Physiology*, vol. 275, no. 3, pp. L533–L544, 1998.
- [30] X. Y. Ji, B. K. Tan, and Y. Z. Zhu, "*Salvia miltiorrhiza* and ischemic diseases," *Acta Pharmacologica Sinica*, vol. 21, no. 12, pp. 1089–1094, 2000.
- [31] J. Han, J. Fan, Y. Horie et al., "Ameliorating effects of compounds derived from *Salvia miltiorrhiza* root extract on microcirculatory disturbance and target organ injury by ischemia and

- reperfusion," *Pharmacology and Therapeutics*, vol. 117, no. 2, pp. 280–295, 2008.
- [32] A. Kunwar and K. I. Priyadarsini, "Free radicals, oxidative stress and importance of antioxidants in human health," *Journal of Medical and Allied Sciences*, vol. 1, no. 2, pp. 53–60, 2011.
- [33] B. B. Mathew, A. Tiwari, and S. K. Jatawa, "Free radicals and antioxidants: a review," *Journal of Pharmacy Research*, vol. 4, no. 12, pp. 4340–4343, 2011.
- [34] R. Kohen and A. Nyska, "Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification," *Toxicologic Pathology*, vol. 30, no. 6, pp. 620–650, 2002.
- [35] GISSI-Prevenzione Investigators, "Dietary supplementation with N-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial," *The Lancet*, vol. 354, no. 9177, pp. 447–455, 1999.
- [36] J. M. McCord, "Therapeutic control of free radicals," *Drug Discovery Today*, vol. 9, no. 18, pp. 781–782, 2004.
- [37] B. J. Day, "Catalytic antioxidants: a radical approach to new therapeutics," *Drug Discovery Today*, vol. 9, no. 13, pp. 557–566, 2004.
- [38] E. Lonn, J. Bosch, S. Yusuf et al., "Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial," *The Journal of the American Medical Association*, vol. 293, no. 11, pp. 1338–1347, 2005.
- [39] T. J. Guzik and D. G. Harrison, "Vascular NADPH oxidases as drug targets for novel antioxidant strategies," *Drug Discovery Today*, vol. 11, no. 11–12, pp. 524–533, 2006.
- [40] J. S. McNally, M. E. Davis, D. P. Giddens et al., "Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress," *The American Journal of Physiology*, vol. 285, no. 6, pp. H2290–H2297, 2003.
- [41] U. Landmesser, S. Dikalov, S. R. Price et al., "Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension," *Journal of Clinical Investigation*, vol. 111, no. 8, pp. 1201–1209, 2003.
- [42] K. Kim, K. Takeda, R. Sethi et al., "Protection from reoxygenation injury by inhibition of  $\text{rac1}$ ," *Journal of Clinical Investigation*, vol. 101, no. 9, pp. 1821–1826, 1998.
- [43] S. Frantz, R. P. Brandes, K. Hu et al., "Left ventricular remodeling after myocardial infarction in mice with targeted deletion of the NADPH oxidase subunit gp91PHOX," *Basic Research in Cardiology*, vol. 101, no. 2, pp. 127–132, 2006.
- [44] M. R. Hoffmeyer, S. P. Jones, C. R. Ross et al., "Myocardial ischemia/reperfusion injury in NADPH oxidase-deficient mice," *Circulation Research*, vol. 87, no. 9, pp. 812–817, 2000.
- [45] Y. Ikeda, L. H. Young, R. Scalia, C. R. Ross, and A. M. Lefer, "PR-39, a proline/arginine-rich antimicrobial peptide, exerts cardioprotective effects in myocardial ischemia-reperfusion," *Cardiovascular Research*, vol. 49, no. 1, pp. 69–77, 2001.
- [46] F. E. Rey, M. E. Cifuentes, A. Kiarash, M. T. Quinn, and P. J. Pagano, "Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular  $\text{O}_2^-$  and systolic blood pressure in mice," *Circulation Research*, vol. 89, no. 5, pp. 408–414, 2001.
- [47] V. Diatchuk, O. Lotan, V. Koshkin, P. Wikstroem, and E. Pick, "Inhibition of NADPH oxidase activation by 4-(2-aminoethyl)benzenesulfonyl fluoride and related compounds," *The Journal of Biological Chemistry*, vol. 272, no. 20, pp. 13292–13301, 1997.
- [48] J. Stolk, T. J. Hiltermann, J. H. Dijkman, and A. J. Verhoeven, "Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 11, no. 1, pp. 95–102, 1994.
- [49] M. Tsai, Y. Lin, and Y. Huang, "Effects of salvianolic acids on oxidative stress and hepatic fibrosis in rats," *Toxicology and Applied Pharmacology*, vol. 242, no. 2, pp. 155–164, 2010.
- [50] S. Gao, Z. Liu, H. Li, P. J. Little, P. Liu, and S. Xu, "Cardiovascular actions and therapeutic potential of tanshinone IIA," *Atherosclerosis*, vol. 220, no. 1, pp. 3–10, 2012.
- [51] D. Karthik, P. Viswanathan, and C. V. Anuradha, "Administration of rosmarinic acid reduces cardiopathology and blood pressure through inhibition of p22phox NADPH oxidase in fructose-fed hypertensive rats," *Journal of Cardiovascular Pharmacology*, vol. 58, no. 5, pp. 514–521, 2011.
- [52] J. A. Holland, R. W. O'Donnell, M. Chang, D. K. Johnson, and L. M. Ziegler, "Endothelial cell oxidant production: effect of NADPH oxidase inhibitors," *Endothelium*, vol. 7, no. 2, pp. 109–119, 1999.
- [53] X. Xie, H. Liu, M. Yang, C. Zuo, Y. Deng, and J. Fan, "Ginsenoside Rb1, a panoxadiol saponin against oxidative damage and renal interstitial fibrosis in rats with unilateral ureteral obstruction," *Chinese Journal of Integrative Medicine*, vol. 15, no. 2, pp. 133–140, 2009.

## Research Article

# Virgin Coconut Oil Prevents Blood Pressure Elevation and Improves Endothelial Functions in Rats Fed with Repeatedly Heated Palm Oil

Badlishah Sham Nurul-Iman,<sup>1,2</sup> Yusof Kamisah,<sup>1</sup>  
Kamsiah Jaarin,<sup>1</sup> and Hj Mohd Saad Qodriyah<sup>1</sup>

<sup>1</sup> Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia

<sup>2</sup> Faculty of Dentistry, Universiti Sains Islam Malaysia, 55100 Kuala Lumpur, Malaysia

Correspondence should be addressed to Hj Mohd Saad Qodriyah; qodryrz@gmail.com

Received 2 November 2012; Revised 13 May 2013; Accepted 20 May 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Badlishah Sham Nurul-Iman et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

This study was performed to explore the effects of virgin coconut oil (VCO) in male rats that were fed with repeatedly heated palm oil on blood pressure, plasma nitric oxide level, and vascular reactivity. Thirty-two male Sprague-Dawley rats were divided into four groups: (i) control (basal diet), (ii) VCO (1.42 mL/kg, oral), (iii) five-times-heated palm oil (15%) (5HPO), and (iv) five-times-heated palm oil (15%) and VCO (1.42 mL/kg, oral) (5HPO + VCO). Blood pressure was significantly increased in the group that was given the 5HPO diet compared to the control group. Blood pressure in the 5HPO + VCO group was significantly lower than the 5HPO group. Plasma nitric oxide (NO) level in the 5HPO group was significantly lower compared to the control group, whereas in the 5HPO + VCO group, the plasma NO level was significantly higher compared to the 5HPO group. Aortic rings from the 5HPO group exhibited attenuated relaxation in response to acetylcholine and sodium nitroprusside as well as increased vasoconstriction to phenylephrine compared to the control group. Aortic rings from the 5HPO + VCO group showed only attenuated vasoconstriction to phenylephrine compared to the 5HPO group. In conclusion, VCO prevents blood pressure elevation and improves endothelial functions in rats fed with repeatedly heated palm oil.

## 1. Introduction

Cardiovascular disease has become the main cause of death worldwide [1]. Hypertension or an increase in blood pressure is among factors that cause cardiovascular complications such as coronary heart disease, atherosclerosis, and stroke [2]. However, an unhealthy lifestyle is the main contributor to the increase in the incidence of hypertension [3].

Previous research has shown that heated palm oil causes a significant increase in blood pressure [4]. Hypertension is related to the overproduction of free radicals and lower antioxidant mechanisms in the body [5]. Repeatedly heated palm oil at a high temperature produces free radicals [6]. The presence of antioxidants such as vitamin E is destroyed during the heating process [7]. When the palm oil is heated repeatedly, not only does it generate free radicals but it also

reduces antioxidant and vitamin contents, which can lead to oxidative stress. Oxidative stress occurs due to an imbalance between the production of free radicals and a decrease in antioxidant activity in the body. Oxidative stress also leads to low-density lipoprotein (LDL) oxidation [8].

Oil that is heated at a high temperature will go through an oxidation process, which causes changes in fatty acid configuration from the cis isomer to the trans. Intake of trans fat correlates with an increase in cardiovascular disease risks [9]. Fatty acids which are oxidized due to repeatedly heated oil cause changes in endothelium function which leads to an impairment in vasodilatation reaction, increase in inflammation and hypertension risks [10] and total serum cholesterol and LDL [6, 11]. Previous research has shown that fried food intake correlates with the decrease in high-density lipoprotein (HDL) level [12].

In this study, repeatedly heated palm oil is used to mimic the situation that happens where people fry foods using the same oil multiple times. This practice is common among Malaysian, as a means to cut expenses. Previous research done by Azman et al. [13] showed that even though night market vendors agreed that repeatedly heated cooking oil is harmful to health, they still continued the practice of using the same cooking oil repeatedly.

Nowadays, virgin coconut oil (VCO) has become popular due to its beneficial effects. VCO has been shown to have anti-inflammatory, analgesic, and antipyretic properties [14]. VCO has been shown to decrease lipid levels in serum and tissue as well as LDL lipid peroxidation [15]. Consumption of VCO enhances antithrombotic effects related to inhibition of platelet coagulation and low cholesterol level [16]. VCO has been known to have higher antioxidant activity compared to refined coconut oil [17]. It has also been proven that VCO enhances antioxidant activity and inhibits lipid peroxidation in rats [18]. Therefore, it is of great interest for us to investigate whether VCO is able to prevent hypertension in male rats given repeatedly heated palm oil.

## 2. Materials and Methods

**2.1. Animals and Experimental Design.** Thirty-two male Sprague-Dawley rats, weighing between 200 and 250 g were obtained from the Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia. They were randomly divided equally into four groups comprising of eight animals each. The ethical approval for this study was obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committee (PP/FAR/2010/QODRIYAH/14-JULY/309-AUGUST-2010-AUGUST-2011). All animal management and procedures were performed in accordance with the recommended guidelines.

The rats were kept in stainless-steel cages at room temperature of  $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$  with a 12-hour light-dark cycle. All rats had free access to food and water throughout the experiment. After 1 week of acclimatization, each group of rats were fed on the following diets: (i) basal diet (commercial rat chow) (control), (ii) basal diet along with 1.42 mL/kg VCO orally (VCO), (iii) basal diet fortified with 15% weight/weight (w/w) five-times-heated palm oil (5HPO), and (iv) basal diet fortified with 15% weight/weight (w/w) five-times-heated palm oil along with 1.42 mL/kg VCO orally (5HPO + VCO) for 16 weeks. Body weight and food intake were measured weekly. Blood pressure was measured at baseline and at intervals of 4 weeks for a total duration of 16 weeks. Blood samples were collected via access to the orbital sinus prior to treatment and at the end of this study. At the end of the study, the animals were then sacrificed, and thoracic aortas were isolated for measurement of vascular reactivity.

**2.2. Virgin Coconut Oil.** The VCO used in this study was purchased from Organic Gain Sdn. Bhd., Bandar Baru Bangi, Selangor, Malaysia. It was administered by oral gavage at a dose of 1.42 mL/kg according to the minimal recommended dose of 10 mL per day in humans [19].

**2.3. Preparation of Diet.** The palm oil (Cap Buruh, Lam Soon Edible Oils, Kuala Lumpur, Malaysia) used was purchased from a local store. In this study, the palm oil was heated five times according to the method described by Owu et al. [20]. The heating process involved using 2.5 L of the oil to fry 1 kg of sweet potatoes in a stainless-steel wok. The temperature of the heated oil reached  $180^{\circ}\text{C}$  for 10 minutes. To heat the oil five times, the oil was cooled for 5 hours between heating, and then, the whole frying process was repeated with a new batch of sweet potatoes without any addition of fresh oil. This protocol was in accordance with earlier experimental procedures used in our laboratory [6]. Standard rat chow (Gold Coin, Kepong, Malaysia) was used. The rat chow was ground and mixed with 15% (w/w) of five-times-heated palm oil. The mixture was made into pellets which were dried overnight at room temperature.

**2.4. Measurement of Blood Pressure.** Systolic blood pressure of the prewarmed conscious rats was measured by the tail-cuff method using PowerLab data acquisition systems (ADInstruments, Castle Hill, NSW, Australia).

**2.5. Measurement of Plasma Nitric Oxide.** Nitric oxide content was indirectly measured by its metabolite nitrite. Blood samples taken were centrifuged to obtain its plasma and kept at  $-70^{\circ}\text{C}$ . Plasma samples of  $50\ \mu\text{L}$  were put into a 96-well microtiter plate and mixed with  $50\ \mu\text{L}$  of modified Griess reagent (Sigma-Aldrich, St. Louis, MO, USA). After 15 minutes of incubation at room temperature in a dark environment, nitrite concentration was measured at 540 nm wave length on an Emax ELISA microplate reader using SoftMax Pro Software (Molecular Devices, Sunnyvale, CA, USA). Nitrite concentration was then determined by plotting a standard curve with increasing concentration of sodium nitrite (Sigma-Aldrich, St. Louis, MO, USA).

**2.6. Measurement of Vascular Reactivity.** The aortic rings were prepared as described by Ajay and Mustafa [21]. The thoracic aorta was dissected, and excess fat and connective tissues were removed. The aorta was cut into ring segments with a width of 3–5 mm and suspended into a 5 mL organ baths containing Krebs solution of the following composition (mM): NaCl 118.0, 2 KCl 4.7,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  2.5,  $\text{KH}_2\text{PO}_4$  1.2,  $\text{MgSO}_4$  1.2, glucose 11.7,  $\text{NaHCO}_3$  25.0, and EDTA 0.026. The bathing solution was continuously provided with a mixture of oxygen and carbon dioxide. The tissue isometric tension (g) was recorded using a force-displacement transducer (FT03E, Grass Instruments, West Warwick, RI, USA) attached to a MacLab recording system (MacLab model 8 S, ADInstruments, Castle Hill, NSW, Australia). The aortic rings were readjusted to a basal tension of 1 g and allowed to equilibrate over 30 to 45 minutes. During this period, the bathing solutions were replaced every 15 minutes as required.

Following the equilibration period, the aortic rings were allowed to achieve contractile response to isotonic KCl solution (high  $\text{K}^+$ , 60 mM). Following the washout of responses to high  $\text{K}^+$ , the rings constricted in response to phenylephrine (PE,  $10^{-7}$  M) induced by an addition of acetylcholine

(Ach,  $10^{-5}$  M) to assess the endothelial integrity. Only the endothelial intact rings with more than 50% relaxation to Ach were further assessed. In addition, these aortic rings were tested for relaxation responses to increasing concentrations of Ach ( $10^{-10}$  M to  $10^{-5}$  M) and sodium nitroprusside (SNP  $10^{-10}$  M to  $10^{-5}$  M) and were recorded in PE ( $10^{-5}$  M) precontracted aortic rings. The contractile responses to increasing concentration of PE ( $10^{-10}$  M to  $10^{-5}$  M) were also recorded in the rings. Different aortic rings with intact endothelium were used in each experiment.

**2.7. Drugs.** The drugs used for the vascular reactivity study included acetylcholine chloride, phenylephrine-HCl (Sigma Chemical Co., St. Louis, MO, USA) and sodium nitroprusside (BDH Limited and BDH Laboratory Supplies, Poole, England).

**2.8. Statistical Analysis.** Results were presented as means  $\pm$  SEM. Normality of the data was determined using the Shapiro-Wilk test. Statistical differences were determined using the paired Student's *t*-test or one-way ANOVA followed by Tukey's HSD post hoc test to identify the differences using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Values of  $P < 0.05$  were considered to be significant.

### 3. Results

**3.1. Body Weight.** There was a significant increase in body weight of all the groups at week 16 compared to base line. There was no significant difference in body weight of the 5HPO group ( $451.63 \text{ g} \pm 15.05$ ) compared to the control group ( $457.00 \text{ g} \pm 11.59$ ). However, body weight in the VCO ( $411.38 \text{ g} \pm 5.33$ ) and 5HPO + VCO ( $431.63 \text{ g} \pm 12.97$ ) groups was significantly lower compared to the control group at week 16 (Figure 1).

**3.2. Food Intake.** The 5HPO ( $154.45 \text{ g} \pm 1.62$ ) and 5HPO + VCO ( $158.86 \text{ g} \pm 1.24$ ) groups showed significantly lower mean food intake compared to the control group ( $169.77 \text{ g} \pm 1.21$ ). There was no significant difference in food intake in the VCO ( $168.59 \text{ g} \pm 1.46$ ) compared to the control group. There was also no significant difference of food intake in the 5HPO + VCO compared to the 5HPO group (Figure 2).

**3.3. Blood Pressure.** Starting from week 8 to week 16, the 5HPO group showed a significant increase in blood pressure compared to the control group. Blood pressure in the 5HPO + VCO group is significantly lower compared to the 5HPO group from week 8 to week 16. Blood pressure in the VCO group ( $75.83 \text{ mmHg} \pm 1.99$ ) is significantly lower compared to the control group ( $98.08 \text{ mmHg} \pm 3.61$ ) at week 8 only (Figure 3).

**3.4. Changes in the Plasma Nitric Oxide Metabolite Level.** The 5HPO group ( $-8.75\% \pm 0.7$ ) showed a significant decrease in nitric oxide level compared to the control group ( $2.12\% \pm 2.6$ ). The VCO ( $14.73\% \pm 0.02$ ) and 5HPO + VCO ( $13.36\% \pm$

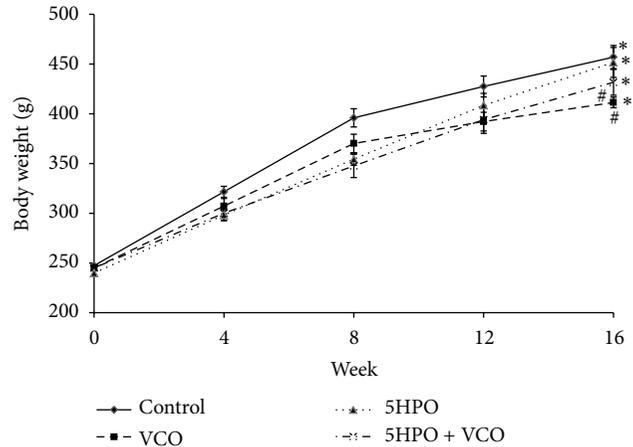


FIGURE 1: Changes in body weight during the study treatment. Data are expressed as mean  $\pm$  SEM. VCO, virgin coconut oil; five-times-heated palm oil, 5HPO. \*Significant difference ( $P < 0.05$ ) at week 16 compared to week 0 for each group. #Significant difference ( $P < 0.05$ ) compared to control at week 16.

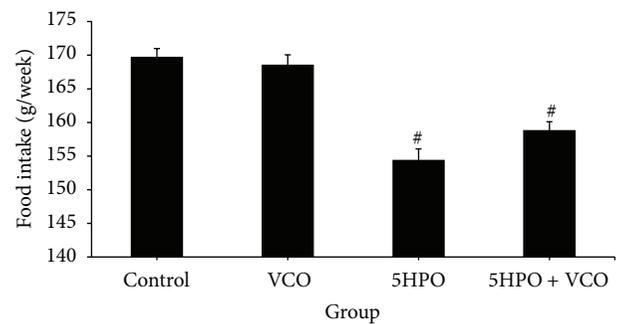


FIGURE 2: Food intake during the study treatment. Data are expressed as mean  $\pm$  SEM. VCO, virgin coconut oil; five-times-heated palm oil, 5HPO. #Significant difference ( $P < 0.05$ ) compared to control.

2.86) groups showed an increase in nitric oxide level compared to the control group. For the 5HPO + VCO group, there was a significant increase in nitric oxide level compared to the 5HPO group (Figure 4).

### 3.5. Vascular Response

**3.5.1. Relaxation in Response to Acetylcholine (Ach).** The percentage of relaxation at  $10^{-5}$  M and  $10^{-6}$  M concentration for 5HPO group was significantly lower compared to the control group. There was no significant difference in the percentage of relaxation in the 5HPO + VCO group compared to the 5HPO group at all concentrations (Figure 5).

**3.5.2. Relaxation in Response to Sodium Nitroprusside (SNP).** Vasodilatation in response to the highest tested concentration ( $10^{-5}$  M) was significantly attenuated in the aortic ring obtained from the 5HPO ( $108\% \pm 1.55$ ) group compared to the control group ( $120\% \pm 1.25$ ). There was no significant

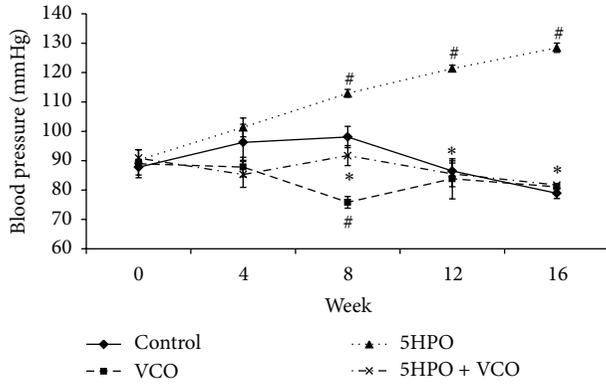


FIGURE 3: Changes in blood pressure during the study treatment. Data are expressed as mean  $\pm$  SEM. VCO, virgin coconut oil; five-times-heated palm oil, 5HPO. #Significant difference ( $P < 0.05$ ) compared to control. \*Significant difference ( $P < 0.05$ ) 5HPO + VCO compared to group 5HPO.

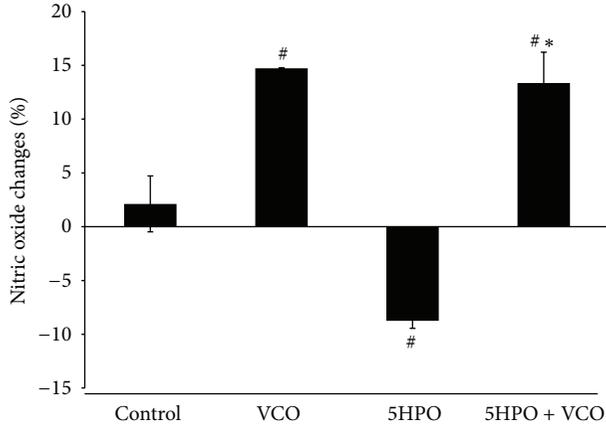


FIGURE 4: Changes in plasma nitric oxide metabolites during the study treatment. Data are expressed as mean  $\pm$  SEM. VCO, virgin coconut oil; five-times-heated palm oil, 5HPO. #Significant difference ( $P < 0.05$ ) compared to control. \*Significant difference ( $P < 0.05$ ) 5HPO + VCO compared to group 5HPO.

difference in vascular relaxation response in the 5HPO + VCO group (115%  $\pm$  0.84) compared to the 5HPO group (Figure 6).

**3.5.3. Contractile Response to Phenylephrine (PE).** The vasoconstriction in response towards PE was significantly augmented in the aortic rings from the 5HPO group compared to the control group at a concentration of  $10^{-6}$  to  $10^{-5}$  M. The aortic rings from the 5HPO + VCO group showed a significant decrease in vasoconstriction compared to the 5HPO group at a concentration of  $10^{-6}$  to  $10^{-5}$  M (Figure 7).

## 4. Discussion

In this study, it was found that food intake in the repeatedly heated oil diet was lower compared to the control group. However, we observed that there was no significant difference

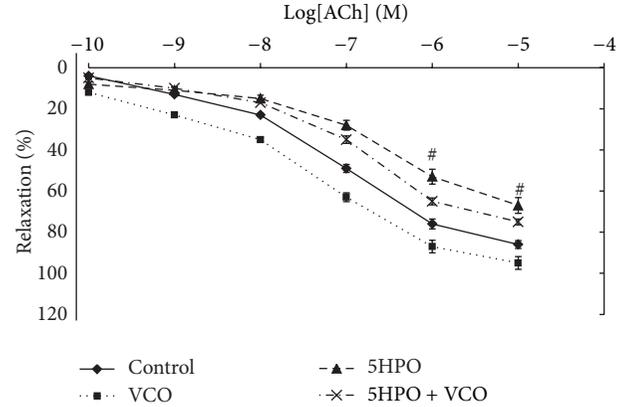


FIGURE 5: Endothelium-dependent relaxation in response to acetylcholine in aortic rings isolated from rats fed with basal diet (control), virgin coconut oil (VCO), five-times-heated palm oil (5HPO), and five-times-heated palm oil along with VCO (5HPO + VCO) at different concentrations. Data are expressed as mean  $\pm$  SEM. #Significant difference ( $P < 0.05$ ) compared to control.

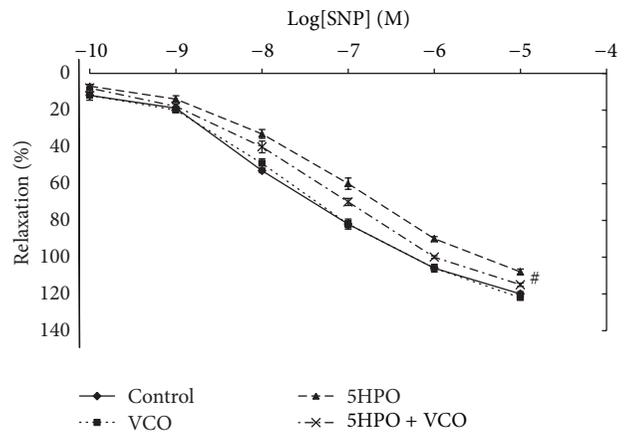


FIGURE 6: Endothelium-independent relaxation in response to sodium nitroprusside in aortic rings isolated from rats fed with basal diet (control), virgin coconut oil (VCO), five-times-heated palm oil (5HPO), and five-times-heated palm oil along with VCO (5HPO + VCO) at different concentrations. Data are expressed as mean  $\pm$  SEM. #Significant difference ( $P < 0.05$ ) compared to control.

in body weight between the 5HPO diet and the control group at week 16. Similar results were also obtained from a study done by Leong et al. [22], using repeated heated oil. Even though the food intake for the VCO and control diet was similar, the VCO diet group was found to have a decreased body weight compared to the control group at week 16. Food intake in the 5HPO + VCO diet was the same as 5HPO diet, but rats from the 5HPO + VCO diet group experienced a reduction in body weight compared to the control group at week 16. This shows that VCO supplementation causes a decrease in body weight. Previous studies in humans have shown that VCO appears to promote a reduction in abdominal obesity [23, 24]. According to a study conducted by St-Onge [25], medium-chain fatty acids (MCFA), compared to long-chain fatty acids,

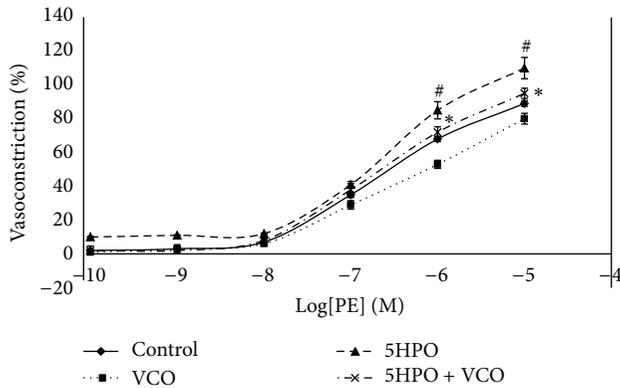


FIGURE 7: Contractile response induced by phenylephrine in aortic rings isolated from rats fed with basal diet (control), virgin coconut oil (VCO), five-times-heated palm oil (5HPO), and five-times-heated palm oil along with VCO (5HPO + VCO) at different concentrations. Data are expressed as mean  $\pm$  SEM. #Significant difference ( $P < 0.05$ ) compared to control. \*Significant difference ( $P < 0.05$ ) 5HPO + VCO compared to group 5HPO.

increase energy expenditure and resulted in faster satiety. MCFA found in VCO may aid as a beneficial replacement for other fats in the diet to help promote fullness and also increase caloric expenditure. The fat content helps to slow down the emptying of the stomach. Apart from that, MCFA are also directly broken down and transported to the liver as fuel. Therefore, VCO is utilized for energy and is less likely to get stored as fat, and this probably explains why it is able to reduce abdominal obesity in humans and body weight in study animals.

Oil that is heated repeatedly contains more saturated fatty acids than unsaturated fatty acids [22]. The body weight increment in rats in the 5HPO diet is not significantly different compared to the control group even though their food intake is lower because saturated fatty acid intake is capable of increasing tissue adiposity. The mechanism involved is due to the decrease of hormone-sensitive lipase and sympathetic activity in adipose tissue. Peroxisome proliferator-activated receptor (PPAR), which is a transcription expression factor, plays a role in proliferation and adiposity differentiation that causes adiposity apoptosis. It is then influenced by saturated fatty acids which lead to adiposity increment (fat tissue growth) that causes body weight increment [26].

The 5HPO diet causes blood pressure elevation. This study shows that feeding with repeatedly heated palm oil causes harm to health in rats by increasing hypertension risk. Previous research also showed that an increment in blood pressure occurs in rats that are administered oxidized oil [6, 27]. This is probably due to the increase in oxidative stress, thus causing changes in the nitric oxide level [6]. Reactive oxygen species (ROS) play an important role in the formation of hypertension [28], and ROS are formed during the heating process. Overproduction of ROS is capable of impairing cells and causing blood pressure elevation. The cell membrane is a structure that is sensitive towards oxidative attack due to the high content of polyunsaturated fatty acid (PUFA). Blood

pressure elevation is due to the production of free radicals that decreases the NO level [29].

VCO supplementation prevents the blood-pressure-raising effect of 5HPO. The blood-pressure-lowering effect of VCO may be attributable to its high polyphenol component [17, 18]. Previous studies have also shown that polyphenol was able to reduce blood pressure in hypertensive subjects [30, 31]. Rats on the VCO diet alone had a transient reduction in blood pressure only at week 8 compared to control. A study by Diebolt et al. [32] showed that short-term administration of polyphenolic compounds reduced blood pressure in normotensive rats. They hypothesized that polyphenol was able to stimulate NO released from the endothelium, giving rise to vasodilation and blood pressure reduction. The reason for the short-term effect is unclear because plasma nitric oxide level was taken at the start and end of the study, not monthly as with the blood pressure measurements. The polyphenol component has also been shown to prevent LDL oxidation, and a diet that contains VCO supplementation increases antioxidant status in rats [15, 18]. The cholesterol level and other lipid parameters in tissue and serum maintained within the normal range as well as increment in HDL concentration due to polyphenol contents in VCO [15].

Intake of the 5HPO diet is found to reduce plasma NO level significantly. Overproduction of free radicals increases inactivation of NO which leads to reduction of NO bioavailability. Hence, a reduction of NO causes blood pressure elevation. Intake of VCO diet shows plasma NO level higher than the control group. It also increases plasma NO levels in rats fed with the 5HPO diet. This is thought to be due to the antioxidant component polyphenol, found in VCO which is responsible for increasing NO bioavailability. Antioxidant contents in VCO are possibly capable of providing protection effects by reducing oxidative stress and thus maintaining the NO bioavailability [33].

In this study, it was found that the relaxation induced by acetylcholine-dependent endothelium was attenuated in rats fed with the 5HPO diet. Release of NO plays a role in determining the balance between vascular smooth muscle relaxation and vasoconstriction. If NO bioavailability decreases, this then leads to attenuation of vascular smooth muscle relaxation and vasoconstriction [34]. The antioxidant protective effects in oil probably deteriorate when palm oil is repeatedly heated.

The SNP is an endothelial vascular vasodilatation agent. SNP induces relaxation by releasing NO into the tissue, while the breakdown of the SNP molecule produces NO which activates guanylate cyclase to increase the formation of cyclic diguanylate monophosphate, which causes vascular smooth muscle relaxation. However, relaxation induced by SNP-independent endothelium was attenuated in rats that were given the 5HPO diet compared to the control group at the highest concentration of  $10^{-5}$  M. This is maybe due to the reduced bioavailability of NO which is involved in vasodilatation [34].

The rats on the 5HPO + VCO diet showed attenuated vasoconstriction induced by phenylephrine-dependent endothelium compared to the 5HPO diet. Free radicals such as anion superoxide are related to the increase

in vascular reactivity including vasoconstriction [35]. The blood-pressure-raising effect of this study was similar to previous research which showed that repeatedly heated oil causes attenuation in vascular reactivity [4]. Repeatedly heated oil also produces toxic products which can increase blood pressure, thus interrupting the endothelium balance. Vitamins and foods that contain antioxidants are capable of improving vascular reactivity and decreasing the bad effects on blood vessels which could prevent hypertension, thus maintaining the endothelium balance. VCO supplementation is capable of improving endothelial function because it is rich in antioxidants.

## 5. Conclusion

This study showed that VCO supplementation is capable of preventing elevation in blood pressure and also decreasing deactivation of nitric oxide in male rats fed with repeatedly heated palm oil. In addition, VCO does not influence relaxation but decreases vasoconstriction of the endothelium.

## Acknowledgments

This study was funded by Grant UKM-FF-03-FRGS0031-2010. The authors would like to thank Miss Juliana Abdul Hamid, Mr Fadhlullah Zuhair, Ms Sinar Suriya Muhammad, and staff members of the Pharmacology Department of Universiti Kebangsaan Malaysia and Universiti Malaya for their technical assistance.

## References

- [1] World Health Organization, *Global Health Risks: Mortality and Burden of Disease Attributable To Selected Major Risks*, World Health Organization, Geneva, Switzerland, 2009.
- [2] R. H. Fagard, "Resistant hypertension," *Heart*, vol. 98, no. 3, pp. 254–261, 2012.
- [3] M. Mohmmerdirfan, K. D. Vikas, and K. Abhay, "A study on effect of lifestyle risk factors on prevalence of hypertension among white collar job people of Surat," *The Internet Journal of Occupational Health*, pp. 2155–7330, 2011.
- [4] X. Leong, M. N. M. Najib, S. Das, M. R. Mustafa, and K. Jaarin, "Intake of repeatedly heated palm oil causes elevation in blood pressure with impaired vasorelaxation in rats," *Tohoku Journal of Experimental Medicine*, vol. 219, no. 1, pp. 71–78, 2009.
- [5] F. Farmand, A. Ehdiaie, C. K. Roberts, and R. K. Sindhu, "Lead-induced dysregulation of superoxide dismutases, catalase, glutathione peroxidase, and guanylate cyclase," *Environmental Research*, vol. 98, no. 1, pp. 33–39, 2005.
- [6] X. F. Leong, A. Aishah, U. Nor Aini, S. Das, and K. Jaarin, "Heated palm oil causes rise in blood pressure and cardiac changes in heart muscle in experimental rats," *Archives of Medical Research*, vol. 39, no. 6, pp. 567–572, 2008.
- [7] S. K. Adam, N. A. Sulaiman, A. G. Mat Top, and K. Jaarin, "Heating reduces vitamin E content in palm and soy oils," *Malaysian Journal of Biochemistry and Molecular Biology*, vol. 15, pp. 76–79, 2007.
- [8] S. Mitra, A. Deshmukh, R. Sachdeva, J. Lu, and J. L. Mehta, "Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy," *American Journal of the Medical Sciences*, vol. 342, no. 2, pp. 135–142, 2011.
- [9] D. C. Klonoff, "Benefits and limitations of self-monitoring blood glucose," *Journal of Diabetes Science and Technology*, vol. 1, no. 1, pp. 130–132, 2007.
- [10] C. Y. Ng, Y. Kamisah, O. Faizah, Z. Jubri, H. M. S. Qodriyah, and K. Jaarin, "Involvement of inflammation and adverse vascular remodelling in the blood pressure raising effect of repeatedly heated palm oil in rats," *International Journal of Vascular Medicine*, vol. 2012, Article ID 404025, 10 pages, 2012.
- [11] S. K. Adam, S. Das, I. N. Soelaiman, N. A. Umar, and K. Jaarin, "Consumption of repeatedly heated soy oil increases the serum parameters related to atherosclerosis in Ovariectomized rats," *Tohoku Journal of Experimental Medicine*, vol. 215, no. 3, pp. 219–226, 2008.
- [12] C. Donfrancesco, C. Lo Noce, O. Brignoli et al., "Italian network for obesity and cardiovascular disease surveillance: a pilot project," *BMC Family Practice*, vol. 9, article 53, 2008.
- [13] A. Azman, S. Mohd Shahrul, S. X. Chan et al., "Level of knowledge, attitude and practice of night market food outlet operators in Kuala Lumpur regarding the usage of repeatedly heated cooking oil," *Medical Journal of Malaysia*, vol. 67, no. 1, pp. 91–101, 2012.
- [14] S. Intahphuak, P. Khonsung, and A. Panthong, "Anti-inflammatory, analgesic, and antipyretic activities of virgin coconut oil," *Pharmaceutical Biology*, vol. 48, no. 2, pp. 151–157, 2010.
- [15] K. G. Nevin and T. Rajamohan, "Beneficial effects of virgin coconut oil on lipid parameters and in vitro LDL oxidation," *Clinical Biochemistry*, vol. 37, no. 9, pp. 830–835, 2004.
- [16] K. G. Nevin and T. Rajamohan, "Influence of virgin coconut oil on blood coagulation factors, lipid levels and LDL oxidation in cholesterol fed Sprague-Dawley rats," *e-SPEN*, vol. 3, no. 1, pp. e1–e8, 2008.
- [17] A. M. Marina, Y. B. Che Man, S. A. H. Nazimah, and I. Amin, "Antioxidant capacity and phenolic acids of virgin coconut oil," *International Journal of Food Sciences and Nutrition*, vol. 60, no. 2, pp. 114–123, 2009.
- [18] K. G. Nevin and T. Rajamohan, "Virgin coconut oil supplemented diet increases the antioxidant status in rats," *Food Chemistry*, vol. 99, no. 2, pp. 260–266, 2006.
- [19] B. Fife, *Coconut Cures: Preventing and Treating Common Health Problems With Coconut*, Avery Trade, New York, NY, USA, 4th edition, 2005.
- [20] D. U. Owu, E. E. Osim, and P. E. Ebong, "Serum liver enzymes profile of Wistar rats following chronic consumption of fresh or oxidized palm oil diets," *Acta Tropica*, vol. 69, no. 1, pp. 65–73, 1998.
- [21] M. Ajay and M. R. Mustafa, "Effects of ascorbic acid on impaired vascular reactivity in aortas isolated from age-matched hypertensive and diabetic rats," *Vascular Pharmacology*, vol. 45, no. 2, pp. 127–133, 2006.
- [22] X. Leong, M. R. Mustafa, S. Das, and K. Jaarin, "Association of elevated blood pressure and impaired vasorelaxation in experimental Sprague-Dawley rats fed with heated vegetable oil," *Lipids in Health and Disease*, vol. 9, article 66, pp. 66–76, 2010.
- [23] K. M. Liau, Y. Y. Lee, C. K. Chen, and A. H. G. Rasool, "An open-label pilot study to assess the efficacy and safety of virgin coconut oil in reducing visceral adiposity," *International Scholarly Research Network Pharmacology*, vol. 2011, 7 pages, 2011.
- [24] M. L. Assuncao, H. S. Ferreira, A. F. dos Santos, C. R. Cabral Jr, and T. M. M. T. Florencio, "Effects of dietary coconut oil on

the biochemical and anthropometric profiles of women presenting abdominal obesity,” *Lipids*, vol. 44, pp. 593–601, 2009.

- [25] M. P. St-Onge, “Dietary fats, teas, dairy, and nuts: potential functional foods for weight control?” *The American journal of clinical nutrition*, vol. 81, no. 1, pp. 7–15, 2005.
- [26] B. L. Wajchenberg, “Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome,” *Endocrine Reviews*, vol. 21, no. 6, pp. 697–738, 2000.
- [27] E. E. Osim, D. U. Owu, and K. M. Etta, “Arterial pressure and lipid profile in rats following chronic ingestion of palm oil diets,” *African Journal of Medicine and Medical Sciences*, vol. 25, no. 4, pp. 335–340, 1996.
- [28] M. Beg, A. Gupta, and V. N. Khanna, “Oxidative stress in essential hypertension and role of antioxidants,” *Indian Academy of Clinical Medicine*, vol. 11, no. 4, pp. 287–293, 2010.
- [29] N. D. Vaziri, X. Q. Wang, F. Oveisi, and B. Rad, “Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats,” *Hypertension*, vol. 36, no. 1, pp. 142–146, 2000.
- [30] J. Barona, J. C. Aristizabal, C. N. Blesso, J. S. Volek, and M. L. Fernandez, “Grape polyphenols reduce blood pressure and increase flow-mediated vasodilation in men with metabolic syndrome,” *The Journal of Nutrition*, vol. 142, no. 9, pp. 1626–1632, 2012.
- [31] R. Moreno-Luna, R. Muñoz-Hernandez, M. L. Miranda et al., “Olive oil polyphenols decrease blood pressure and improve endothelial function in young women with mild hypertension,” *American Journal of Hypertension*, vol. 25, no. 12, pp. 1299–1304, 2012.
- [32] M. Diebolt, B. Bucher, and R. Andriantsitohaina, “Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression,” *Hypertension*, vol. 38, no. 2, pp. 159–165, 2001.
- [33] A. Carr and B. Frei, “The role of natural antioxidants in preserving the biological activity of endothelium-derived nitric oxide,” *Free Radical Biology and Medicine*, vol. 28, no. 12, pp. 1806–1814, 2000.
- [34] A. E. Abdel Moneim, M. A. Dkhil, and S. Al-Quraishy, “Effects of flaxseed oil on lead acetate-induced neurotoxicity in rats,” *Biological Trace Element Research*, vol. 144, no. 1–3, pp. 904–913, 2011.
- [35] M. McIntyre, D. F. Bohr, and A. F. Dominiczak, “Endothelial function in hypertension: the role of superoxide anion,” *Hypertension*, vol. 34, no. 4, pp. 539–545, 1999.

## Research Article

# QSYQ Attenuates Oxidative Stress and Apoptosis Induced Heart Remodeling Rats through Different Subtypes of NADPH-Oxidase

Yong Wang,<sup>1</sup> Chun Li,<sup>1</sup> Yuli Ouyang,<sup>1</sup> Tianjiao Shi,<sup>1</sup> Xiaomin Yang,<sup>1</sup> Junda Yu,<sup>1</sup> Qi Qiu,<sup>2</sup> Jing Han,<sup>1</sup> Yan Wu,<sup>1</sup> Binghua Tang,<sup>1</sup> and Wei Wang<sup>1</sup>

<sup>1</sup> Beijing University of Chinese Medicine, Bei San Huan Dong Lu 11, Chao Yang District, 100029 Beijing, China

<sup>2</sup> Capital Medical University Beijing Anzhen Hospital, Beijing, China

Correspondence should be addressed to Wei Wang; wangwei.8@sina.cn

Received 4 March 2013; Revised 8 May 2013; Accepted 11 May 2013

Academic Editor: Keji Chen

Copyright © 2013 Yong Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

We aim to investigate the therapeutic effects of QSYQ, a drug of heart failure (HF) in clinical practice in China, on a rat heart failure (HF) model. 3 groups were divided: HF model group (LAD ligation), QSYQ group (LAD ligation and treated with QSYQ), and sham-operated group. After 4 weeks, rats were sacrificed for cardiac injury measurements. Rats with HF showed obvious histological changes including necrosis and inflammation foci, elevated ventricular remodeling markers levels (matrix metalloproteinases-2, MMP-2), deregulated ejection fraction (EF) value, increased formation of oxidative stress (Malondialdehyde, MDA), and up-regulated levels of apoptotic cells (caspase-3, p53 and tunnel) in myocardial tissue. Treatment of QSYQ improved cardiac remodeling through counter-acting those events. The improvement of QSYQ was accompanied with a restoration of NADPH oxidase 4 (NOX4) and NADPH oxidase 2 (NOX2) pathways in different patterns. Administration of QSYQ could attenuate LAD-induced HF, and AngII-NOX2-ROS-MMPs pathway seemed to be the critical potential targets for QSYQ to reduce the remodeling. Moreover, NOX4 was another key targets to inhibit the p53 and Caspase3, thus to reduce the hypertrophy and apoptosis, and eventually provide a synergetic cardiac protective effect.

## 1. Introduction

Heart failure (HF) after myocardial infarction (MI) is a common clinical syndrome with high morbidity and mortality [1]. It has been suggested that HF is closely correlated with cardiac remodeling [2]. A variety of known factors are involved in the development and progression of HF [3]; during these factors, reactive oxygen species (ROS) and apoptosis were considered as the most critical pathological changes [4].

More and more pieces of evidence have indicated that the activated oxidative stress contributes significantly to the deterioration of cardiovascular function and eventually leads to myocardial remodeling [5]. Experimental and clinical heart failure trials documented an increased production of ROS, like superoxide, malondialdehyde, and hydroxyl radicals. Different sources of increased ROS production

were found in the failing heart, including NAD(P)H oxidase (NOX) [6]. Two NOX isoforms, NOX2 and NOX4, are deeply investigated. Studies using NOX2 activity deficiency mice indicated that NOX2 activation contributes to angiotensin-II-induced cardiomyocyte hypertrophy [7]. In contrast, NOX4, which is constitutively active at a low level, can be induced by pressure overload and mediates cardiac hypertrophy mainly by apoptosis [8]. The development to target individual NOX isoforms may be important for the achievement of therapeutic efficacy in heart failure [9].

Apoptosis is another critical pathological change in HF. In particular, apoptotic cell death in infarcted tissue is reported to play an important role in progression of cardiac remodeling in HF [10]. Hayakawa et al. have reported that inhibition of cell apoptosis significantly improved left ventricular remodeling and heart failure in the chronic stage [11]. Especially, p53-caspase-3 mediated apoptosis is considered to

be the critical pathway during the HF [12]. Therefore not in vivo detection of apoptosis only may prove clinically useful information in the diagnosis and prognosis but also indicates that p53 pathway may be the potential target for the drugs [13]. Furthermore, apoptosis can be triggered by NAD(P)H oxidase-ROS pathway, which makes HF more complicated.

QiShenYiQi (QSYQ), a formula long been used for the routine treatment of coronary heart disease (CHD) or chronic heart failure (CHF) in China, has benefit efficacy in clinical [14]. It consists of six Chinese herbs (Radix Astragali Mongolici, *Salvia miltiorrhiza* bunge, Flos Lonicerae, *Scrophularia*, Radix Aconiti Lateralis Preparata, and Radix Glycyrrhizae), and is widely produced in China in accordance with the China Pharmacopoeia standard of quality control [13]. Our previous study found that QSYQ ameliorated myocardial hypertrophy and remodeling by inhibiting the expression of AngII in LAD rats [15]. However, little is known about the exact targets of QSYQ acting on myocardial remodeling. The purpose of the present study is to investigate whether QSYQ can act on HF in improving left ventricular remodeling associated with oxidative stress and apoptosis.

## 2. Materials and Methods

**2.1. Animals and Grouping.** Studies were performed in accordance with the China Physiological Society's "Guiding Principles in the Care and Use of Animals" and with approval of the Animal Care Committee of Beijing Medical Center. A total of 60 male SD rats (weighted  $240 \pm 10$  g) in SPF grade were selected (purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd.) into the study. Rats were housed in a standard animal room with food and water ad libitum under controlled conditions of humidity and temperature ( $25 \pm 1.2^\circ\text{C}$ ), under a 12 h light : 12 h dark lighting schedule.

**2.2. HF Model Preparation.** HF model was established as before [14]. Briefly, pentobarbital-anesthetized rats were fixed on the operating table. The thoracic cavity was opened to expose the heart, and the left coronary arteries (LADs) were ligated. In the sham group, the sutures were passed under the LADs without ligation. Animals were housed routinely following surgery. After ECG testing, rats that averaged QT-interval prolongation in three precordial leads were included into the model and QSYQ pharmacologic study. Then they were divided into 2 groups randomly, 8 in model group and 8 in QSYQ group. Meanwhile, 8 in sham-operated group were investigated together. The QSYQ group was treated for 28 days by daily irrigation stomach with total daily dosages of 2.33 g/kg of the concentrated QSYQ (Beijing university of Chinese Medicine, Beijing, China) dissolved in water as we did before [15, 16]. The sham-operated group and model group received the same volume water via irrigation stomach as the QSYQ vehicle. At the end of the study, all animals were anaesthetized using Isoflurane (Abraxis BioScience, Richmond Hill, ON, Canada) following an overnight fast. Heart tissue samples were excised parallel to

coronary sulcus, 3 mm apart from cardiac apex. All samples were frozen in liquid nitrogen immediately for further examinations.

**2.3. Preparation and Dose Consideration of Concentrated QSYQ.** The QSYQ used in the present study was manufactured by Beijing University of Chinese Medicine (Beijing, China) using the six Chinese herbs at a composition of 460 g Radix Astragali Mongolici, 230 g *salvia miltiorrhiza* bunge, 160 g Flos Lonicerae, 160 g *scrophularia*, 140 g Radix Aconiti Lateralis Preparata, and 90 g Radix Glycyrrhizae as before [15, 16]. Briefly, following extraction with 95% ethanol, the residue of Radix Astragali Mongolici was mixed with all *salvia miltiorrhiza* bunge, Flos Lonicerae, *Scrophularia*, and Radix Glycyrrhizae, followed by extraction with hot water (twice, 2 hr each). The water extract was then concentrated to form a paste, and the ethanol was added for 24 hr, collected the filtration to form the final product. In present study, dosage of 2.33 g/kg was established as the same content at our previous study [15, 16].

**2.4. Echocardiographic Assessment of Left Ventricular Function.** Echocardiography was used to detect the left ventricular end-systolic diameter (LVEDs), left ventricular end-diastolic diameter (LVEDd), ejection fraction (EF), fractional shortening (FS), and other indicators. Vevo 2100 Imaging System (VisualSonics, Canada) with RMV 710B probe (21 MHz probe) was employed, which generates two-dimensional images at a frame rate of 300 to 500 frames/s. The LV dimension (LVD) was measured using m-model fractional shortening, and FS% was calculated using the following equation:  $\text{FS}\% = [(\text{LVEDd} - \text{LVEDs})/\text{LVEDd}] \times 100$ .

**2.5. Histology and Immunohistochemistry (IHC).** The ventricles were fixed in 4% paraformaldehyde, paraffin embedded hearts were sectioned at 200  $\mu\text{m}$  intervals from base to apex, and serial sections of 4 mm were cut and placed on polylysine-coated glass slides. Tissue sections were deparaffinized and stained with Masson's trichrome reagent. An avidin-biotin-peroxidase complex commercial method (cell and tissue staining kit, R&D Systems, Inc., USA) was used for immunohistochemistry. Briefly, 4 mm thick paraffin wax sections were mounted on slides, which were dried for 30 minutes in an oven ( $60\text{--}70^\circ\text{C}$ ) and deparaffinized in xylene. The slides were then placed in changes of ethanol for 2 minutes each. Washing in buffer solution was performed between steps. The slides were then placed in 3% hydrogen peroxide for 15 minutes and then were subsequently incubated in avidin block for 15 minutes, biotin block for 15 minutes, primary antibody (p53, caspase-3, Phoenix Pharmaceuticals Inc., Germany; 1:200) for 12 hours at  $4^\circ\text{C}$ , and biotinylated secondary antibody for 1 hour. The reagent incubation was performed with streptavidin peroxidase for 15 minutes. A 1-minute Mayer's hematoxylin counterstain was used. The slides were dehydrated, cleared with xylene, and mounted with permanent mounting medium. Finally pictures were analyzed by IPP 6.0 software.

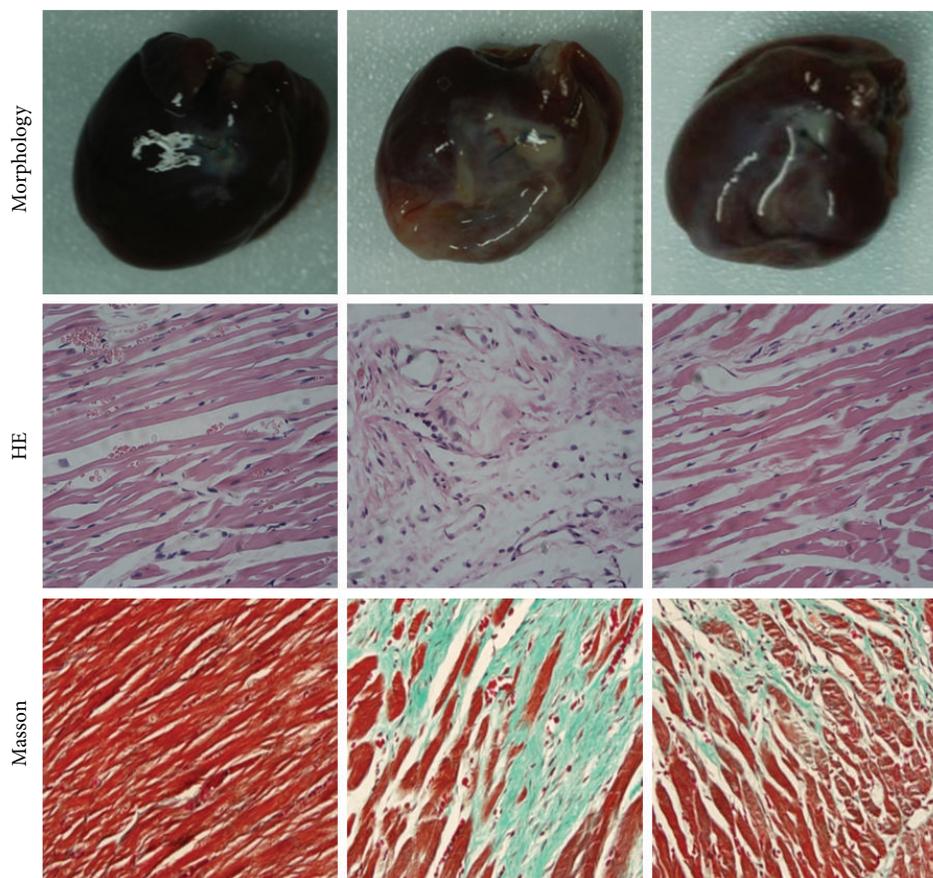


FIGURE 1: Morphology, HE, and Masson results in different groups.

**2.6. Determination of Plasma Superoxide Dismutase (SOD), Malondialdehyde (MDA), and NOX2 by Radioimmunoassay (RIA).** The plasma was homogenized in saline containing enzyme inhibitor (0.3 M EDTA-Na 10 ul, 0.34 M 8-hydroxyquinoline 10 ul, and 0.32 M dimercaptopropanol 5 ul) (1 ml blood) on ice. The homogenate was centrifuged at 8000 ×g for 10 min. The supernatant was used for determination of SOD, MDA, and NOX2 using an RIA kit (Beijing Kangyuan Ruide Biotechnology Co. Ltd., Beijing, China) following the instructions of the company.

**2.7. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL).** The cell apoptosis rate in the myocardium was determined by TUNEL according to the manufacturer's instructions (Roche Applied Science, South San Francisco, CA, USA). Six micrographs were randomly selected, and the numbers of healthy or apoptotic cardiomyocytes were counted. The TUNEL-positive percentage of apoptotic cells was considered as the percentage of the total numbers of cells [17].

**2.8. Measurement of Indicators by Western Blot.** Total protein from myocardium tissues was extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE). Proteins were then transferred to a nitrocellulose (NC) filter membrane, blocked, and probed sequentially with primary antibodies against NOX4 and matrix metalloproteinase-2 (MMP-2). After incubation in the primary antibody, the membrane was incubated in an appropriate secondary antibody. After washing, the bound antibody complexes were detected using an electrochemiluminescence reagent, taking the GAPDH as internal reference.

**2.9. Statistical Analysis.** Statistical analysis was performed with the SPSS program package (SPSS version 17.0). All data were presented as mean ± standard deviation (SD). Statistical analysis was carried out on three or more groups using one-way analysis of variance (ANOVA) and Dunnett's test. The values of  $P < 0.05$  were considered statistically significant.

### 3. Results

**3.1. QSYQ Suppressed Heart Failure after MI.** In order to investigate the roles of QSYQ in protecting HF, we initially examined the heart morphological changes in different groups. 28 days after LAD ligation, hearts in model group rats presented with abnormal enlarged ventricular cavity (Figure 1). Pathological examination showed that the cardiac myocytes exhibited an irregular shape and arrangement

TABLE 1: Echocardiography of rats in each group.

Group	N	LVEDd (CM)	LVEDs (CM)	FS (%)	EF (%)
Sham	8	0.740 ± 0.047**	0.350 ± 0.055**	52.470 ± 5.423**	87.41 ± 4.271**
Model	8	1.050 ± 0.107 <sup>▲▲</sup>	0.850 ± 0.078 <sup>▲▲</sup>	18.710 ± 1.675 <sup>▲▲</sup>	42.86 ± 3.110 <sup>▲▲</sup>
QSYQ	8	0.950 ± 0.128 <sup>▲▲</sup>	0.700 ± 0.203 <sup>▲▲*</sup>	28.071 ± 11.879 <sup>▲▲*</sup>	57.38 ± 18.519 <sup>▲▲*</sup>

<sup>▲▲</sup>  $P < 0.01$ , versus sham-operated group; \*  $P < 0.05$ , \*\*  $P < 0.01$ , versus model group.

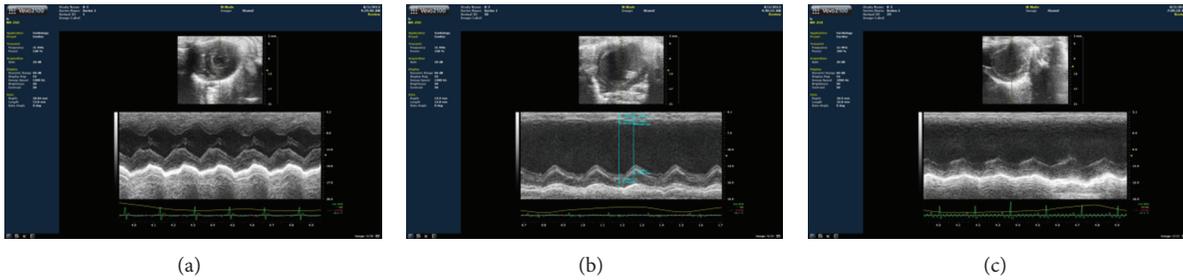


FIGURE 2: Cardiac function detected by echocardiography. (a) EF value, FS value, LVEDd, and LVEDs in sham-operated group. (b) Increase in EF value, FS value and decrease in LVEDd, LVEDs in sham-operated rats. (c) Improvements in EF and FS in QSYQ group.

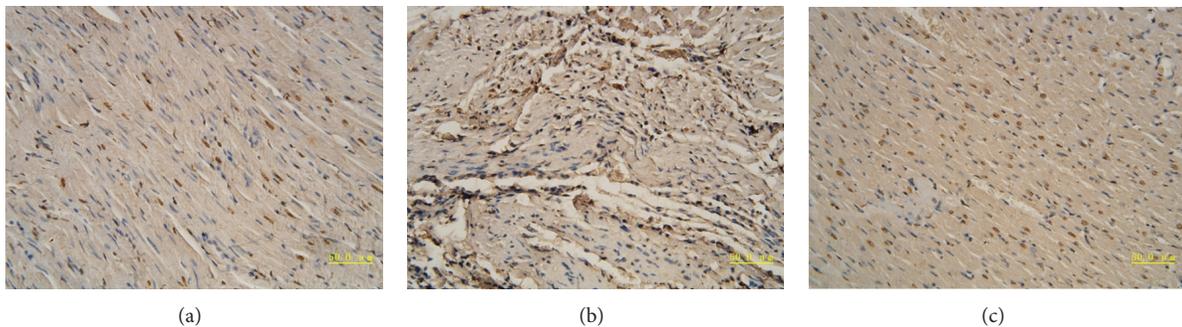


FIGURE 3: QSYQ inhibited cell apoptosis in the rat with HF. TUNEL analysis was carried out 28 days after the end of drug treatments. (a) The TUNEL-positive cells (apoptotic cells) in sham group. (b) Model; (c) QSYQ.

with myocardial fibrosis. Masson trichrome staining showed fibrotic areas were significantly greater in model group than sham (Figure 1). Moreover, the number of cardiac myocytes was greatly reduced. Notably, QSYQ treatment significantly suppressed those events in the model (Figure 1) and lessened the fibrotic areas.

**3.2. QSYQ Upregulated Cardiac Function Related Parameters.** 28 days after surgery, echocardiography showed that EF and FS values in the model group were significantly different ( $P < 0.05$ ). EF value of model group rats dropped down to 50.97% compared with sham-operated group, accompanied by increase in LVEDd and LVEDs, suggesting a change of cardiac hypertrophy in this stage. After treated by QSYQ for 28 days, the EF value and FS value recovered by 33.88% and 46.50%, respectively; compared with model group, LVEDs was also less than model group, with a reduction of 17.65%, but LVEDd had no significant change with model rats. The ventricular wall in QSYQ group was still thicker than sham-operated group (Table 1, Figure 2).

**3.3. QSYQ Treatment Inhibited Apoptosis of HF after MI.** Cell apoptosis is one of the major outcomes of HF. Our previous study motivated us to further investigate the impacts of QSYQ on myocardial cell apoptosis. With the TUNEL assay, we found that increased numbers of apoptotic myocardial cells were presented in HF rats, whereas the QSYQ treatment dramatically decreased cell apoptosis rate (Figure 3).

To further confirm the results, p53 and caspase-3, which are the major indicators of apoptosis, were detected by IHC, respectively. As revealed in Figures 4, 5, and 6, compared with sham, p53 was significantly up-regulated as well as the caspase-3. Combined with TUNEL, all evidence indicates elevated degree of apoptotic changes in model group rats. QSYQ greatly suppressed cell apoptosis by p53 and caspase-3. Altogether, these results demonstrated that QSYQ can definitely inhibit cell apoptosis in HF.

**3.4. QSYQ Treatment Inhibited Oxidative Stress Levels in HF.** The RIA of SOD showed that the plasma SOD in model group decreased by 32.88% ( $P < 0.05$ ) compared with

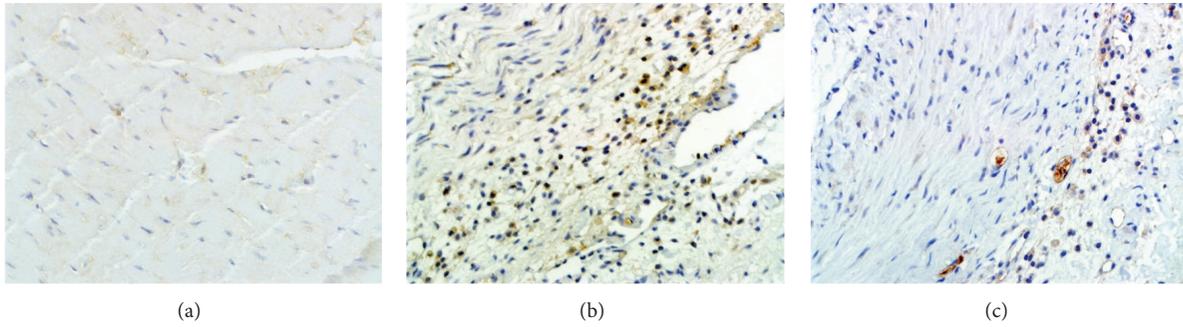


FIGURE 4: IHC results of p53 in different groups (×400): (a) sham; (b) model; (c) QSYQ.

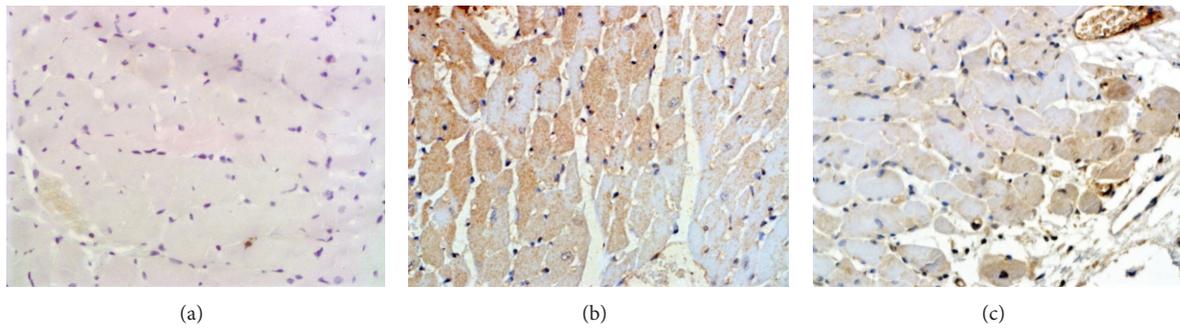


FIGURE 5: IHC results of caspase-3 in different groups (×400): (a) sham; (b) model; (c) QSYQ.

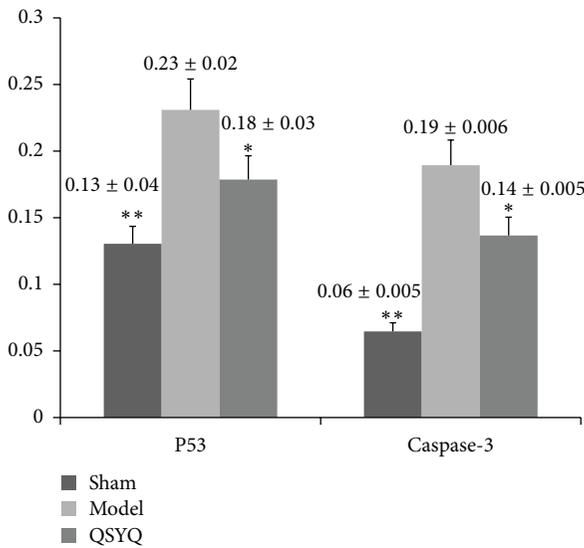


FIGURE 6: Quantification of apoptotic cell death. \* $P < 0.05$  compared with model group, \*\* $P < 0.01$  compared with model group.

sham; the level of SOD in QSYQ group showed a 51.83% upregulation compared with model group ( $P < 0.05$ ), which had no statistical significance when compared to the sham (Table 2). Results of MDA showed reverse changes. MDA in model group increased by 33.62% ( $P < 0.05$ ) compared with sham, while the level of MDA after QSYQ treatments showed

a 31.68% downregulation compared with model group ( $P < 0.05$ ), which is almost same to the level of sham (Table 2). NOX2 showed a similar outcome as MDA. Its level in model group is up-regulated by 29.79%, and a 24.45% reduction was detected in QSYQ group compared with model group.

The Western blot showed that; the NOX4 in model group increased by 62.00% ( $P < 0.05$ ) compared with sham, the level of NOX4 in QSYQ group showed a 35.80% reduction compared with model group ( $P < 0.05$ ) (Figure 7).

**3.5. QSYQ Treatment Inhibited the Level of MMP-2.** MMP-2, as a critical prognosis for heart failure [18], was also detected in present study. The Western blot of the cardiac MMP-2 in model group increased ( $1.53 \pm 0.215$ ,  $P < 0.01$ ) compared with sham-operated group ( $1.00 \pm 0.000$ ), while, treated by QSYQ for 28 days, the level of MMP-2 showed a reduction compared with model group ( $1.06 \pm 0.198$ ,  $P < 0.05$ ), which had no statistical significance compared to the sham (Figure 8).

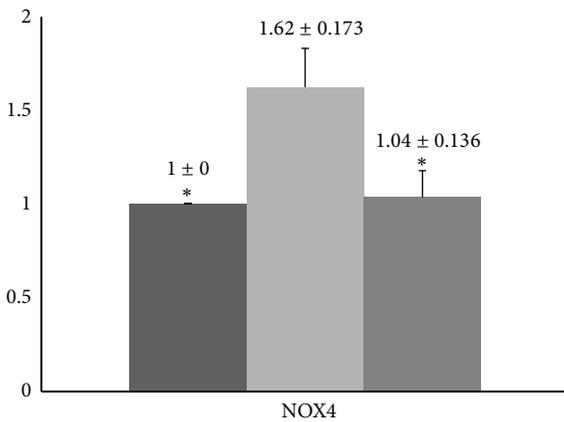
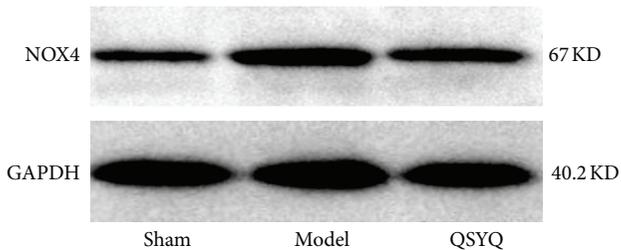
#### 4. Discussion

A variety of stimuli, such as oxidative stress [19] and inflammatory factors [20], have been suggested to trigger apoptosis and eventually caused myocardial modeling during HF. First, the stressed endoplasmic reticulum (ER) induces the activation of oxidative stress including ROS and NADPH [21] and subsequently leads to cardiac myocytes apoptosis [22]. There is evidence indicating that the expressions of NOX2 and NOX4 are markedly changed, accompanied by the

TABLE 2: Levels of oxidative stress indicators in different groups.

Group	N	SOD (U/mgprot)	MDA (nmol/mgprot)	NOX2 (ng/mL)
Sham	8	145.870 ± 14.807**	5.740 ± 0.992*	5.640 ± 0.701*
Model	8	97.940 ± 3.388▲▲	7.670 ± 0.896▲	7.320 ± 1.341▲
QSYQ	8	148.700 ± 32.041**	5.240 ± 1.736*	5.530 ± 0.335*

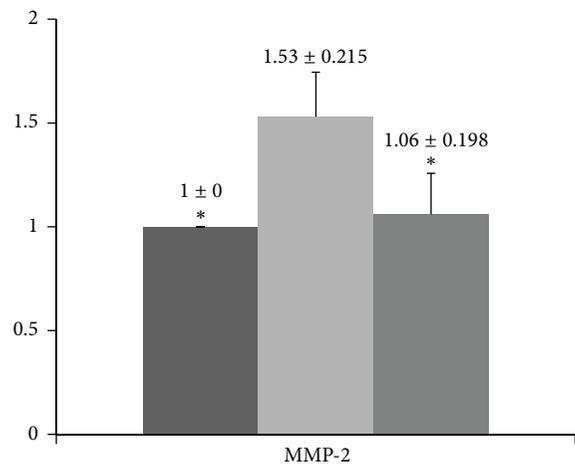
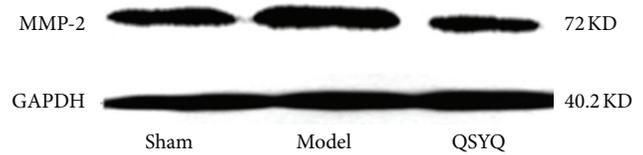
Compared with model, \* $P < 0.05$ , \*\* $P < 0.01$ ; compared with control, ▲ $P < 0.05$ , ▲▲ $P < 0.01$ .



■ Sham  
 ■ Model  
 ■ QSYQ

FIGURE 7: The Western blot results of NADPH oxidase (NOX4) in different groups. QSYQ: QiShenYiQi.

activation of apoptosis [23]. Consistent with previous studies, here we found changed expressions of SOD and MDA in HF model, suggesting that induction of oxidative stress occurred during HF after MI [24]. Moreover, the two isoforms of NADPH oxidases were believed to cause apoptosis by inducing ROS [25]. NOX2 and NOX4 are major sources of ROS in endothelial cells and are implicated both in vasodilator dysfunction and in the modulation of redox-sensitive signaling pathways [26], and the activation of NOX2 and NOX4 showed distinct functions. NOX2 is normally quiescent and is acutely activated by stimuli such as G-protein-coupled receptor (GPCR) agonists (e.g., angiotensin II, endothelin-1) and cytokines to initiate enzyme activity [27]. Some studies indicated that NOX2 activation contributes to angiotensin-II-induced cardiomyocyte hypertrophy. NOX2 contributes to myocyte death under stress situations and plays important roles in postmyocardial infarction remodeling, in part by modulating matrix metalloprotease activity. In contrast to NOX2, NOX4 is constitutively active at a low level and



■ Sham  
 ■ Model  
 ■ QSYQ

FIGURE 8: The Western blot results of MMP-2 in different groups. QSYQ: QiShenYiQi.

induces other effects in the heart under chronic stress, for example, by inducing the cell death signaling [28]. Therefore, NOX4 was regarded as an inducible isoform. That is to say, NOX2 plays an important role in mediating angiotensin-II-induced cardiac hypertrophy through MMPs, while NOX4 mediates cardiac hypertrophy and heart failure in response to pressure overload mainly by apoptosis [29].

Interestingly, in present study, the QSYQ can significantly downregulate the level of both oxidative stress and apoptosis, indicating a synergetic efficacy on HF. Moreover, NOX2 and NOX4 showed significant increase in model group. Both echocardiographic and MMP-2 results all indicated a cardiac hypertrophy after being operated for 28 days, while QSYQ can improve cardiac remodeling through counter-acting these events. Combined with our previous study and the references [16, 27], AngII-NOX2-ROS-MMPs pathway seems to be the critical target for QSYQ to reduce the remodeling. Furthermore, NOX4 is another potential targets to inhibit the p53 and caspase-3 [26], thus to reduce the apoptosis

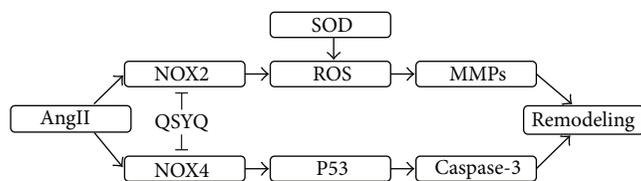


FIGURE 9: Potential mechanism of QSYQ attenuates oxidative stress and cell death signaling pathways in heart failure rats.

and hypertrophy and eventually provide a synergetic cardiac protective effect (Figure 9).

To sum up, this paper presents a multitarget pharmacological study of Chinese herbal formula. The TCM with multiple chemical components targets on multiple proteins [30], which may produce greater synergetic efficacy and fewer side effects [31]. And the results also show “therapeutic” QSYQ administration which can reduce the oxidative stress and apoptosis in NOX2 and NOX4 pathway, respectively, further proved the beneficial effects of QSYQ. In conclusion, administration of QSYQ could attenuate LAD-induced HF, oxidative stress, and apoptosis partly through the NOX2 and NOX4 pathways, which can treat CHD efficiently and safely.

But some problems still exist. For example, how the NOX4 affected the p53 pathway was not investigated. Moreover, whether the NOXs shared same pathway, or the interactive effects between them were not taken into consideration. Improvements should be made in our future work.

## Acknowledgments

The authors thank the reviewers for their excellent comments. Y. Wang, C. Li, and Y. Ouyang contributed equally to this work. Grants are provided from the National Department Public Benefit Research Foundation of China (no. 200807007), the Creation for Significant New Drugs Project of China (no. 2012ZX09103-201-011), the National Natural Science Foundation of China (no. 81202788), and National Science and Technology Pillar Program (no. 2012BAI29B07).

## References

- [1] D. Lloyd-Jones, R. J. Adams, T. M. Brown et al., “Heart disease and stroke statistics-2010 update: a report from the American Heart Association,” *Circulation*, vol. 121, pp. e46–e215, 2010.
- [2] A. V. Ambardekar and P. M. Buttrick, “Reverse remodeling with left ventricular assist devices a review of clinical, cellular, and molecular effects,” *Circulation: Heart Failure*, vol. 4, no. 2, pp. 224–233, 2011.
- [3] Y. Wang, W. J. Chuo, C. Li et al., “Energy metabolism disorder and myocardial injury in chronic myocardial ischemia with Qi deficiency and blood stasis syndrome based on 2-DE proteomics,” *Chinese Journal of Integrative Medicine*, 2012.
- [4] A. Blum, “Heart failure—new insights,” *The Israel Medical Association Journal*, vol. 11, no. 2, pp. 105–111, 2009.
- [5] A. Rashikh, S. J. Ahmad, K. K. Pillai, K. Kohli, and A. K. Najmi, “Aliskiren attenuates myocardial apoptosis and oxidative stress in chronic murine model of cardiomyopathy,” *Biomedicine and Pharmacotherapy*, vol. 66, no. 2, pp. 138–143, 2012.
- [6] H. Tsutsui, S. Kinugawa, and S. Matsushima, “Oxidative stress and heart failure,” *American Journal of Physiology*, vol. 301, no. 6, pp. 2181–2190, 2011.
- [7] M. Zhang, A. Perino, A. Ghigo, E. Hirsch, and A. M. Shah, “NADPH oxidases in heart failure: poachers or gamekeepers?” *Antioxidants & Redox Signaling*, vol. 18, no. 9, pp. 1024–1041, 2013.
- [8] K. Schröder, M. Zhang, S. Benkhoff et al., “Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase,” *Circulation Research*, vol. 110, no. 9, pp. 1217–1225, 2012.
- [9] A. V. Maksimenko and A. V. Vavaev, “Antioxidant enzymes as potential targets in cardioprotection and treatment of cardiovascular diseases. Enzyme antioxidants: the next stage of pharmacological counterwork to the oxidative stress,” *Heart International*, vol. 7, no. 1, pp. 14–19, 2012.
- [10] Y. Hojo, T. Saito, and H. Kondo, “Role of apoptosis in left ventricular remodeling after acute myocardial infarction,” *Journal of Cardiology*, vol. 60, no. 2, pp. 91–92, 2012.
- [11] K. Hayakawa, G. Takemura, M. Kanoh et al., “Inhibition of granulation tissue cell apoptosis during the subacute stage of myocardial infarction improves cardiac remodeling and dysfunction at the chronic stage,” *Circulation*, vol. 108, no. 1, pp. 104–109, 2003.
- [12] X. Feng, X. Liu, W. Zhang, and W. Xiao, “p53 directly suppresses BNIP3 expression to protect against hypoxia-induced cell death,” *The EMBO Journal*, vol. 30, no. 16, pp. 3397–3415, 2011.
- [13] A. Abbate, R. Bussani, M. S. Amin, G. W. Vetovec, and A. Baldi, “Acute myocardial infarction and heart failure: role of apoptosis,” *International Journal of Biochemistry and Cell Biology*, vol. 38, no. 11, pp. 1834–1840, 2006.
- [14] G. Dai, B. Zhang, and Z. Guo, “Application of central randomized system in project of clinical trial for secondary prevention of myocardial infarction by Qishen Yiqi Drop Pill,” *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 27, no. 7, pp. 653–656, 2007.
- [15] Y. Wang, Z. Liu, C. Li et al., “Drug target prediction based on the herbs components: the study on the multitargets pharmacological mechanism of Qishenkeli acting on the coronary heart disease,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 698531, 10 pages, 2012.
- [16] Y. Wang, C. Li, Y. Ouyang et al., “Cardioprotective effects of Qishenyiqi mediated by angiotensin II type 1 receptor blockade and enhancing angiotensin-converting enzyme 2,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 978127, 9 pages, 2012.
- [17] X. J. Song, C. Y. Yang, B. Liu et al., “Atorvastatin inhibits myocardial cell apoptosis in a rat model with post-myocardial infarction heart failure by downregulating ER stress response,” *International Journal of Medical Sciences*, vol. 8, no. 7, pp. 564–572, 2011.
- [18] A. Shirakabe, K. Asai, N. Hata et al., “Immediate administration of atorvastatin decreased the serum MMP-2 level and improved the prognosis for acute heart failure,” *Journal of Cardiology*, vol. 59, no. 3, pp. 374–382, 2012.
- [19] J. R. Burgoyne, H. Mongue-Din, P. Eaton, and A. M. Shah, “Redox signaling in cardiac physiology and pathology,” *Circulation Research*, vol. 111, no. 8, pp. 1091–1106, 2012.
- [20] Y. Jiang, Y. Miao, L. Yang et al., “Effect of chinese herbal drug-containing serum for activating-blood and dispelling-toxin on ox-LDL-induced inflammatory factors’ expression in endothelial cells,” *Chinese Journal of Integrative Medicine*, vol. 18, no. 1, pp. 30–33, 2012.

- [21] J. Kuroda and J. Sadoshima, "NADPH oxidase and cardiac failure," *Journal of Cardiovascular Translational Research*, vol. 3, no. 4, pp. 314–320, 2010.
- [22] M. Bao, W. Dai, Y. Li, and C. Hu, "Rutaecarpine prevents hypoxia-reoxygenation-induced myocardial cell apoptosis via inhibition of NADPH oxidases," *Canadian Journal of Physiology and Pharmacology*, vol. 89, no. 3, pp. 177–186, 2011.
- [23] A. Nabebaccus, M. Zhang, and A. M. Shah, "NADPH oxidases and cardiac remodelling," *Heart Failure Reviews*, vol. 16, no. 1, pp. 5–12, 2011.
- [24] R. Cangemi, A. Celestini, M. Del Ben et al., "Role of platelets in NOX2 activation mediated by TNF $\alpha$  in heart failure," *Internal and Emergency Medicine*, vol. 28, 2012.
- [25] K. T. Moe, N. O. Yin, T. M. Naylynn et al., "Nox2 and Nox4 mediate tumour necrosis factor- $\alpha$ -induced ventricular remodelling in mice," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 12, pp. 2601–2613, 2011.
- [26] R. Dworakowski, S. P. Alom-Ruiz, and A. M. Shah, "NADPH oxidase-derived reactive oxygen species in the regulation of endothelial phenotype," *Pharmacological Reports*, vol. 60, no. 1, pp. 21–28, 2008.
- [27] A. van der Vliet, "NADPH oxidases in lung biology and pathology: host defense enzymes, and more," *Free Radical Biology and Medicine*, vol. 44, no. 6, pp. 938–955, 2008.
- [28] R. P. Brandes, N. Weissmann, and K. Schröder, "NADPH oxidases in cardiovascular disease," *Free Radical Biology and Medicine*, vol. 49, no. 5, pp. 687–706, 2010.
- [29] Y. Maejima, J. Kuroda, S. Matsushima, T. Ago, and J. Sadoshima, "Regulation of myocardial growth and death by NADPH oxidase," *Journal of Molecular and Cellular Cardiology*, vol. 50, no. 3, pp. 408–416, 2011.
- [30] T. Chen, X. Z. Zhou, R. S. Zhang, and L. W. Zhang, "Discovery of regularities in the use of herbs in chinese medicine prescriptions," *Chinese Journal of Integrative Medicine*, vol. 18, no. 2, pp. 88–92, 2012.
- [31] A. S. Ferreira and A. J. Lopes, "Chinese medicine pattern differentiation and its implications for clinical practice," *Chinese Journal of Integrative Medicine*, vol. 17, no. 11, pp. 818–823, 2011.

## Review Article

# Evidence-Based Chinese Medicine for Hypertension

**Jie Wang and Xingjiang Xiong**

*Department of Cardiology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100053, China*

Correspondence should be addressed to Xingjiang Xiong; [5administration@163.com](mailto:5administration@163.com)

Received 15 October 2012; Accepted 11 May 2013

Academic Editor: Keji Chen

Copyright © 2013 J. Wang and X. Xiong. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Hypertension is an important worldwide public-health challenge with high mortality and disability. Due to the limitations and concerns with current available hypertension treatments, many hypertensive patients, especially in Asia, have turned to Chinese medicine (CM). Although hypertension is not a CM term, physicians who practice CM in China attempt to treat the disease using CM principles. A variety of approaches for treating hypertension have been taken in CM. For seeking the best evidence of CM in making decisions for hypertensive patients, a number of clinical studies have been conducted in China, which has paved the evidence-based way. After literature searching and analyzing, it appeared that CM was effective for hypertension in clinical use, such as Chinese herbal medicine, acupuncture, moxibustion, cupping, qigong, and Tai Chi. However, due to the poor quality of primary studies, clinical evidence is still weak. The potential benefits and safety of CM for hypertension still need to be confirmed in the future with well-designed RCTs of more persuasive primary endpoints and high-quality SRs. Evidence-based Chinese medicine for hypertension still has a long way to go.

## 1. Introduction

In global health politics, cardiovascular disease is the elephant in the room; it is a massive problem that few want to acknowledge and even fewer want to tackle [1]. Cardiovascular disease (CVD) is as the leading cause of death worldwide, accounting for an estimated 30% and 10% of all deaths and disability, respectively [2, 3]. It is reported that, approximately 62% of strokes and 49% of myocardial infarctions are caused by high blood pressure (BP) [4]. Hypertension is an important worldwide public-health challenge because of its high frequency and concomitant risks of cardiovascular and kidney disease [5]. It affects about 972 million adults worldwide [5] and is attributable each year for 7.6 million excess deaths and loss of 92 million disability-adjusted life years (DALYs) [2]. The purpose of antihypertensive treatment is to prevent the occurrence of CVD, by means of strict control of BP [6]. However, hypertension in most adults remains untreated or uncontrolled. BP control in the population is far from optimal, and SBP/DBP values <140/90 mmHg are achieved in no more than 25% of patients with treated hypertension worldwide [1]. Effective treatment of hypertension is limited by availability, cost, and adverse

effects of antihypertensive medications [6]. Thus, due to the limitations and concerns with current available hypertension treatments, a certain proportion of the population, especially in Asia, has turned to complementary and alternative medicine (CAM) [7–11], including Chinese medicine (CM) [12–15], in searching for a treatment modality with potential efficacy and few adverse effects. CAM is becoming increasingly popular and frequently used among patients with CVD, but these therapies lack demonstrated efficacy and safety for treating cardiovascular disease including hypertension [16]. Further research is essential in all areas of CAM to confirm its usefulness as an adjunct therapy [17, 18].

Chinese medicine, a system of ancient medical practice that differs in substance, methodology, and philosophy to modern medicine, plays an important role in health maintenance for the peoples of Asia and is becoming more frequently used in countries in the West [19]. It has been used to treat symptoms related to hypertension for more than 2500 years [20, 21]. Today, CM is commonly used to treat hypertension in China and the West [22–25]. And until now, the efficacy of CM for treating hypertension is suggested by a large number of published case series and uncontrolled trials

[26–30]. Six randomized controlled trials [31–36] reported significant reductions in BP relative to randomly assigned control groups treated for 4 to 12 weeks, whereas the other six trials [37–42] reported negative results of CM relative to control subjects. For seeking the best evidence of CM in making decisions for hypertensive patients, a number of clinical studies have been conducted in China to gain credibility with the researchers' unremitting efforts. Thus, it is helpful to review the current research status of clinical study of evidence-based Chinese medicine for hypertension.

The purpose of the paper is to review multiple approaches of Chinese medicine therapies for the treatment of hypertension. The literature available through both English and Chinese search engines that discusses the potential uses of Chinese medicine therapies to treat hypertension is reviewed. The English language literature is searched through the Cochrane Central Register of Controlled Trials (CENTRAL) in the Cochrane Library (September, 2012), MEDLINE (1959–2012), PUBMED (1959–2012), and EMBASE (1980–2012) databases. The Chinese language literature was searched through Chinese National Knowledge Infrastructure (CNKI) (1980–2012), Chinese Scientific Journal Database (VIP) (1989–2012), Chinese Biomedical Literature Database (CBM) (1978–2012), and WANFANG (1998–2012) databases. The following search terms were used individually or combined: “traditional Chinese medicine,” “Chinese medicine,” “Chinese herbal medicine,” “herb,” “blood pressure,” “hypertension,” “essential hypertension.” Chinese terms that were used in search were equivalent to those used to search from English language databases. Finally, 15 systematic reviews (SRs) and meta-analysis [43–57] were collected and reviewed for this study, with 5 articles in English [47, 53–56] and 10 articles in Chinese [43–46, 48–52, 57]. To our knowledge, this is the first systematic English review of the evidence-based Chinese herbs for the treatment of hypertension.

## 2. The Understanding of Hypertension from the Perspective of Chinese Medicine

Different from Western medicine (WM), Chinese medicine (CM) has formed a unique way to diagnose and treat diseases [58]. Great efforts have been made by China's ancient ancestors through meticulous observation of nature, the cosmos, and the human body. And a series of traditional medical practices were originated in China including Chinese herbal medicine (CHM), acupuncture, moxibustion, cupping, qigong, Tai Chi (shadow boxing exercise), diet, and exercise therapy.

As we know, blood pressure is the diagnostic gold standard in conventional medicine. Thus, there is no concept and diagnosis of hypertension in ancient China. Although hypertension is not a CM term, physicians who practice CM in China attempt to treat the disease using CM principles. According to the typical signs and symptoms of the disease, it falls into the category of “vertigo or headache” in CM [21]. CM has long been used to treat hypertension-related symptoms in clinical practice for centuries. CM approaches

hypertension as it does for other diseases under the guidance of holistic concept and treatment based on syndrome differentiation and formula syndrome differentiation [59, 60]. CM has been widely used to certain syndromes and formula syndromes in hypertension, such as fire syndrome, *Banxia Baizhu Tianma Tang* (decoction of *Pinellia ternata*, *Atractylodes*, and *Gastrodia elata*) syndrome [61]. Physicians who prescribe Chinese herbs and formulas recently realized that patient with hypertension exhibit the same pathological changes as those that are characteristic of fire syndrome and *Banxia Baizhu Tianma Tang* (Decoction of *Pinellia ternata*, *Atractylodes* and *Gastrodia elata*) syndrome. Moreover, increasing evidence indicates that, Chinese herbs and formulas that improve fire syndrome and *Banxia Baizhu Tianma Tang* (Decoction of *Pinellia ternata*, *Atractylodes*, and *Gastrodia elata*) syndrome are useful in treating hypertensive patients in China [21].

In our previous studies, hypertension could be divided into the following three major types on the basis of the stage and symptoms of the disease in CM. The first one is fire syndrome which could be found in various stages of hypertension. It can also be further divided into four types such as liver fire, heart fire, stomach fire, and intestinal fire. The second one is phlegm-fluid retention syndrome which often appears in the later stage of the disease. In light of the disease location, it could be divided into three types such as fluid retention in up *jiao* syndrome, fluid retention in middle *jiao* syndrome, and fluid retention in down *jiao* syndrome. The last one is deficiency syndrome. The most common deficiency syndromes are spleen deficiency syndrome and kidney deficiency syndrome. The recommended treatment program of hypertension by Chinese herbal formulas is shown in Table 1.

A variety of approaches for treating hypertension have been taken in CM. Among them, Chinese herbal therapy is the most commonly used. Furthermore, acupuncture, moxibustion, cupping, qigong, Tai Chi (Shadow boxing exercise), and CM external therapy (including bath foot, acupoint application, and thorn collaterals bloodletting) could also be used in the treatment of the disease.

## 3. Paving the Way for Evidence-Based Chinese Medicine for Hypertension

Evidence-based medicine (EBM), a new paradigm for medical practice, quickly developed in the 1990s. According to the book of “Evidence-based Medicine: How to Practice and Teach EBM” written by Dr. Sackett, one of the pioneers in EBM, EBM makes the explicit, judicious, and conscientious use of the best evidence in making decisions for preventing diseases, promoting the recovery and improving life quality [62]. It has brought great impacts on the efficacy and safety of previous widely accepted strategies of therapeutic, rehabilitative, and preventive regimens by the evidences from a series of systematic reviews and meta-analysis. That is to say clinical experience is unreliable and all medical interventions should be based on rigorous research evidences [63]. Eugene Braunwald, a famous cardiologist, also advocated that current

TABLE 1: Recommended treatment program of hypertension by Chinese herbal formulas.

Syndrome	Clinical signs	Treatment principles	Classical formula
<b>Fire syndrome</b>			
Liver fire syndrome	Vertigo, headache, facial flushing with perspiration, conjunctival congestion, bitter taste in the mouth, thirst, irritability and restlessness, wiry-rapid-powerful pulse or powerful cunkou pulse alone, or wiry and long pulse even well beyond the cunkou pulse	Calming liver and suppressing liveryang hyperactivity	Tianma Gouteng decoction, Zhengan Xifeng decoction, Jianling decoction, and Longdan Xiegan decoction
Heart fire syndrome	Facial flushing with perspiration, bitter taste in the mouth, thirst, insomnia, red tip of the tongue, and rapid pulse	Clearing heart fire	Zhi-zi-chi decoction, Sanhuang Xiexin decoction, and Huanglian Jiedu decoction
Stomach fire syndrome and intestine fire syndrome	Dry mouth, thirst with desire for cold drinks, easy to starve, foul breath, abdominal distension and pain, smelly stool, constipation, red tongue, yellow dry fur, right guan pulse powerful alone, or strength and deep-hidden-powerful pulse	Clearing stomach-intestine fire, promoting digestion, relaxing bowels, and relieving constipation	Da Chai Hu decoction, Baohe pill, Baihu decoction, Houpu Dahuang decoction, Gegen Qinlian decoction, and Zeng Ye decoction
<b>Phlegm-fluid retention syndrome</b>			
Phlegm and dampness syndrome	Obesity, dizziness, sticky mouth, thirst without a desire to drink, chest distress, nausea, vomiting, anorexia, abdominal distension, loose stools, sleepiness, greasy tongue coating, and slippery pulse	Dispelling phlegm and eliminating dampness	Erchen decoction, Pingwei powder, Wendan decoction, Banxia Baizhu Tianma decoction, and Xiao Xianxiang decoction
Fluid retention syndrome	Dizziness aggravated by change in body position, thirst without a desire to drink or not being thirsty, chest distress, palpitation, gastric distension, abdominal distension, poor appetite, lumbar heaviness, weakness and heaviness in the lower extremities, edema, daytime sleepiness, abnormal leucorrhea, dysuria, greasy fur, swollen tongue, and deep pulse	Dissipating excessive fluid	Banxia baizhu tianma decoction, Wuling powder, Zhuling decoction, Zexie decoction, and Fuling Guizhi Baizhu Gancao decoction
<b>Deficiency syndrome</b>			
Spleen deficiency syndrome	Fatigue, shortness of breath, stomach pain, poor appetite, abdominal distension, and loose stools	Reinforcing spleen	Fuling Guizhi Baizhu Gancao decoction, Si jun Zi decoction, and Liu Jun Zi decoction
Kidney deficiency syndrome	Tiredness in the loins and legs, tinnitus and dizziness, sexual dysfunction, dysuria, weakness and fatigue, and weak chi pulse	Reinforcing kidney	Liuwei Dihuang pill and Shenqi pill

Each CHM under the classical formula is composed of multiple herbs.

cardiology practice should be evidence based and global in scope [64].

There is close relationship between CM and EBM [65]. Due to the shortage of objective and quantitative criteria in evaluating therapeutic effect and safety in CM, it is urgent to formulate a scientific way. The emergence of EBM had just provided an appropriate method to solve this critical issue. As the applications of CM in the treatment of hypertension are increasing, more and more concern on the efficacy and safety are aroused [22]. Whether CM is equal or superior to WM, how CM plays the role in enhancing efficacy and reducing toxicity, and how to optimize the therapeutic regimen by combination of CM and WM, all these problems are not clear currently. All of these issues warrant further investigation and need more evidences. Here, the paper reviews the background of CM for the treatment of hypertension.

According to historical records in CM classics, the earliest evidence of Chinese herbal medicine used in China is of two graves from the Han Era (206 B.C. to 220 A.D.) [66]. There are a large number of clinical trials about classical famous prescriptions for the treatment of “vertigo or headache” since ancient time. Although patients with “vertigo or headache” may not necessarily be fully consistent with the diagnosis of hypertension, previous widely used formulae still have a good clinical effect in the treatment of hypertension today. Physicians in ancient China realized that there is a certain connection between a special pattern and a corresponding herb or formula in the clinical practice. And they recorded the treatment process. It is considered as the “clinical trial” in ancient time. After then, the “clinical trial” was tested and repeated by the successors for hundreds or even thousands of years. Thus, it is a unique clinical trial. In the trial,

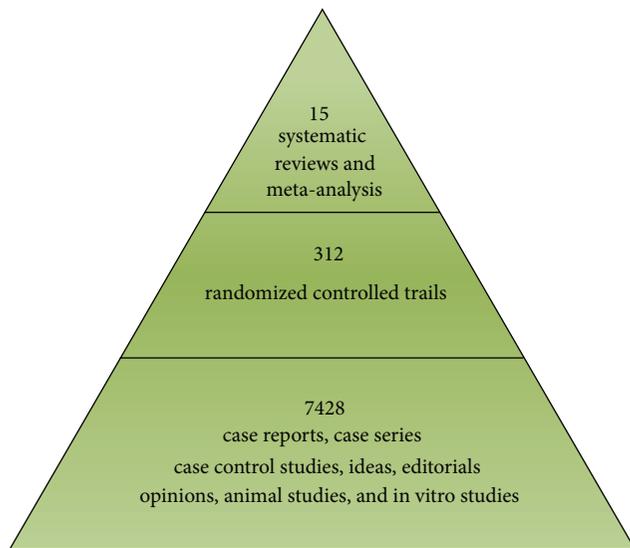


FIGURE 1: Number of studies on CM for hypertension published in Cochrane Library, PubMed, EMBASE, CNKI, VIP, CBM, and WANFANG.

the special pattern is also known as “formula syndrome” or “herb syndrome” [60], which is the indication of Chinese herbs and formulas [59]. The corresponding formula is called classical formulae. These classical formulae in the treatment of hypertension included *Tianma Gouteng Yin* (decoction of *Gastrodia* and *Uncaria*), *Banxia Baizhu Tianma Tang* (decoction of *Pinellia ternata*, *Atractylodes* and *Gastrodia elata*), *Longdan Xiegan Tang* (decoction of radix gentianae for purging liver fire), *Da Chai Hu Tang* (Major *Bupleurum* Decoction), *Zexie Tang* (Decoction of American water Plantain), and *Liu Wei Dihuang Wan* (Pill of *Rehmannia*) [21].

In order to obtain supportive evidence of CM herbs and approaches that are thought to exert hypotensive effect, we retrieved the data primarily via the Internet (Cochrane Library, PubMed, EMBASE, CNKI, VIP, CBM, and WANFANG) up to September 30, 2012. All the case reports, case series, case control studies, ideas, editorials, opinions, animal studies, in vitro studies, randomized controlled trials, systematic reviews, and meta-analysis based on CM for essential hypertension were included. There were no restrictions on population characteristics, language, and publication type. Duplicated publications reporting that the same groups of participants were excluded. We could see from Figure 1 that there are a large number of researches in the field of CM for hypertension in the past 50 years, especially in the past 30 years.

As shown in the first stage, 7428 studies reported the effectiveness of CM for hypertension ranging from case control studies, case reports, case series, ideas, editorials, opinions, animal studies, and in vitro studies to controlled observational studies. Among the herbal therapies, it includes three categories of formulae. The first one is individually prescribed decoctions, which have been used in most outpatients and inpatients. The second one is currently effective practice formula or experienced prescriptions from famous

CM doctors. The third one is the modified classical formulae. The last one is the classical formulae or well-known Chinese medicine formula. In recent decades, the proprietary Chinese medicines (PCMs) for treatment of hypertension are mainly originated from the last three categories. The PCMs have been tested in a large number of clinical trials.

With increasing awareness and practice of EBM, 312 randomized controlled trials (RCTs) have been conducted to evaluate the effectiveness of CM for hypertension as shown in the second stage.

Regarding clinical effect and its evaluation in clinical researches on CM for hypertension, systematic reviews (SRs) and meta-analysis are important approaches to get the best available evidence. In the third stage of Figure 1, there are 15 SRs and meta-analysis [43–57] collected after literature searches.

#### 4. Exploring the Differences in Response to Treatment from SR

As shown in Table 2, there are 15 SRs and meta-analysis of CM for hypertension published in Chinese or English. Here, these published findings are analyzed to explore the range and role of CM for the treatment of hypertension.

**4.1. Chinese Herbal Medicine.** There are 9 SR of Chinese herbal medicines published whether on individually prescribed decoctions or classical formulae for hypertension. The results are shown in Table 1 [43–51]. Comprehensive evaluations of the clinical efficacy of the Chinese herbal medicine were conducted in 3 SRs [43–45]. Chinese herbal medicine, which could clear fire, suppress liver yang hyperactivity, remove blood stasis, fluid and phlegm, nourish kidney, and reinforce spleen *qi*, were all included in the analysis. The result showed that Chinese herbal medicine the use of alone may be beneficial to reduce BP in patients with hypertension, and no significant difference was found between CM and WM [44, 45]. Combination therapies, just CM combined with WM, showed better results than those of WM for treating hypertension [45]. However, another SR reported negative results that total effective rate and efficiency of CM are lower than that of single WM as for BP controlling [43]. Owing to the lack of data from high-quality RCT, the efficacy needs to be further studied [43–45].

Tianma Gouteng Yin Formula (TGYF), a famous prescription noted in *Za Bing Zheng Zhi Xin Yi* (New Meanings in Syndrome and Therapy of Miscellaneous Diseases), contains eleven commonly used herbs (*Gastrodia Elata*, *Uncaria*, *Abalone Shell*, *Eucommia Ulmoides Oliv*, *Achyranthes Root*, *Loranthus Parasiticus*, *Gardenia*, *Scutellaria Baicalensis Georgi*, *Leonurus Japonicus*, *Poria Cocos*, and *caulis polygoni multiflori*). It could suppress liver yang hyperactivity, clear heat, activate blood, and nourish the kidney; it has been widely used to treat hypertension-related signs and symptoms in clinical practice for centuries in China. 2 SRs [46, 47] assess the efficacy and safety of TGYF for treating primary hypertension. One SR [46] showed that TGYF combined with enalapril showed additional better effects

TABLE 2: The characteristics of systematic reviews and meta-analysis of CM for hypertension.

Intervention	Title	Authors	Year published	Trials	Participants included	Authors' comments
Chinese Herbal medicine	Meta-analysis of effectiveness of antihypertension of 442 traditional Chinese herbal decoctions	Ding and Zhou [43]	2012	7	808	Limited evidence suggested that total effective rate and efficiency of CM are lower than those of single WM as for BP controlling. But owing to lack of data from high-quality RCT, the efficacy need to be further studied.
	Quantitative analysis of clinical controlled trials of traditional Chinese medicine and systematic evaluation of randomized controlled trials involving traditional Chinese medicine for essential hypertension	Hu [44]	2009	24	1660	CM combined with WM showed better results than WM for treating hypertension. However, due to the generally low quality of the trials, large sample, multicenter double blind RCTs with strict design are warranted.
	Meta-analysis of traditional Chinese medicine for essential hypertension	Ren et al. [45]	2006	11	1010	CM may be beneficial to reduce BP in patients with hypertension.
	Systematic review and meta-analysis of Tianma Gouteng Yin combined with enalapril for essential hypertension	Dong et al. [46]	2011	6	543	Tianma Gouteng Yin combined with enalapril showed additional better effects than enalapril for hypertension. No serious adverse event is reported. Due to the low methodological quality and potential bias of trials, large-sample, multicenter, randomized, double-blind, controlled trials are warranted.
	Tianma Gouteng Yin Formula for treating primary hypertension <sup>E</sup>	Zhang et al. [47]	2012	0	0	The review could not find any randomized controlled clinical trials that compared Tianma Gouteng Yin Formula (TGYF) to placebo or no treatment. The authors cannot draw a conclusion that TGYF may be beneficial for hypertension. Well-designed randomized controlled studies need to be conducted and published.
	Systematic review of clinical evidence about calm the liver and subdue yang therapy on the hypertension disease with the syndrome of upper hyperactivity of liver yang	Xu and Li [48]	2012	8	944	The calm the liver and subdue yang therapy for treating hypertension disease with syndrome of upper hyperactivity of liver yang has curative effect and high safety. However, owing to lack of data from high-quality RCT and potential publication bias, the positive findings should be interpreted conservatively.
	Systematic review of replenishing kidney <i>qi</i> method for essential hypertension with kidney <i>qi</i> deficiency syndrome	Shi and Zhang [49]	2012	5	457	The replenishing kidney <i>qi</i> therapy for treating hypertension with kidney <i>qi</i> deficiency syndrome has curative effect and high safety. High-quality and large-scale RCTs are needed to further prove the results of the study because of the low quality of the included studies.

TABLE 2: Continued.

Intervention	Title	Authors	Year published	Trials	Participants included	Authors' comments
	Systematic review on treatment of essential hypertension from spleen and kidney deficiency	Liu and Li [50]	2011	15	1661	Treatment of essential hypertension from the spleen and kidney deficiency was effective, and the level of safety is reliable. However, the quality of most trials was low.
	Effects of Chinese medicine on elderly isolated systolic hypertension: a meta-analysis	Li and Yang [51]	2012	17	1323	Chinese medicine is effective on treating isolated systolic hypertension of the old, as well as reducing symptoms and pulse pressure.
Acupuncture	The effect of acupuncture therapy on essential hypertension: a systematic review of long-term effect	Zhao et al. [52]	2011	18	1460	Although it shows a tendency that acupuncture can improve the conditions of essential hypertension, a reliable conclusion cannot be drawn from the present data because of the defects in methodological quality and insufficient numbers of trials. It is necessary to perform more multicentral RCTs of high quality in the future.
Moxibustion	Moxibustion for hypertension: a systematic review <sup>E</sup>	Kim et al. [53]	2010	4	240	There is insufficient evidence to suggest that moxibustion is an effective treatment for hypertension. Rigorously designed trials are warranted to answer the many remaining questions.
Cupping	Cupping for hypertension: a systematic review <sup>E</sup>	Lee et al. [54]	2010	2	76	The evidence is not significantly convincing to suggest that cupping is effective for treating hypertension. Further research is required to investigate whether it generates any specific effects for that condition.
Qigong	Qigong for hypertension: a systematic review of randomized clinical trials <sup>E</sup>	Lee et al. [55]	2007	12	1332	There is some encouraging evidence of qigong for lowering SBP, but the conclusiveness of these findings is limited. Rigorously designed trials are warranted to confirm these results.
	Clinical effect of Qigong practice on essential hypertension: a meta-analysis of randomized controlled trials <sup>E</sup>	Guo et al. [56]	2008	9	908	Self-practiced qigong for less than 1 year is better in decreasing BP in patients with essential hypertension than in no-treatment controls, but is not superior to that in active controls. More methodologically strict studies are needed to prove real clinical benefits of qigong and to explore its potential mechanism.
Tai Chi	Systematic review of Tai Chi for essential hypertension	Li and Xu [57]	2011	5	318	Tai Chi is effective on treating essential hypertension. However, different exercise time of Tai Chi has an impact on hypertensive patients. More RCTs of high quality are warranted to prove benefits of Tai Chi on hypertensive patients with different stages.

CM: Chinese medicine; WM: Western medicine; RCT: randomized controlled trial; BP: blood pressure; SBP: systolic blood pressure; and E: in English.

than enalapril for hypertension. No serious adverse event is reported. However, the other SR could not find any randomized controlled clinical trials that compared TGYF to placebo or no treatment [47]. Authors advised that well-designed randomized controlled studies need to be conducted and published.

Liver yang hyperactivity syndrome, kidney deficiency syndrome, and spleen deficiency syndrome are very common in CM. Aiming to improve these different syndromes, treatment principles of calming the liver and replenishing kidney and spleen were used, respectively. 3 SRs [48–50] assess the efficacy and safety of treatment based on Chinese medicine principles for hypertension. All SR showed curative effect and high safety of CM.

When referring to elderly isolated systolic hypertension, it indicated that CM is effective on treating isolated systolic hypertension of the old, as well as reducing symptoms and pulse pressure [51]. Authors also gave conclusions that the evidence for the favorable results in the trials is limited, and these findings should be carefully interpreted due to the low methodological quality.

**4.2. Acupuncture.** Acupuncture is considered an ancient practice of TCM that began thousands of years ago. It has been reported to have potential effectiveness for treating cardiovascular diseases including hypertension, with few reported adverse effects [67, 68]. Several features of acupuncture make it an attractive therapeutic alternative with increasing popularity [36]. Although the results of the trials showed a tendency that acupuncture can improve the conditions of essential hypertension, a reliable conclusion cannot be drawn from the present data because of the defects in methodological quality and insufficient numbers of trials [52]. Thus, evidence of efficacy in lowering blood pressure from controlled trials has been scant. It is necessary to perform more multicentral RCTs of high quality in the future.

**4.3. Moxibustion.** Moxibustion, a traditional medical intervention of CM, involves the application of ignited mugwort (*Artemisia vulgaris*) directly or indirectly at acupuncture points or other specific parts of the body to treat or prevent diseases [69]. The mechanism of moxibustion maybe related to the combination of heat (burning pain and heat stress), tar (extract), aroma (fume), and psychological stress [70]. According to the theory of CM, a possible explanation for how moxibustion works is that heat could increase *qi* circulation and relieves *qi* stagnation by stimulating the acupuncture points to regulate the function of meridians and visceral organs [71]. A SR on the effects of moxibustion on hypertension revealed no evidence that moxibustion is beneficial to people with hypertension [53]. Differences between specific and nonspecific effects should be examined in a future study, and rigorously designed trials are warranted to answer the many remaining questions.

**4.4. Cupping.** Cupping therapy, as a part of CM, is widely used in treating pain and many other complaints for millennia [72]. A glass cup is utilized to create suction over a painful area or an acupuncture point after incisions are

made to the skin. By doing so, the skin is pulled into the cup without drawing blood. Therefore, negative pressure acts on the skin and irritates subcutaneous muscles. It is often used to lower BP and relieve hypertension-related symptoms such as headaches and anxiety [73]. A SR on the effect of cupping on hypertension revealed no significantly convincing evidence to suggest that cupping is effective for treating hypertension. Further research is required to investigate whether it generates any specific effects for that condition [54].

**4.5. Qigong.** Qigong, as an ancient Chinese healing art, is widely used in Asia and has been officially recognized as a standard medical technique in Chinese hospitals. It involves exercises for posture, coordination of different breathing patterns, movement, and meditation [74]. According to the theory of CM, it could increase the healthy flow of *qi* throughout the body to heal itself. It is claimed that qigong has potential beneficial effects on various disorders, including cardiovascular disease [75]. Several RCTs have claimed that qigong has therapeutic effects on blood pressure in patients with hypertension [76–78]. 2 SRs on the effects of qigong on hypertension revealed some encouraging evidence of qigong for lowering BP. However, the conclusiveness of these findings is limited. Rigorously designed trials are warranted to confirm these results [55, 56].

**4.6. Tai Chi.** Tai Chi (also known as Tai Chi Quan or Shadow Boxing), originated in ancient China, is a Chinese conditioning exercise well known for its graceful movement. It has been practiced for centuries in the East for health promotion and longevity. In recent years, there has been a growing interest and prevalence in Tai Chi exercise in western societies. During the practice, it combines deep diaphragmatic breathing with continuous body motions to achieve a harmonious balance between body and mind. Previous researches have indicated that Tai Chi exercise may improve health-related fitness (including cardiorespiratory function, muscular strength, balance, and flexibility), quality of life, and psychological well-being. Recent studies also suggest that it may have beneficial effects for patients with cardiovascular conditions and some cardiovascular risk factors [79, 80], including hypertension [81]. There are few trials on the effectiveness of Tai Chi in the management of hypertension. A SR [57] including 5 randomized clinical trials with 318 hypertensive patients reported some positive findings for Tai Chi on treating essential hypertension. It is also pointed out that different exercise time of Tai Chi has an impact on hypertension. However, more RCTs of high quality are warranted to prove benefits of Tai Chi on hypertensive patients with different stages.

## 5. Providing Evidence of Safety

It is widely accepted that herbal medicine is undoubtedly safe for various diseases in China. However, with the increasing reports of liver toxicity and other adverse events associated with Chinese herbal medicines [82–84], the safety of CM

needs to be monitored rigorously and reported appropriately in the future clinical trials [85–87]. According to our review, safety evaluation of CM is not the highlight. Inadequate reporting on adverse events is found either in the included SRs or in the original RCTs. Most of the adverse effects of CM were mentioned as “low adverse effect” or “none obvious.” Only six of the fifteen SRs reported the adverse effect of CM briefly, providing limited information [43, 46, 48–50, 56]. Among them, four SRs about Chinese herbal medicine [43, 46, 48, 50] reported nine specific symptoms in treatment group including headache, dizziness, dry mouth, dry cough, abdominal distension, and constipation. However, all these adverse events could be tolerated by participants in the trials. The other two SRs reported no serious adverse event in replenishing kidney *qi* group [49] and qigong group [56]. The rest nine SRs did not mention whether they had monitored adverse effects at all [44, 45, 47, 51–55, 57]. It is generally believed that acupuncture, moxibustion, cupping, qigong, and Tai Chi have reliable safety. Unfortunately, included SRs about these approaches provided insufficient evidence. Therefore, conclusions about the safety of CM cannot be made from this paper and needs to be further proven, due to the limited, inadequate recording, and reporting of adverse events.

## 6. Discussion

Considerable progress has been made by domestic and foreign clinical experts and researchers for the treatment of hypertension [88–90]. Oral antihypertensive drugs are a milestone in the therapy of essential hypertension. However, the current awareness, control, and mortality rates of hypertension are still far from optimal [91, 92]. Only 25% of patients could achieve the goal, and recurrent cardiovascular events still occur in those who take antihypertensive drugs. What's more, numerous adverse reactions, including headache, dizziness, orthostatic hypotension, and decreased sexual function, limit the clinical practice of antihypertensive drugs [93]. Thus, traditional medicine (TM) has got increasing popularity with people all over the world [94–97]. CM has made great contributions to the health and well-being of the people for its unique advantages in preventing and curing diseases, rehabilitation, and health care [98–103]. Over the past 30 years, the study of CM for treating hypertension is the most active area of researches worldwide [104–108]. Significant progress has been made from theory and experiments to clinic fields based on the inheritance and innovation of thoughts of CM for hypertension [109–113]. A recent study investigated the multiprotective mechanisms of Chinese herbal formulas for treating hypertension from the perspective of modern science, including smoothly controlling BP, reducing blood pressure variability (BPV), protecting target organs, regulating renin-angiotensin-aldosterone system (RAAS), reversing risk factors, improving endothelial function, blocking calcium channels, improving life quality and clinical symptoms, and reversing uncontrollable factors of BP [114]. Therefore, much attention has been paid to the holistic, multitarget, and

multidimensional pharmacological studies of CM currently.

The systematic reviews indicated the potential benefit of CM for hypertension in terms of some outcome measures, but none of them drew a definite conclusion due to the poor quality of primary studies. Poor methodology and reporting quality of SRs about CM have caused widespread concern [115, 116]. According to preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement, it was found that most of the included reviews have poor quality [117]. If the reviews are poorly designed and reported, misleading conclusions about current clinical practice would be given. So, the reporting of SR should be in accordance with PRISMA in future researches. The included literature must be scrutinized and selected strictly in order to avoid the potential bias. Process of extracting data such as study design, allocation sequence, allocation concealment, blinding, intention to treat analysis, and drop outs should be conducted rigorously. Also, how to evaluate the validity of the primary studies is an important aspect. It is well known that the primary goal of the treatment for essential hypertension is to reduce mortality and prevent progression to heart disease and other complications of hypertension. The secondary endpoints are mainly blood pressure, blood lipid, and traditional Chinese medicine syndromes [118]. Our overview showed that there is a lack of definite data on the primary endpoints, whereas the secondary endpoints were most commonly adopted in clinical trials. Therefore, the persuasion of conclusions about CM for hypertension would be reduced greatly. Although it appeared that CM was effective for hypertension in clinical use, such as Chinese herbal medicine, acupuncture, moxibustion, cupping, qigong, and Tai Chi, most SRs were inconclusive that CM had a definite effect owing to the poor evidence. As weak recommendations result from low quality evidence, high quality evidence of CM for hypertension is warranted in further RCTs to guide clinical practice either for hypertensive patients or physicians.

In conclusion, evidence-based Chinese medicine for hypertension still has a long way to go [119, 120]. The potential benefits and safety of CM for hypertension still need to be confirmed in the future with well-designed RCTs of more persuasive primary endpoints and high-quality SRs. Although the development of evidence-based CM for hypertension will be full of challenge, we have full confidence.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Acknowledgments

This work was supported in part by the National Basic Research Program of China (973 Program, 2003CB517103) and the National Natural Science Foundation Project of China (90209011).

## References

- [1] S. MacMahon, M. H. Alderman, L. H. Lindholm, L. S. Liu, R. A. Sanchez, and Y. K. Seedat, "Blood-pressure-related disease is a global health priority," *The Lancet*, vol. 371, no. 9623, pp. 1480–1482, 2008.
- [2] C. M. Lawes, S. Vander Hoorn, and A. Rodgers, "Global burden of blood-pressure-related disease, 2001," *The Lancet*, vol. 371, no. 9623, pp. 1513–1518, 2008.
- [3] World Health Organization, *World Health Statistics 2006*, World Health Organization, Geneva, Switzerland, 2006.
- [4] C. Farsang, L. Naditch-Brule, A. Avogaro et al., "Where are we with the management of hypertension? From science to clinical practice," *The Journal of Clinical Hypertension*, vol. 11, no. 2, pp. 66–73, 2009.
- [5] P. M. Kearney, M. Whelton, K. Reynolds, P. Muntner, P. K. Whelton, and J. He, "Global burden of hypertension: analysis of worldwide data," *The Lancet*, vol. 365, no. 9455, pp. 217–223, 2005.
- [6] A. V. Chobanian, G. L. Bakris, H. R. Black et al., "Seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure," *Hypertension*, vol. 42, no. 6, pp. 1206–1252, 2003.
- [7] H. Xu and K. J. Chen, "Complementary and alternative medicine: is it possible to be mainstream?" *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 403–404, 2012.
- [8] K. J. Chen, K. K. Hui, M. S. Lee, and H. Xu, "The potential benefit of complementary/alternative medicine in cardiovascular diseases," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 125029, 1 pages, 2012.
- [9] M. J. Wood, R. L. Stewart, H. Merry, D. E. Johnstone, and J. L. Cox, "Use of complementary and alternative medical therapies in patients with cardiovascular disease," *American Heart Journal*, vol. 145, no. 5, pp. 806–812, 2003.
- [10] J. Wang, X. J. Xiong, B. Feng, and H. Xu, "Cardiovascular effects of salvianolic acid B," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 247948, 2013.
- [11] G. Y. Yeh, R. B. Davis, and R. S. Phillips, "Use of complementary therapies in patients with cardiovascular disease," *American Journal of Cardiology*, vol. 98, no. 5, pp. 673–680, 2006.
- [12] K. J. Chen, "Clinical service of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 14, no. 3, pp. 163–164, 2008.
- [13] H. Xu and K. J. Chen, "Integrative medicine: the experience from China," *The Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 3–7, 2008.
- [14] K. J. Chen and H. Xu, "The integration of traditional Chinese medicine and Western medicine," *European Review*, vol. 11, no. 2, pp. 225–235, 2003.
- [15] J. Wang and X. J. Xiong, "Current situation and perspectives of clinical study in integrative medicine in China," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.
- [16] J. A. Astin, "Why patients use alternative medicine: results of a national study," *Journal of the American Medical Association*, vol. 279, no. 19, pp. 1548–1553, 1998.
- [17] H. M. Arthur, C. Patterson, and J. A. Stone, "The role of complementary and alternative therapies in cardiac rehabilitation: a systematic evaluation," *European Journal of Cardiovascular Prevention and Rehabilitation*, vol. 13, no. 1, pp. 3–9, 2006.
- [18] M. C. Lin, R. Nahin, M. E. Gershwin, J. C. Longhurst, and K. K. Wu, "State of complementary and alternative medicine in cardiovascular, lung, and blood research: executive summary of a workshop," *Circulation*, vol. 103, no. 16, pp. 2038–2041, 2001.
- [19] F. Cheung, "TCM: made in China," *Nature*, vol. 480, no. 7378, pp. S82–S83, 2011.
- [20] K. J. Chen and L. Z. Li, "Study of traditional Chinese medicine—which is after all the right way?" *Chinese Journal of Integrative Medicine*, vol. 11, no. 4, pp. 241–242, 2005.
- [21] J. Wang and X. J. Xiong, "Control strategy on hypertension in Chinese medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 284847, 6 pages, 2012.
- [22] E. Ernst, "Complementary/alternative medicine for hypertension: a mini-review," *Wiener Medizinische Wochenschrift*, vol. 123, pp. 386–391, 2005.
- [23] R. Nahas, "Complementary and alternative medicine approaches to blood pressure reduction: an evidence-based review," *Canadian Family Physician*, vol. 54, no. 11, pp. 1529–1533, 2008.
- [24] R. A. Bell, C. K. Suerken, J. G. Grzywacz, W. Lang, S. A. Quandt, and T. A. Arcury, "CAM use among older adults age 65 or older with hypertension in the United States: general use and disease treatment," *The Journal of Alternative and Complementary Medicine*, vol. 12, no. 9, pp. 903–909, 2006.
- [25] N. M. Kaplan, "Acupuncture for hypertension: can 2500 years come to an end?" *Hypertension*, vol. 48, no. 5, p. 815, 2006.
- [26] Y. M. Gong, R. X. Guo, and L. S. Zhang, "Application of jiang ya paste onto yongquan acupoint for treatment of primary hypertension," *Journal of Traditional Chinese Medicine*, vol. 15, no. 2, pp. 112–113, 1995.
- [27] G. K. Wei, J. M. He, and Z. G. Chen, "Treatment of 104 cases suffering from cervico-spinal hypertension with rotation-reduction method—observation of the long-term effect," *Journal of Traditional Chinese Medicine*, vol. 9, no. 4, pp. 266–268, 1989.
- [28] H. Q. Huang and S. Z. Liang, "Acupuncture at otoacupoint heart for treatment of vascular hypertension," *Journal of Traditional Chinese Medicine*, vol. 12, no. 2, pp. 133–136, 1992.
- [29] Y. J. Chiu, A. Chi, and I. A. Reid, "Cardiovascular and endocrine effects of acupuncture in hypertensive patients," *Clinical and Experimental Hypertension*, vol. 19, no. 7, pp. 1047–1063, 1997.
- [30] J. Dong, "Acupuncture treatment of hypertension: a report of 70 cases," *International Journal of Clinical Acupuncture*, vol. 7, pp. 173–175, 1996.
- [31] H. Li, L. T. Liu, W. M. Zhao et al., "Effect of traditional and integrative regimens on quality of life and early renal impairment in elderly patients with isolated systolic hypertension," *Chinese Journal of Integrative Medicine*, vol. 16, no. 3, pp. 216–221, 2010.
- [32] G. W. Zhong, M. J. Chen, Y. H. Luo et al., "Effect of Chinese herbal medicine for calming Gan and suppressing hyperactive yang on arterial elasticity function and circadian rhythm of blood pressure in patients with essential hypertension," *Chinese Journal of Integrative Medicine*, vol. 17, no. 6, pp. 414–420, 2011.
- [33] J. Park, S. Hong, T. Park et al., "P02. 136. A randomized controlled trial for the use of qigong in the treatment of pre and mild essential hypertension," *BMC Complementary and Alternative Medicine*, vol. 12, supplement 1, p. P192, 2012.
- [34] M. S. Lee, H. J. Lim, and M. S. Lee, "Impact of qigong exercise on self-efficacy and other cognitive perceptual variables in patients with essential hypertension," *The Journal of Alternative and Complementary Medicine*, vol. 10, no. 4, pp. 675–680, 2004.
- [35] A. F. Walker, G. Marakis, A. P. Morris, and P. A. Robinson, "Promising hypotensive effect of hawthorn extract: a randomized double-blind pilot study of mild, essential hypertension," *Phytotherapy Research*, vol. 16, no. 1, pp. 48–54, 2002.

- [36] F. A. Flachskampf, J. Gallasch, O. Gefeller et al., "Randomized trial of acupuncture to lower blood pressure," *Circulation*, vol. 115, no. 24, pp. 3121–3129, 2007.
- [37] P. M. Stavro, M. Woo, T. F. Heim, L. A. Leiter, and V. Vuksan, "North American Ginseng exerts a neutral effect on blood pressure in individuals with hypertension," *Hypertension*, vol. 46, no. 2, pp. 406–411, 2005.
- [38] P. M. Stavro, M. Woo, L. A. Leiter, T. F. Heim, J. L. Sievenpiper, and V. Vuksan, "Long-term intake of North American Ginseng has no effect on 24-hour blood pressure and renal function," *Hypertension*, vol. 47, no. 4, pp. 791–796, 2006.
- [39] E. A. Macklin, P. M. Wayne, L. A. Kalish et al., "Stop hypertension with the acupuncture research program (SHARP): results of a randomized, controlled clinical trial," *Hypertension*, vol. 48, no. 5, pp. 838–845, 2006.
- [40] J. J. Li, Z. L. Lu, W. R. Kou et al., "Beneficial impact of Xuezhikang on cardiovascular events and mortality in elderly hypertensive patients with previous myocardial infarction from the China Coronary Secondary Prevention Study (CCSPS)," *Journal of Clinical Pharmacology*, vol. 49, no. 8, pp. 947–956, 2009.
- [41] J. J. Li, Z. L. Lu, W. R. Kou et al., "Long-term effects of Xuezhikang on blood pressure in hypertensive patients with previous myocardial infarction: data from the Chinese Coronary Secondary Prevention Study (CCSPS)," *Clinical and Experimental Hypertension*, vol. 32, no. 8, pp. 491–498, 2010.
- [42] J. J. Li, Z. L. Lu, W. R. Kou et al., "Impact of Xuezhikang on coronary events in hypertensive patients with previous myocardial infarction from the China Coronary Secondary Prevention Study (CCSPS)," *Annals of Medicine*, vol. 42, no. 3, pp. 231–240, 2010.
- [43] H. S. Ding and X. F. Zhou, "Meta-analysis of effectiveness of anti-hypertension of 442 traditional Chinese herbal decoctions," *Shi Yong Yi Yuan Lin Chuang Za Zhi*, vol. 9, no. 4, pp. 192–194, 2012.
- [44] Y. X. Hu, *Quantitative analysis of clinical controlled trials of traditional Chinese medicine and systematic evaluation of randomized controlled trials involving traditional Chinese medicine for essential hypertension [M.S. thesis]*, Guangzhou University of Chinese Medicine, Guangzhou, China, 2009.
- [45] Y. Ren, A. H. Ou, X. Z. Lin, and Y. R. Lao, "Meta-analysis of traditional Chinese medicine for essential hypertension," *Shanxi Zhong Yi*, vol. 27, no. 7, pp. 794–796, 2006.
- [46] D. X. Dong, S. L. Yao, N. Yu, and B. Yang, "Systematic review and meta-analysis of Tianma Gouteng Yin combined with enalapril for essential hypertension," *Zhongguo Zhong Yi Ji Zheng*, vol. 20, no. 5, pp. 762–764, 2011.
- [47] H. W. Zhang, J. Tong, G. Zhou, H. Jia, and J. Y. Jiang, "Tianma Gouteng Yin Formula for treating primary hypertension," *Cochrane Database of Systematic Reviews*, no. 6, Article ID CD008166, 2012.
- [48] W. J. Xu and Y. L. Li, "Systematic review of clinical evidence about calm the liver and subdue yang therapy on the hypertension disease with syndrome of upper hyperactivity of liver yang," *Zhonghua Zhong Yi Yao Za Zhi*, vol. 27, no. 3, pp. 736–739, 2012.
- [49] M. Shi and Y. H. Zhang, "Systematic review of replenishing kidney qi method for essential hypertension with kidney qi deficiency syndrome," *Shandong Zhong Yi Za Zhi*, vol. 31, no. 4, pp. 236–238, 2012.
- [50] L. Liu and Y. L. Li, "Systematic review on treatment of essential hypertension from spleen and kidney deficiency," *Zhonghua Zhong Yi Yao Za Zhi*, vol. 26, no. 8, pp. 1700–1703, 2011.
- [51] D. N. Li and C. H. Yang, "Effects of Chinese medicine on elderly isolated systolic hypertension: a meta-analysis," *Liaoning Zhong Yi Za Zhi*, vol. 39, no. 5, pp. 812–815, 2012.
- [52] R. Zhao, L. X. Fu, J. Xiong, S. Li, and Z. L. Wang, "The effect of acupuncture therapy on essential hypertension: a systematic review of long-term effect," *Zhen Jiu Lin Chuang Za Zhi*, vol. 27, no. 3, pp. 46–51, 2011.
- [53] J. I. Kim, J. Y. Choi, H. Lee, M. S. Lee, and E. Ernst, "Moxibustion for hypertension: a systematic review," *BMC Cardiovascular Disorders*, vol. 10, article 33, 2010.
- [54] M. S. Lee, T. Y. Choi, B. C. Shin, J. I. Kim, and S. S. Nam, "Cupping for hypertension: a systematic review," *Clinical and Experimental Hypertension*, vol. 32, no. 7, pp. 423–425, 2010.
- [55] M. S. Lee, M. Pittler, R. L. Guo, and E. Ernst, "Qigong for hypertension: a systematic review of randomized clinical trials," *Journal of Hypertension*, vol. 25, no. 8, pp. 1525–1532, 2007.
- [56] X. F. Guo, B. Zhou, T. Nishimura, S. Teramukai, and M. Fukushima, "Clinical effect of Qigong practice on essential hypertension: a meta-analysis of randomized controlled trials," *The Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 27–37, 2008.
- [57] H. G. Li and Z. W. Xu, "Systematic review of Tai Chi for essential hypertension," *Wen Ti Yong Pin Yu Ke Ji*, vol. 22, no. 7, pp. 35–37, 2011.
- [58] J. T. Cheng, "Review: drug therapy in Chinese traditional medicine," *The Journal of Clinical Pharmacology*, vol. 40, no. 5, pp. 445–450, 2000.
- [59] J. Wang, P. Q. Wang, and X. J. Xiong, "Current situation and re-understanding of syndrome and formula syndrome in Chinese medicine," *Internal Medicine*, vol. 2, no. 3, Article ID 1000113, 2012.
- [60] X. J. Xiong, F. Y. Chu, H. X. Li, and Q. Y. He, "Clinical application of the TCM classic formulae for treating chronic bronchitis," *Journal of Traditional Chinese Medicine*, vol. 31, no. 1, pp. 69–72, 2011.
- [61] H. Xu and K. J. Chen, "Progress, difficulty and countermeasure in treating hypertensive disease with integrated Chinese and Western medicine," *Shi Jie Zhong Yi Yao*, vol. 2, no. 1, pp. 3–5, 2007.
- [62] D. L. Sackett, S. E. Straus, W. S. Richardson, W. Rosenberg, and R. B. Hanynes, *Evidence Based Medicine: How To Practice and Teach EBM*, Churchill Livingstone, London, UK, 2nd edition, 2000.
- [63] H. Xu and K. J. Chen, "Making evidence-based decisions in the clinical practice of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 6, pp. 483–485, 2010.
- [64] E. Braunwald, "The rise of cardiovascular medicine," *European Heart Journal*, vol. 33, no. 7, pp. 838–845, 2012.
- [65] J. Y. Wang, "Significance of evidence-based medicine in the assessment of Chinese medicine clinical efficacy," *Chinese Journal of Integrative Medicine*, vol. 16, no. 5, pp. 392–393, 2010.
- [66] L. Isaacs, Alternative forms of medicine are becoming more popular, <http://www.smudailymustang.com/?p=27201>.
- [67] X. Xu, "Acupuncture in an outpatient clinic in China: a comparison with the use of acupuncture in North America," *Southern Medical Journal*, vol. 94, no. 8, pp. 813–816, 2001.
- [68] V. Napadow and T. J. Kaptchuk, "Patient characteristics for outpatient acupuncture in Beijing, China," *The Journal of Alternative and Complementary Medicine*, vol. 10, no. 3, pp. 565–572, 2004.

- [69] S. Y. Kim, Y. Chae, S. M. Lee, H. Lee, and H. J. Park, "The effectiveness of moxibustion: an overview during 10 years," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 306515, 19 pages, 2011.
- [70] H. Yamashita, Y. Ichiman, and Y. Tanno, "Changes in peripheral lymphocyte subpopulations after direct moxibustion," *The American Journal of Chinese Medicine*, vol. 29, no. 2, pp. 227–235, 2001.
- [71] M. S. Lee, J. W. Kang, and E. Ernst, "Does moxibustion work? An overview of systematic reviews," *BMC Research Notes*, vol. 3, article 284, 2010.
- [72] R. Lauche, H. Cramer, C. Hohmann et al., "The effect of traditional cupping on pain and mechanical thresholds in patients with chronic nonspecific neck pain: a randomized controlled pilot study," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 429718, 10 pages, 2012.
- [73] J. I. Kim, M. S. Lee, D. H. Lee, K. Boddy, and E. Ernst, "Cupping for treating pain: a systematic review," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 467014, 7 pages, 2011.
- [74] M. S. Lee, S. S. Hong, H. J. Lim, H. J. Kim, W. H. Woo, and S. R. Moon, "Retrospective survey on therapeutic efficacy of Qigong in Korea," *The American Journal of Chinese Medicine*, vol. 31, no. 5, pp. 809–815, 2003.
- [75] K. M. Sancier and D. Holman, "Multifaceted health benefits of medical qigong," *The Journal of Alternative and Complementary Medicine*, vol. 10, no. 1, pp. 163–165, 2004.
- [76] M. S. Lee, M. S. Lee, E. S. Choi, and H. T. Chung, "Effects of Qigong on blood pressure, blood pressure determinants and ventilatory function in middle-aged patients with essential hypertension," *The American Journal of Chinese Medicine*, vol. 31, no. 3, pp. 489–497, 2003.
- [77] M. S. Lee, H. J. Lim, and M. S. Lee, "Impact of qigong exercise on self-efficacy and other cognitive perceptual variables in patients with essential hypertension," *The Journal of Alternative and Complementary Medicine*, vol. 10, no. 4, pp. 675–680, 2004.
- [78] M. S. Lee, M. S. Lee, H. J. Kim, and E. S. Choi, "Effects of qigong on blood pressure, high-density lipoprotein cholesterol and other lipid levels in essential hypertension patients," *International Journal of Neuroscience*, vol. 114, no. 7, pp. 777–786, 2004.
- [79] G. Y. Yeh, C. Wang, P. M. Wayne, and R. Phillips, "Tai Chi exercise for patients with cardiovascular conditions and risk factors: a systematic review," *Journal of Cardiopulmonary Rehabilitation and Prevention*, vol. 29, no. 3, pp. 152–160, 2009.
- [80] M. S. Lee, M. H. Pittler, R. E. Taylor-Piliae, and E. Ernst, "Tai Chi for cardiovascular disease and its risk factors: a systematic review," *Journal of Hypertension*, vol. 25, no. 9, pp. 1974–1975, 2007.
- [81] J. C. Tsai, W. H. Wang, P. Chan et al., "The beneficial effects of Tai Chi Chuan on blood pressure and lipid profile and anxiety status in a randomized controlled trial," *The Journal of Alternative and Complementary Medicine*, vol. 9, no. 5, pp. 747–754, 2003.
- [82] X. J. Xiong and J. Wang, "Discussion of related problems in herbal prescription science based on objective indications of herbs," *Journal of Chinese Integrative Medicine*, vol. 8, no. 1, pp. 20–24, 2010.
- [83] H. Xu and K. J. Chen, "Herb-drug interaction: an emerging issue of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 3, pp. 195–196, 2010.
- [84] X. J. Xiong, J. Wang, and Q. Y. He, "Application status and safety countermeasures of traditional Chinese medicine injections," *Journal of Chinese Integrative Medicine*, vol. 8, no. 4, pp. 307–311, 2010.
- [85] H. Xu and K. J. Chen, "Integrating traditional medicine with biomedicine towards a patient-centered healthcare system," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 83–84, 2011.
- [86] X. J. Xiong, J. Wang, and Q. Y. He, "Thinking about reducing adverse reactions based on idea of formula corresponding to syndromes," *Zhongguo Zhong Yao Za Zhi*, vol. 35, no. 4, pp. 536–538, 2010.
- [87] Z. Y. Shen and X. Chen, "Analysis on 99 cases of adverse reactions of Chinese patent drugs," *African Journal of Microbiology Research*, vol. 6, no. 8, pp. 1742–1746, 2012.
- [88] Y. F. Wu, R. Huxley, L. M. Li et al., "Prevalence, awareness, treatment, and control of hypertension in China data from the China National Nutrition and Health Survey 2002," *Circulation*, vol. 118, no. 25, pp. 2679–2686, 2008.
- [89] K. Sliwa, S. Stewart, and B. J. Gersh, "Hypertension: a global perspective," *Circulation*, vol. 123, no. 24, pp. 2892–2896, 2011.
- [90] G. Mancina, G. De Backer, A. Dominiczak et al., "Guidelines for the management of arterial hypertension: the task force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC)," *Journal of Hypertension*, vol. 25, no. 6, pp. 1105–1187, 2007.
- [91] M. H. Alderman and T. Ogihara, "Global challenge for overcoming high blood pressure: fukuoka statement, 19 October 2006," *Journal of Hypertension*, vol. 25, no. 3, p. 727, 2007.
- [92] M. Ezzati, A. D. Lopez, A. Rodgers, S. Vander Hoorn, and C. J. L. Murray, "Selected major risk factors and global and regional burden of disease," *The Lancet*, vol. 360, no. 9343, pp. 1347–1360, 2002.
- [93] D. Yach, C. Hawkes, C. L. Gould, and K. J. Hofman, "The global burden of chronic diseases: overcoming impediments to prevention and control," *Journal of the American Medical Association*, vol. 291, no. 21, pp. 2616–2622, 2004.
- [94] M. Y. Liu and K. J. Chen, "Convergence: the tradition and the modern," *Chinese Journal of Integrative Medicine*, vol. 18, no. 3, pp. 164–165, 2012.
- [95] X. J. Xiong, "Study on the history of formulas corresponding to syndromes," *Journal of Chinese Integrative Medicine*, vol. 8, no. 6, pp. 581–588, 2010.
- [96] A. P. Lu and K. J. Chen, "Integrative medicine in clinical practice: from pattern differentiation in traditional Chinese medicine to disease treatment," *Chinese Journal of Integrative Medicine*, vol. 15, no. 2, p. 152, 2009.
- [97] A. P. Lu, Z. X. Bian, and K. J. Chen, "Bridging the traditional Chinese medicine pattern classification and biomedical disease diagnosis with systems biology," *Chinese Journal of Integrative Medicine*, vol. 18, no. 12, pp. 883–890, 2012.
- [98] J. Wang, B. Feng, X. C. Yang et al., "Tianma gouteng yin as adjunctive treatment for essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 706125, 18 pages, 2013.
- [99] X. J. Xiong, X. C. Yang, W. Liu et al., "Banxia baizhu tianma decoction for essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 271462, 10 pages, 2012.

- [100] K. J. Chen, Y. H. Xie, and Y. Liu, "Profiles of traditional Chinese medicine schools," *Chinese Journal of Integrative Medicine*, vol. 18, no. 7, pp. 534–538, 2012.
- [101] X. J. Xiong and J. Wang, "Experience of diagnosis and treatment of exogenous high-grade fever," *Journal of Chinese Integrative Medicine*, vol. 9, no. 6, pp. 681–687, 2011.
- [102] X. G. Sun, W. K. Wu, and Z. P. Lu, "Chinese integrative medicine: translation toward person-centered and balanced medicine," *Chinese Journal of Integrative Medicine*, vol. 18, no. 1, pp. 3–6, 2012.
- [103] G. Dobos and I. Tao, "The model of Western integrative medicine: the role of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 11–20, 2011.
- [104] X. J. Xiong, X. C. Yang, B. Feng et al., "Zhen gan xi feng decoction, a traditional Chinese herbal formula, for the treatment of essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 982380, 9 pages, 2013.
- [105] J. Wang, X. C. Yang, B. Feng et al., "Is Yangxue Qingnao Granule combined with antihypertensive drugs, a new integrative medicine therapy, more effective than antihypertensive therapy alone in treating essential hypertension?" *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 540613, 8 pages, 2013.
- [106] J. Wang, K. W. Yao, X. C. Yang et al., "Chinese patent medicine liu wei di huang wan combined with antihypertensive drugs, a new integrative medicine therapy, for the treatment of essential hypertension: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 714805, 7 pages, 2012.
- [107] A. M. Wu, D. M. Zhang, Y. H. Gao et al., "The correlation between high-sensitivity C-reactive protein, matrix metalloproteinase 9, and traditional Chinese medicine syndrome in patients with hypertension," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 780937, 8 pages, 2013.
- [108] S. L. Chen, X. Y. Liu, W. M. Xu, W. Y. Mei, and X. L. Chen, "Clinical study of Western medicine combined with Chinese medicine based on syndrome differentiation in the patients with polarized hypertension," *Chinese Journal of Integrative Medicine*, vol. 18, no. 10, pp. 746–751, 2012.
- [109] J. S. Janicki and S. P. Levick, "The convergence of ancient Chinese medicine with modern therapeutics to prevent cardiac fibrosis," *American Journal of Hypertension*, vol. 25, no. 2, p. 139, 2012.
- [110] W. Liu, J. Chen, T. Xu, W. Tian, Y. Li, and W. R. Li, "Qiliqiangxin improves cardiac function in spontaneously hypertensive rats through the inhibition of cardiac chymase," *American Journal of Hypertension*, vol. 25, no. 2, pp. 250–260, 2012.
- [111] Y. Lei, "Train of thought and strategy over strengthening the research of treating hypertension by integrative Chinese and Western medicine," *Chinese Journal of Integrative Medicine*, vol. 10, no. 1, pp. 4–6, 2004.
- [112] Z. X. Shi, "Train of thought about treatment of high blood pressure with integrative traditional and Western medicine," *Chinese Journal of Integrative Medicine*, vol. 10, no. 1, pp. 2–4, 2004.
- [113] Y. H. Zhao, Y. H. Xu, Y. Guan, and P. Xiang, "Effects of Yinian Jiangya Decoction containing serum on cytokines secretion of vascular endothelium of spontaneously hypertensive rats," *Chinese Journal of Integrative Medicine*, vol. 16, no. 4, pp. 344–347, 2010.
- [114] X. J. Xiong, X. C. Yang, Y. M. Liu, Y. Zhang, P. Q. Wang, and J. Wang, "Chinese herbal formulas for treating hypertension in traditional Chinese medicine: perspective of modern science," *Hypertension Research*, 2013.
- [115] Z. Junhua, S. Hongcai, G. Xiumei et al., "Methodology and reporting quality of systematic review/meta-analysis of traditional Chinese medicine," *The Journal of Alternative and Complementary Medicine*, vol. 13, no. 8, pp. 797–805, 2007.
- [116] E. Manheimer, S. Wieland, E. Kimbrough, K. Cheng, and B. M. Berman, "Evidence from the Cochrane Collaboration for traditional Chinese medicine therapies," *The Journal of Alternative and Complementary Medicine*, vol. 15, no. 9, pp. 1001–1014, 2009.
- [117] D. Moher, A. Liberati, J. Tetzlaff, and D. G. Altman, "Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement," *PLOS Medicine*, vol. 6, no. 7, Article ID e1000097, 2009.
- [118] J. Wang and X. J. Xiong, "Outcome measures of Chinese herbal medicine for hypertension: an overview of systematic reviews," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 697237, 7 pages, 2012.
- [119] C. M. Witt, W. J. Huang, L. X. Lao, and B. M. Berman, "Which research is needed to support clinical decision-making on integrative medicine?—can comparative effectiveness research close the gap?" *Chinese Journal of Integrative Medicine*, vol. 18, no. 10, pp. 723–729, 2012.
- [120] J. L. Tang, "Some reflections on the evaluation of clinical effectiveness of Chinese medicine in China," *Chinese Journal of Integrative Medicine*, vol. 16, no. 5, pp. 390–391, 2010.

## Research Article

# Effectiveness of Yoga for Hypertension: Systematic Review and Meta-Analysis

Marshall Hagins,<sup>1</sup> Rebecca States,<sup>1</sup> Terry Selfe,<sup>2,3</sup> and Kim Innes<sup>2,4</sup>

<sup>1</sup> Department of Physical Therapy, Long Island University, Brooklyn Campus, One University Plaza, Brooklyn, NY 10021, USA

<sup>2</sup> Department of Epidemiology, West Virginia University School of Public Health, Morgantown, WV 26506-9190, USA

<sup>3</sup> Center for the Study of Complementary and Alternative Therapies, University of Virginia Health System, Charlottesville, VA 22908-0782, USA

<sup>4</sup> Department of Physical Medicine and Rehabilitation, Center for the Study of Complementary and Alternative Therapies, University of Virginia Health System, Charlottesville, VA 22908-0782, USA

Correspondence should be addressed to Marshall Hagins; [mhagins@liu.edu](mailto:mhagins@liu.edu)

Received 16 November 2012; Revised 25 April 2013; Accepted 25 April 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 Marshall Hagins et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Objectives.** To systematically review and meta-analyze the effectiveness of yoga for reducing blood pressure in adults with hypertension and to assess the modifying influences of type and length of yoga intervention and type of comparison group. **Methods.** Academic Search Premier, AltHealthWatch, BIOSIS/Biological Abstracts, CINAHL, Cochrane Library, Embase, MEDLINE, PsycINFO, PsycARTICLES, Natural Standard, and Web of Science databases were screened for controlled studies from 1966 to March 2013. Two authors independently assessed risk of bias using the Cochrane Risk of Bias Tool. **Results.** All 17 studies included in the review had unclear or high risk of bias. Yoga had a modest but significant effect on systolic blood pressure (SBP) ( $-4.17$  [ $-6.35, -1.99$ ],  $P = 0.0002$ ) and diastolic blood pressure (DBP) ( $-3.62$  [ $-4.92, -1.60$ ],  $P = 0.0001$ ). Subgroup analyses demonstrated significant reductions in blood pressure for (1) interventions incorporating 3 basic elements of yoga practice (postures, meditation, and breathing) (SBP:  $-8.17$  mmHg [ $-12.45, -3.89$ ]; DBP:  $-6.14$  mmHg [ $-9.39, -2.89$ ]) but not for more limited yoga interventions; (2) yoga compared to no treatment (SBP:  $-7.96$  mmHg [ $-10.65, -5.27$ ]) but not for exercise. **Conclusion.** Yoga can be preliminarily recommended as an effective intervention for reducing blood pressure. Additional rigorous controlled trials are warranted to further investigate the potential benefits of yoga.

## 1. Introduction

Current estimates suggest that over 76 million US adults suffer from hypertension [1] and that blood pressure is well controlled in less than 50% of these individuals [2]. Uncontrolled hypertension is thought to be responsible for 62% of cerebrovascular disease and 49% of ischemic heart disease [3] and is estimated to cost the United States \$93.5 billion in health care services, medications, and missed days of work in 2010 [4]. The cost of drugs, drug interactions, and nonadherence with the drug regimen all contribute to current high rates of uncontrolled hypertension. Alternative, less expensive methods to reduce blood pressure that have lower risk of drug interactions and which may convey the benefits of long-term adherence are much needed.

Yoga is one such alternative healthcare practice thought to improve blood pressure control [5–7]. There is no single definition of the practice of yoga, that is universally accepted although it is generally described as an ancient tradition (originating 5,000 to 8,000 years ago) [8–10] that incorporates postures, breath control, and meditation, as well as specific ethical practices [11, 12]. The number of yoga practitioners continues to rise, with current estimates indicating at least 15.8 million people in the United States (6.9% of Americans) practice yoga [13]. Most relevant to the issue of blood pressure control is that yoga is increasingly being suggested by American health care providers as a means of enhancing health [13]. Of the many benefits ascribed to yoga practice, blood pressure control is among the most studied [7]. While several reviews regarding the potential benefits of yoga for

reducing blood pressure and other cardiovascular disease risk factors have been published [5, 7, 14–17], most have stated that the quality of the studies are generally poor. Additionally, few reviews have specifically focused on blood pressure control, and meta-analyses are lacking. Thus, the degree to which yoga may decrease blood pressure as well as the potential modifying effects of type of yoga intervention and type of comparison group remain unclear. To address these gaps, this paper presents a systematic review and meta-analysis of controlled studies (randomized and nonrandomized) examining the effects of yoga practice on systolic and diastolic blood pressure in individuals with prehypertension or hypertension.

## 2. Methods

**2.1. Literature Search.** Methods of the analysis and inclusion criteria were specified in advance but were not documented in a publicly available protocol. A systematic literature search was carried out using the databases Academic Search Premier, AltHealthWatch, Biosis/Biological Abstracts, CINAHL Plus with Full Text, Cochrane Library, Embase, MEDLINE, PsycINFO, PsycARTICLES, Natural Standard, and Web of Science. Additional studies were identified by searching bibliographies of reviews, all studies included in this review, and select uncontrolled studies of yoga and blood pressure. Search terms included yoga or yogi\* or yama or niyama or pratyahara or dharana or dhyana or samadhi or asana combined with “blood pressure” or hypertension or hypertensive or systolic or diastolic.

### 2.2. Inclusion/Exclusion Criteria

- (1) *Types of studies:* Peer reviewed, English language, controlled studies (either a randomized controlled trial (RCT) or a non-RCT) published between January 1966 and March 2013 were included. Cross-sectional studies, case series, dissertations, and abstracts/posters were not included.
- (2) *Participants:* Adults (mean age  $\geq 18$  years) with prehypertension or hypertension.
- (3) *Interventions:* Given the large variability in practices associated with the term “yoga,” only papers that explicitly labeled the intervention as “yoga” were examined. Consequently, we excluded studies that reported on the effects of any form of meditation, mindfulness-based stress reduction, or relaxation response which the authors did not specifically label as yoga. Studies of Transcendental Meditation (TM), a form of yogic meditation, were excluded, since a comprehensive review and meta-analysis regarding the effects of TM on blood pressure has been recently conducted [18]. In addition, we excluded studies only examining immediate changes following a single yoga session. We also excluded studies examining only practices rarely performed currently but historically associated with yoga such as bloodletting, starvation, and cleansing of the stomach.

- (4) *Outcome measures:* Blood pressure (mmHg) was the only outcome of interest (systolic and diastolic). Studies which did not provide blood pressure data (effect size and/or variability estimates) were excluded.

**2.3. Data Extraction.** Abstracts were initially examined by a single investigator (MH). Independent extraction of data on potentially eligible articles was performed by two authors (MH/RS) using predefined data fields. Disagreements between reviewers were resolved by discussion to achieve consensus. Blood pressure values with standard deviation or standard error as well as participant health status, type of yoga intervention, type of comparison group, demographic characteristics, number of participants enrolled and completing the study, location of the study, reporting of adverse events, and methods for measurement of blood pressure were gathered from each paper. Systolic and diastolic blood pressures (mmHg) were the only measures of treatment effect investigated by meta-analysis. Mean posttest values, or change scores when available, were used for analysis. Where no standard deviations were available they were calculated from the standard error. For otherwise eligible studies that did not provide blood pressure values, corresponding authors were contacted by email in an effort to obtain the information needed for inclusion in this review.

**2.4. Risk of Bias.** The risk of bias for each study was determined independently, but unblinded, by the same two authors using the criteria of the Cochrane Risk of Bias Tool. Disagreements were resolved by discussion to achieve consensus [36]. Studies which had unclear or high risk of bias in one or more key domains (selection, detection, attrition, reporting but not *performance* bias) were considered at high risk of bias.

**2.5. Data Analysis/Assessment of Heterogeneity.** Reference Manager (RevMan) Version 5.1 from the Cochrane Collaboration [37] was used to analyze all data and construct forest plots, as well as to evaluate heterogeneity across studies and to perform sensitivity and subgroup analyses. Statistical heterogeneity across studies was tested using  $\text{Tau}^2$ ,  $\text{Chi}^2$ , and the method proposed by Higgins and Thompson [38]. Given the broad nature of the research question and the variability within the target studies by type of yoga and type of comparison group we expected a large degree of heterogeneity. Consequently we planned use of a random effects model for all comparisons [39].

**2.6. Subgroup and Sensitivity Analysis.** A primary methodological concern was whether controlled but nonrandomized studies should be included in the meta-analysis given that such studies by definition suffer from selection bias. Consequently, sensitivity analyses were conducted to assess potential variation by presence or absence of random participant allocation. In an effort to be maximally inclusive of relevant data we included studies whose populations were not explicitly hypertensive but was composed of individuals with cardiac health related issues (e.g., diabetes, metabolic

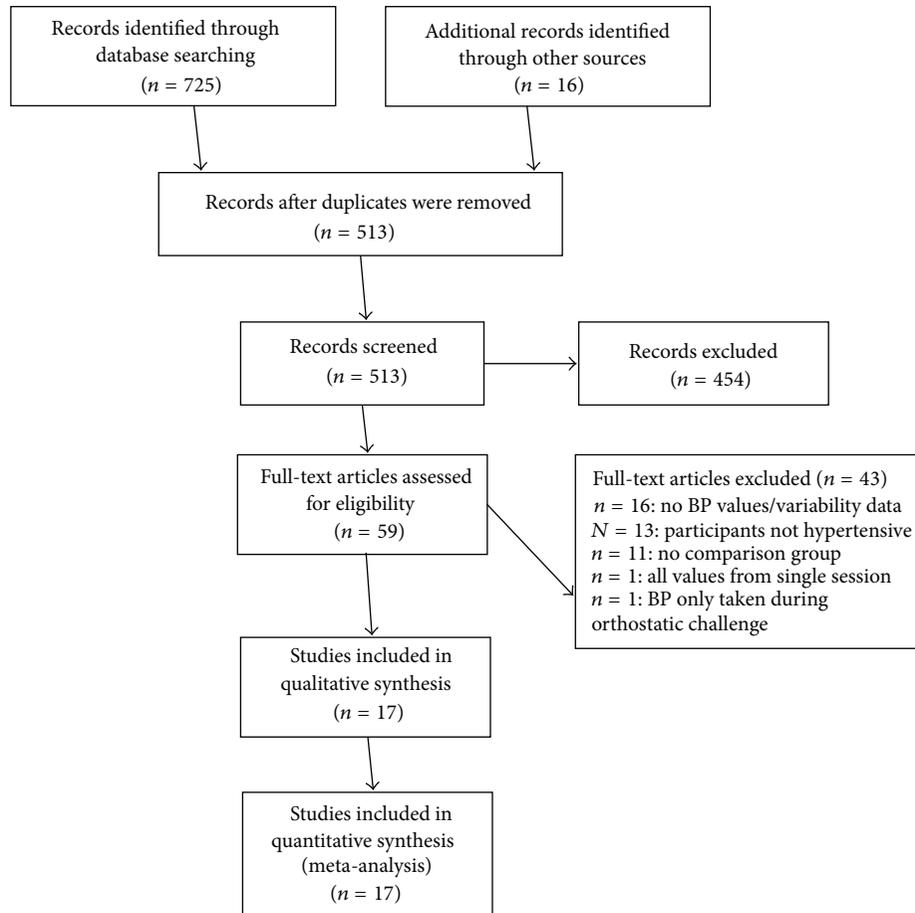


FIGURE 1: Flow Diagram of article selection.

syndrome) with a majority of the study participants currently hypertensive. Consequently, sensitivity analyses were conducted to assess potential variation by presence or absence of study inclusion criteria that required participants to be hypertensive.

We hypothesized a priori that variation in intervention practices would likely contribute substantial heterogeneity to the outcomes. Consequently, subgroup analyses were performed based on duration of the yoga intervention and on yoga practice components included in the intervention. Yoga interventions were divided into 3 categories: (1) those that incorporated postures, meditation, and breathing (“3-element yoga”); (2) those that included fewer than the 3 yoga practices just described; (3) yoga using any combination of the three elements plus one or more additional intervention(s). We also categorized yoga intervention by total time of practice, distinguishing between studies where total time of practice was shorter or longer than the mean duration across all studies. Finally, we performed a subgroup analysis based on comparison group, as we expected between-group effects to vary depending on the control condition. For this subgroup analysis, we used three categories of comparison groups: (1) usual care, no treatment, or wait list; (2) exercise; and (3) attention control or active, nonexercise comparator.

### 3. Results

**3.1. Literature Search.** The initial database searching located 725 potentially eligible articles; an additional 16 papers were identified through other sources, bringing the total number of articles for preliminary review to 741 (Figure 1). Of these, 228 were excluded as duplicates, and 454 for failure to meet inclusion criteria after review of the abstract. Of the remaining 59 articles a full text review yielded 16 studies meeting our full eligibility criteria. An additional 15 studies did not report blood pressure values or variability data, but met all remaining eligibility requirements [32, 36, 40–52]; the primary authors of these studies were contacted to request data. Only one author agreed to provide data and this study [32] was included in the analysis, bringing the total eligible articles to 17. Several papers examined more than one comparison group. These studies were considered independent trials [53] and consequently 22 trials within the 17 studies were identified for analysis.

**3.2. Study Characteristics.** Characteristics of each study are detailed in Table 1. Most studies were conducted in India ( $n = 8$ ) and the USA ( $n = 6$ ), with the remaining conducted in The Netherlands ( $n = 1$ ), Brazil ( $n = 1$ ), and

TABLE 1: Characteristics of studies ( $n = 17$ ), randomized, nonrandomized controlled trials.

Author/date/ location	Sample size (yoga, control)	% com- pleted (yoga, controls)	Study population (categorization)	Yoga intervention description (categorization)	Comparison group(s) (categorization)	Yoga frequency/duration of session and total sessions	Total time in minutes	BP measure	Adverse events
Randomized controlled trials									
Cade et al. [19] 2010 USA	34, 26	85.3, 80.8	HIV infected adults with moderate CVD risk, 83% with hypertension, 18–70 yrs., 47% male, most on multiple medications related to HIV status and CVD risk including BP meds, unclear control of changes in BP meds during study	P, M, B; Ashtanga Vinyasa; encouraged to practice at least one time per week at home/no homework compliance measures [1]	usual care [1]	2.5 wk/60 mins/20 wks	3000	NR	NR
Cohen et al. [20] 2008 USA	14, 12	85.7, 100	Underactive, overweight adults, with metabolic syndrome, 30–65 yrs., 25% males, 59% on at least one BP med., no reported control for BP meds during study	P, M, B; “Restorative” warm up of stretches and breathing exercises followed by 10 poses. Home practice: 3x week for 30 minutes each/home diary for compliance [1]	No treatment [1]	Intro class 180 mins + 2x wk/90 mins/5 weeks + 1x wk/5 wks + reported mean 117 mins × 10 wks	2700	S	None
Cohen et al. [21] 2011 USA	46, 32	56.5, 96.8	Hypertensive adults, 22–69 yrs., 50% males, none on BP meds by exclusion at recruitment	P, M, B; Iyengar yoga. Home practice during weeks 6–12 one time per day for 25 minutes/home diary for compliance [1]	Enhanced usual care; motivational and behavioral components of life style modifications, for example, reduction of weight and ingestion of sodium and alcohol [3]	2x wk/70 mins/6 wks + 1x wk/6 wks	1260	Am	3 (7%)
McCaffrey et al. [22] 2005 Thailand	32, 29	84.4, 93	Hypertensive adults, age range not reported/mean = 56 yrs., 35% male, none on BP meds by exclusion at recruitment, controlled for those who began BP meds by dropping from study	P, M, B; unspecified type of yoga it appears to be independent practice rather than classes using booklets based on yogic principles for guidance. No information about training in yoga practice. As appears that all practice was at home (no group classes)—no additional home practice [1]	Usual care [1]	3x wk/63 mins/8 wks	1512	NR	NR

TABLE 1: Continued.

Author/date/ location	Sample size (yoga, control)	% com- pleted (yoga, controls)	Study population (categorization)	Yoga intervention description (categorization)	Comparison group(s) (categorization)	Yoga frequency/duration of session and total sessions	Total time in minutes	BP measure	Adverse events
van Montfrans et al. [23] 1990 The Netherlands	19, 23	94.7, 73.9	Hypertensive adults, 24–60 yrs., 51% male, none on BP meds by exclusion at recruitment, no reported control for BP meds during study	P, M, B; multimodality program. Hatha yoga plus progressive relaxation and autogenic training for 8 weeks followed by 10 months of independent practice 2x day with cassette tape. All practice was at home except first 8 weeks so no additional home practice [3]	Education about stress and hypertension. Relaxation in comfortable chair [3]	1x wk/60 mins/8 wks plus home practice of 7x/wk/30 mins/40 wks	480	Am	NR
Murugesan et al. [24] 2000 India	11, 11, 11	100, 100, 100*	Hypertensive adults, 35–65 yrs., gender not reported, none on BP meds by exclusion at recruitment, one comparison group used BP meds	P, M, B; unspecified type of yoga. List of asanas provided plus Om recitation and meditation. No home practice [1]	No treatment [1], medication [3]	12x wk/60 mins/11 wks	7920	S	NR
Patel and North [25] 1975 USA	18, 18	94.4, 94.4	Hypertensive adults, 34–75 yrs., 38% male, 94% on BP meds at enrollment, no reported control for BP meds during study	Not reported if P, M, B; multimodality, unspecified type of yoga. Yoga plus education regarding hypertension, “yoga relaxation methods,” “transcendental meditation,” and skin resistance biofeedback. “Instructed to practice relaxation and meditation twice per day.” No homework compliance measures [3]	No treatment [1]	2x wk/30 mins/6 wks	360	S	NR
Saptharishi et al. [26] 2009 India	27, 30, 28, 28	77.8, 96.7, 96.4, 89.3	Young pre- and hypertensive adults, age range not reported/mean of all groups 22 yrs., 67% male, BP meds status not a recruitment criterion and not reported	P, B; unspecified type of yoga; postures and breath practices as per reference to previous paper. It appears that only practice is home practice “encouraged to practice yoga.” No compliance measures reported [2]	No treatment [1] walking program [2], reduction of salt intake [3]	5x wk/45 mins/8 wks	1800	S	NR

TABLE 1: Continued.

Author/date/ location	Sample size (yoga, control)	% com- pleted (yoga, controls)	Study population (categorization)	Yoga intervention description (categorization)	Comparison group(s) (categorization)	Yoga frequency/duration of session and total sessions	Total time in minutes	BP measure	Adverse events
Subramanian et al. [27] 2011 India	25, 25, 23, 25	100, 100, 100, 84	Young pre- and hypertensive adults, age range not reported/mean of all groups 23 yrs., 65% male, BP meds status not a recruitment criterion and not reported	P, B; unspecified type of yoga; postures and breath practices as per reference to previous paper. It appears that only practice is home practice "encouraged to practice yoga." No compliance measures reported [2]	No treatment [1] walking program [2], reduction of salt intake [3]	5x wk/45 mins/8 wks	1800	S	NR
Non randomized controlled trials									
Deepa et al. [28] 2012 India	15, 15	100, 100*	Hypertensive adults, 45–65 yrs., 53% male, 100% on BP medication	P, M, B; Yoga Nidra: it begins with single sitting pose and single breath exercise followed by 45 mins of corpse pose meditation led by instructor. No home practice as this occurred 2x/day [1]	Usual care, in this case, continued medication [1]	10x wk/60 mins/12 wks	7200	S	NR
Hegde et al. [29] 2011 India	60, 63	95, 100	Adults with Type 2 diabetes, 40–75 yrs., gender not reported, BP meds status and recruitment criterion not reported	P; unspecified type of yoga—19 asanas described only. No home practice described [2]	Usual care [1]	Class length and frequency not reported: class sessions occurred over 3 months	NR	NR	None
Jain et al. [30] 2010 India	57, 30	100, 100	Adults, hypertension status not described (although mean BP values suggest pre-hypertension of both groups), yoga group 30–60 yrs., age of control group not reported, 60% male in yoga group, gender not reported in control group, BP meds status and recruitment criterion not reported	P, M; unspecified type of yoga, Surya Namaskar + "Sharir Sanchalan", and "Bhajan Cassette" No home practice as this occurred daily [2]	No description of any kind for control group [1]	7x wk/90 mins/18 weeks	11340	S	NR
Lakkireddy et al. [31] 2013 USA	52, 49	94, 100	Adults with paroxysmal atrial fibrillation, 39% with known hypertension, (mean BP values across groups suggest pre-hypertension) 18–80 yrs., 47% male, BP meds not a recruitment criteria but reported and controlled for during the interventions	P, M, B; iyengar: home practice encouraged with DVD provided but no compliance measures for homework [1]	Wait list control, same participants for yoga and control group [1]	3x wk (median value)/60 mins/12 wks.	2160	NR	None

TABLE 1: Continued.

Author/date/location	Sample size (yoga, control)	% completed (yoga, controls)	Study population (categorization)	Yoga intervention description (categorization)	Comparison group(s) (categorization)	Yoga frequency/duration of session and total sessions	Total time in minutes	BP measure	Adverse events
Mizuno and Montêiro [32] 2013 Brazil	17, 16	100, 100	Hypertensive adults, age range not reported/mean(SD) yoga group = 67 (7) and control group = 62 (12) yrs., 15% male, majority of participants on blood pressure medication, meds controlled for in study	P, M, B; Unspecified type of yoga, although reference for asanas is Iyengar text; Pranayama, then asana, end with breathing meditation [1]	Usual care [1]	3x wk/90 mins/16 wks	4320	NR	None (PC)
Niranjan et al. [33] 2009 India	16, 16	100, 100	Hypertensive adults, age not reported, gender not reported; BP meds status and recruitment criterion not reported	P, M, B; Unspecified type of yoga, chanting, prayer, asana, breathing exercises, ending with Savasana. No home practice described [1]	Standard exercise, warm up, stationary bike 30 mins, cool down total = 45 mins; intensity not described [2]	4x wk/60 mins/36 wks	8640	NR	NR
Patel [34] 1975 USA	20, 20	100*	Hypertensive adults, age range not reported/mean = 57 yrs., 31% male, 64% on BP meds at enrollment, no reported control for BP meds during study	Not reported if P, M, B; Multimodality, unspecified type of yoga. Yoga plus 'psycho-physical relaxation exercise based on yogic principles and reinforced by bio-feedback instruments.' No home practice [3]	No treatment [1]	3x wk/30 mins/12 wks	1080	NR	NR
Selvamurthy et al. [35] 1998 India	10, 10	100, 100	Hypertensive adults, 100% male, age range not reported/groups divided by age with mean of yoga 50 yrs. and mean of control group 34 yrs., BP meds gradually withdrawn on all participants prior to study onset	P; Unspecified type of yoga; described several specific asanas. No homework practice [1]	Tilt table [3]	Frequency/time in class not reported. Class sessions occurred over 3 weeks	NR	S	NR

Yoga intervention categorization: P: postures; B: breathing; M: meditation; 1 = P + M + B, 2 = any 2 of these or less; 3 = ( $\pm P \pm M \pm B$ )  $\pm$  other interventions.

Comparison group categorization: 1 = no intervention or usual care, 2 = exercise or exercise + additional intervention, 3 = nonexercise intervention.

BP: blood pressure; measurement methods: S: sphygmomanometer; M: machine; Am: ambulatory blood pressure, and NR: not reported.

Males within study based on enrollment data, if not available, data of participants that completed study was used.

Adverse event: NR: not reported; PC: per personal communication with corresponding author.

\*Number of participants at completion not reported/estimate assumes 100% completion.

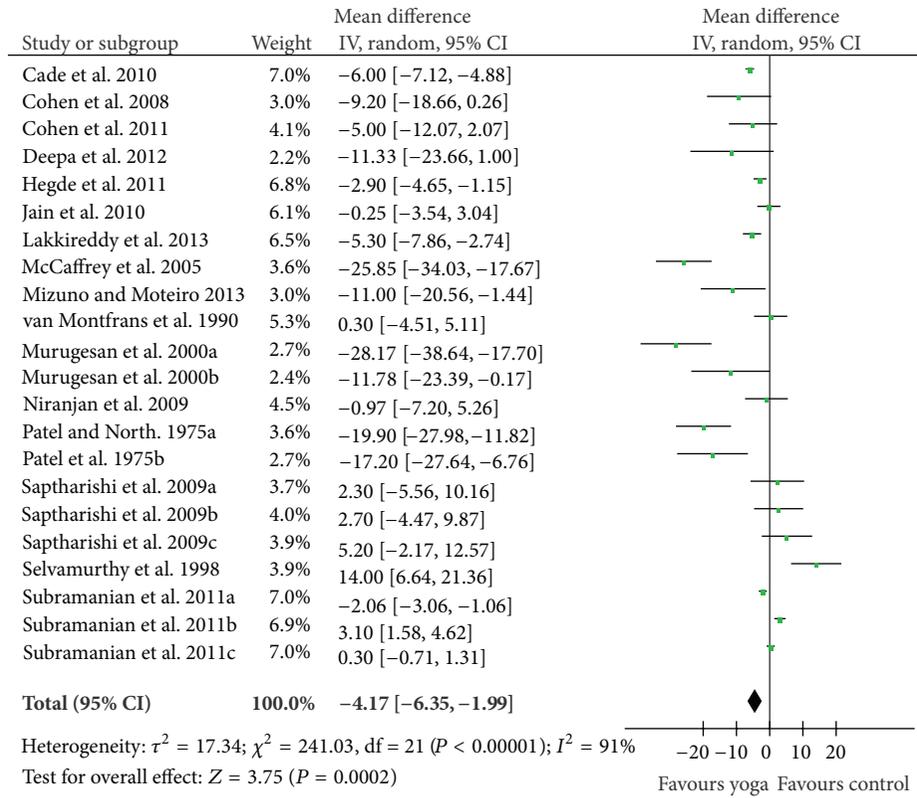
Thailand ( $n = 1$ ). The total number of enrolled participants examined across all included studies was 1013, with 473 (46.7%) assigned to the yoga group and 540 (53.3%) assigned to the comparison group. The total number of participants completing the studies was 943 (yoga = 427; controls = 516) with mean drop-out rates of 9.7% and 4.4% for the yoga and comparison groups, respectively. Of the studies that reported gender ( $n = 14$ ), approximately 38% of study participants were male (some studies reported percentages and did not clarify if gender applied to enrolled participants or to those completing the study). The mean study sample size (using number of participants who completed the study) was 55.4 ( $\pm 31.8$ ), ranging from 20 to 120 participants. Ten (58.8%) of the 22 studies incorporated three elements of yoga (postures, meditation, and breathing) with no additional interventions, while 4 (23.5%) used two or fewer of the elements, and 3 (17.6%) used various elements of yoga in combination with additional interventions. Within the 22 trials three categories of comparison groups were identified: 13 (59%) no treatment or usual care; 3 (13.6%) exercise; 5 (22.7%) various types of nonyoga, nonexercise interventions. Potential adverse events were not reported in 12 (70.1%) of the studies, the absence of adverse events were reported in 4 (23.5%) of the studies, and one study (5.8%) [21] reported three adverse events within the yoga group. The mean length of time used for yoga practice was 58.9 ( $\pm 56.1$ ) hours; 12 studies had fewer hours and 5 had more hours than the average.

**3.2.1. Risk of Bias.** Categorization of the risk of bias at the individual study level is presented in Figure 2. No studies achieved a low risk of bias as all had an unclear or high risk of bias within at least one major domain. *Sequence Generation and Treatment Allocation:* 15 of the 17 studies had unclear or high risk of selection bias as 8 of the studies were nonrandomized and 7 failed to describe sequence generation or allocation. *Blinding of participants:* all studies had high risk of bias for blinding of intervention. Due to the required participatory nature of yoga this category was not considered a primary domain for risk of bias. *Blinding of outcome assessors:* all studies had an unclear risk of bias for outcome assessment with the exception of two which reported blinding (low risk of bias) [21, 23]. *Attrition bias* varied across groups. Eight of 17 studies were assigned unclear or high risk of attrition bias as 3 [21, 23, 28] had high drop-out rates and/or no report of intention-to-treat analysis (high risk of bias) and in 5 studies the drop-out rates exceeded 15%, but were comparable between groups (unclear risk of bias). In the remaining studies ( $n = 9$ ) both intervention and comparison groups had dropout rates of 15% or less or conducted an intention to treat analysis (low risk of bias); and *Selective reporting:* as only one outcome (blood pressure) was examined within this review and studies were only included if these values were described in the report. *Other bias:* all studies had low risk of other biases except one assigned high risk of bias as baseline values differed significantly between groups [35] and one assigned unclear risk of bias as [22] as values were inconsistent between text and tables.

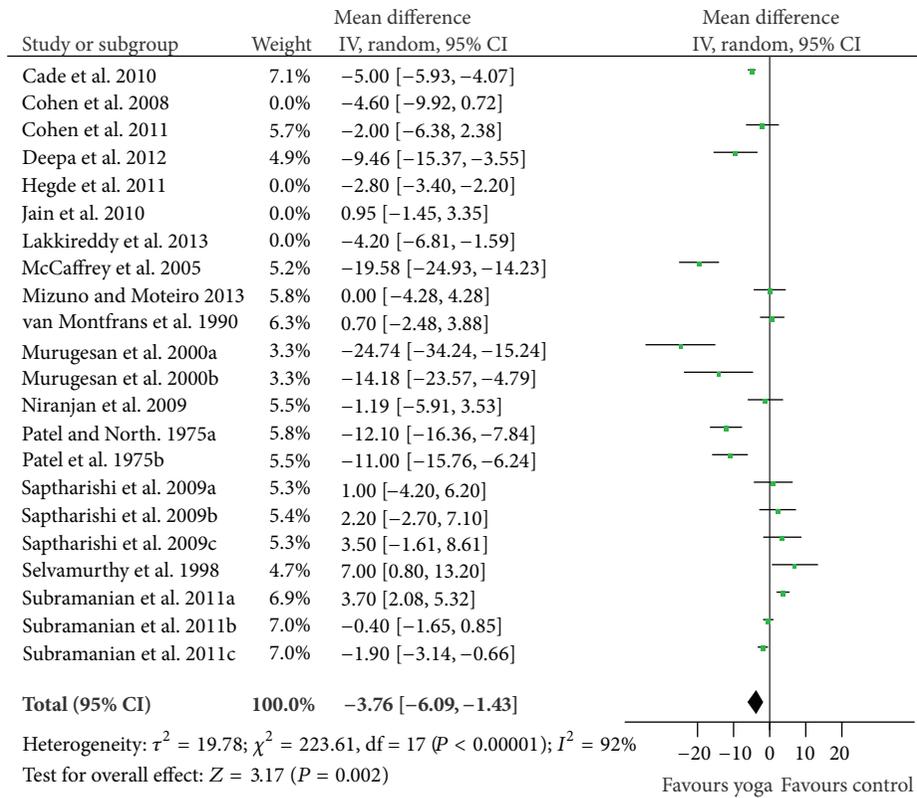
	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Cade 2010	?	?	-	?	?	+	+
Cohen 2008	?	?	-	?	+	+	+
Cohen 2011	?	?	-	+	-	+	+
Deepa 2012	-	-	-	?	-	+	+
Hegde 2011	-	-	-	?	+	+	+
Jain 2010	-	-	-	?	+	+	+
Lakkireddy 2013	-	-	-	?	+	+	+
McCaffrey 2005	+	?	-	?	?	+	?
Mizuno 2013	-	-	-	?	+	+	+
Montfrans 1990	?	?	-	+	-	+	+
Murugesan 2000	?	?	-	?	?	+	+
Niranjan 2009	-	-	-	?	+	+	+
Patel 1975a	-	-	-	?	?	+	+
Patel 1975b	?	?	-	?	+	+	+
Saptharishi 2009	+	?	-	?	+	+	+
Selvamurthy 1998	-	-	-	?	+	+	-
Subramanian 2011	?	?	-	?	?	+	+

FIGURE 2: Risk of bias summary.

**3.2.2. Effects of Yoga on Blood Pressure.** As illustrated in Figures 3(a) and 3(b), yoga had a modest but significant effect on both systolic ( $Z = 3.75, (P = 0.0002); -4.17$  mmHg  $[-6.35, -1.99]$ ) and diastolic blood pressure ( $Z = 3.86, (P = 0.0001); -3.26$  mmHg  $[-4.92, -1.60]$ ). There was substantial heterogeneity present across the included studies:



(a) Systolic



(b) Diastolic

FIGURE 3: Forest plots of overall effect of yoga on prehypertension and hypertension: (a) systolic, and (b) diastolic.

TABLE 2: Results of subgroup analyses: effect sizes, number of trials, and number of participants per subgroup.

Subgroup category	Number of trials	Number of participants	Effect size (confidence interval), mmHg	
			Systolic	Diastolic
Type of yoga intervention*				
(1) P, M, B	11	431	-8.17 (-12.75, -3.89)	-6.14 (-9.39, -2.89)
(2) 2 or less of PMB	8	403	0.19 (-1.70, 2.07)	0.38 (-1.55, 2.32)
(3) ( $\pm P \pm M \pm B$ ) + other intervention	3	109	-11.87 (-26.43, 2.70)	-7.35 (-16.20, 1.50)
Type of comparison group*				
(1) No intervention or usual care	13	656	-7.96 (-10.65, -5.27)	-5.52 (-7.92, -3.11)
(2) Exercise or exercise + additional intervention	3	97	2.87 (1.42, 4.31)	-0.30 (-1.47, 0.87)
(3) Non-exercise intervention	6	190	1.14 (-3.37, 5.66)	-0.35 (-3.56, 2.86)
Length of yoga intervention				
(1) $\leq$ mean (58.9 hours)	16	728	-3.11 (-5.49, -0.73)	-2.55 (-2.95, 2.15)
(2) $>$ mean (58.9 hours)	6	215	-9.73 (-17.66, -1.79)	-1.83 (3.59, -0.07)

Types of yoga intervention: P: postures; B: breathing; M: meditation; 1 = P + M + B, 2 = any 2 of these or less; 3: ( $\pm P \pm M \pm B$ ) + Other intervention.

Length of yoga intervention: 16 trials (12 studies) were categorized as being of short duration as they fell below the mean value across all studies of 58.9 hours; 6 trials (5 studies) were categorized as being of long duration.

\*Significant effect of subgroup differences,  $P < 0.001$ .

$\text{Tau}^2 = 17.34$ ;  $\text{Chi}^2 = 241.03$ ,  $\text{df} = 21$ , ( $P < 0.00001$ ),  $I^2 = 91\%$  for systolic and  $\text{Tau}^2 = 11.17$ ;  $\text{Chi}^2 = 234.96$ ,  $\text{df} = 21$ , ( $P < 0.00001$ ),  $I^2 = 91\%$  for diastolic.

**3.3. Sensitivity Analysis.** Sensitivity analysis was performed by comparing the meta-analysis from all 17 studies with a meta-analysis of the RCTs only ( $n = 9$ ). A second sensitivity analysis was performed by comparing the meta-analysis from all 17 studies with a meta-analysis of the studies which focused on cardiac related health issues but did not have hypertension as an explicit inclusion criteria, although the majority of the participants had hypertension ( $n = 5$ ) [19, 20, 24–26]. For both sensitivity analyses, no substantive differences in either the direction or magnitude of effect size were created by removing the identified studies. Consequently, the findings of all 17 studies were pooled for these analyses.

The number of trials, number of participants, and effect sizes for subgroups is reported in Table 2. Subgroup analyses for systolic and diastolic blood pressure indicated a significant modifying effect of type of yoga intervention ( $\text{Chi}^2 = 14.30$ ,  $P = 0.0008$  and  $\text{Chi}^2 = 13.14$ ,  $P = 0.001$ , resp.) and type of comparison group ( $\text{Chi}^2 = 48.30$ ,  $P = 0.00001$  and  $\text{Chi}^2 = 14.89$ ,  $P = 0.0006$ , resp.) but not for duration of yoga practice ( $\text{Chi}^2 = 2.45$ ,  $P = 0.12$  and  $\text{Chi}^2 = 0.61$ ,  $P = 0.43$ , resp.). The subgroup analysis for type of yoga intervention suggests that incorporating three elements of practice (posture, meditation, and breathing) is associated with significant reductions in blood pressure whereas yoga interventions using two or fewer elements of yoga practice or that combine yoga practice with additional interventions are not (Table 2). The subgroup analysis regarding type of comparison group suggests that RCTs comparing yoga to usual care showed that yoga had a significant effect on blood pressure compared to no treatment but not when compared to exercise or other types of treatment (Table 2).

## 4. Discussion

When the results of all 17 studies (22 trials) examined in this review are pooled, yoga was associated with a small but significant decline in both systolic and diastolic blood pressure ( $-4.17$  and  $-3.26$  mmHg, resp.). Further, yoga's effects on blood pressure varied by type of yoga intervention and by comparison group, but not by duration of yoga practice. These subgroup differences may partially explain the high degree of heterogeneity found across all studies. The level of overall blood pressure reduction achieved by yoga is similar to that of other lifestyle modifications advocated by current guidelines, including exercise [27] and reduced intake of sodium and alcohol [3]. While the overall declines resulting from yoga practice were modest, even small reductions in blood pressure have been shown to reduce risk for coronary heart disease and stroke [29, 30].

When the analysis was restricted to studies using interventions incorporating three elements of yoga practice (postures, meditation, and breathing), larger reductions of  $-8.17$  (systolic) and  $-6.14$  (diastolic) mmHg were observed. Declines of this magnitude are of clear clinical and prognostic significance [3]. To our knowledge, this is the first study to provide preliminary evidence supporting increases in blood pressure reduction associated with specific methods of yogic practice.

Yoga was also associated with a significant decline in systolic ( $-7.96$  mmHg) and diastolic blood pressure ( $-5.52$  mmHg) relative to no treatment, but not when compared to exercise or other types of interventions. It is well known that exercise and some of the other active interventions used within the included studies decrease blood pressure relative to no treatment [27, 29] in the range of 3–9 mmHg (systolic). Given that their effects are comparable in magnitude and direction to those observed with yoga, it is not surprising that we found no significant benefit of yoga when it was compared to an alternate active treatment.

**4.1. External and Internal Validity.** The participants of studies included in this report were male and female adults with prehypertension or hypertension with or without cardiovascular disease. The findings of this report are thus applicable to the majority of individuals with elevated blood pressure. Most studies assessed gentle yoga programs of relatively short duration that could be readily implemented in this clinical population.

Unfortunately, overall quality of studies included in this meta-analysis was poor. All had either unclear or high risk of bias on one or more primary domains. The most common risk of bias was the failure to blind (or to report blinding of) participants. However, studies requiring active participation in an instructor-led intervention cannot be blinded and consequently we did not consider this a primary domain reflecting study quality. However, only 2 of the 17 studies reported blinding of outcome assessors, an entirely feasible method for active intervention studies. In addition, 8 of 17 studies had high or unclear risk of attrition bias and 15 of 17 studies had high or unclear risk of selection bias.

**4.2. Strengths and Weaknesses.** This is the first meta-analytic review to examine the effects of yoga on blood pressure. Strengths of this study include the systematic literature search using multiple databases and based on criteria defined a priori, assessment of studies by multiple authors, a priori decisions regarding appropriate subgroup analyses, and use of well-established meta-analysis procedures for our analyses. One limitation of the current study is we did not assess other potentially contributing factors such as style of yoga, qualifications of instructors or teaching styles, practice environment, participant characteristics such as physical fitness and yoga experience, as well as blood pressure assessment procedures, and other methodological issues. Additional limitations are the restriction to English-language publications, to the selected database sources, and to studies that reported complete blood pressure values.

Exclusion of studies that used yogic interventions but did not label the intervention as such may also have introduced bias. Because there are no universally accepted standards for what constitutes yoga practice, reviews such as this one must necessarily create criteria to define yoga for the purposes of analysis. In this review we excluded studies of certain therapies that, while not defined by the authors as “yoga,” could arguably be viewed as yogic practices. These included, for example, studies of certain meditation techniques that, while generally considered yogic practices, were not described as such. Given that there is already considerable evidence suggesting that meditation is effective in lowering blood pressure; [18, 31, 33] exclusion of these studies may have biased our subgroup analysis of effects by yoga program type. Thus, our findings suggesting that programs incorporating three core elements of yoga (postures, meditation, and breath control) led to significant blood pressure reductions while yoga programs using two or less elements of yoga did not lead to significant reductions in blood pressure reduction should be interpreted with caution. In addition, although some studies included in this review were of reasonably long duration (189 hours) [25], the majority of studies

( $n = 10$ ) were less than 50 hours. Future studies should consider methods, as far as are feasible, which more closely resemble suggested yogic practice (many months to years of practice). Given that the studies within this report had substantial potential risk of bias across multiple domains, future studies should focus on the use of well-designed RCTs which blind outcome assessors, use intention to treat analyses, fully report adverse events, and incorporate measures of treatment expectancy.

## 5. Conclusion

The current study is the first meta-analysis to examine the effects of yoga on blood pressure among individuals with prehypertension or hypertension. Overall, yoga was associated with a modest but significant reduction in blood pressure ( $\approx 4$  mmHg, systolic and diastolic) in this population. Subgroup analyses demonstrated larger, more clinically significant reductions in blood pressure for (1) interventions incorporating 3 basic elements of yoga practice (postures, meditation, and breathing) ( $\approx 8$  mmHg, systolic;  $\approx 6$  mmHg, diastolic) but not for more limited yoga interventions; (2) yoga compared to no treatment ( $\approx 8$  mmHg, systolic; 6 mmHg, diastolic) but not compared to exercise. These reductions are of clear clinical significance and suggest that yoga may offer an effective intervention for reducing blood pressure among people with prehypertension or hypertension. As none of the included studies had methodologies with low risk of bias in primary domains additional rigorous controlled trials are warranted to further investigate the potential benefits of yoga for improving blood pressure in these populations and to determine optimal yoga program design and dosing.

## Funding

This work was funded by the National Institute of General Medical Sciences: 1SC3GM088049-01A1.

## References

- [1] V. L. Roger, A. S. Go, D. M. Lloyd-Jones et al., “On behalf of the American Heart Association statistics committee and stroke statistics subcommittee. Heart disease and stroke statistics—2012 update: a report from the American Heart Association,” *Circulation*, vol. 125, no. 1, pp. 188–197, 2012.
- [2] C. Gillespie, E. V. Kuklina, P. A. Briss, N. A. Blair, and Y. Hong, “Vital signs: prevalence, treatment, and control of hypertension, United States, 1999–2002 and 2005–2008,” *Morbidity and Mortality Weekly Report*, vol. 60, no. 4, pp. 103–108, 2011.
- [3] A. V. Chobanian, G. L. Bakris, H. R. Black et al., “The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report,” *Journal of the American Medical Association*, vol. 289, no. 19, pp. 2560–2572, 2003.
- [4] “High blood pressure facts,” Centers for Disease Control and Prevention Website, 2012, <http://www.cdc.gov/bloodpressure/facts.htm>.
- [5] N. R. Okonta, “Does yoga therapy reduce blood pressure in patients with hypertension?: an integrative review,” *Holistic Nursing Practice*, vol. 26, pp. 137–141, 2012.

- [6] K. E. Innes and H. K. Vincent, "The influence of yoga-based programs on risk profiles in adults with type 2 diabetes mellitus: a systematic review," *Evidence-Based Complementary and Alternative Medicine*, vol. 4, no. 4, pp. 469–486, 2007.
- [7] K. E. Innes, C. Bourguignon, and A. G. Taylor, "Risk indices associated with the insulin resistance syndrome, cardiovascular disease, and possible protection with yoga: a systematic review," *Journal of the American Board of Family Practice*, vol. 18, no. 6, pp. 491–519, 2005.
- [8] J. D. Walters, *The Art and Science of Raja Yoga: Fourteen Steps to Higher Awareness*, Motilal Banarsidass, Delhi, India, 2002.
- [9] G. Feuerstein, *The Yoga Tradition: Its History, Literature, Philosophy, and Practice*, Bhavana Books, New Delhi, India, 2002.
- [10] R. P. Brown and P. L. Gerbarg, "Sudarshan Kriya yogic breathing in the treatment of stress, anxiety, and depression—part I: neurophysiologic model," *Journal of Alternative and Complementary Medicine*, vol. 11, no. 1, pp. 189–201, 2005.
- [11] M. C. Baldwin, "Psychological and physiological influences of hatha yoga training on healthy, exercising adults (yoga, stress, wellness)," *Dissertation Abstracts International Section A*, vol. 60, p. 1031, 1999.
- [12] V. S. Cowen and T. B. Adams, "Physical and perceptual benefits of yoga asana practice: results of a pilot study," *Journal of Bodywork and Movement Therapies*, vol. 9, no. 3, pp. 211–219, 2005.
- [13] J. Dvivedi, H. Kaur, and S. Dvivedi, "Effect of 1 week '61-points relaxation training' on cold pressor test induced stress in premenstrual syndrome," *Indian Journal of Physiology and Pharmacology*, vol. 52, no. 3, pp. 262–266, 2008.
- [14] S. Hutchinson and E. Ernst, "Yoga therapy for coronary heart disease: a systematic review," *Focus on Alternative and Complementary Therapies*, vol. 8, p. 144, 2003.
- [15] J. A. Raub, "Psychophysiological effects of Hatha Yoga on musculoskeletal and cardiopulmonary function: a literature review," *Journal of Alternative and Complementary Medicine*, vol. 8, no. 6, pp. 797–812, 2002.
- [16] S. R. Jayasinghe, "Yoga in cardiac health (a review)," *European Journal of Cardiovascular Prevention and Rehabilitation*, vol. 11, no. 5, pp. 369–375, 2004.
- [17] A. Bussing, A. Michalsen, S. B. Khalsa, S. Telles, and K. J. Sherman, "Effects of yoga on mental and physical health: a short summary of reviews," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 165410, 7 pages, 2012.
- [18] J. W. Anderson, C. Liu, and R. J. Kryscio, "Blood pressure response to transcendental meditation: a meta-analysis," *American Journal of Hypertension*, vol. 21, no. 3, pp. 310–316, 2008.
- [19] W. T. Cade, D. N. Reeds, K. E. Mondy et al., "Yoga lifestyle intervention reduces blood pressure in HIV-infected adults with cardiovascular disease risk factors," *HIV Medicine*, vol. 11, no. 6, pp. 379–388, 2010.
- [20] B. E. Cohen, A. A. Chang, D. Grady, and A. M. Kanaya, "Restorative yoga in adults with metabolic syndrome: a randomized, controlled pilot trial," *Metabolic Syndrome and Related Disorders*, vol. 6, no. 3, pp. 223–229, 2008.
- [21] D. L. Cohen, L. T. Bloedon, R. L. Rothman et al., "Iyengar yoga versus enhanced usual care on blood pressure in patients with prehypertension to stage I hypertension: a randomized controlled trial," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 546428, 8 pages, 2011.
- [22] R. McCaffrey, P. Ruknui, U. Hatthakit, and P. Kasetsoomboon, "The effects of yoga on hypertensive persons in Thailand," *Holistic Nursing Practice*, vol. 19, no. 4, pp. 173–180, 2005.
- [23] G. A. van Montfrans, J. M. Karemaker, W. Wieling, and A. J. Dunning, "Relaxation therapy and continuous ambulatory blood pressure in mild hypertension: a controlled study," *British Medical Journal*, vol. 300, no. 6736, pp. 1368–1372, 1990.
- [24] R. Murugesan, N. Govindarajulu, and T. K. Bera, "Effect of selected yogic practices on the management of hypertension," *Indian Journal of Physiology and Pharmacology*, vol. 44, no. 2, pp. 207–210, 2000.
- [25] C. Patel and W. R. S. North, "Randomised controlled trial of yoga and bio feedback in management of hypertension," *The Lancet*, vol. 2, no. 7925, pp. 93–95, 1975.
- [26] L. G. Saptharishi, M. B. Soudarssanane, D. Thiruselvakumar et al., "Community-based randomized controlled trial of non-pharmacological interventions in prevention and control of hypertension among young adults," *Indian Journal of Community Medicine*, vol. 34, no. 4, pp. 329–334, 2009.
- [27] H. Subramanian, M. B. Soudarssanane, R. Jayalakshmy et al., "Non-pharmacological interventions in hypertension: a community-based cross-over randomized controlled trial," *Indian Journal of Community Medicine*, vol. 36, pp. 191–196, 2011.
- [28] T. Deepa, G. Sethu, and N. Thirrunavukkarasu, "Effect of yoga and meditation on mild to moderate essential hypertensives," *Journal of Clinical and Diagnostic Research*, vol. 6, pp. 21–26, 2012.
- [29] S. V. Hegde, P. Adhikari, S. Kotian, V. J. Pinto, S. D'Souza, and V. D'Souza, "Effect of 3-month yoga on oxidative stress in type 2 diabetes with or without complications," *Diabetes Care*, vol. 34, no. 10, pp. 2208–2210, 2011.
- [30] S. Jain, M. Jain, and C. S. Sharma, "Effect of yoga and relaxation techniques on cardiovascular system," *Indian Journal of Physiology and Pharmacology*, vol. 54, no. 2, pp. 183–185, 2010.
- [31] D. Lakkireddy, D. Atkins, J. Pillarisetti et al., "Effect of yoga on arrhythmia burden, anxiety, depression, and quality of life in paroxysmal atrial fibrillation: the YOGA My Heart Study," *Journal of the American College of Cardiology*, vol. 61, pp. 1177–1182, 2013.
- [32] J. Mizuno and H. L. Monteiro, "An assessment of a sequence of yoga exercises to patients with arterial hypertension," *Journal of Bodywork and Movement Therapies*, vol. 17, pp. 35–41, 2013.
- [33] M. Niranjan, K. Bhagyalakshmi, B. Ganaraja, P. Adhikari, and R. Bhat, "Effects of yoga and supervised integrated exercise on heart rate variability and blood pressure in hypertensive patients," *Journal of Chinese Clinical Medicine*, vol. 4, no. 3, pp. 139–143, 2009.
- [34] C. Patel, "12-month follow up of yoga and bio feedback in the management of hypertension," *The Lancet*, vol. 1, no. 7898, pp. 62–64, 1975.
- [35] W. Selvamurthy, K. Sridharan, U. S. Ray et al., "A new physiological approach to control essential hypertension," *Indian Journal of Physiology and Pharmacology*, vol. 42, no. 2, pp. 205–213, 1998.
- [36] L. Gordon, E. Y. Morrison, D. A. McGrowder et al., "Changes in clinical and metabolic parameters after exercise therapy in patents with type 2 diabetes," *Archives of Medical Science*, vol. 4, no. 4, pp. 427–437, 2008.
- [37] *Review Manager (RevMan) [Computer Program]. Version 5.1*, The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark, 2011.
- [38] J. P. T. Higgins and S. G. Thompson, "Quantifying heterogeneity in a meta-analysis," *Statistics in Medicine*, vol. 21, no. 11, pp. 1539–1558, 2002.

- [39] J. Deeks and J. P. Higgins, "Analysing data and undertaking meta-analysis," in *Cochrane Handbook for Systematic Reviews of Interventions*, J. P. Higgins and S. Green, Eds., pp. 243–296, John Wiley & Sons, Chichester, UK, 2008.
- [40] P. R. Pullen, *The Benefits of Yoga Therapy for Heart Failure Patients*, Georgia State University, 2009.
- [41] K. M. Chen, J. T. Fan, H. H. Wang, S. J. Wu, C. H. Li, and H. S. Lin, "Silver yoga exercises improved physical fitness of transitional frail elders," *Nursing Research*, vol. 59, no. 5, pp. 364–370, 2010.
- [42] A. U. Latha and K. V. Kaliappan, "Yoga, pranayama, thermal biofeedback techniques in the management of stress and high blood pressure," *Journal of Indian Psychology*, vol. 9, pp. 36–46, 1991.
- [43] D. Haber, "Health promotion to reduce blood pressure level among older blacks," *Gerontologist*, vol. 26, no. 2, pp. 119–121, 1986.
- [44] D. Haber, "Yoga as a preventive health care program for white and black elders: an exploratory study," *International Journal of Aging and Human Development*, vol. 17, no. 3, pp. 169–176, 1983.
- [45] M. Mourya, A. S. Mahajan, N. P. Singh, and A. K. Jain, "Effect of slow- and fast-breathing exercises on autonomic functions in patients with essential hypertension," *Journal of Alternative and Complementary Medicine*, vol. 15, no. 7, pp. 711–717, 2009.
- [46] D. Khatri, K. C. Mathur, S. Gahlot, S. Jain, and R. P. Agrawal, "Effects of yoga and meditation on clinical and biochemical parameters of metabolic syndrome," *Diabetes Research and Clinical Practice*, vol. 78, no. 3, pp. e9–e10, 2007.
- [47] S. C. Chung, M. M. Brooks, M. Rai, J. L. Balk, and S. Rai, "Effect of sahaja yoga meditation on quality of life, anxiety, and blood pressure control," *Journal of Alternative and Complementary Medicine*, vol. 18, pp. 589–596, 2012.
- [48] A. Pal, N. Srivastava, S. Tiwari et al., "Effect of yogic practices on lipid profile and body fat composition in patients of coronary artery disease," *Complementary Therapies in Medicine*, vol. 19, no. 3, pp. 122–127, 2011.
- [49] L. Skoro-Kondza, S. See Tai, R. Gadelrab, D. Drincevic, and T. Greenhalgh, "Community based yoga classes for type 2 diabetes: an exploratory randomised controlled trial," *BMC Health Services Research*, vol. 9, article 33, pp. 1–8, 2009.
- [50] A. Broota, R. Varma, and A. Singh, "Role of relaxation in hypertension," *Journal of the Indian Academy of Applied Psychology*, vol. 21, pp. 29–36, 1995.
- [51] A. K. Chaudhary, H. N. Bhatnagar, L. K. Bhatnagar, and K. Chaudhary, "Comparative study of the effect of drugs and relaxation exercise (yoga shavasana) in hypertension," *The Journal of the Association of Physicians of India*, vol. 36, no. 12, pp. 721–723, 1988.
- [52] J. Yogendra, H. J. Yogendra, S. Ambardekar et al., "Beneficial effects of Yoga lifestyle on reversibility of ischaemic heart disease: caring heart project of international board of Yoga," *Journal of Association of Physicians of India*, vol. 52, pp. 283–289, 2004.
- [53] R. Sharma, N. Gupta, and R. L. Bijlani, "Effect of yoga based lifestyle intervention on subjective well-being," *Indian Journal of Physiology and Pharmacology*, vol. 52, no. 2, pp. 123–131, 2008.

## Research Article

# Serum Containing Tao-Hong-Si-Wu Decoction Induces Human Endothelial Cell VEGF Production via PI3K/Akt-eNOS Signaling

DengKe Yin,<sup>1,2,3</sup> ZhuQing Liu,<sup>1,3</sup> DaiYin Peng,<sup>1,3</sup> Ye Yang,<sup>1,3</sup> XiangDong Gao,<sup>2</sup>  
Fan Xu,<sup>3</sup> and Lan Han<sup>1,3</sup>

<sup>1</sup> School of Pharmacy, Anhui University of Traditional Chinese Medicine, Hefei 230031, China

<sup>2</sup> State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China

<sup>3</sup> Anhui Provincial Key Laboratory for Chinese Medicine Research and Development, Hefei 230038, China

Correspondence should be addressed to DaiYin Peng; pengdy111@yahoo.cn

Received 23 December 2012; Revised 11 April 2013; Accepted 21 April 2013

Academic Editor: Myeong Soo Lee

Copyright © 2013 DengKe Yin et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Tao-Hong-Si-Wu decoction (TSD) is a famous traditional Chinese medicine (TCM) and widely used for ischemic disease in China. TSD medicated serum was prepared after oral administration of TSD (1.6 g/kg) twice a day for 3 days in rats. TSD medicated serum induced human umbilical vein endothelial cells (HUVECs) proliferation, VEGF secretion, and nitric oxide (NO) production. These promoted effects of TSD were partly inhibited by treatment with PI3K inhibitor (LY294002) or eNOS inhibitor (L-NAME), respectively, and completely inhibited by treatment with LY294002 and L-NAME simultaneously. Western blot analysis findings further indicated that TSD medicated serum upregulated p-Akt and p-eNOS expressions, which were significantly inhibited by LY294002 or L-NAME and completely inhibited by both LY294002 and L-NAME; these results indicated that TSD medicated serum induced HUVECs VEGF expression via PI3K/Akt-eNOS signaling. TSD medicated serum contains hydroxysafflor yellow A, ferulic acid, and ligustilide detected by UPLC with standards, so these effect of TSD medicated serum may be associated with these three active compounds absorbed in serum.

## 1. Introduction

Ischemic diseases, especially ischemic heart disease, remain a major and well-researched challenge for humans. In the process of angiogenesis, modulation of endothelial cells plays a key role in such processes as proliferation, migration, and assembly. Numerous regulatory angiogenic factors have been identified, and their molecular modulations have been associated with several angiogenic disorders [1, 2]. Therapeutic angiogenesis is the clinical use of methods to enhance or promote the development of collateral blood vessels in ischemic tissue and is critical to ischemic diseases such as myocardial infarction and stroke. Angiogenesis is the formation of new blood vessels from preexisting capillaries in embryonic development, wound healing, and cardiovascular disease [3]. Although increasing evidence indicates that angiogenesis is a highly sophisticated and coordinated process, the activation of endothelial cells and release of angiogenic factors are

the most important steps. The survey and development of new agents promoting angiogenesis via growth factors have become a focus of therapeutic strategies for these ischemic diseases [4].

Tao-Hong-Si-Wu decoction (TSD) is a famous traditional Chinese medicine, first recorded in *Yizong jinjian* (Golden Mirror of Medicine, 1749) by Wu Qian, and widely used for blood stasis syndrome with a history of several centuries. The formula mainly consists of six plant materials (Table 1). Traditional Chinese medicine practitioners described the function of TSD as “promoting blood circulation to remove blood stasis.” In clinical practice, TSD could open the blood vessels and promote blood flow in circulation to relieve woman’s irregular menses disorder and is also used to treat cardiovascular diseases such as hypertension and angina. Furthermore, it can increase blood flow of the microcirculation thereby regulating diabetic neuropathies and glucocorticoid-induced avascular necrosis of the femoral head [5].

TABLE 1: The recipe of Tao-Hong-Siwu-Tang (TSD).

Components	Ratio
Shu Di Huang ( <i>Rehmannia glutinosa</i> Libosch)	4
Bai Shao ( <i>Paeonia lactiflora</i> Pallas)	3
Dang Gui ( <i>Angelica sinensis</i> (Oliv.) Diels)	3
Chuan Xiong ( <i>Ligusticum chuanxiong</i> Hort.)	2
Tao Ren ( <i>Prunus persica</i> (L.))	3
Hong Hua ( <i>Carthamus tinctorius</i> L)	2

Many researchers believed that serum pharmacology is more scientific and more befitting for Chinese traditional medicine than traditional pharmacology in which crude drugs are directly added into the culture system of cells or organs in vitro [6, 7]. Medicine or medicine compounds are orally administered to animal, blood is collected to separate to the serum after a definitive period of time, and the drug serum is ready for experimental analysis in vitro. Although TSD has been widely used in ischemic disease, the effects of TSD on the critical step of angiogenesis, endothelial cell activation, has not been clarified. The aim of this study is to investigate the effect of TSD on endothelial cell proliferation and release of VEGF with the method of serum pharmacology.

## 2. Materials and Methods

### 2.1. Materials

**2.1.1. Composition and Preparation of TSD.** TSD consists of six medicinal plants as shown in Table 1. Six herbs were purchased from Hefei He Yi Tang Traditional Chinese Medicines Limited Liability Company and identified by Professor Dequn Wang in the School of Pharmacy, Anhui University of Traditional Chinese Medicine.

TSD were prepared according to the following procedure: six medicinal materials were mixed in proportion and were macerated for 6 h with ten times (v/w) 75% ethanol. The medical solution was heated to boiling then refluxed for 1.5 h and filtrate was collected. The residue was refluxed again for 1.5 h, with eight times (v/w) 75% ethanol; then filtrate was collected again and mixed with previous collected filtrate and condensed and dried at 65°C. The yield of dried powder was 18.27% according to the original herbs. The doses were presented as such powder suspended in the distilled water.

**2.1.2. Reagent.** Other drugs and reagents used in this study are as follows: Akt, p-Akt, and p-eNOS antibody were purchased from Abzoom biolabs, Inc., import packing. Anti-PIP3 antibody was purchased from Echelon Biosciences. LY294002 was purchased from Gibco Company, and L-NAME was purchased from Beijing Grandsky Company. The VEGF and Ang-1 kits were purchased from the R&D Company, and the NO kit was purchased from Nanjing Jiancheng Bioengineering Institute. Reagents and chemicals standard for ultrahigh performance liquid chromatography (UPLC)

detection: CH<sub>3</sub>CN and H<sub>3</sub>PO<sub>4</sub>: TEDIA (Fairfield, OH, USA); gallic acid, paeoniflorin, ferulic acid, tetramethylpyrazine, ligustilide, and hydroxysafflor yellow A were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

**2.1.3. Animals.** Twenty-eight clean healthy Sprague-Dawley rats (all females), weighing 200–220 g, were provided by the Laboratory Animal Center of Anhui Medical University and housed in a cage at 23 ± 1°C with a 12-hour light-dark cycle. Food and water were freely available. The protocol was performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals (National Research Council of USA, 1996) and related ethical regulations of our university.

**2.1.4. Umbilical Cord.** Healthy sterile umbilical cords were provided by Obstetrics and Gynecology of Anhui Provincial Maternal and Child Health Hospital and Obstetrics and Gynecology of First Affiliated Hospital, Anhui Medical University.

### 2.2. Methods

**2.2.1. Cell Culture.** HUVECs were isolated from healthy umbilical cords obtained through local hospitals under approval of the appropriate institutional review board (IRB). The process was as follows: untraumatized umbilical cord segments, 15–20 cm in length, the umbilical vein was cannulated and perfused with Dulbecco's phosphate-buffered saline (PBS, with calcium and magnesium) to remove all traces of blood; the vein lumen was filled with 1 mg/mL collagenase I; after a 18 min incubation at 37°C, the contents of the vein were gently flushed out with an equal volume of M199 supplemented with 20% fetal bovine serum (FBS) (Hyclone) and centrifuged at 1000 r/min for 10 minutes, and cells were resuspended in M199 (containing 20% FBS, penicillin 100 ku/L streptomycin 100 ku/L, basic fibroblast growth factor (bFGF) (PeproTech Inc.) 10 ng/mL, L-glutamine 2 mmol/L), packaging into 1% gelatin-coated flasks for culturing and incubating at 37°C and 5% CO<sub>2</sub>. After 24 h, the medium was changed to remove residual blood cells and nonadherent cells, the medium was changed every 2-3 days, and cultured flasks were covered by cells.

**2.2.2. Preparation of TSD Medicated Serum.** The 28 Sprague-Dawley rats were randomly divided into TSD ( $n = 14$ ) and blank control ( $n = 14$ ) groups. Rats in the TSD group received intragastric administration of TSD (1.6 g/kg) twice a day for 3 days. The control group received intragastric injection of physiological saline twice a day for 3 days. One hour after the last administration, rats were intraperitoneally anesthetized by amobarbital and blood was sampled from the abdominal aorta and centrifuged. The serum was aliquoted into 10 mL ampoules and preserved at –80°C for future use.

**2.2.3. Plasma Samples Preparation for UPLC Analysis.** To tube containing 2 mL plasma, 6 mL methanol were added, and mixture was then vortexed for 2 min. The sample was



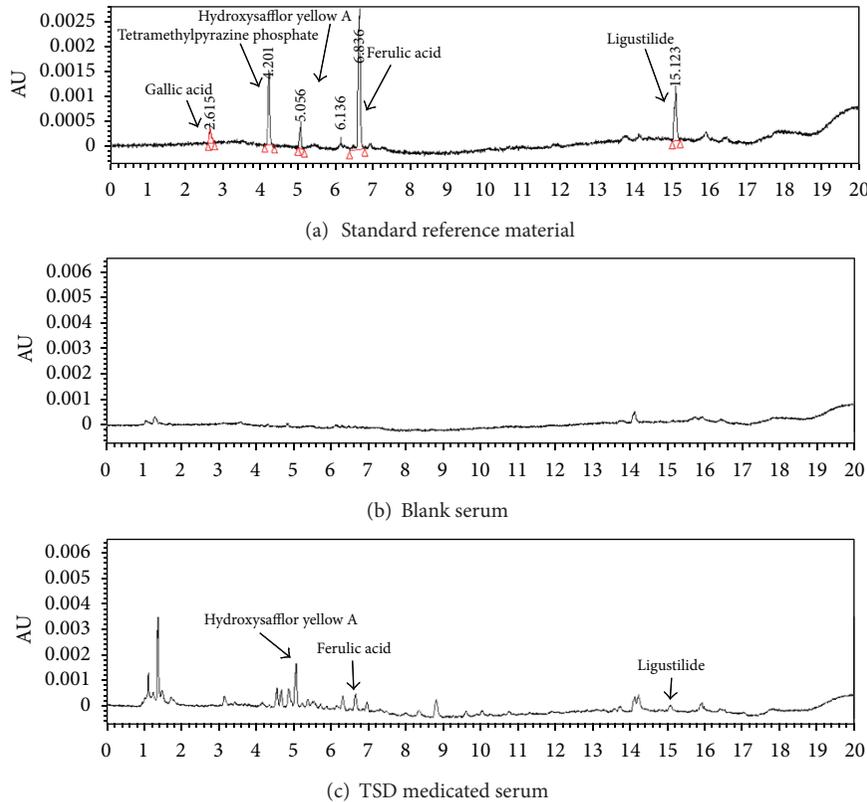


FIGURE 1: UPLC pattern of TSD medicated Serum.

proliferation was promoted by TSD medicated serum in different concentration (5%–10%) compared with the same concentration of blank serum (Figure 2); 10% TSD medicated serum significantly promoted HUVECs proliferation compared with 10% blank serum on cell proliferation ( $P < 0.01$ ). However, the promoted effect on HUVECs proliferation were significantly inhibited by LY294002 ( $20 \mu\text{M}$ ) or L-NAME ( $750 \mu\text{M}$ ) (Figure 3).

**3.3. TSD Medicated Serum Induced NO Production in HUVECs.** Supernatants were used to detect the TSD medicated serum on NO production. Compared with the same concentration of the blank serum, NO production was promoted by TSD medicated serum (concentration of 10% serum). While LY294002 ( $20 \mu\text{M}$ ) or L-NAME ( $750 \mu\text{M}$ ) was incubated with endothelial cells could significantly inhibit TSD medicated serum induced NO production, there was no significantly difference between group of blank serum with two inhibitors and TSD medicated serum with two inhibitors ( $P > 0.05$ ); these results indicated that the production of NO promoted by TSD medicated serum was completely inhibited by two inhibitors coincubated with HUVECs (Figure 4).

**3.4. TSD Medicated Serum Induced Expression of VEGF in HUVECs.** VEGF is known to be a key activator of angiogenesis. In order to clarify the effect of TSD medicated serum on expression of VEGF in HUVECs, the supernatant

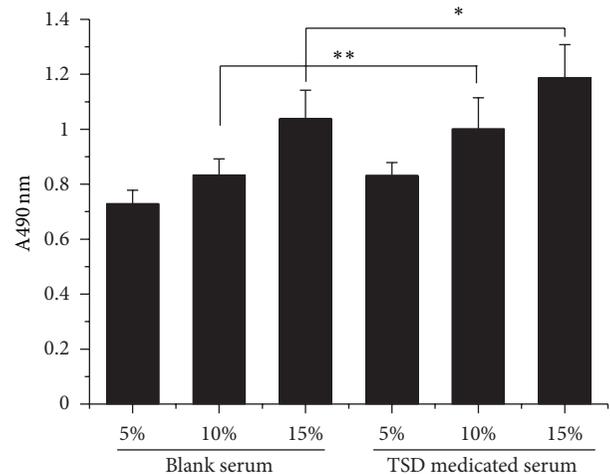


FIGURE 2: TSD medicated serum-induced HUVECs proliferation. Cells were treated with blank serum or TSD medicated serum for 24 h, and the proliferations of HUVECs were determined by MTT assay. The data were expressed as mean  $\pm$  SD,  $n = 8$ . \* $P < 0.05$  compared with 15% blank serum, \*\* $P < 0.01$  compared with 10% blank serum.

in conditioned medium was determined by ELISA. Treatment with TSD medicated serum (concentration of 10% serum) could promote the expression of VEGF in HUVECs

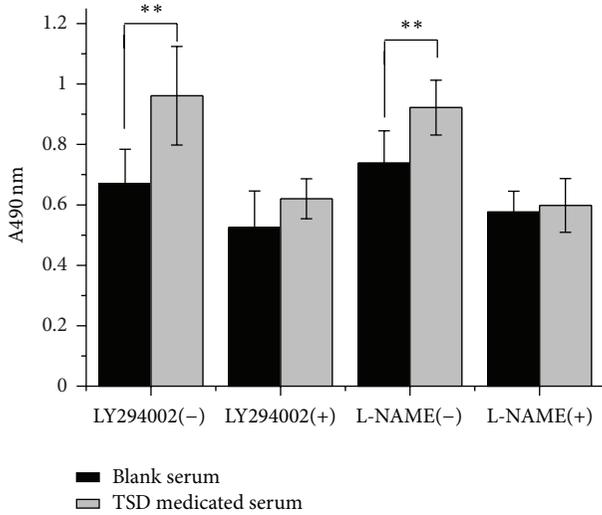


FIGURE 3: The effect of LY294002 and L-NAME on TSD medicated serum induced HUVECs proliferation. Cells were treated with 10% blank serum or 10% TSD medicated serum for 24 h in presence of LY294002 or L-NAME or not. The proliferations of HUVECs were determined by MTT assay. The data were expressed as mean ± SD, n = 8. \*\*P < 0.01.

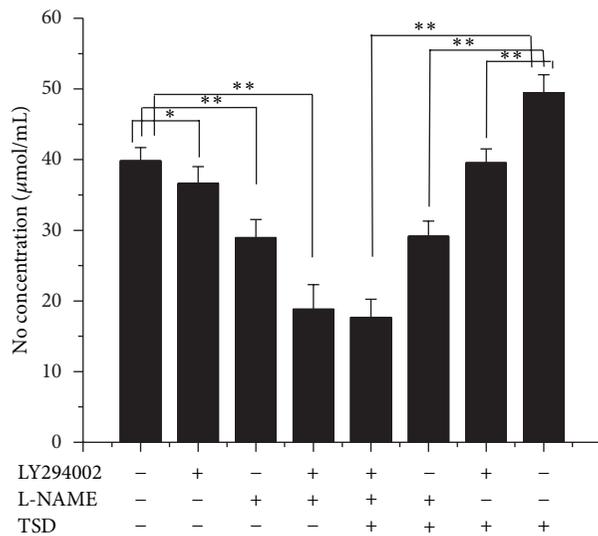


FIGURE 4: The effect of LY294002 and L-NAME on TSD medicated serum induced NO production in HUVECs. Cells were treated with 10% blank serum or 10% TSD medicated serum for 24 h in presence of LY294002 or L-NAME or not; the production of NO was determined by measuring the stable end product of NO, NO<sup>2-</sup>, and NO<sup>3-</sup>. The data were expressed as mean ± SD, n = 8. \*P < 0.05 and \*\*P < 0.01.

compared with HUVECs treatment with 10% blank serum; while these promoted effect of TSD medicated serum was significantly inhibited by LY294002 (20 µM) or L-NAME (750 µM), especially, when the two inhibitors coincubated with HUVECs, the upregulation of TSD medicated serum was completely inhibited (Figure 5).

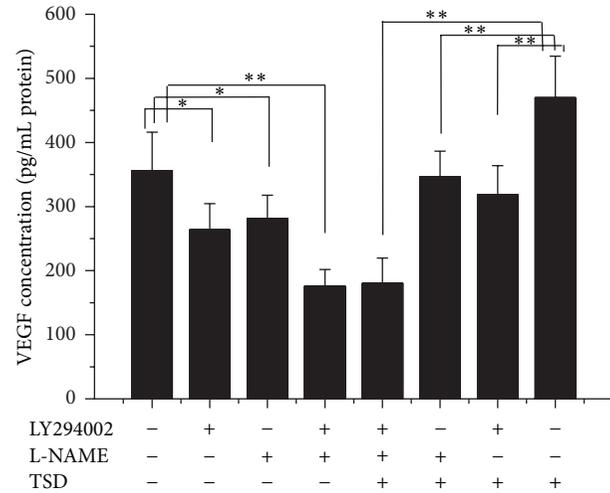


FIGURE 5: The effect of LY294002 and L-NAME on TSD medicated serum induced VEGF production in HUVECs. Cells were treated with 10% blank serum or 10% TSD medicated serum for 24 h in presence of LY294002 or L-NAME or not; the production of NO was determined by ELISA according to the manual. The data were expressed as mean ± SD, n = 8. \*P < 0.05 and \*\*P < 0.01.

3.5. TSD Medicated Serum Induced the Expression of p-Akt and p-eNOS in PI3K/Akt-eNOS Pathways. With the concentration of serum increased, the protein expression of p-Akt and p-eNOS was increased in HUVECs treated with blank serum or TSD medicated serum, while total Akt expression did not significantly changed (Figure 6). There was more expression of p-Akt and p-eNOS in TSD medicated serum than the same serum concentration of blank serum (Figure 6). LY294002 (20 µM) significantly decreased the expression of p-Akt in HUVECs compared with groups without inhibitor, as well as L-NAME (750 µM) decreased the expression of p-eNOS (Figure 7). The expression of p-Akt and p-eNOS in groups of 10% TSD medicated serum without inhibitors was much more than in groups of 10% blank serum without inhibitors. While in presence of LY294002 and L-NAME, the upregulated effect of medicated serum on expression of p-Akt and p-eNOS was completely inhibited (Figure 7).

3.6. TSD Medicated Serum Enhanced the Level of PIP3. In PI3K/Akt signaling, PIP2 activated PIP3 by PI3K, which caused Akt activation. In order to clarify the effect of TSD medicated serum on PI3K, the level of PIP3 was analyzed by flow cytometry with anti-PIP3 antibody. 10% TSD medicated serum enhanced the level of PIP3 in HUVECs compared with 10% blank serum-treated HUVECs; the enhanced level of PIP3 was completely inhibited by PI3K inhibitor (LY294002) (Figure 8).

#### 4. Discussion

Cardiovascular disease is the leading cause of death worldwide and is often associated with partial or full occlusion of the blood vessel network in the affected organs. Restoring

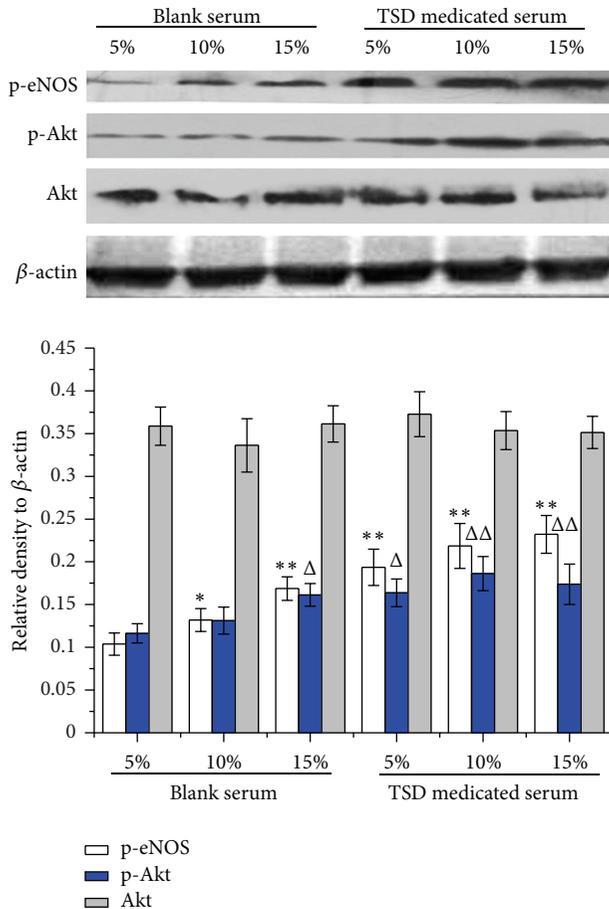


FIGURE 6: Effects of different concentrations of TSD medicated serum on expression of p-Akt, total Akt and p-eNOS. Cells were treated with blank serum (5–10%) or TSD medicated serum (5–10%) for 24 h. Cells were lysed, and the expression was determined by western blot with specific primary antibody to p-Akt, total Akt, and p-eNOS; bands were displayed with secondary antibody and ECL system. Data were expressed as mean  $\pm$  SD,  $n = 3$ . \* $P < 0.05$  and \*\* $P < 0.01$  compared with p-eNOS of 5% blank serum group,  $\Delta P < 0.05$  and  $\Delta\Delta P < 0.01$  compare with p-Akt of 5% blank serum group.

blood supply is critical for the successful treatment of cardiovascular disease [8]. Endothelial cell dysfunction is the main reason for diverse cardiovascular disease; insufficient angiogenesis is caused by the inadequate production of angiogenesis growth factors and/or excessive amounts of angiogenesis inhibitors [9]. Angiogenic factors bind to their receptors on endothelial cells, and promoting endothelial cells proliferation is the critical step in angiogenesis. Vascular endothelial growth factor (VEGF) is now known as a multifunctional peptide capable of inducing receptor-mediated endothelial cell proliferation and angiogenesis both in vivo and in vitro. It has a crucial role in embryonic vascular development and in adult pathophysiology [10, 11].

Direct activation of the angiogenic signal pathways and production of angiogenic factors including VEGF increase

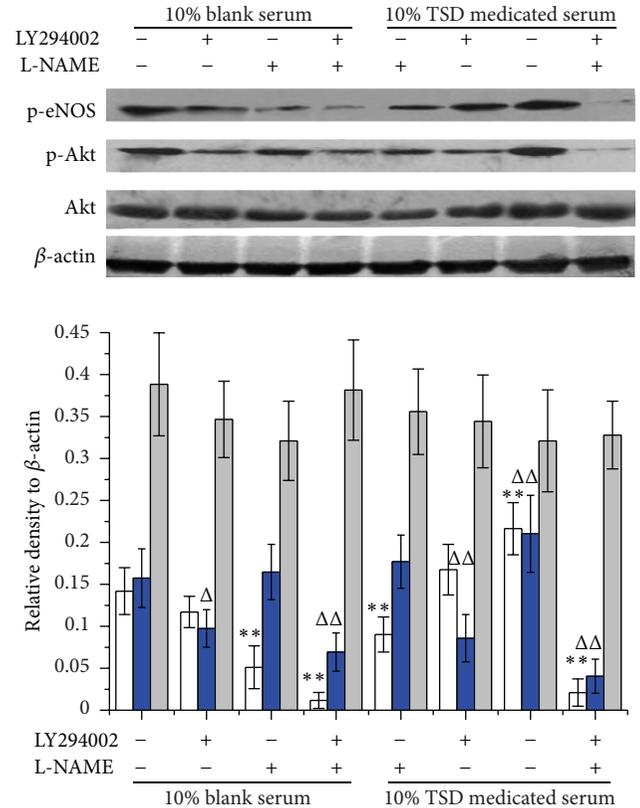


FIGURE 7: Effects of 10% TSD medicated serum on expression of p-Akt, total Akt, and p-eNOS in presence of LY294002 and L-NAME. Cells were treated with 10% blank serum or 10% TSD medicated serum for 24 h in presence of LY294002, L-NAME, or both. Cells were lysed, and the expression was determined by western blot with specific primary antibody to p-Akt, total Akt, and p-eNOS; bands were displayed with secondary antibody and ECL system. Data were expressed as mean  $\pm$  SD,  $n = 3$ . \* $P < 0.05$  and \*\* $P < 0.01$  compared with p-eNOS of 10% blank serum group,  $\Delta P < 0.05$  and  $\Delta\Delta P < 0.01$  compare with p-Akt of 10% blank serum group.

neovascularization [12]. VEGF promote angiogenesis by activating multiple signal pathways, such as the MEK/ERK pathway for endothelial cell proliferation, the PI3K/Akt/eNOS pathway for endothelial cell survival, and p125<sup>FAK</sup>/Src/p38 MAPK signaling system for endothelial cell migration [12, 13]. Our data showed that TSD medicated serum promotes the expression of VEGF in HUVECs, indicating that TSD may indirectly activate the angiogenic signal pathways. Indeed, we confirmed that TSD medicated serum induced the proliferation of HUVECs by PI3K/Akt-eNOS dependent pathways, which are essential for the activation of endothelial cells induced by direct angiogenic factors [12].

Endothelial dysfunction characterized by a decrease in the bioavailability of vasodilator like a nitric oxide (NO) has been observed in individuals with vascular complications [14]. In particular, endothelium-derived NO is a cerebrovascular protector and an important endogenous mediator of vascular homeostasis and blood flow [15]. The loss of endothelial NO impairs vascular function, in part

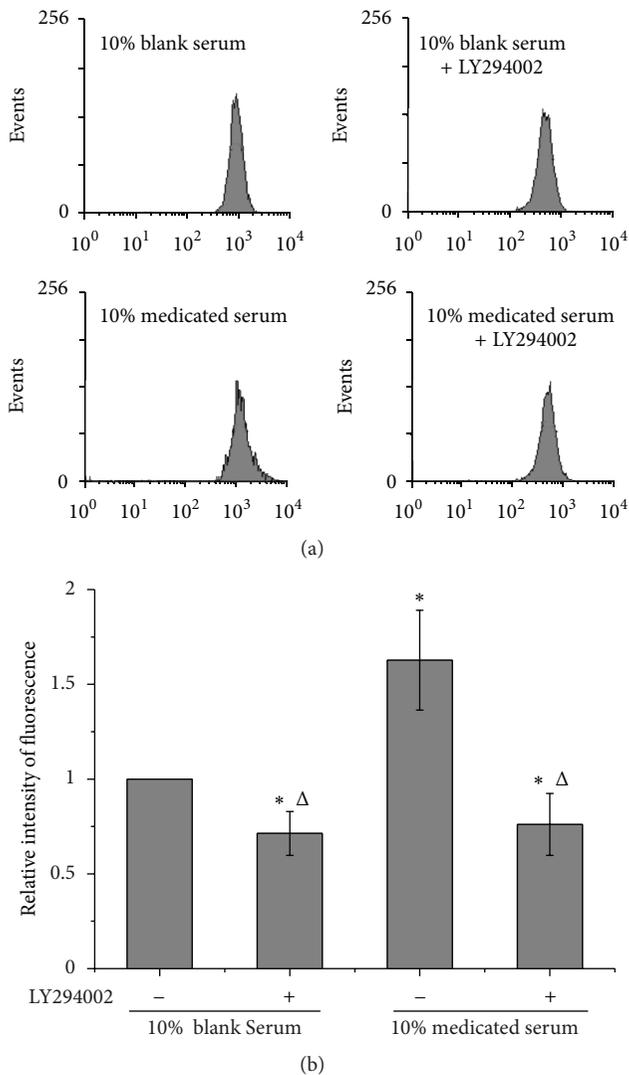


FIGURE 8: Effects of 10% TSD medicated serum on the levels of PIP3. Cells were treated with 10% blank serum or 10% TSD medicated serum for 24 h in presence of LY294002 or not. The levels of PIP3 were analysis by flow cytometry with anti-PIP3 antibody and FITC conjugated secondary antibody. (a) is the representative figure of flow cytometry. (b) is the bar graph of relative intensity of fluorescence in three independent experiments. Data were expressed as mean  $\pm$  SD. \* $P < 0.05$  compared with 10% blank serum and  $\Delta P < 0.05$  compared with 10% medicated serum.

by promoting vasoconstriction, platelet aggregation, smooth muscle cell proliferation, and leukocyte adhesion [16, 17]. In endothelial cells, NO is synthesized from the substrate L-arginine via eNOS, and the phosphorylation of a specific serine residue (Ser-1177) in eNOS is important for its enzymatic activity [18]. VEGF is known to induce the release of NO from endothelial cells, and vascular endothelium inducible NO synthase production is amplified during VEGF-induced angiogenesis [19]. The role of NO in VEGF-induced angiogenesis has been shown in NOS knocked out mice as well as after NOS inhibition, both result in reduction of angiogenesis

[20–22]. NO has also a regulatory effect on VEGF production [23–25].

In these experiment, TSD medicated serum increased the expression of VEGF in HUVECs as well as production of NO. It have been demonstrated that VEGF enhances the expression of eNOS in native and cultured endothelial cells, an effect that may be important in the process of VEGF-induced angiogenesis [21, 26]. Inhibitors of NOS suppress angiogenesis, and the proliferative effect of VEGF is decreased in the presence of NOS inhibitor [27]. Our results indicated that PI3K inhibitor suppressed the promoted effect of TSD medicated serum on expression of VEGF and production of NO, as well as eNOS inhibitor decreased the expression of VEGF and production of NO. Simultaneously treated with PI3K inhibitor and eNOS inhibitor, the effect of TSD medicated serum on VEGF expression and NO production of endothelial cell was completely inhibited, this also proved that TSD medicated serum activated the HUVECs by PI3K/Akt-eNOS pathways. In order to obtain the direct evidence on PI3K/Akt-eNOS pathways, the effect of TSD medicated serum on expression of p-eNOS, p-Akt and Akt was investigated by western blot. The expression of p-eNOS and p-Akt was significantly increased in groups of TSD medicated serum compared with blank serum groups, but had no significant influence on Akt expression. PI3K is the upstream molecular in PI3K/Akt-eNOS pathway, and PI3K activation could catalyze the PIP2 to PIP3, so the level of PIP3 was used to indicated the activity of PI3K; the results also indicated that TSD medicated serum have promoted effect on PI3K. These results also clarified that TSD medicated serum has an effect on PI3K/Akt-eNOS pathways.

TSD consists of six medicinal plants, in which Danggui and Chuanxiong could affect VEGF expression in rat myocardial infarction, promote endothelial cell proliferation, and stimulate quantity of vessels on chick embryo chorioallantoic membrane (CAM) [28]. Because the active compounds of these medicinal plants should be absorbed and translated into serum, the active compounds could play their therapeutic effect. Using UPLC with standard compounds, we found that TSD medicated serum containing Hydroxysafflor yellow A (HSYA), ferulic acid, and ligustilide. Previously, studies indicated that HSYA could protect HUVECs from hypoxia-induced injuries [29], enhance the survival of endothelial cell, and induce the express of VEGF [30]. HSYA also has a therapeutic effect on focal cerebral ischaemia in vivo [31, 32]; HSYA protects the myocardium against ischaemia-reperfusion injury through enhanced nitric oxide production by eNOS activation [33]. Ferulic acid promotes endothelial cells proliferation through upregulation VEGF [34] and augments angiogenesis [35]. Ligustilide also has protective effects against ischaemia-reperfusion [36] and alleviates brain damage of chronic cerebral hypoperfusion [37]. So we could speculate that the effect of TSD on endothelial cell is mainly associated with these three active compounds absorbed in serum.

## Conflict of Interests

The authors declare no conflict of interests.

## Authors' Contribution

ZhuQing Liu collaborated equally to the present work.

## Acknowledgments

This paper was supported by the National Natural Science Foundation of China (81073091, 81102682), the Natural Science Program of Department of Education of Anhui Province (KJ2011A189), and the Open Project Program of State Key Laboratory of Natural Medicines (SKLNMKF201218).

## References

- [1] S. Kofler, T. Nickel, and M. Weis, "Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation," *Clinical Science*, vol. 108, no. 3, pp. 205–213, 2005.
- [2] M. Papetti and I. M. Herman, "Mechanisms of normal and tumor-derived angiogenesis," *The American Journal of Physiology*, vol. 282, no. 5, pp. C947–C970, 2002.
- [3] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine*, vol. 1, no. 1, pp. 27–31, 1995.
- [4] T. P. Fan, J. C. Yeh, K. W. Leung, P. Y. K. Yue, and R. N. S. Wong, "Angiogenesis: from plants to blood vessels," *Trends in Pharmacological Sciences*, vol. 27, no. 6, pp. 297–309, 2006.
- [5] C. J. Wu, J. T. Chen, T. L. Yen et al., "Neuroprotection by the Traditional Chinese Medicine, Tao-Hong-Si-Wu-Tang, against middle cerebral artery occlusion-induced cerebral ischemia in rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 803015, 9 pages, 2011.
- [6] W. Bochu, Z. Liancai, and C. Qi, "Primary study on the application of serum pharmacology in Chinese traditional medicine," *Colloids and Surfaces B*, vol. 43, no. 3-4, pp. 194–197, 2005.
- [7] Z. G. Peng, H. S. Chen, Z. M. Guo, B. Dong, G. Y. Tian, and G. Q. Wang, "Anti-HIV activities of *Achyranthes bidentata* polysaccharide sulfate in vitro and in vivo," *Yao Xue Xue Bao*, vol. 43, no. 7, pp. 702–706, 2008.
- [8] L. Deveza, J. Chol, and F. Yang, "Therapeutic angiogenesis for treating cardiovascular diseases," *Theranostics*, vol. 2, no. 8, pp. 801–814, 2012.
- [9] J. Davignon and P. Ganz, "Role of endothelial dysfunction in atherosclerosis," *Circulation*, vol. 109, no. 23, pp. III27–III32, 2004.
- [10] P. Carmeliet and D. Collen, "Molecular analysis of blood vessel formation and disease," *The American Journal of Physiology*, vol. 273, no. 5, pp. H2091–H2104, 1997.
- [11] G. Neufeld, T. Cohen, S. Gengrinovitch, and Z. Poltorak, "Vascular endothelial growth factor (VEGF) and its receptors," *FASEB Journal*, vol. 13, no. 1, pp. 9–22, 1999.
- [12] R. Muñoz-Chápuli, A. R. Quesada, and M. A. Medina, "Angiogenesis and signal transduction in endothelial cells," *Cellular and Molecular Life Sciences*, vol. 61, no. 17, pp. 2224–2243, 2004.
- [13] S. J. Lee, S. Namkoong, Y. M. Kim et al., "Fractalkine stimulates angiogenesis by activating the Raf-1/MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways," *The American Journal of Physiology*, vol. 291, no. 6, pp. H2836–H2846, 2006.
- [14] Y. Kang, M. Hu, Y. Zhu, X. Gao, and M. W. Wang, "Antioxidative effect of the herbal remedy Qin Huo Yi Hao and its active component tetramethylpyrazine on high glucose-treated endothelial cells," *Life Sciences*, vol. 84, no. 13-14, pp. 428–436, 2009.
- [15] R. D. Rudic and W. C. Sessa, "Nitric oxide in endothelial dysfunction and vascular remodeling: clinical correlates and experimental links," *The American Journal of Human Genetics*, vol. 64, no. 3, pp. 673–677, 1999.
- [16] R. M. J. Palmer, A. G. Ferrige, and S. Moncada, "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor," *Nature*, vol. 327, no. 6122, pp. 524–526, 1987.
- [17] L. J. Ignarro, "Biosynthesis and metabolism of endothelium-derived nitric oxide," *Annual Review of Pharmacology and Toxicology*, vol. 30, pp. 535–560, 1990.
- [18] Z. Yang and X. F. Ming, "Recent advances in understanding endothelial dysfunction in atherosclerosis," *Clinical Medicine and Research*, vol. 4, no. 1, pp. 53–65, 2006.
- [19] R. M. Tuder, B. E. Flook, and N. F. Voelkel, "Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia. Modulation of gene expression by nitric oxide," *Journal of Clinical Investigation*, vol. 95, no. 4, pp. 1798–1807, 1995.
- [20] T. Murohara, J. R. Horowitz, M. Silver et al., "Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin," *Circulation*, vol. 97, no. 1, pp. 99–107, 1998.
- [21] M. Ziehe, L. Morbidelli, R. Choudhuri et al., "Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis," *Journal of Clinical Investigation*, vol. 99, no. 11, pp. 2625–2634, 1997.
- [22] Y. Tsurumi, T. Murohara, K. Krasinski et al., "Reciprocal relation between VEGF and NO in the regulation of endothelial integrity," *Nature Medicine*, vol. 3, no. 8, pp. 879–886, 1997.
- [23] J. Dulak, A. Józkowicz, A. Dembinska-Kiec et al., "Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 3, pp. 659–666, 2000.
- [24] J. Dai, L. Peng, K. Fan et al., "Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells," *Oncogene*, vol. 28, no. 38, pp. 3412–3422, 2009.
- [25] I. Shiojima and K. Walsh, "Role of Akt signaling in vascular homeostasis and angiogenesis," *Circulation Research*, vol. 90, no. 12, pp. 1243–1250, 2002.
- [26] M. Ziche, "Role of nitric oxide in the angiogenesis of avascular tissue," *Osteoarthritis and Cartilage*, vol. 7, no. 4, pp. 403–405, 1999.
- [27] M. Ziche, L. Morbidelli, E. Masini et al., "Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P," *Journal of Clinical Investigation*, vol. 94, no. 5, pp. 2036–2044, 1994.
- [28] H. Meng, J. Guo, J. Y. Sun et al., "Angiogenic effects of the extracts from Chinese herbs: Angelica and ChuanXiong," *The American Journal of Chinese Medicine*, vol. 36, no. 3, pp. 541–554, 2008.
- [29] D. B. Ji, L. Y. Zhang, C. L. Li, J. Ye, and H. B. Zhu, "Effect of Hydroxysafflor yellow A on human umbilical vein endothelial cells under hypoxia," *Vascular Pharmacology*, vol. 50, no. 3-4, pp. 137–145, 2009.
- [30] D. B. Ji, M. C. Zhu, B. Zhu et al., "Hydroxysafflor yellow A enhances survival of vascular endothelial cells under hypoxia via upregulation of the HIF-1 $\alpha$ -VEGF pathway and regulation

- of Bcl-2/Bax,” *Journal of Cardiovascular Pharmacology*, vol. 52, no. 2, pp. 191–202, 2008.
- [31] H. Zhu, Z. Wang, C. Ma et al., “Neuroprotective effects of hydroxysafflor yellow a: in vivo and in vitro studies,” *Planta Medica*, vol. 69, no. 5, pp. 429–433, 2003.
- [32] H. B. Zhu, L. Zhang, Z. H. Wang et al., “Therapeutic effects of hydroxysafflor yellow A on focal cerebral ischemic injury in rats and its primary mechanisms,” *Journal of Asian Natural Products Research*, vol. 7, no. 4, pp. 607–613, 2005.
- [33] Y. N. Liu, Z. M. Zhou, and P. Chen, “Evidence that hydroxysafflor yellow A protects the heart against ischaemia-reperfusion injury by inhibiting mitochondrial permeability transition pore opening,” *Clinical and Experimental Pharmacology and Physiology*, vol. 35, no. 2, pp. 211–216, 2008.
- [34] J. Wang, Z. Yuan, H. Zhao et al., “Ferulic acid promotes endothelial cells proliferation through upregulating cyclin D1 and VEGF,” *Journal Ethnopharmacology*, vol. 137, no. 2, pp. 992–997, 2011.
- [35] C. M. Lin, J. H. Chiu, I. H. Wu, B. W. Wang, C. M. Pan, and Y. H. Chen, “Ferulic acid augments angiogenesis via VEGF, PDGF and HIF-1 $\alpha$ ,” *Journal of Nutritional Biochemistry*, vol. 21, no. 7, pp. 627–633, 2010.
- [36] X. M. Wu, Z. M. Qian, L. Zhu et al., “Neuroprotective effect of ligustilide against ischaemia-reperfusion injury via up-regulation of erythropoietin and down-regulation of RTP801,” *The British Journal of Pharmacology*, vol. 164, no. 2, pp. 332–343, 2011.
- [37] Z. Feng, Y. Lu, X. Wu et al., “Ligustilide alleviates brain damage and improves cognitive function in rats of chronic cerebral hypoperfusion,” *Journal of Ethnopharmacology*, vol. 144, no. 2, pp. 313–321, 2012.

## Research Article

# The Cardioprotective Effects of Citric Acid and L-Malic Acid on Myocardial Ischemia/Reperfusion Injury

Xilan Tang,<sup>1,2,3</sup> Jianxun Liu,<sup>1</sup> Wei Dong,<sup>2,3</sup> Peng Li,<sup>1</sup> Lei Li,<sup>1</sup> Chengren Lin,<sup>1</sup>  
Yongqiu Zheng,<sup>1</sup> Jincai Hou,<sup>1</sup> and Dan Li<sup>1</sup>

<sup>1</sup> Experimental Research Center, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China

<sup>2</sup> Key Laboratory of Modern Preparation of TCM, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China

<sup>3</sup> Beijing University of Chinese Medicine, Beijing 100029, China

Correspondence should be addressed to Jianxun Liu; liujx0324@sina.com

Received 26 December 2012; Revised 3 April 2013; Accepted 4 April 2013

Academic Editor: Hao Xu

Copyright © 2013 Xilan Tang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Organic acids in Chinese herbs, the long-neglected components, have been reported to possess antioxidant, anti-inflammatory, and antiplatelet aggregation activities; thus they may have potentially protective effect on ischemic heart disease. Therefore, this study aims to investigate the protective effects of two organic acids, that is, citric acid and L-malic acid, which are the main components of *Fructus Choerospondiatis*, on myocardial ischemia/reperfusion injury and the underlying mechanisms. In *in vivo* rat model of myocardial ischemia/reperfusion injury, we found that treatments with citric acid and L-malic acid significantly reduced myocardial infarct size, serum levels of TNF- $\alpha$ , and platelet aggregation. *In vitro* experiments revealed that both citric acid and L-malic acid significantly reduced LDH release, decreased apoptotic rate, downregulated the expression of cleaved caspase-3, and upregulated the expression of phosphorylated Akt in primary neonatal rat cardiomyocytes subjected to hypoxia/reoxygenation injury. These results suggest that both citric acid and L-malic acid have protective effects on myocardial ischemia/reperfusion injury; the underlying mechanism may be related to their anti-inflammatory, antiplatelet aggregation and direct cardiomyocyte protective effects. These results also demonstrate that organic acids, besides flavonoids, may also be the major active ingredient of *Fructus Choerospondiatis* responsible for its cardioprotective effects and should be attached great importance in the therapy of ischemic heart disease.

## 1. Introduction

Ischemic heart disease is a leading cause of mortality of the clinical cardiovascular diseases and remains a major public health threat worldwide. Myocardial damage in ischemic heart disease is likely due to ischemia/reperfusion injury. Myocardial ischemia/reperfusion can lead to cardiomyocyte loss by several pathological mechanisms, which contain free radical formation, inflammatory response and endothelial dysfunction, platelet aggregation and microembolization, necrosis and apoptosis, and so forth [1]. Therefore, a pharmacologic approach to ischemia/reperfusion injury remains a longstanding challenge in medicine.

*Fructus Choerospondiatis*, a widely known Mongolian herb derived from the dried mature fruit of *Choerospondias axillaris* (Roxb.) Burt et Hill, with efficacy of “activating vital energy and blood circulation” and “nourishing heart

for tranquilization” [2], according to traditional Chinese medicine theory, has been used extensively as a remedy for ischemic heart disease and achieved good clinical efficacy. It consists of several ingredients, including organic acids, phenolic acids, and flavonoids. Previous studies have been focused on flavonoids, which were always considered to be the main active constituents responsible for the pharmacological actions of *Fructus Choerospondiatis* [3]. However, recent pharmaceutical chemistry studies showed that the content of total flavonoids (mainly quercetin) in *Fructus Choerospondiatis* was very low and only accounted for 0.0003% of its water-soluble extracts [4], whereas the content of total organic acids was in significant amounts, up to 8.13%. And citric acid and L-malic acid are two main organic acids of *Fructus Choerospondiatis*, the content of which accounted for 26.36% and 22.95% of total organic acids, respectively [5].

Organic acids, which are also widely distributed in fresh fruits, vegetables, and spices besides Chinese herbs, were always considered to be weak in activity and were usually discarded in the extraction process. Therefore, they have been long neglected and their pharmacological actions have not been sufficiently studied. Recent research indicated that some organic acids have various pharmacological effects, including anti-inflammatory response [6, 7], antiplatelet aggregation [8], antioxidant [9, 10], and reducing cell apoptosis and so on. Therefore, we hypothesized that organic acids might have protective effect on myocardial ischemia/reperfusion injury.

In the present study, we investigated the protective effects of citric acid and L-malic acid on myocardial ischemia/reperfusion and its possible mechanisms. To the best of our knowledge, the finding that citric acid and L-malic acid have protective effects on myocardial ischemia/reperfusion injury by anti-inflammatory, antiplatelet aggregation, and direct cardiomyocyte protective effects is therefore particularly significant for providing important insights into the understanding of cardioprotective effects of organic acids in traditional Chinese medicine.

## 2. Materials and Methods

**2.1. Animals.** Male adult Wistar rats (210–240 g body weight) were provided by the Experimental Animal Research Institute, Chinese Academy of Medical Sciences (clean degree, certificate no. SCXK (Jing) 2009-0007). Neonatal Sprague-Dawley rats (SPF degree, 1 to 2 days old) were purchased from Beijing Vital River Laboratory Animal Technology Co.Ltd., China (certificate no. SCXK (Jing) 2012-0001). Rats were housed under standard conditions and supplied with drinking water and food *ad libitum*. All animal experiments in this study were performed in accordance with China Academy of Chinese Medical Sciences Guide for Laboratory Animals that conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publications no. 85-23, revised 1996).

**2.2. Reagents and Chemicals.** Lactate dehydrogenase (LDH) assay kit was purchased from Beijing Zhongsheng Biological Technology Co., Ltd. (batch no. 110391). Rat tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) Quantikine ELISA kit was obtained from R&D (Catalog no. RTA00, USA). FITC-annexin V/propidium iodide apoptosis detection kit was from BD Biosciences (Catalog no. 556547, USA). Nitroblue tetrazolium (N-BT) (Ultra Pure Grade, 0329) and anti-cleaved caspase-3 antibody (Catalog no. C8487) were products of Sigma Chemical Co. (USA). The antibodies for anti-p-Akt (Ser473, no. 4060) and anti-Akt (no. 9272) were from Cell Signaling (USA). Citric acid (batch no. 111679-200401) and L-malic acid (batch no. 190014-201001) were purchased from National Institutes for Food and Drug Control (Beijing, China). All chemicals used were of analytical grade.

**2.3. Drug Pretreatment and Myocardial Ischemia/Reperfusion Protocols.** The studies of citric acid and L-malic acid on myocardial ischemia/reperfusion were performed independently although the experimental designs of them were

identical. All animals were randomly assigned to six groups ( $n = 10$  for each group). Vehicle or drugs were fed once daily (10 mL/kg) for 3 consecutive days prior to the experiment and treated as follows.

Group 1: sham group. Rats were orally administered 0.9% saline.

Group 2: ischemia/reperfusion (I/R) model control group. Rats were also orally administered 0.9% saline.

Group 3: diltiazem (Tanabe Seiyaku Co., Ltd., Tianjin)-pretreated group as positive control. Rats were orally administered diltiazem at a dose of 16 mg/kg.

Group 4: clopidogrel (Sanofi Winthrop Industrie)-pretreated group as another positive control. Rats were orally administered clopidogrel at a dose of 13.5 mg/kg.

Group 5: citric acid- or L-malic acid-pretreated group. Rats were orally administered citric acid or L-malic acid at a dose of 500 mg/kg.

Group 6: citric acid- or L-malic acid-pretreated group. Rats were orally administered citric acid or L-malic acid at a dose of 250 mg/kg.

Myocardial ischemia/reperfusion injury rat model was constructed by LAD ligation for 40 min followed by 2 h reperfusion at 1 h after the last drug treatment as previously described [11]. Rats were anesthetized with 3.5% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd., China) (350 mg/kg, i.p.). The neck was opened with a ventral midline incision. The trachea was exposed and cannulated to establish artificial respiration provided by a rodent ventilator (ALC-V8S, China) with oxygen at a breath ratio of 1:2 and at a frequency of 70 breaths/min with tidal volume of 9.0 mL. Myocardial ischemia was produced by exteriorizing the heart through a left thoracic incision and placing a 5-0 silk suture and making a plastic tubing at the distal 1/3 of the left anterior descending coronary artery. After 40 min of ischemia, the plastic tubing was cut and the myocardium was reperfused for 2 h.

**2.4. Measurement of Myocardial Infarct Size.** Myocardial infarct size was evaluated by N-BT staining as previously described [12]. Briefly, at the end of 2 h reperfusion, rats were anesthetized with 3.5% chloral hydrate and then sacrificed. The hearts were quickly excised and sliced into 5 sections from the position under the ligation line. The slices were weighed and then immediately incubated in N-BT staining solution dissolved in saline at 25°C for 15 min. The infarcted size, noninfarcted size, and total heart size were measured by multimedia color pathological image analytical system (MPIAS-500, Beijing). The infarction percentage of the ventricle, infarction percentage of the heart, infarction area, and infarction weight were calculated.

**2.5. Determination of Inflammatory Cytokine Activity and Platelet Maximum Aggregation Rate.** The serum levels of TNF- $\alpha$  were measured using ELISA kits according to the manufacturer's instructions. The absorbance at 450 nm was

measured on a microplate reader (BioTek SYNERGY 4, USA).

Blood was collected from the abdominal aorta and anti-coagulated with citrate (3.8%, 1:9, v/v). Platelet-rich plasma (PRP) was obtained by centrifugation at 1000 rpm at 25°C for 10 min and the remaining blood was further centrifuged at 3000 rpm at 25°C for 10 min to prepare platelet-poor plasma (PPP). The platelet concentration was adjusted to  $4 \times 10^8$  platelets/mL. The platelet agonist adenosine diphosphate disodium (Shanghai Institute of Biochemistry, Chinese Academy of Sciences) (1 mmol/L, 10  $\mu$ L) was used to stimulate platelet aggregation. The level of platelet aggregation was measured using an aggregometer (BS634, Beijing) according to the method reported by Born and Cross [13].

**2.6. Cell Culture, Hypoxia/Reoxygenation (H/R), and Drug Treatment.** Primary cultures of neonatal rat cardiomyocytes were prepared as previously described with some modifications [14, 15]. In brief, the hearts were removed and washed with cold PBS. The atria and aorta were discarded. The ventricles were minced with scissors into 1 mm<sup>3</sup> fragments. The tissue fragments were digested by gentle shaking at 37°C in PBS containing 0.625 g/L trypsin (Gibco) and 0.5 g/L collagenase II (Gibco). The digestion was conducted for 5–8 times, 10 min each. The dispersed cells were incubated on a 100 mm culture dish for 1 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The nonadherent cells were harvested and then seeded into gelatin-coated 6-well plates and incubated in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% newborn calf serum (TBD21HY, Tianjin), penicillin (100 U/mL), streptomycin (100 U/mL), and 5-bromo-2'-deoxyuridine (0.1 mmol/L, sigma), which was used to inhibit cardiac fibroblasts growth.

Hypoxia/reoxygenation-induced cardiomyocytes injury was performed as described previously [15]. Before hypoxia, cardiomyocytes were washed three times with glucose-free Tyrode's solution (in mmol/L: NaCl 136.9, KCl 2.68, NaH<sub>2</sub>PO<sub>4</sub> 0.42, NaHCO<sub>3</sub> 11.9, MgCl<sub>2</sub> 1.05, and CaCl<sub>2</sub> 1.8). Cells were incubated with glucose-free Tyrode's solution (2 mL/well) saturated with 95% N<sub>2</sub> and 5% CO<sub>2</sub> for 15 min. Cells were placed into a hypoxia chamber which was then ventilated with 95% N<sub>2</sub> and 5% CO<sub>2</sub> for 15 min and maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 3 h. Reoxygenation was accomplished by replacing the glucose-free Tyrode's solution with normal cell medium under normoxic conditions. Reoxygenation time varied depending on the experimental objectives: 2 h reoxygenation was performed for measurement of LDH release, whereas 6 h reoxygenation was performed for flow cytometry assay and measurements of cleaved caspase-3, Akt, and p-Akt expressions.

In the normal control group, cells were cultured with Tyrode's solution that contained 5.5 mmol/L glucose for 3 h and reoxygenated with DMEM for 2 h or 6 h. In the treatment groups, diltiazem (positive control, final concentration: 45  $\mu$ g/mL), citric acid, and L-malic acid (final concentrations: 200, 100, 50, 25  $\mu$ g/mL) were dissolved with dimethylsulfoxide (DMSO) and added, respectively, into the medium with the ratio of 1:1000 at the start of hypoxia and reoxygenation.

For the normal control group and model control group, equivalent volumes of DMSO were added.

**2.7. LDH Release.** The hypoxia and reoxygenation supernatants were collected. After 2 h reoxygenation, cells were lysed by freeze thawing in distilled water. LDH activities were measured using the enzymatic reaction kinetics monitoring method according to the manufacturer's instructions.

The total LDH activity was obtained from adding LDH activities in the hypoxia and reoxygenation supernatants and the cell lysate together. The LDH release rate was calculated by dividing the sum of LDH activities in the hypoxia and reoxygenation supernatants into the total LDH activity.

**2.8. Flow Cytometry Analysis.** Apoptosis was assessed by FITC-annexin V/propidium iodide apoptosis detection kit according to the manufacturer's protocol. Briefly, at the end of 6 h reoxygenation, cells were harvested with trypsin (0.25%) and centrifugation (1000 rpm for 5 min). Cells were washed twice with cold PBS and then resuspended in 200  $\mu$ L binding buffer at a concentration of  $1 \times 10^5$  cells/mL. Cells were incubated with 5  $\mu$ L FITC-annexin V and 5  $\mu$ L propidium iodide (PI) for 15 min in the dark at room temperature (25°C). Samples were analyzed by flow cytometry (Epics Elite, Beckman Coulter) immediately. Approximately 10 000 cells were counted for each sample and data were analyzed by using Expo32 software.

**2.9. Western Blot Analysis.** The expression levels of cleaved caspase-3, Akt, and p-Akt were measured by western blotting. Cells were washed with prewarmed PBS and then lysed at 4°C with ice-cold RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and phosphatase inhibitors mixture (#P1260, Applygen Technologies Inc.)) for 30 min. Cell lysates were then centrifuged at 12000 g at 4°C for 5 min and protein concentrations in the supernatants were determined by BCA protein assay kit (Beyotime Biotechnology).

Samples with equivalent amounts of total protein (20  $\mu$ g) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked in 5% BSA for 1 h and then incubated overnight at 4°C with primary antibody (rabbit anti-cleaved caspase 3, rabbit anti-Akt, and rabbit anti-p-Akt at 1:500, 1:1000, and 1:2000 dilution, and mouse anti- $\alpha$ -actin (Beijing Biosynthesis Biotechnology, China) at 1:2000 dilution). The membranes were washed six times in 1  $\times$  Tris-buffer saline-Tween 20 (TBST) buffer and then incubated with horseradish-peroxidase-(HRP-) conjugated goat anti-rabbit or mouse secondary antibodies (1:40000 dilution) for 1 h at room temperature. After excess antibodies were removed by washing, bands were detected with an enhanced chemiluminescence (ECL) system (Thermo, USA) and visualized with the Chemi Doc XRS+ gel documentation system (Bio-Rad, USA) and analyzed by using Image lab 3.0 software (Bio-Rad, USA). The expression levels of  $\alpha$ -actin served as an internal control for protein loading.

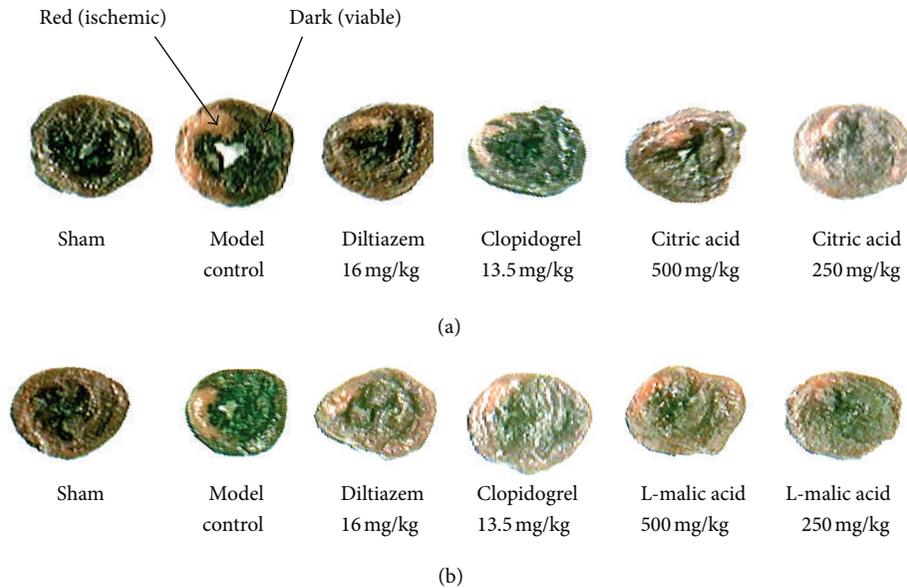


FIGURE 1: A representative N-BT staining of infarct size. The normal myocardium was stained dark, and the ischemic area was stained red. (a) Citric acid; (b) L-malic acid.

TABLE 1: The effect of citric acid on myocardial ischemia/reperfusion injury in rats ( $\bar{x} \pm s$ ,  $n = 10$ ).

Groups	Dosage (mg/kg)	Infarction of the ventricle (%)	Infarction of the heart (%)	Infarction area (mm <sup>2</sup> )	Infarction weight (g)
Sham	—	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.000 ± 0.000
Model control	—	17.30 ± 3.58 <sup>##</sup>	11.98 ± 2.20 <sup>##</sup>	49.75 ± 9.14 <sup>##</sup>	0.086 ± 0.017 <sup>##</sup>
Diltiazem	16	8.23 ± 2.50 <sup>**</sup>	5.33 ± 1.11 <sup>**</sup>	21.90 ± 4.78 <sup>**</sup>	0.038 ± 0.007 <sup>**</sup>
Clopidogrel	13.5	11.35 ± 2.71 <sup>**</sup>	8.52 ± 1.77 <sup>**</sup>	35.17 ± 9.74 <sup>**</sup>	0.064 ± 0.015 <sup>*</sup>
Citric acid	500	12.16 ± 4.27 <sup>**</sup>	8.80 ± 3.41 <sup>**</sup>	36.18 ± 15.23 <sup>**</sup>	0.064 ± 0.026 <sup>*</sup>
	250	11.80 ± 3.67 <sup>**</sup>	8.83 ± 3.09 <sup>**</sup>	33.97 ± 10.99 <sup>**</sup>	0.063 ± 0.022 <sup>**</sup>

<sup>##</sup> $P < 0.01$  versus sham, <sup>\*\*</sup> $P < 0.01$ , <sup>\*</sup> $P < 0.05$  versus model control.

**2.10. Statistical Analysis.** All data were presented as the mean ± SD. The data analyses were performed using one-way ANOVA analysis followed by Student-Newman-Keuls test for multiple comparisons. In all cases, values of  $P < 0.05$  were considered statistically significant.

### 3. Results

**3.1. Effects of Citric Acid and L-Malic Acid on Myocardial Infarct Size.** As illustrated in Figure 1(a) and Table 1, no myocardial infarction was observed in the sham group, while myocardial ischemia/reperfusion resulted in significant myocardial infarcts ( $P < 0.01$ ). Diltiazem and clopidogrel, which were used as positive controls, significantly reduced infarction percentage of the ventricle, infarction percentage of the heart, infarction area, and infarction weight, as compared with the model control ( $P < 0.01$  or  $P < 0.05$ ). Compared with the model control group, treatments with citric acid at the doses of 500 mg/kg and 250 mg/kg significantly reduced infarction percentage of the ventricle, infarction percentage of the heart, infarction area, and infarction weight ( $P < 0.01$  or  $P < 0.05$ ).

A similar result was shown in Figure 1(b) and Table 2. Myocardial ischemia/reperfusion resulted in substantial

myocardial infarcts, which were significantly reduced by treatments with diltiazem and clopidogrel ( $P < 0.01$  or  $P < 0.05$ ). Compared with the model control group, treatment with L-malic acid at the dose of 250 mg/kg significantly decreased infarction percentage of the ventricle, infarction percentage of the heart, infarction area, and infarction weight ( $P < 0.01$ ), and treatment with L-malic acid at the dose of 500 mg/kg significantly decreased infarction percentage of the ventricle and infarction area ( $P < 0.01$  and  $P < 0.05$ , resp.) but had only a tendency to reduce infarction percentage of the heart and infarction weight ( $P = 0.056$  and  $P = 0.095$ , resp.).

**3.2. Effects of Citric Acid and L-Malic Acid on TNF- $\alpha$  Production following Myocardial Ischemia/Reperfusion.** Figure 2(a) showed that myocardial ischemia/reperfusion injury significantly increased the level of serum TNF- $\alpha$  compared with the sham group ( $26.71 \pm 6.44$  versus  $11.84 \pm 1.67$  pg/mL,  $P < 0.05$ ). Compared with the model control group, pretreatment with clopidogrel significantly reduced serum TNF- $\alpha$  level by 45.7% ( $14.51 \pm 3.02$  pg/mL,  $P < 0.01$ ), and pretreatments with citric acid at the doses of 500 mg/kg and 250 mg/kg significantly reduced serum TNF- $\alpha$  levels by 15.2% ( $22.66 \pm$

TABLE 2: The effect of L-malic acid on myocardial ischemia/reperfusion injury in rats ( $\bar{x} \pm s$ ,  $n = 10$ ).

Groups	Dosage (mg/kg)	Infarction of the ventricle (%)	Infarction of the heart (%)	Infarction area (mm <sup>2</sup> )	Infarction weight (g)
Sham	—	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.000 ± 0.000
Model control	—	9.03 ± 3.32 <sup>##</sup>	6.00 ± 1.67 <sup>##</sup>	32.01 ± 11.84 <sup>##</sup>	0.051 ± 0.014 <sup>##</sup>
Diltiazem	16	6.86 ± 1.76 <sup>**</sup>	4.68 ± 1.39 <sup>*</sup>	25.11 ± 6.48 <sup>*</sup>	0.041 ± 0.012 <sup>*</sup>
Clopidogrel	13.5	6.51 ± 1.58 <sup>**</sup>	4.75 ± 1.19 <sup>*</sup>	23.67 ± 5.46 <sup>*</sup>	0.038 ± 0.010 <sup>*</sup>
L-malic acid	500	6.71 ± 1.23 <sup>**</sup>	4.98 ± 0.92	25.37 ± 4.55 <sup>*</sup>	0.043 ± 0.009
	250	6.22 ± 1.15 <sup>**</sup>	4.48 ± 0.93 <sup>**</sup>	22.70 ± 4.26 <sup>**</sup>	0.036 ± 0.007 <sup>**</sup>

<sup>##</sup> $P < 0.01$  versus sham, <sup>\*\*</sup> $P < 0.01$ , <sup>\*</sup> $P < 0.05$  versus model control.

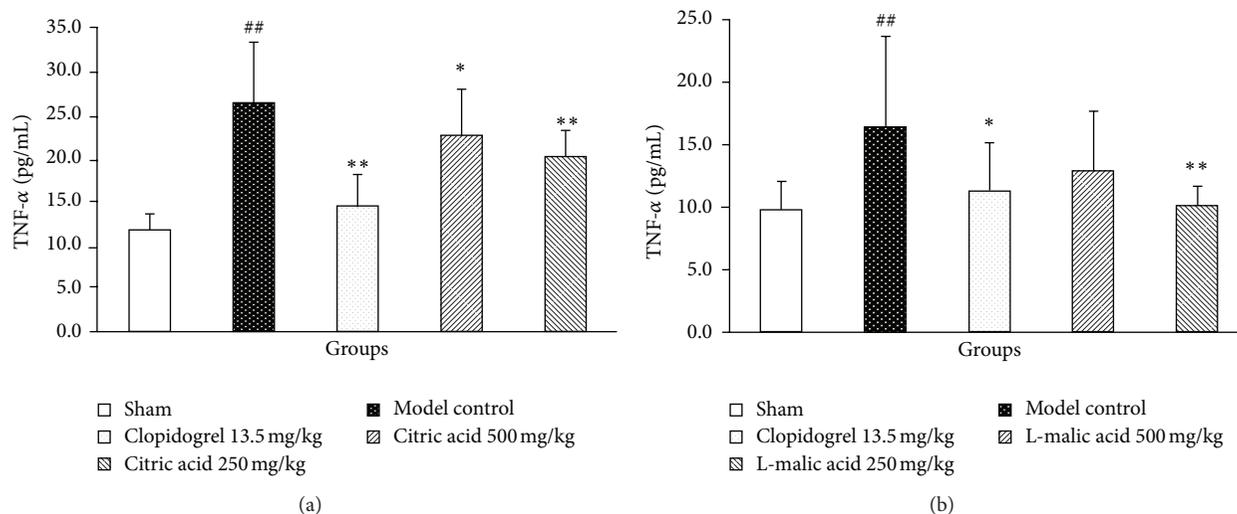


FIGURE 2: Effects of citric acid (a) and L-malic acid (b) on serum levels of TNF- $\alpha$  following myocardial ischemia/reperfusion. Data are shown as mean  $\pm$  SD. <sup>##</sup> $P < 0.01$  versus sham, <sup>\*\*</sup> $P < 0.01$ , <sup>\*</sup> $P < 0.05$  versus model control ( $n = 10$ ).

5.22 pg/mL,  $P < 0.05$ ) and 23.3% ( $20.49 \pm 2.71$  pg/mL,  $P < 0.01$ ), respectively.

Similarly, Figure 2(b) showed that the level of serum TNF- $\alpha$  in the model control group was significantly increased ( $16.42 \pm 7.27$  versus  $9.85 \pm 2.25$  pg/mL in the sham group,  $P < 0.05$ ). Compared with the model control group, pretreatment with clopidogrel reduced serum TNF- $\alpha$  level by 30.8% ( $11.36 \pm 3.73$  pg/mL,  $P < 0.05$ ), and pretreatment with L-malic acid at the dose of 500 mg/kg had only a tendency to decrease serum TNF- $\alpha$  level ( $12.98 \pm 4.63$  pg/mL,  $P = 0.129$ ), while pretreatment with L-malic acid at the dose of 250 mg/kg significantly reduced serum TNF- $\alpha$  level by 37.9% ( $10.20 \pm 1.50$  pg/mL,  $P < 0.01$ ).

**3.3. Effects of Citric Acid and L-Malic Acid on Platelet Aggregation Induced by ADP following Myocardial Ischemia/Reperfusion.** We measured the effects of citric acid and L-malic acid on platelet aggregation induced by one of the classical endogenous agonists ADP. As shown in Figure 3(a), compared with the sham group, myocardial ischemia/reperfusion significantly increased platelet aggregation rate induced by ADP ( $57.53 \pm 7.47\%$  versus  $46.81 \pm 6.18\%$ ,  $P < 0.05$ ). Compared with the model control group, pretreatment with clopidogrel significantly reduced platelet aggregation rate ( $2.12 \pm 3.44\%$ ,  $P < 0.01$ ), and pretreatments with citric acid at the doses of 500 mg/kg and 250 mg/kg significantly decreased

platelet aggregation rate ( $35.19 \pm 13.29\%$  and  $27.50 \pm 14.08\%$ , resp.,  $P < 0.01$  each).

A similar result was shown in Figure 3(b), the platelet aggregation rate in the model control group was significantly increased ( $66.43 \pm 8.66\%$  versus  $53.06 \pm 5.27\%$  in the sham group,  $P < 0.05$ ). Compared with the model control group, the platelet aggregation rate for the clopidogrel group was  $3.36 \pm 4.15\%$  ( $P < 0.01$ ), and that for L-malic acid at the doses of 500 mg/kg and 250 mg/kg groups was  $47.02 \pm 17.09\%$  ( $P < 0.01$ ) and  $57.58 \pm 8.09\%$  ( $P = 0.149$ ), respectively.

**3.4. Effects of Citric Acid and L-Malic Acid on H/R-Induced Cardiomyocyte Necrosis.** LDH leakage from cells was widely used as a reliable marker of cellular injury. The degree of LDH release was closely related to cardiomyocyte necrosis [16, 17]. Thus, we explored the protective effects of citric acid and L-malic acid on H/R-induced cardiomyocyte injury *in vitro* by detecting LDH release. Figure 4(a) showed that after cardiomyocytes were subjected to 3 h hypoxia followed by 2 h reoxygenation, a significant LDH release was induced ( $40.76 \pm 2.88\%$  versus  $14.57 \pm 0.96\%$  in the normal control group,  $P < 0.01$ ), which were significantly inhibited by diltiazem ( $23.25 \pm 2.61\%$ ,  $P < 0.01$ ) and citric acid at the concentration of 200  $\mu$ g/mL ( $31.07 \pm 5.54\%$ ,  $P < 0.01$ ).

A similar result was shown in Figure 4(b), the LDH release rate in the model control group was significantly

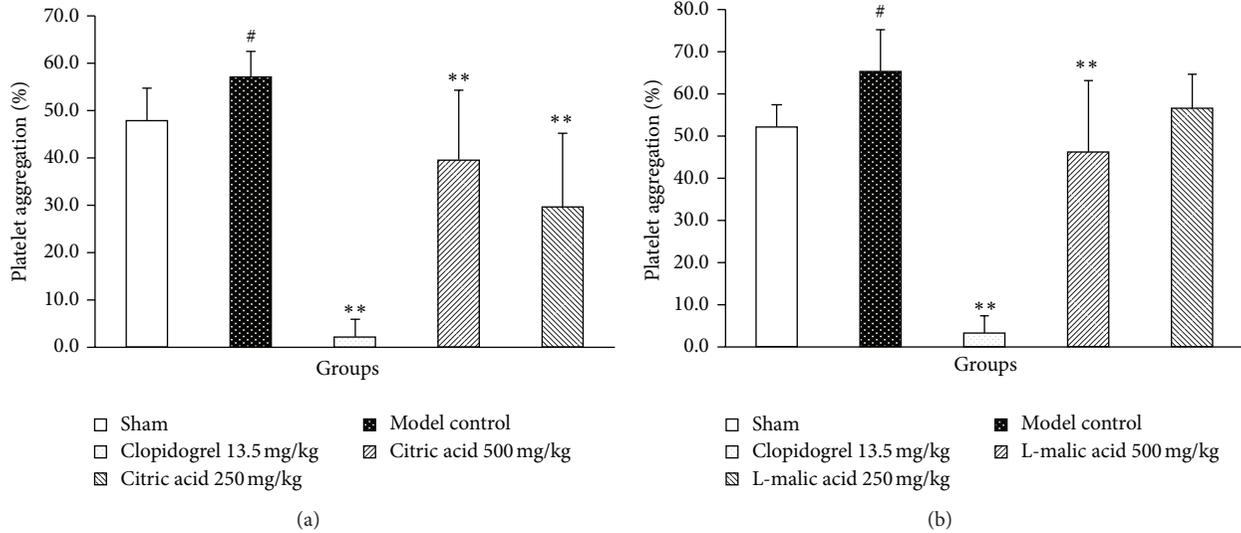


FIGURE 3: Effects of citric acid (a) and L-malic acid (b) on platelet aggregation induced by ADP following myocardial ischemi-reperfusion. Data are shown as mean  $\pm$  SD. <sup>#</sup> $P < 0.05$  versus sham, <sup>\*\*</sup> $P < 0.01$  versus model control ( $n = 10$ ).

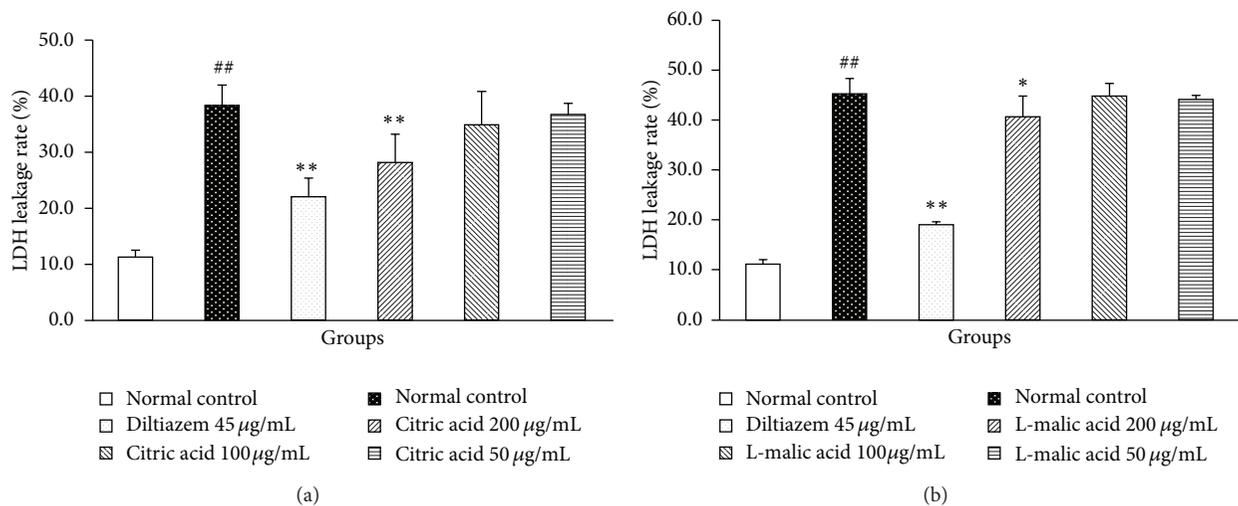


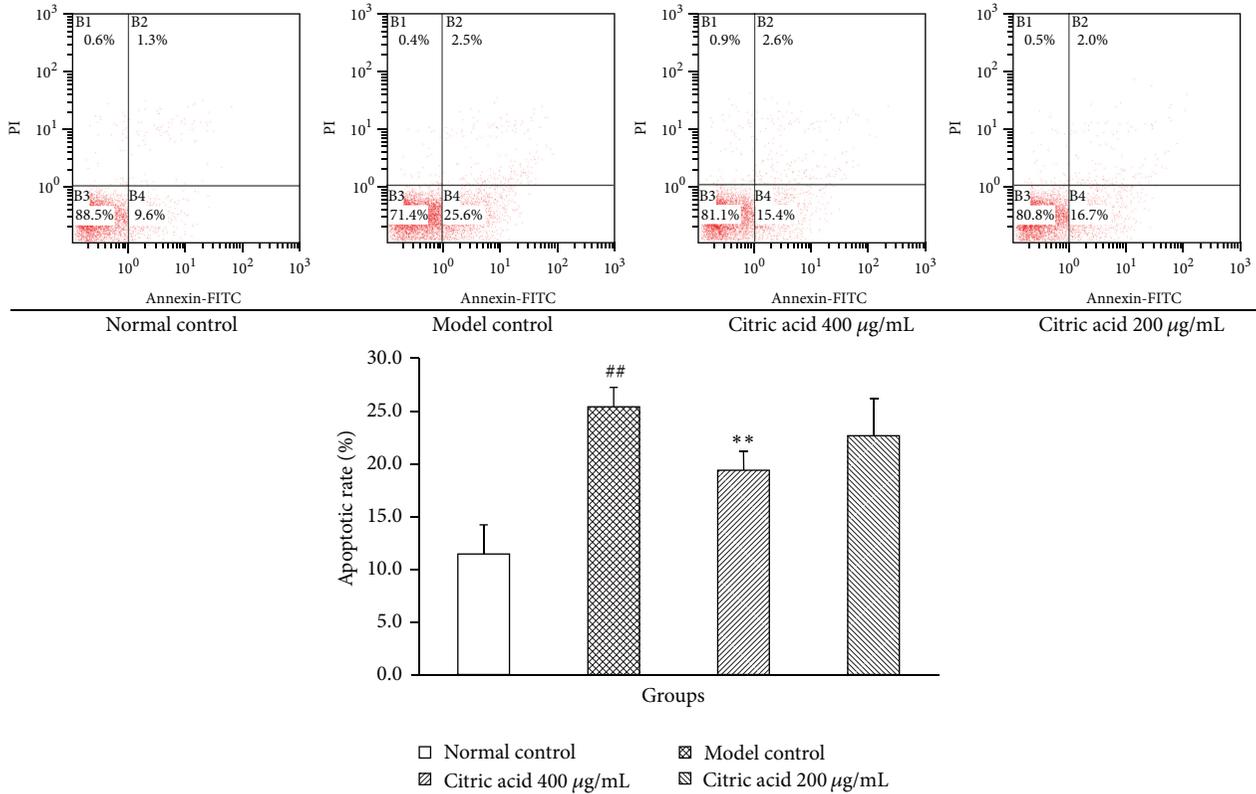
FIGURE 4: Effects of citric acid (a) and L-malic acid (b) on LDH release. Cardiomyocytes were subjected to 3 h hypoxia followed by 2 h reoxygenation with or without treatment. LDH activities in the hypoxia and the reoxygenation media and in the cell lysates were measured. Data are shown as mean  $\pm$  SD. <sup>##</sup> $P < 0.01$  versus normal control, <sup>\*\*</sup> $P < 0.01$ , <sup>\*</sup> $P < 0.05$  versus model control ( $n = 3$ ).

increased ( $45.31 \pm 3.00\%$  versus  $11.23 \pm 0.86\%$  in normal control group,  $P < 0.01$ ). Compared with the model control group, the LDH release rate for diltiazem group was  $19.12 \pm 0.57\%$  ( $P < 0.01$ ), and that for L-malic acid at the concentration of 200  $\mu$ g/mL group was  $40.69 \pm 4.03\%$  ( $P < 0.05$ ).

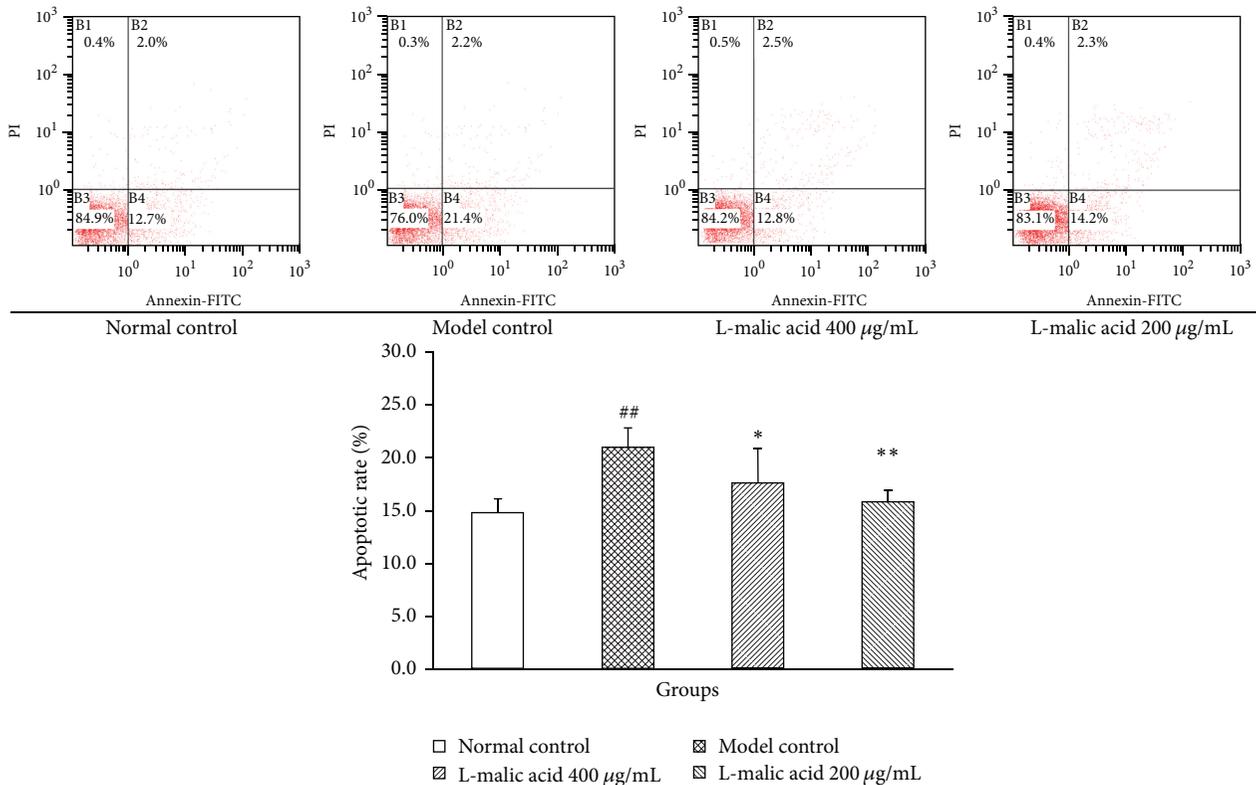
**3.5. Effects of Citric Acid and L-Malic Acid on H/R-Induced Cardiomyocyte Apoptosis.** Based on the previous results that treatments with citric acid and L-malic acid below concentration of 200  $\mu$ g/mL could not decrease LDH release, we chose to use citric acid and L-malic acid at concentrations of 400  $\mu$ g/mL and 200  $\mu$ g/mL to observe whether citric acid and L-malic acid could decrease H/R-induced cardiomyocyte apoptosis. As data shown in Figure 5(a), after

cardiomyocytes were subjected to 3 h hypoxia followed by 6 h reoxygenation injury, the number of apoptotic cells was significantly increased as compared with the normal control group ( $25.45 \pm 1.81\%$  versus  $11.48 \pm 2.74\%$ ,  $P < 0.01$ ). In contrast, treatments with citric acid at concentrations of 400  $\mu$ g/mL and 200  $\mu$ g/mL reduced the number of apoptotic cells to  $19.43 \pm 1.69\%$  ( $P < 0.01$ ) and  $22.70 \pm 3.47\%$  ( $P = 0.179$ ), respectively.

Similarly, Figure 5(b) showed that the number of apoptotic cells in the model control group was significantly increased ( $22.13 \pm 1.69\%$  versus  $15.65 \pm 1.34\%$  in the normal control group,  $P < 0.01$ ), which was significantly reduced by treatments with L-malic acid at concentrations of 400  $\mu$ g/mL ( $18.63 \pm 3.17\%$ ,  $P < 0.05$ ) and 200  $\mu$ g/mL ( $16.70 \pm 0.62\%$ ,  $P < 0.01$ ), respectively.



(a)



(b)

FIGURE 5: Effects of citric acid (a) and L-malic acid (b) on H/R-induced cardiomyocyte apoptosis. Cardiomyocytes were subjected to 3 h hypoxia and 6 h reoxygenation in the presence or absence of citric acid or L-malic acid. Data are shown as mean  $\pm$  SD. <sup>##</sup>  $P < 0.01$  versus normal control, <sup>\*\*</sup>  $P < 0.01$ , <sup>\*</sup>  $P < 0.05$  versus model control ( $n = 3$ ).

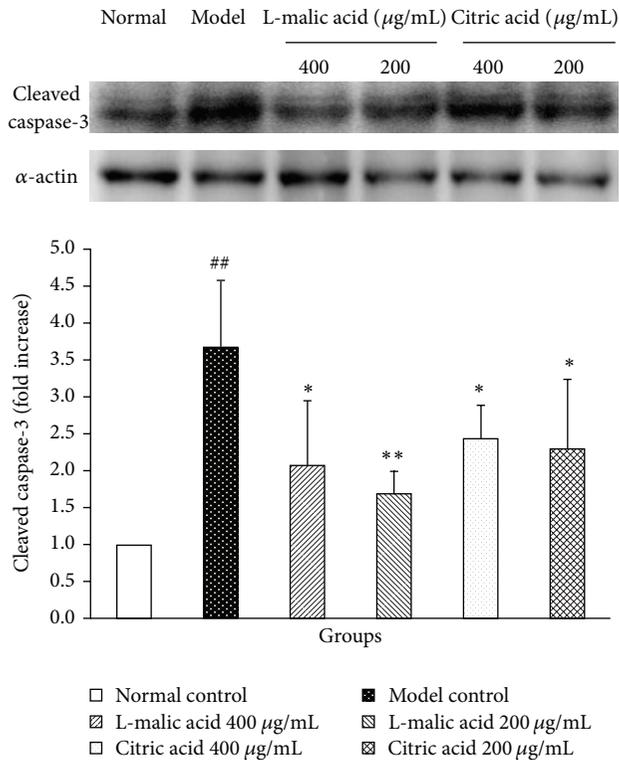


FIGURE 6: Effects of citric acid and L-malic acid on expression levels of cleaved caspase-3 (fold increase relative to normal control levels) after cardiomyocytes subjected to 3 h hypoxia followed by 6 h reoxygenation. Data are shown as mean  $\pm$  SD. <sup>##</sup> $P < 0.01$  versus normal control, <sup>\*\*</sup> $P < 0.01$ , <sup>\*</sup> $P < 0.05$  versus model control. Results are representative of three independent experiments.

**3.6. Effects of Citric Acid and L-Malic Acid on Expression of Cleaved Caspase-3.** Next we investigated the effects of citric acid and L-malic acid on expression levels of cleaved caspase-3, the activated form of caspase-3. As shown in Figure 6, western blot analysis revealed that the expression of cleaved caspase-3 was significantly upregulated (2.69-fold,  $P < 0.01$ ) after cardiomyocytes were subjected to 3 h hypoxia followed by 6 h reoxygenation, which was significantly downregulated by treatments with citric acid at the concentrations of 400  $\mu\text{g}/\text{mL}$  (33.60%,  $P < 0.05$ ) and 200  $\mu\text{g}/\text{mL}$  (37.40%,  $P < 0.05$ ) and treatments with L-malic acid at concentrations of 400  $\mu\text{g}/\text{mL}$  (43.63%,  $P < 0.05$ ) and 200  $\mu\text{g}/\text{mL}$  (53.93%,  $P < 0.01$ ), respectively.

**3.7. Effects of Citric Acid and L-Malic Acid on Expression Levels of Akt and p-Akt.** The PI3K/Akt pathway plays a critical role in survival after myocardial ischemia/reperfusion injury. Phosphorylation of Akt S473 represents its maximal activation [18, 19]. To determine whether Akt was involved in citric acid and L-malic acid protection from cardiomyocyte injury, we further detected the expressions of Akt and phospho-Akt (Ser 473). As illustrated in Figure 7, western blotting results showed that total Akt was comparable in all groups. The densities of phosphorylated Akt were normalized against total Akt. We found that H/R-induced cardiomyocyte

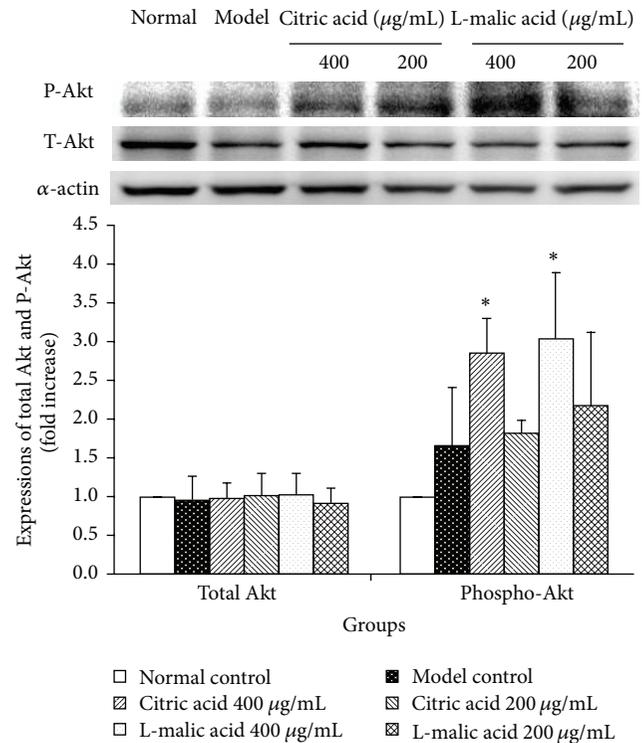


FIGURE 7: Citric acid and L-malic acid activate PI3K and phosphorylation of Akt. A representative western blot analysis of total Akt and phosphorylation of Akt at Ser473 after cardiomyocytes were subjected to 3 h hypoxia followed by 6 h reoxygenation. Data are shown as mean  $\pm$  SD. <sup>\*</sup> $P < 0.05$  versus model control. Results are representative of three independent experiments.

injury by itself resulted in a 0.67-fold increase in Akt phosphorylation, while treatments with both citric acid at the concentration of 400  $\mu\text{g}/\text{mL}$  and L-malic acid at the concentration of 400  $\mu\text{g}/\text{mL}$  further significantly upregulated the expression levels of phosphorylated Akt after cardiomyocytes hypoxia/reoxygenation injury compared with the model control group (0.71-fold and 0.82-fold, resp.,  $P < 0.05$  each). Treatments with both citric acid at the concentration of 200  $\mu\text{g}/\text{mL}$  and L-malic acid at the concentration of 200  $\mu\text{g}/\text{mL}$  had a tendency to increase the expression levels of phosphorylated Akt (9.0% and 30.54%, resp.,  $P = 0.763$  and  $P = 0.337$ , resp.), without significant differences.

#### 4. Discussion

In the present study, we reported for the first time the *in vivo* data demonstrating that pretreatments with both citric acid and L-malic acid significantly ameliorated the I/R-induced cardiac injury, including reduced myocardial infarct size, decreased inflammatory cytokine TNF- $\alpha$  activity, and inhibited ADP-induced platelet aggregation. Furthermore, *in vitro* experiments revealed that both citric acid and L-malic acid protected cardiomyocyte damage from necrosis and apoptosis during cardiomyocyte hypoxia/reoxygenation injury possibly via a mechanism involving PI3K/Akt survival pathway.

In recent years, traditional Chinese medicine has been greatly developed in many countries due to its high quality and safety [20–22], and considerable attention has focused on the material basis of Chinese medicine studies. The material basis of *Fructus Choerospondiatis* responsible for its cardioprotective effects has been always considered to be flavonoids (mainly quercetin). However, our data *in vivo* demonstrated that both citric acid (500, 250 mg/kg) and quercetin (40, 20 mg/kg) significantly ameliorated the I/R-induced cardiac injury (data unpublished), and the *in vitro* experiments confirmed that both organic acids (citric acid, L-malic acid, succinic acid, and tartaric acid; concentration: 400  $\mu\text{g}/\text{mL}$ ) and flavonoids (quercetin and kaempferol; concentrations: 12.5, 25, 50, and 100  $\mu\text{g}/\text{mL}$ ) significantly decreased LDH release rate of cardiomyocytes injured by hypoxia/reoxygenation [23, 24]. Although the dosage of organic acids used in these studies was 10~30 times higher than that of flavonoids, the content of total organic acids in *Fructus Choerospondiatis* was nearly 30 000 times higher than that of total flavonoids. Therefore, our results still furnish strong evidence that organic acids may also be the major active ingredients of *Fructus Choerospondiatis* responsible for its cardioprotective effects.

The extent of myocardial damage is closely related to prognosis. Therefore, determination of infarct size is the strongest determinant of prognosis of ischemic heart disease [25]. The results showed that pretreatments with both citric acid and L-malic acid significantly reduced I/R-induced myocardial infarct size and thus protected the infarcted myocardium.

Inflammatory responses and platelet aggregation have been implicated in myocardial ischemia/reperfusion injury [26]. Within minutes after reperfusion, inflammatory cascade is triggered and copious amounts of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 are produced and released [27]. These proinflammatory cytokines (particularly TNF- $\alpha$ ), as important factors in cardiac dysfunction, activate neutrophils and endothelial cells and aggravate myocardial ischemia/reperfusion injury [28, 29]. Platelets play a critical role in the process of myocardial ischemia/reperfusion injury. After reperfusion, platelets are immediately activated and increased, and platelet aggregability will aggravate myocardial ischemia/reperfusion injury in turn, which may be related to endothelial dysfunction and platelet-derived p-selectin, and so forth [30]. The results showed that both citric acid and L-malic acid decreased TNF- $\alpha$  level and inhibited platelet aggregation on myocardial ischemia/reperfusion injury. These data *in vivo* provided direct evidence that organic acids protected ischemia myocardium may be partly due to inhibition of inflammation and platelet aggregation.

Cardiomyocyte necrosis and apoptosis are the major contributors to myocardial ischemia/reperfusion injury [31]. Cardiomyocyte loss, caused by both necrosis and apoptosis, is the main feature of myocardial ischemia/reperfusion injury [32]. Necrosis and apoptosis are two distinct types of cell death with different characteristics. Necrosis leads to membrane lysis, release of cellular contents, and resulting inflammation, while apoptosis is characterized by cell

shrinkage, membrane blebbing, and nuclear condensation and degradation [33]. Necrotic cells are mainly found in the central zone of the infarct, while apoptotic cells are more apparent at the marginal zone [33]. It may be beneficial for attenuating necrosis and apoptosis to prevent cardiomyocyte loss caused by myocardial ischemia/reperfusion injury. Thus, after an initial investigation of the effects of citric acid and L-malic acid on myocardial ischemia/reperfusion injury in *in vivo* rat model, we further observed their cardioprotective effects in cellular level. The concentrations of citric acid and L-malic acid (400  $\mu\text{g}/\text{mL}$  and 200  $\mu\text{g}/\text{mL}$ ) used in this study had been evaluated on cytotoxicity, as determined by MTT assay. There were no significant differences between citric acid or L-malic acid at the concentrations below 500  $\mu\text{g}/\text{mL}$  and the control group [23]. LDH is a stable cytosolic enzyme present in mammalian cells and LDH release is an indication of cell membrane integrity. The amount of LDH released from cells is proportional to the extent of membrane damage and cell necrosis [34]. The data showed that H/R injury induced significant LDH release, but treatments with citric acid at the concentration of 200  $\mu\text{g}/\text{mL}$  and L-malic acid at the concentration of 200  $\mu\text{g}/\text{mL}$  could significantly reduce cardiomyocyte LDH release rate.

Furthermore, we studied the effects of citric acid and L-malic acid on hypoxia/reoxygenation-induced apoptosis by flow cytometry analysis. The results showed that H/R injury significantly increased the number of apoptotic cardiomyocytes, while treatments with citric acid at the concentration of 400  $\mu\text{g}/\text{mL}$  or L-malic acid at the concentrations of 400  $\mu\text{g}/\text{mL}$  and 200  $\mu\text{g}/\text{mL}$  significantly reduced the number of apoptotic cells. The cleavage of caspase-3 is often identified as the important step in the apoptotic signaling pathway activation process and it is considered to be a potential molecular therapeutic target for preventing cardiomyocyte apoptosis [35]. We next investigated the expression of cleaved caspase-3 and found that the cleaved caspase-3 was significantly upregulated by hypoxia/reoxygenation-induced cardiomyocyte injury while significantly downregulated by treatments with citric acid at the concentrations of 400  $\mu\text{g}/\text{mL}$  and 200  $\mu\text{g}/\text{mL}$  or L-malic acid at the concentrations of 400  $\mu\text{g}/\text{mL}$  and 200  $\mu\text{g}/\text{mL}$  relative to the model control group.

Akt is a potent cell survival factor and an important downstream kinase of PI3K. The phosphorylation and activation of Akt play a pivotal role in myocardial ischemia/reperfusion injury [36]. Considerable evidence suggests that the activation of Akt reduced myocardial infarct size [17, 37]. To investigate whether Akt is involved in citric acid and L-malic acid-induced cardioprotection, we evaluated the expression of Akt and its activated, phosphorylated form (phospho-Akt) at Ser473 after hypoxia/reoxygenation-induced cardiomyocyte injury. Our results showed that treatments with citric acid and L-malic acid significantly upregulated the expression of phosphorylated Akt. All data *in vitro* concluded that the cardioprotection of citric acid and L-malic acid contributed to preventing cardiomyocyte from necrosis and apoptosis, which may have the PI3K/Akt survival pathway involved.

In conclusion, the present study demonstrates that citric acid and L-malic acid have protective effects on

myocardial ischemia/reperfusion injury; the underlying mechanism may be associated with their anti-inflammatory, anti-platelet aggregation and direct cardiomyocyte protective effects. Based on these findings, we concluded that organic acids may also be the major active ingredients of *Fructus Choerospondiatis* responsible for its cardioprotective effects but not only flavonoids now.

## Conflict of Interests

The authors declare that there is no conflict of interests.

## Acknowledgments

This research was supported by grants from the National Natural Science Foundation of China (Grant nos. 81073085; 81001662) and the National Science & Technology Major Project of China (Grant nos. 2012zx09301002-004-002; 2012zx0 9103201-049).

## References

- [1] A. T. Turer and J. A. Hill, "Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy," *The American Journal of Cardiology*, vol. 106, no. 3, pp. 360–368, 2010.
- [2] National Pharmacopoeia Committee, *Chinese Pharmacopoeia*, vol. 1, Chemical Industry Press, Beijing, China, 2005.
- [3] J. Ao, H. Feng, and F. Xia, "Transforming growth factor and nuclear factor kappa B mediated prophylactic cardioprotection by total flavonoids of *fructus chorspondiatis* in myocardial ischemia," *Cardiovascular Drugs and Therapy*, vol. 21, no. 4, pp. 235–241, 2007.
- [4] G. L. Qu, "The study on pharmacodynamic material basis of *Fructus Choerospondiatis* and *Radix et Rhizoma Rhodiola Crenulatae*," Postdoctoral Research Report, The Chinese Academy of Traditional Chinese Medicine, 2012.
- [5] X. G. Liu and Y. S. Chen, "Component analysis of *fructus chorspondiatis*," *Chinese Wild Plant Resources*, vol. 19, no. 3, pp. 35–40, 2000 (Chinese).
- [6] K. K. Dharmappa, R. V. Kumar, A. Nataraju, R. Mohamed, H. V. Shivaprasad, and B. S. Vishwanath, "Anti-inflammatory activity of oleanolic acid by inhibition of secretory phospholipase A<sub>2</sub>," *Planta Medica*, vol. 75, no. 3, pp. 211–215, 2009.
- [7] K. Takada, T. Nakane, K. Masuda, and H. Ishii, "Ursolic acid and oleanolic acid, members of pentacyclic triterpenoid acids, suppress TNF- $\alpha$ -induced E-selectin expression by cultured umbilical vein endothelial cells," *Phytomedicine*, vol. 17, no. 14, pp. 1114–1119, 2010.
- [8] K. Y. Kim, K. M. Lim, J. Y. Noh et al., "Novel antiplatelet activity of protocatechuic acid through the inhibition of high shear stress-induced platelet aggregation," *Journal of Pharmacology and Experimental Therapeutics*, vol. 343, no. 3, pp. 704–711, 2012.
- [9] X. Wang, X. L. Ye, R. Liu et al., "Antioxidant activities of oleanolic acid *in vitro*: possible role of Nrf2 and MAP kinases," *Chemico-Biological Interactions*, vol. 184, no. 3, pp. 328–337, 2010.
- [10] R. Vari, M. D'Archivio, C. Filesi et al., "Protocatechuic acid induces antioxidant/detoxifying enzyme expression through JNK-mediated Nrf2 activation in murine macrophages," *Journal of Nutritional Biochemistry*, vol. 22, no. 5, pp. 409–417, 2011.
- [11] Z. Wang, M. Li, W. K. Wu, H. M. Tan, and D. F. Geng, "Ginsenoside Rb1 preconditioning protects against myocardial infarction after regional ischemia and reperfusion by activation of phosphatidylinositol-3-kinase signal transduction," *Cardiovascular Drugs and Therapy*, vol. 22, no. 6, pp. 443–452, 2008.
- [12] J. X. Liu, X. Z. Li, X. B. Ma et al., "Cardio-protective effects of Corocalm on acute myocardial ischemia/reperfusion injury in rats," *Chinese Journal of Integrative Medicine*, vol. 12, no. 3, pp. 199–202, 2006.
- [13] G. V. Born and M. J. Cross, "The aggregation of blood platelets," *The Journal of Physiology*, vol. 168, pp. 178–195, 1963.
- [14] X. O. Wang, S. Ma, and G. X. Qi, "Effect of hypoxia-inducible factor 1- $\alpha$  on hypoxia/reoxygenation-induced apoptosis in primary neonatal rat cardiomyocytes," *Biochemical and Biophysical Research Communications*, vol. 417, no. 4, pp. 1227–1234, 2012.
- [15] P. Li, J. H. Fu, J. K. Wang, J. G. Ren, and J. X. Liu, "Extract of paris polyphylla simth protects cardiomyocytes from anoxia-reoxia injury through inhibition of calcium overload," *Chinese Journal of Integrative Medicine*, vol. 17, no. 4, pp. 283–289, 2011.
- [16] J. Zhang, A. Liu, R. Hou et al., "Salidroside protects cardiomyocyte against hypoxia-induced death: a HIF-1 $\alpha$ -activated and VEGF-mediated pathway," *European Journal of Pharmacology*, vol. 607, no. 1–3, pp. 6–14, 2009.
- [17] H. J. Pan, D. Y. Li, F. Fang et al., "Salvianolic acid A demonstrates cardioprotective effects in rat hearts and cardiomyocytes after ischemia/reperfusion injury," *Journal of Cardiovascular Pharmacology*, vol. 58, no. 5, pp. 535–542, 2011.
- [18] M. P. Wymann, M. Zvelebil, and M. Laffargue, "Phosphoinositide 3-kinase signalling—which way to target?" *Trends in Pharmacological Sciences*, vol. 24, no. 7, pp. 366–376, 2003.
- [19] Y. C. Li, C. H. Yeh, M. L. Yang, and Y. H. Kuan, "Luteolin suppresses inflammatory mediator expression by blocking the Akt/NF $\kappa$ B pathway in acute lung injury induced by lipopolysaccharide in mice," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 383608, 8 pages, 2012.
- [20] N. Robinson, "Integrative medicine-traditional Chinese medicine, a model?" *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 21–25, 2011.
- [21] H. Xu and K. Chen, "Complementary and alternative medicine: is it possible to be mainstream?" *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 403–404, 2012.
- [22] G. Dobos and I. Tao, "The model of western Integrative medicine: the role of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 11–20, 2011.
- [23] X. L. Tang, J. X. Liu, L. Li et al., "Cardioprotective effects of total organic acids in *Fructus Choerospondiatis* on myocardial ischemia-reperfusion injury," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 19, no. 4, pp. 168–172, 2013 (Chinese).
- [24] X. L. Tang, J. X. Liu, P. Li et al., "Protective effects of kaempferol and quercetin on hypoxia/reoxygenation and peroxidation injury in neonatal cardiomyocytes," *Pharmacology and Clinics of Chinese Materia Medica*, vol. 18, no. 5, pp. 243–246, 2012 (Chinese).
- [25] J. Hallén, "Troponin for the estimation of infarct size: what have we learned?" *Cardiology*, vol. 121, no. 3, pp. 204–212, 2012.
- [26] M. Akhlaghi and B. Bandy, "Mechanisms of flavonoid protection against myocardial ischemia-reperfusion injury," *Journal of Molecular and Cellular Cardiology*, vol. 46, no. 3, pp. 309–317, 2009.

- [27] J. Vinten-Johansen, R. Jiang, J. G. Reeves, J. Mykytenko, J. Deneve, and L. J. Jobe, "Inflammation, proinflammatory mediators and myocardial ischemia-reperfusion injury," *Hematology/Oncology Clinics of North America*, vol. 21, no. 1, pp. 123–145, 2007.
- [28] P. Kleinbongard, G. Heusch, and R. Schulz, "TNF $\alpha$  in atherosclerosis, myocardial ischemia/reperfusion and heart failure," *Pharmacology and Therapeutics*, vol. 127, no. 3, pp. 295–314, 2010.
- [29] N. G. Frangogiannis, C. W. Smith, and M. L. Entman, "The inflammatory response in myocardial infarction," *Cardiovascular Research*, vol. 53, no. 1, pp. 31–47, 2002.
- [30] Y. Xu, Y. Huo, M. C. Toufektsian et al., "Activated platelets contribute importantly to myocardial reperfusion injury," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 290, no. 2, pp. H692–H699, 2006.
- [31] M. I. Oerlemans, S. Koudstaal, S. A. Chamuleau, D. P. de Kleijn, P. A. Doevendans, and J. P. Sluijter, "Targeting cell death in the reperfused heart: pharmacological approaches for cardioprotection," *International Journal of Cardiology*, vol. 165, no. 3, pp. 410–422, 2013.
- [32] Y. Fujio, T. Nguyen, D. Wencker, R. N. Kitsis, and K. Walsh, "Akt promotes survival of cardiomyocytes *in vitro* and protects against Ischemia-reperfusion injury in mouse heart," *Circulation*, vol. 101, no. 6, pp. 660–667, 2000.
- [33] A. Hamacher-Brady, N. R. Brady, and R. A. Gottlieb, "The interplay between pro-death and pro-survival signaling pathways in myocardial ischemia/reperfusion injury: apoptosis meets autophagy," *Cardiovascular Drugs and Therapy*, vol. 20, no. 6, pp. 445–462, 2006.
- [34] L. Parhamifar, H. Andersen, S. M. Moghimi et al., "Lactate dehydrogenase assay for assessment of polycation cytotoxicity," *Methods in Molecular Biology*, vol. 948, pp. 13–22, 2013.
- [35] S. A. Lakhani, A. Masud, K. Kuida et al., "Caspases 3 and 7: key mediators of mitochondrial events of apoptosis," *Science*, vol. 311, no. 5762, pp. 847–851, 2006.
- [36] L. Ji, F. Fu, L. Zhang et al., "Insulin attenuates myocardial ischemia/reperfusion injury via reducing oxidative/nitrative stress," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 298, no. 4, pp. E871–E880, 2010.
- [37] T. Matsui, J. Tao, F. del Monte et al., "Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia *in vivo*," *Circulation*, vol. 104, no. 3, pp. 330–335, 2001.

## Research Article

# A Systems Biology Approach to Characterize Biomarkers for Blood Stasis Syndrome of Unstable Angina Patients by Integrating MicroRNA and Messenger RNA Expression Profiling

Jie Wang and Gui Yu

Department of Cardiology, Guang'anmen Hospital, China Academy of Chinese Medical Science, Beixiang 5, Xicheng District, Beijing 100053, China

Correspondence should be addressed to Gui Yu; [cliffyugui@sina.com](mailto:cliffyugui@sina.com)

Received 4 February 2013; Revised 22 March 2013; Accepted 29 March 2013

Academic Editor: Hao Xu

Copyright © 2013 J. Wang and G. Yu. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Blood stasis syndrome (BSS) has been considered to be the major type of syndromes in unstable angina (UA) patients. The aim of this study was to find the systems biology-based microRNA (miRNA) and mRNA expression biomarkers for BSS of UA. We identified 1081 mRNAs and 25 miRNAs differentially expressed between BSS of UA patients and healthy controls by microarrays. We used DAVID, miRTrail, and the protein-protein interactions method to explore the related pathways and networks of differentially expressed miRNAs and mRNAs. By combining the results of pathways and networks, we found that the upregulation of miR-146b-5p may induce the downregulation of CALR to attenuate inflammation and the upregulation of miR-199a-5p may induce the downregulation of TP53 to inhibit apoptosis in BSS of UA patients. The expression patterns of miR-146b-5p, miR-199a-5p, CALR, and TP53 were confirmed by qRT-PCR in an independent validation cohort including BSS of UA patients, non-BSS of UA patients, and healthy controls. miR-146b-5p, miR-199a-5p, CALR, and TP53 could be significant biomarkers of BSS of UA patients. The systems biology-based miRNA and mRNA expression biomarkers for the BSS of UA may be helpful for the further stratification of UA patients when deciding on interventions or clinical trials.

## 1. Introduction

Unstable angina (UA) constitutes a clinical syndrome subset of the acute coronary syndrome (ACS) and is associated with an increased risk of cardiac death and subsequent myocardial infarction (MI) [1]. UA is diagnosed by electrocardiographic (ECG) ST-segment depression or prominent T-wave inversion and negative biomarkers of necrosis and in an appropriate clinical setting (chest discomfort or angina equivalent). Its pathophysiological origins relate to disruption or erosion of an atherosclerotic plaque and a subsequent cascade of pathological processes that decrease coronary blood flow [2]. UA remains a severe burden on society and family in both industrialized and developing countries. Although anti-ischemic, antiplatelet, and anticoagulant/antithrombotic therapies and early standard coronary revascularization procedures have been used for fighting against UA, there are some drawbacks of current therapeutics, such as aspirin

resistance and adverse effects of statin [3, 4]. New thoughts need to be present for developing efficient diagnosis and optimal therapeutics to combat UA.

Traditional Chinese medicine (TCM) is rapidly gaining attention in the world as sources for discovering new cardiovascular drugs [5–9]. However, simply copying TCM therapy to the treatment of UA is not feasible. Some systematic reviews have shown that the benefits of standard TCM therapies given to UA patients are not always obvious [10, 11]. We think that the main problem is that these clinical trials did not consider the further stratification of UA patients based on TCM syndrome differentiation. Since a single disease can have several kinds of syndromes, it should be treated with different therapies instead of one single therapy [12–14]. According to TCM theory, blood stasis (“*Xueyu*” in Chinese Mandarin) is one of the key pathogenesis of UA. This has been confirmed by a binary logistic regression analysis of a multicenter prospective research on TCM Syndromes in 815

cases of UA [15]. Blood stasis syndrome (BSS) refers to a condition in which any pathological change is characterized by retarded or impeded blood flow. The local manifestations of BSS include mass formation, ecchymosis or petechiae, and stabbing or pricking pain fixed in location and accompanied by tenderness. The general manifestations of BSS include darkish complexion, skin texture becoming thickened, lack of smooth feeling, dark purple tongue with purple spots, and choppy or irregular pulse. Since BSS is one of the most common syndromes in TCM, lots of research has been done about it [16–21]. The grading system in quantifying BSS diagnosis standards has been built by clinical investigation and multifactorial analysis, and regressive analysis as well as differential analysis in China since 1988 [16, 22]. The grading system can be adapted to all kinds of BSS patients, including UA patients.

Among the classic teachings of TCM, it is stated that “when blockage is opened, pain is relieved; when blockage is not opened, pain persists.” If a UA patient has BSS, the treatment should be promoting blood circulation and removing blood stasis (PBCRBS). The concept of PBCRBS is similar to the modern revascularization method, but it is a kind of macroscopic thinking achieved by drugs rather than microscopic mechanism. The benefit of PBCRBS in UA patients with BSS has been demonstrated by increasing clinical evidences in recent years [23, 24]. It again confirms the importance of considering the further stratification of UA based on TCM syndrome differentiation in clinical trials. More importantly, it gives valuable impetus to the hypothesis that BSS of UA in TCM may have its own specific biomarkers. Exploring these biomarkers may facilitate further risk stratification of UA and provide some new therapeutic targets for treating UA.

MicroRNAs (miRNAs) are endogenous, nonprotein-coding, single-stranded, small RNAs that are generally regarded as negative regulators of gene expression by inhibiting translation and/or promoting messenger RNA (mRNA) degradation [25]. In various cardiovascular diseases, gain-and loss-of-function studies using *in vitro* and *in vivo* models have revealed pathogenic and protective functions of miRNAs; therefore they emerge as interesting novel candidates for the development of miRNA-based therapeutic strategies in cardiovascular disease [26]. Besides their function, recent studies have been demonstrated that miRNAs can circulate in the blood of cardiovascular-diseased patients in a remarkably stable form [27]. The discovery of circulating miRNAs opens up intriguing possibilities to use the circulating miRNAs' patterns as biomarker for cardiovascular diseases. Therefore, circulating miRNAs' patterns are likely to provide supplementary information for investigating biomedical mechanisms of BSS of UA patients and characterizing its biomarkers.

The purpose of this study was to investigate circulating miRNAs' patterns of BSS of UA patients by a systems biology approach. MiRNA and mRNA expression profilings of peripheral blood mononuclear cells (PBMCs) of BSS of UA patients were compared to ones of PBMCs of healthy controls to identify the differentially expressed miRNAs and mRNAs by using a gene expression oligonucleotide microarray and a microRNA microarray. Bioinformatics analysis was used

to find critically deregulated miRNAs and mRNAs involved in pathogenesis of BSS of UA and potential biomarkers. The expression patterns of miRNA and mRNA biomarkers in BSS of UA patients were independently validated by real-time quantitative polymerase chain reaction (qRT-PCR) in an independent validation cohort including BSS of UA patients, non-BSS of UA patients, and healthy controls.

## 2. Materials and Methods

**2.1. Patients and Controls.** Patients with UA undergoing clinically indicated coronary angiography were consecutively recruited into the study in Guang, Anmen Hospital, Beijing, China. The diagnosis of coronary artery disease (CAD) was confirmed in all patients by coronary angiography showing at least one vessel disease (>50% narrowing of luminal diameter). UA patients were eligible to participate if they had met the American College of Cardiology/American Heart Association (ACC/AHA) criteria for UA [1]. All UA patients ( $n = 65$ ) had experienced ischemic chest pain within the preceding 48 h, including angina pectoris with an accelerating pattern, or prolonged duration (>20 min), or recurrent episodes at rest or within minimal effort, but with no evidence of enzymatic criteria. Transient ST-T segment depression and/or T-wave inversion were present in all cases. In patients undergoing percutaneous coronary intervention, all blood samples were taken before this procedure.

UA patients were diagnosed as BSS or non-BSS according to the grading system in quantifying BSS diagnosis standards. It had 33 items for diagnosis, including symptoms, signs, and laboratory tests (Table 1). Each item had an assigned point. If the grade of 33 items of the diagnostic scale of BSS in a patient was more than 19, the patient could be diagnosed as BSS. If the grade of 33 items in a patient was less than 19, the patient could be diagnosed as non-BSS. The severity of BSS of patients could be assessed by their grades. Although BSS was the major syndrome type of UA, UA also had other syndromes, such as phlegm, qi, or blood deficiency. This study just included UA patients who had only BSS in BSS group. The diagnosis about BSS of UA patients were made by 3 appointed TCM practitioners. Patients were included in the study only if the 3 practitioners reported consistent results. This ensured that all of the selected patients had typical manifestations of BSS.

Patients who had received thrombolytic therapy in the previous month were excluded from the study. Patients with stable angina, MI, heart failure, valvular heart disease, dilated cardiomyopathy, malignant tumor, advanced liver disease, renal failure, autoimmune diseases, and other inflammatory diseases and women who were pregnant and breast-feeding were excluded from the study. Control subjects ( $n = 20$ ) were healthy volunteers, recruited from the same population and the same area of China as the patients' group. Total RNAs isolated from 5 BSS of UA patients and 5 healthy volunteers were used for gene expression oligonucleotide microarray and microRNA microarray. The results obtained from bioinformatics analysis of microarray were then prospectively tested in a validation cohort of 30 BSS of UA patients,

TABLE 1: The grading system in quantifying blood stasis syndrome diagnosis standards.

Signs and symptom	Point
Purple tongue	(less severe) 8, (more severe) 10
Resistance to pressure in lower abdomen	(less severe) 8, (more severe) 10
Choppy pulse	10
Dark stool (Melena)	10
Pathogenic nodules	10
Distended veins under tongue	(less severe) 8, (more severe) 10
Irregular pulse	8
No pulse	10
Distended veins in abdominal wall	10
Hypodermal ecchymoses	(less severe) 8, (more severe) 10
Dark menstrual blood with clots	(less severe) 8, (more severe) 10
Persistent angina pectoris	10
General fixed pain	8
Dark red lips and gums	6
Small vessels	5
Numb extremities	5
Surgery history	5
Mucosal membrane of palate (+)	(less severe) 4, (more severe) 5
Paralysis in extremities	(less severe) 5, (more severe) 7
Psychiatric abnormality	(Irritability) 4, (Mania) 8
Rough skin	(less severe) 4, (more severe) 5
Complete blood viscosity (+)	10
Blood plasma viscosity (+)	5
External clot net weight (+)	10
External clot total weight (+)	8
Increase in platelet aggregation	10
Abnormality in blood clot elasticity	8
Microcirculation obstruction	10
Hemodynamics obstruction	10
Decrease in fiber dissolution activity	10
Resistance in platelet release	10
Pathogenic scan (+) for blood stasis	10
Blood vessel obstruction by new technology analysis	10

Grades <19 points are categorized as non-BSS. Grades 20–49 points are categorized as less severe BSS. Grades >50 points are categorized as more severe BSS [16, 22].

30 non-BSS of UA patients, and 15 healthy volunteers. The study complied with the Declaration of Helsinki and was approved by the local ethics committee. All individuals gave their written informed consent to participate in the study.

**2.2. Plasma Collection and RNA Isolation.** Whole blood samples (10 mL) were drawn from each of the 85 participants (35 UA patients with BSS, 30 non-BSS of UA patients and 20 healthy volunteers) with 19-gauge needle for clean venipuncture of an antecubital vein on the following morning after arrival. Timing of phlebotomy of UA patients compared with onset of chest pain was <24 h. Blood was drawn into EDTA-containing tubes and PBMCs were isolated by performing density gradient centrifugation with Ficoll (Invitrogen, Carlsbad, CA, USA). Total RNAs were extracted from PBMCs using Trizol reagent (Invitrogen) according to the manufacturer's instruction. RNA quantity and purity were assessed using NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). Pass criteria for absorbance ratios were established at  $A_{260}/A_{280} \geq 1.8$  and  $A_{260}/A_{230} \geq 1.5$  indicating acceptable RNA purity. RNA Integrity Number (RIN) values were ascertained using Agilent RNA 6000 Nano assay (Agilent Technologies, Santa Clara, CA, USA). Pass criteria for RIN value were established at  $\geq 6$  indicating acceptable RNA integrity. Genomic DNA contamination was evaluated by gel electrophoresis. The RNA samples were stored at  $-80^{\circ}\text{C}$  until analysis.

**2.3. mRNA Expression Profiling.** Total RNAs of 10 participants (5 UA patients with BSS and 5 healthy volunteers) were used for mRNA expression profiling by the Human Whole Genome OneArray v5 (Phalanx Biotech Group, Hsinchu, Taiwan). It contained 30275 DNA oligonucleotide probes, and each probe was a 60-mer designed in the sense direction. Among the probes, 29187 probes corresponded to the annotated genes in RefSeq v38 and Ensembl v56 database. Besides, 1088 control probes were also included. Fluorescent aRNA targets were prepared from 1 or 2.5  $\mu\text{g}$  total RNA samples using OneArray Amino Allyl aRNA Amplification Kit (Phalanx Biotech Group) and Cy5 dyes (Amersham Pharmacia, Piscataway, NJ, USA). Fluorescent targets were hybridized to the Human Whole Genome OneArray with Phalanx hybridization buffer using Phalanx Hybridization System. After 16 hrs hybridization at  $50^{\circ}\text{C}$ , nonspecific binding targets were washed away by three different washing steps (Wash I  $42^{\circ}\text{C}$  5 minutes; Wash II  $42^{\circ}\text{C}$  5 minutes,  $25^{\circ}\text{C}$  5 minutes; Wash III rinse 20 times), and the slides were dried by centrifugation and scanned by Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The intensities of each probe were obtained by GenePix 4.1 software (Molecular Devices). The raw intensity of each spot was loaded into Rosetta Resolver System (Rosetta Biosoftware, Seattle, WA, USA) to process data analysis. The error model of Rosetta Resolver System could remove both systematic and random errors from the data. Those probes with background signals were filtered out. Probes that passed the criteria were normalized by 50% median scaling normalization method. Normalized spot intensities were transformed to mRNA expression  $\log_2$  ratios between the UA patients with BSS and healthy controls.

**2.4. MicroRNA Expression Profiling.** We performed miRNA expression profiling in the same set of samples (5 UA patients with BSS and 5 healthy volunteers) that were used in

the analysis of mRNA microarray. MiRNA microarray analysis was performed by using the Human miRNA OneArray v4 (Phalanx Biotech Group). It contained triplicated 1884 unique miRNA probes from Human (miRBase Release v18) each printed in technical triplicate, and 144 experimental control probes. Small RNA was pre-enriched by Nanoseplook (Pall Corporation, Port Washington, NY, USA) from 2.5  $\mu$ g total RNA samples and labeled with miRNA ULS Labeling Kit (Kreatech Diagnostics, Vierweg, Amsterdam, The Netherlands). Labeled miRNA targets were hybridized to the Human miRNA OneArray v4 with OneArray Hybridization System. After 16 hrs hybridization at 37°C, nonspecific binding targets were washed away by three different washing steps (Wash I 37°C 5 minutes; Wash II 37°C 5 minutes, 25°C 5 minutes; Wash III rinse 20 times), and the slides were dried by centrifugation and scanned by an Axon 4000B scanner (Molecular Devices). The Cy5 fluorescent intensities of each probe were analyzed by GenePix 4.1 software (Molecular Devices). The raw intensity of each probe was processed by R program. Probes that passed the criteria were normalized by 75% median scaling normalization method. Normalized spot intensities were transformed to miRNA expression  $\log_2$  ratios between the UA patients with BSS and healthy controls.

## 2.5. Integrated Bioinformatics Analysis of the mRNA and MicroRNA Expression Profiles

**2.5.1. Identification of Differentially Expressed mRNAs and miRNAs.** The expressions of mRNAs with  $\log_2$  ratio  $\geq 1$  or  $\log_2$  ratio  $\leq -1$  and  $P$  value  $< 0.05$  were defined as differential mRNAs. The expressions of miRNAs with  $\log_2$  ratio  $\geq 0.8$  or  $\log_2$  ratio  $\leq -0.8$  and  $P$  value  $< 0.05$  were defined as differential miRNAs. Hierarchical clustering analysis combined with a heatmap was applied to evaluate the overall reproducibility and variation of 5 samples within each group and the differences between the 2 groups. An average linkage hierarchical clustering was performed with clustering software Cluster3.0 and Java TreeView-1.1.6r2 was applied to generate the heatmap [28, 29].

**2.5.2. KEGG Pathway Analysis Using DAVID.** There were thousands of deregulated genes; we cannot analyze the function of all these deregulated genes one by one, so we could only focus on the main functions of deregulated genes. We used DAVID Bioinformatics Resources 6.7 (the Database for Annotation, Visualization, and Integrated Discovery) [30], a comprehensive set of functional annotation tools for understanding the biological meaning behind large lists of genes, to identify enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways [31] information for differential mRNAs between UA patients with BSS and healthy controls. EASE Score, a modified Fisher Exact  $P$  Value, was used to measure the gene enrichment in annotation pathways and Benjamini-Hochberg False Discovery Rate was used as testing correction. The threshold of EASE Score was set 0.1. Because miRNAs were usually negatively correlated with target mRNAs and differentially expressed miRNAs were mainly upregulated, pathways enriched of downregulated

mRNAs were chose for further analysis and downregulated mRNAs were marked in green on these pathways by Search & Color Pathway in KEGG [31]. MiRNAs which could regulate downregulated genes of these pathways were predicted by one of the 2 algorithms of DIANA-microT and TargetsCan [32, 33]. If these predicted miRNAs were among the actually upregulated miRNAs, they were marked in red on these pathways.

**2.5.3. Integrated miRNA/mRNA Network Analysis.** MiRNAs were usually negatively correlated with target mRNAs and differentially expressed miRNAs in BBS with UA group were mainly upregulated, so upregulated miRNAs and downregulated mRNAs were uploaded into the miRTrail, a knowledge-based tool for integrative network analysis that allowed for studying the interactions between microRNAs and their target mRNAs [34]. For each upregulated miRNA, the target mRNAs were predicted by the respective web-resource microCosm and the prediction threshold was set at 0.01. The predicted target mRNAs were compared with the actually downregulated mRNAs in order to find the overlap between them. The miRNAs that could regulate these overlap mRNAs were ranked based on the number of deregulated target mRNAs and the top 5% were selected. The interactive network of selected miRNAs and actually deregulated target mRNAs was visualized by the network analyzers and viewers BiNA [35].

**2.5.4. Protein-Protein Interactions Network Analysis.** In the integrated miRNA/mRNA network, there were hundreds of upregulated mRNAs. To further understand the function of these miRNAs, we use a method of combing the protein-protein interactions (PPIs) network and hubs. Protein-Protein Interactions (PPIs) were commonly understood as physical contacts with molecular docking between proteins that occur in a cell or in a living organism *in vivo* [36]. Each of these interactions was specifically adapted to carry out certain biological functions. A PPIs network was represented as proteins as nodes and interactions between nodes are edges. To further understand the function of the upregulated miRNAs, a PPIs network for mRNAs in the miRNA/mRNA interactive network was built by Reactome FI, a Cytoscape plugin [37]. This plugin accessed the Reactome Functional Interaction (FI) network, a highly reliable, manually curated pathway-based protein functional interaction network covering close to 50% of human proteins, and allowed you to construct an FI subnetwork based on a set of genes by using linker genes. The PPIs network was visualized using the Cytoscape software [38].

Hubs were highly connected nodes in a network and vital for the proper function of a network [39]. CytoHubba, a Cytoscape plugin, was used to find the hubs of the PPIs network [40]. It evaluated node essentiality by topological characters. The degree of a node was the number of links incident to this node in a network and it was chose for topological analysis in the study. Top 10 essential nodes ranked by degree scores were selected as hubs from the network.

TABLE 2: The forward primers of miRNAs for amplification.

Accession	Name		Sequence
MIMAT0002809	hsa-miR-146b-5p	Forward	5'-TGAGAACTGAATTCCATAGGCT-3'
MIMAT0000231	hsa-miR-199a-5p	Forward	5'-CCCAGTGTTCAGACTACCTGTTC-3'
X07425.1	Human U6 snRNA	Forward	5'-CGCAAGGATGACACGCAAATTC-3'

TABLE 3: The primers of mRNAs for amplification.

Accession	Name		Sequence
NM_004343.3	CALR	Forward	5'-GGCTATGTGAAGCTGTTTCCTAAT-3'
		Reverse	5'-GTTCTTGCCCTTGTAGTTGAAGAT-3'
BC003596.1	TP53	Forward	5'-GGAGTAGGACATACCAGCTTAGATTT-3'
		Reverse	5'-TACCTAGAATGTGGCTGATTGTAAAC-3'
NG_007073.2	GAPDH	Forward	5'-CAAAGTTGTCATGGATGACC-3'
		Reverse	5'-CCATGGAGAAGGCTGGG-3'

**2.6. Reverse Transcription and qRT-PCR of miRNA and mRNA.** Combining pathway analysis with network analysis, hsa-miR-146b-5p and has-miR-199a-5p seemed to be the most involved miRNAs in UA patients with BSS. Thus, to support the robustness of our analysis, differences in expression of the 2 miRNAs and 2 downregulated target mRNAs (CALR and TP53) were validated in an independent cohort of 30 UA patients with BSS, 30 non-BSS of UA patients, and 15 healthy controls by qRT-PCR. To assay for miRNAs, 1  $\mu$ g of purified total RNA generated cDNA using the QuantiMir RT Kit, following the manufacturer's instructions (System Biosciences, Mountain View, CA, USA). qRT-PCR was performed using 2X SYBR Green qPCR Mastermix (Roche Applied Science, Indianapolis, IN, USA) 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The mature sequences of hsa-miR-146b-5p and has-miR-199a-5p were used as the forward primers, and the 3' universal reverse primer provided from the QuantiMir RT Kit (System Biosciences) was used as the reverse primer. The human U6 RNA was amplified in parallel as the internal control. All the miRNA forward primer sequences were listed in Table 2. The Mastermix contents included 10  $\mu$ L 2X SYBR Green qPCR Mastermix buffer, 2  $\mu$ L miRNA-specific forward primer, 1  $\mu$ L universal reverse primer, 1  $\mu$ L diluted QuantiMir cDNA and 6  $\mu$ L RNase-free water. The thermal cycling conditions were at 95°C for 5 minutes, followed by 40 cycles of at 95°C for 30 seconds, at 55°C for 30 seconds, 72°C for 50 seconds, and a final extension at 72°C for 8 minutes. For mRNA expression analysis, 2  $\mu$ g of purified total RNA generated cDNA using RevertAid First Strand cDNA Synthesis Kit, following the manufacturer's instructions (Thermo Scientific). qRT-PCR was performed using 2X SYBR Green qPCR Mastermix (Roche Applied Science) 7900HT Fast Real-Time PCR System (Applied Biosystems). GAPDH was used as the internal control. All the mRNA primer sequences were listed in Table 3. The Mastermix contents included 10  $\mu$ L 2X SYBR Green qPCR Mastermix buffer, 1  $\mu$ L mRNA-specific forward primer, 1  $\mu$ L mRNA-specific reverse primer, 1  $\mu$ L cDNA, and 7  $\mu$ L RNase-free water. The thermal cycling conditions were at 95°C for 5 minutes, followed by 35 cycles

of at 95°C for 35 seconds, at 54°C for 35 seconds, 72°C for 50 seconds and a final extension at 72°C for 8 minutes. The reaction products were analyzed by electrophoresis in 3% agarose gels to confirm specificity. Analysis was performed by relative standard curve method for quantification [41].

**2.7. Statistics.** All results for continuous variables were expressed as means  $\pm$  SEM, if not stated otherwise. For group-wise comparisons, Man-Whitney test (2 groups), ANOVA, Kruskal-Wallis test ( $n$  groups), or Student's  $t$ -test (2 groups) were used as appropriate. For categorical variables, the Chi-square test or Fischer's exact test was used. All tests were performed 2-sided and a significance level of  $P < 0.05$  was considered to indicate statistical significance. For all statistical analyses, the statistical software SPSS 16.0 (Statistical Package for the Social Sciences, Chicago, IL, USA) for Windows was used. GraphPad Prism 5 (GraphPad software, San Diego, CA, USA) was used to draw bar and box charts.

### 3. Results

**3.1. Basic Clinical Characteristics of Subjects.** A total of 85 subjects were studied. 35 UA patients with BSS and 30 UA patients with non-BSS had angiographically documented CAD. 20 healthy volunteers were selected as the healthy controls. 5 UA patients with BSS and 5 healthy volunteers were used for mRNA and miRNA microarray analysis. The clinical characteristics of the 2 groups of microarray analysis were summarized in Table 4. 30 UA patients with BSS, 30 UA patients with non-BSS and 15 healthy volunteers were used for qRT-PCR. The clinical characteristics of the 3 groups of qRT-PCR were summarized in Table 5. There were no significant differences in age, percentage of males, BMI (body mass index), percentage of active smoker, history of type 2 diabetes mellitus, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, and calcium-channel blockers medication between UA patients with BSS and healthy control group in microarray analysis. There were significant differences in other clinical parameters between the two

TABLE 4: Clinical characteristics of subjects of microarray analysis.

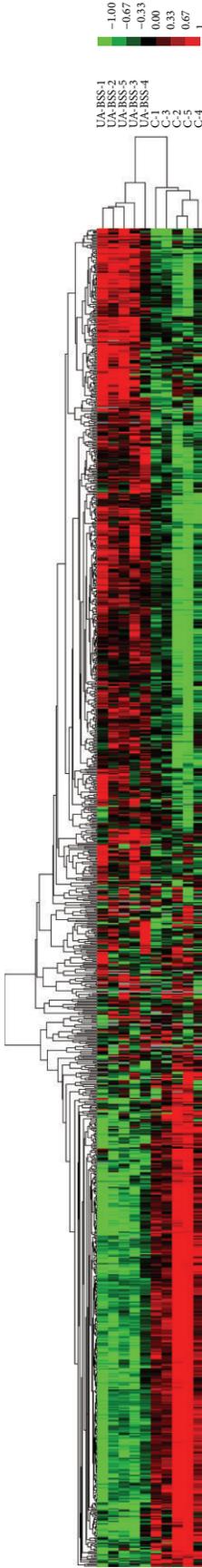
	UA with BSS ( <i>n</i> = 5)	Controls ( <i>n</i> = 5)	<i>P</i> value
Male gender ( <i>n</i> )	3 (60%)	3 (60%)	
Age (years)	61.60 ± 4.93	61.00 ± 4.36	0.596
BMI (kg/m <sup>2</sup> )	23.18 ± 3.89	23.24 ± 1.62	0.975
Active smoker ( <i>n</i> )	1 (20%)	1 (20%)	
BSS grades	72.80 ± 10.47	0	
Number of vessels ( <i>n</i> )	3.40 ± 0.89	0	
Hypertension ( <i>n</i> )	4 (80%)	0	0.048
Type 2 diabetes mellitus ( <i>n</i> )	0	0	
Total cholesterol (mmol/L)	3.96 ± 1.45	4.02 ± 0.79	0.347
LDL cholesterol (mmol/L)	2.33 ± 1.01	2.51 ± 0.60	0.743
HDL cholesterol (mmol/L)	1.33 ± 0.35	1.30 ± 0.30	0.874
Triglycerides (mmol/L)	1.15 ± 0.50	1.09 ± 0.34	0.815
CRP (mg/L)	3.64 ± 3.25	0.94 ± 0.09	0.018
Concurrent medication ( <i>n</i> )			
Antiplatelet	100%	0	0.008
Beta-blocker	4 (80%)	0	0.048
ACEI/ARB	4 (80%)	0	0.048
Calcium-channel blockers	3 (60%)	0	0.167
Nitrates	4 (80%)	0	0.048
Statin	4 (80%)	0	0.048

Data represents means ± SEM. Abbreviations: ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; BMI: body mass index; BSS: blood stasis syndrome; HDL: high-density protein; CRP: C-reactive protein; LDL: low-density protein.

TABLE 5: Clinical characteristics of subjects of qRT-PCR.

	UA with BSS ( <i>n</i> = 30)	UA with non-BSS ( <i>n</i> = 30)	Controls ( <i>n</i> = 15)	<i>P</i> value
Male gender ( <i>n</i> )	15 (50%)	15 (50%)	8 (53.3%)	0.974
Age (years)	57.06 ± 5.62	57.67 ± 6.43	56.87 ± 6.70	0.896
BMI (kg/m <sup>2</sup> )	24.58 ± 1.52	24.10 ± 2.13	23.63 ± 1.52	0.231
Active smoker ( <i>n</i> )	17 (56.7%)	18 (60%)	6 (40%)	0.429
BSS Grades	73.93 ± 2.91	14.03 ± 2.22	0	
Number of vessels ( <i>n</i> )	1.53 ± 0.94	1.57 ± 0.95	0	
Hypertension ( <i>n</i> )	22 (73.3%)	23 (76.7%)	0	0.000
Type 2 diabetes mellitus ( <i>n</i> )	14 (46.7%)	15 (50%)	0	0.001
Total cholesterol (mmol/L)	4.19 ± 0.83	4.19 ± 0.95	3.71 ± 0.76	0.172
LDL cholesterol (mmol/L)	2.60 ± 0.59	2.55 ± 0.58	2.37 ± 0.54	0.436
HDL cholesterol (mmol/L)	1.13 ± 0.21	1.12 ± 0.21	1.31 ± 0.29	0.032
Triglycerides (mmol/L)	1.79 ± 0.89	1.89 ± 1.04	1.03 ± 0.27	0.001
CRP (mg/L)	3.93 ± 2.21	3.98 ± 2.26	2.73 ± 2.05	0.122
Concurrent medication ( <i>n</i> )				
Antiplatelet	100%	28 (93.3%)	0	0.000
Beta-blocker	20 (66.7%)	20 (66.7%)	0	0.000
ACEI/ARB	18 (60%)	16 (53.3%)	0	0.000
Calcium-channel blockers	13 (43.3%)	15 (50%)	0	0.001
Nitrates	22 (73.3%)	20 (66.7%)	0	0.000
Statin	26 (86.7%)	24 (80%)	0	0.000

Data represents means ± SEM. Abbreviations: ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; BMI: body mass index; BSS: blood stasis syndrome; HDL: high-density protein; CRP: C-reactive protein; LDL: low-density protein.



(a)

FIGURE 1: Continued.

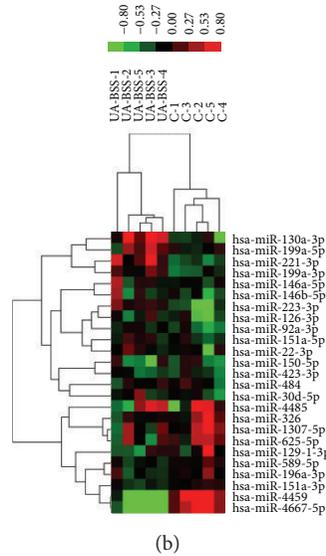


FIGURE 1: Heat maps of mRNAs and miRNAs differentially expressed between UA patients with BSS and healthy controls. (a) Heat map and cluster analysis of the 1,206 differentially expressed probes between UA patients with BSS and healthy control. Red and green represented, respectively, differentially upregulated and downregulated mRNAs in UA patients with BSS. (b) Heat map and cluster analysis of the 25 differentially expressed miRNAs between UA patients with BSS and healthy control. Red and green represented, respectively, differentially upregulated and downregulated miRNAs in UA patients with BSS. Gray was for missing values. UA-BSS: unstable angina patients with Blood stasis syndrome; C: control group.

groups in microarray analysis ( $P < 0.05$ ). There were no significant differences in age, percentage of males, BMI (body mass index), percentage of active smoker, total cholesterol, LDL cholesterol and CRP among UA patients with BSS, 30 UA patients with non-BSS and healthy control group in qRT-PCR analysis. There were significant differences in other clinical parameters among the 3 groups in qRT-PCR analysis ( $P < 0.05$ ). There were no significant differences in HDL cholesterol, triglycerides (TG), hypertension, concurrent medication, and number of vessels between UA patients with BSS and UA patients with non-BSS in qRT-PCR analysis. There was significant difference in BSS grades between UA patients with BSS and UA patients with non-BSS in qRT-PCR analysis ( $P < 0.05$ ).

**3.2. Identification of Differentially Expressed mRNAs and miRNAs.** A list of 1081 mRNAs was identified as differentially expressed between UA patients with BSS and the healthy controls (Figure 1(a)): 673 (56%) overexpressed and 408 (44%) underexpressed. The 1081 mRNAs were corresponding to 1206 nonunique probes because multiple probes in the microarray platform could be representative of a single mRNA. A list of 25 miRNAs was identified as differentially expressed between UA patients with BSS and the healthy control (Figure 1(b), Table 6): 23 (92%) overexpressed and 2 (8%) underexpressed.

**3.3. Pathway Analysis Using DAVID.** The KEGG pathway analysis of upregulated and downregulated mRNAs between UA patients with BSS and the healthy controls using DAVID

was shown in Tables 7 and 8. Upregulated genes were enriched in 7 pathways and downregulated genes were enriched in 6 pathways. Among 7 pathways enriched of upregulated genes, NOD-like receptor signaling pathway, apoptosis pathway, and cytokine-cytokine receptor interaction pathway were closely related with UA. In the NOD-like receptor signaling pathway, ERBB2IP, BIRC2, BIRC3, TNFAIP3, RIPK2, CASP8, TAB2, CXCL1 and IL1B were upregulated. (Figure 2) In the apoptosis pathway, IL1A, IL1B, IL1RAP, IRAK3, PRKAR2B, BIRC2, BIRC3, BCL2, and CASP8 were upregulated. (Figure 3) In the cytokine-cytokine receptor interaction pathway, IL1A, IL1B, IL1RAP, IL7R, IL21R, CCRI, CXCR4, CXCL1, CXCL3, CXCL5, PF4V1, CCL20, IFNG, IFNGRI, OSM and ACVR2A were upregulated. (Figure 4) Among 6 pathways enriched of downregulated genes, the antigen processing and presentation pathway and p53 signaling pathway were closely related with UA, so the 2 pathways were chose for next analysis. Upregulated miRNAs which could regulate downregulated genes of the 2 pathways were analyzed by one of the 2 algorithms of DIANA-mT and Targetscan and mapped on the 2 pathways. In the antigen processing and presentation pathway, CALR, HLA-DRB1, HLA-DRB5, KLRC1, KLRC3, KIR3DS1 and KIR2DS3 were downregulated. CALR could be the target gene of miR-146a-5p, miR-146b-5p, miR-326, miR-589 and miR-625. HLA-DRB1 and HLA-DRB5 could be the target gene of miR-129-3p. KLRC1, KLRC3, KIR3DS1 and KIR2DS3 could be the target gene of miR-223. (Figure 5(a)) In the p53 signaling pathway, TP53, CDK4, STEAP3, SHISA5 and SESN2 were downregulated. TP53 could be the target gene of

TABLE 6: Differentially expressed miRNAs in UA patients with BSS versus healthy controls.

ID	Name	UA with BSS/control ratio	P value
PH_mr_0001929	hsa-miR-221-3p	1.58	0.0440
PH_mr_0000996	hsa-miR-130a-3p	1.55	0.0091
PH_mr_0001948	hsa-miR-199a-5p	1.41	0.0438
PH_mr_0003258	hsa-miR-223-3p	1.38	0.0324
PH_mr_0002734	hsa-miR-199a-3p	1.36	0.0171
PH_mr_0000023	hsa-miR-126-3p	1.27	0.0443
PH_mr_0004749	hsa-miR-4485	1.24	0.0443
PH_mr_0000176	hsa-miR-146a-5p	1.18	0.0328
PH_mr_0000566	hsa-miR-151a-5p	1.17	0.0387
PH_mr_0000751	hsa-miR-146b-5p	1.15	0.0227
PH_mr_0000446	hsa-miR-22-3p	1.15	0.0421
PH_mr_0001186	hsa-miR-150-5p	0.95	0.0165
PH_mr_0008005	hsa-miR-1307-5p	0.94	0.0051
PH_mr_0001511	hsa-miR-151a-3p	0.94	0.0147
PH_mr_0000436	hsa-miR-92a-3p	0.94	0.0301
PH_mr_0001750	hsa-miR-589-5p	0.94	0.0188
PH_mr_0001629	hsa-miR-484	0.88	0.0406
PH_mr_0001844	hsa-miR-326	0.87	0.0068
PH_mr_0001893	hsa-miR-30d-5p	0.86	0.0228
PH_mr_0000911	hsa-miR-129-1-3p	0.86	0.0379
PH_mr_0001753	hsa-miR-196a-3p	0.85	0.0159
PH_mr_0003163	hsa-miR-423-3p	0.83	0.0203
PH_mr_0002404	hsa-miR-625-5p	0.80	0.0360
PH_mr_0004525	hsa-miR-4459	-0.88	0.0225
PH_mr_0004803	hsa-miR-4667-5p	-1.02	0.0419

TABLE 7: The pathways related to the upregulated mRNAs between UA patients with BSS and healthy controls.

KEGG pathway	Number of genes	P value
hsa04621: NOD-like receptor signaling pathway	9	0.0024
hsa04210: apoptosis	9	0.0182
hsa03040: spliceosome	11	0.0228
hsa04640: hematopoietic cell lineage	8	0.0469
hsa05130: pathogenic Escherichia coli infection	6	0.0672
hsa03018: RNA degradation	6	0.0672
hsa04060: cytokine-cytokine receptor interaction	16	0.0790

miR-129-3p, miR-130a, miR-1307, miR-151-3p, miR-199a-3p, miR-199a-5p, miR-22, miR-221, miR-223, miR-30d, miR-326, miR-484, miR-589, miR-625 and miR-92a. CDK4 could be the target gene of miR-326 and miR-625. STEAP3 could be the target gene of miR-129-3p, miR-1307, miR-199a-3p, miR-199a-5p, miR-223, miR-326, miR-625 and miR-92a. SESN2 could be the target gene of miR-130a, miR-150, miR-199a-5p, miR-22, miR-221, miR-223, miR-326, miR-484 and miR-589 (Figure 5(b)).

TABLE 8: The pathways related to the downregulated mRNAs between UA patients with BSS and healthy controls.

KEGG pathway	Number of genes	P value
hsa05332: graft-versus-host disease	5	0.0121
hsa04612: antigen processing and presentation	7	0.0121
hsa04115: p53 signaling pathway	5	0.0722
hsa00280: valine, leucine, and isoleucine degradation	4	0.0812
hsa00510: N-Glycan biosynthesis	4	0.0901
hsa00532: chondroitin sulfate biosynthesis	3	0.0913

3.4. *Integrated miRNA/mRNA Network Analysis.* 23 upregulated miRNAs and 408 downregulated mRNAs were uploaded into miRTrail. 4250 target mRNAs were predicted by microCosm. 115 mRNAs were found to be the overlap between the predicted target mRNAs and the actually downregulated mRNAs. 6 upregulated miRNAs were selected according to the top 5% of the rank based on the number of deregulated target mRNAs. The interactive network of 115 downregulated mRNAs and 6 upregulated miRNAs was

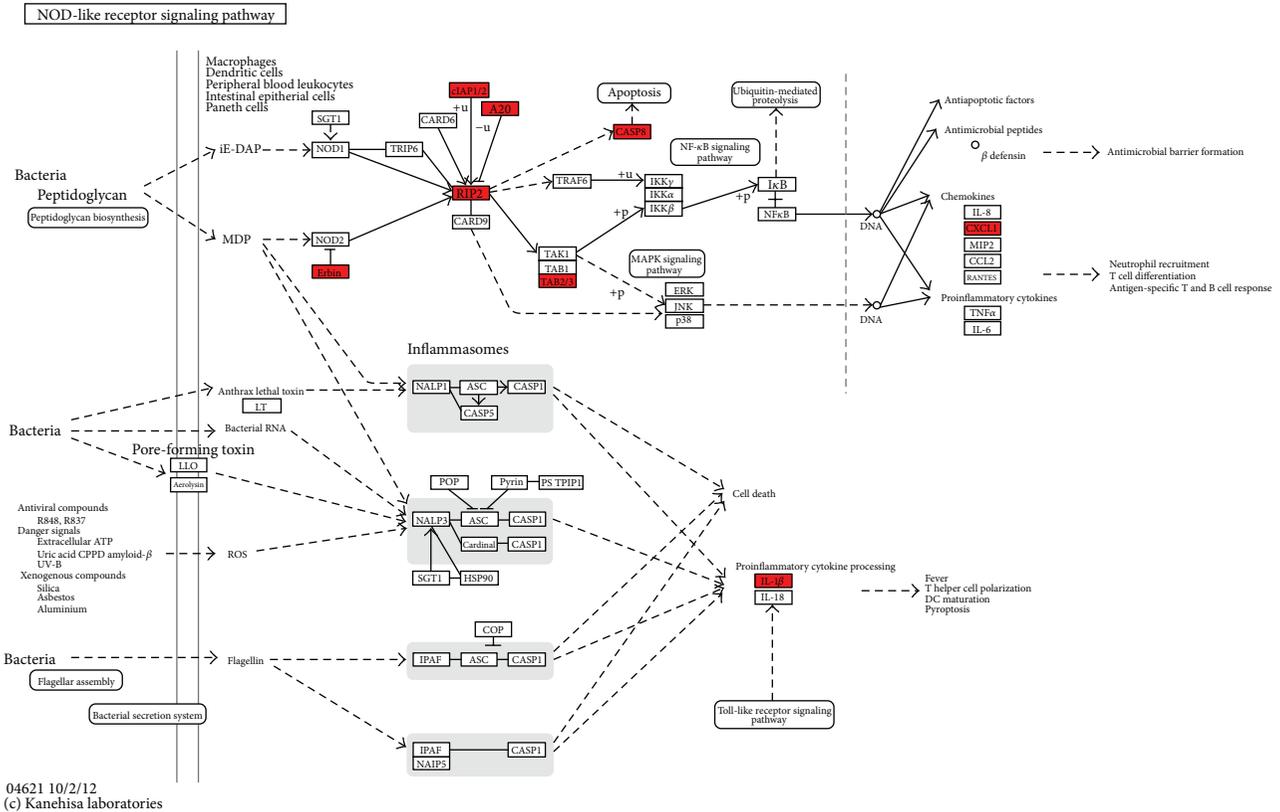


FIGURE 2: NOD-like receptor signaling pathway. Upregulated mRNAs were depicted in red by Search & Color Pathway in KEGG.

visualized by BiNA. The 6 upregulated miRNAs were miR-146b-5p, miR-199a-3p, miR-199a-5p, miR-326, miR-423-3p and miR-484 (Figure 6).

**3.5. Protein-Protein Interactions Network Analysis.** Based on the integrated miRNA/mRNA network analysis results, a PPIs network for 115 downregulated mRNAs was built by Reactome FI plugin (Figure 7). It included 124 nodes and 291 edges. Hubs of the PPIs network were found by CytoHubba. Hubs were MAPK14, AKT1, EP300, HDAC1, TP53, E2F1, SMAD3, GNB1, MYC, and SRC (Figure 8). The degrees of hubs were listed in Table 9.

**3.6. qRT-PCR of miRNA and mRNA.** Representative qRT-PCR results for hsa-miR-146b-5p, has-miR-199a-5p, CALR, and TP53 were reported in Figure 9. Hsa-miR-146b-5p and has-miR-199a-5p were upregulated, while CALR and TP53 were downregulated in UA patients with BSS compared to UA patients with non-BSS or the healthy control ( $P < 0.05$ ). These results confirmed our bioinformatics analysis.

**4. Discussion**

In this study, using a systems biology approach we exploited critically deregulated miRNAs and mRNAs involved in pathogenesis of BSS of UA and potential biomarkers for

TABLE 9: The degrees of hubs of the PPI network.

Genes	Degree	Genes	Degree
MAPK14	20	E2F1	14
AKT1	18	SMAD3	14
EP300	17	GNB1	14
HDAC1	17	MYC	14
TP53	16	SRC	14

BSS of UA. Our integration strategy started by extracting differentially expressed mRNAs and miRNAs between UA patients with BSS and the healthy control from the microarray analysis and then took them to integrative bioinformatics analysis. The robustness of our analysis was confirmed by qRT-PCR.

**4.1. mRNA Expression Data.** Regarding thousands of deregulated genes, we cannot analyze the function of all these deregulated genes one by one, so we only focused on the function of deregulated genes enriched in KEGG pathways. The function of deregulated genes in the pathways related with UA would be discussed in the following part to understand the biomedical mechanisms of BSS of UA.

Among upregulated genes of NOD-like receptor signaling pathway (Figure 2), RIPK2 was a key mediator of the activation of apoptosis pathway [42]. Apoptosis was known

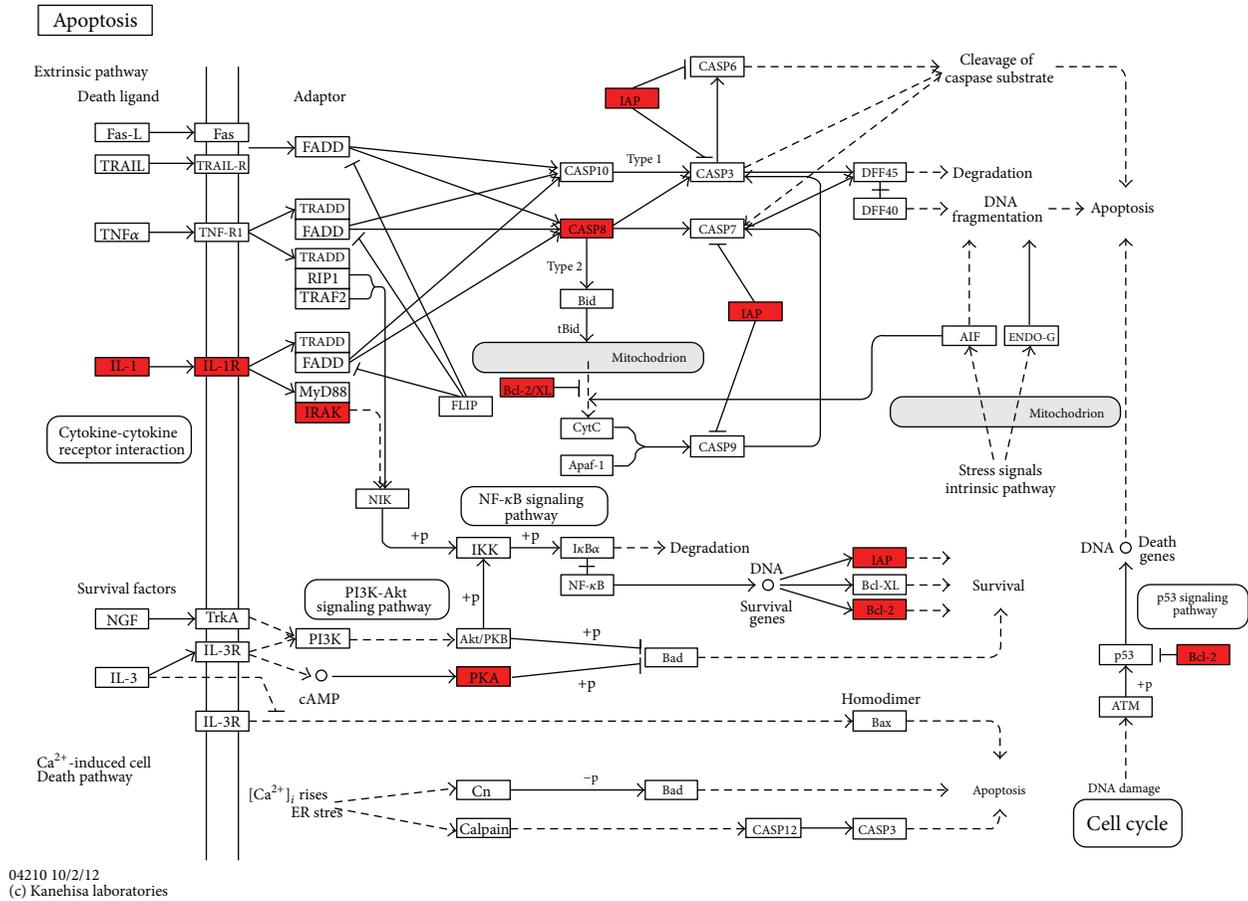
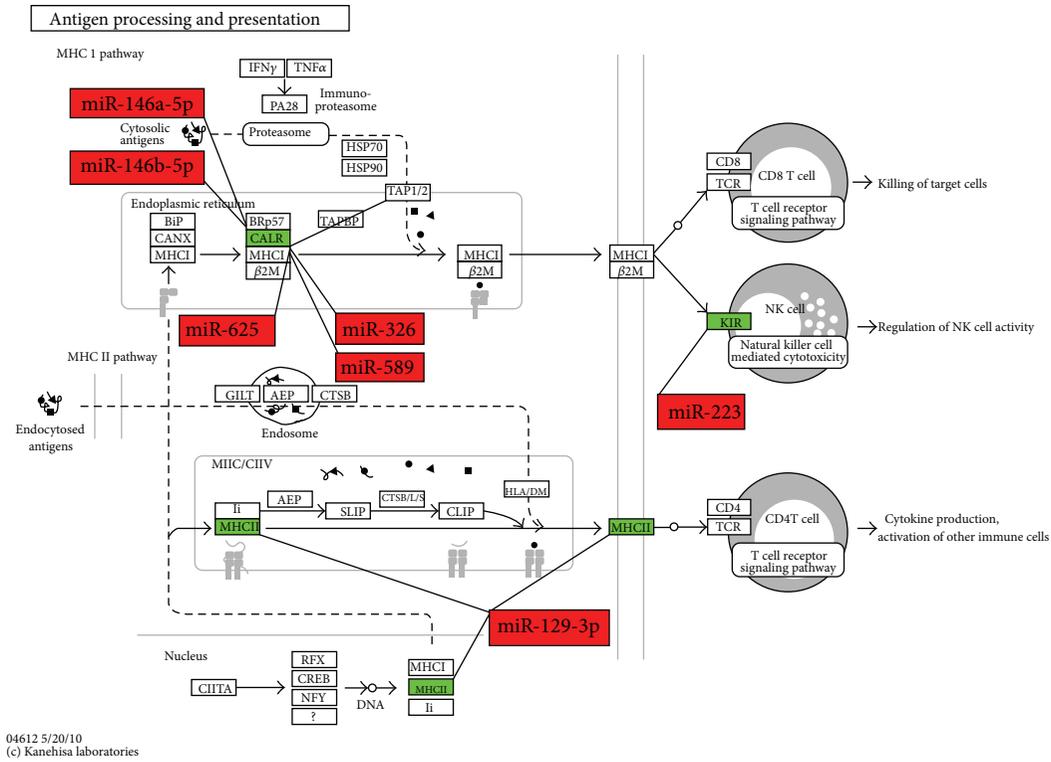


FIGURE 3: Apoptosis pathway. Upregulated mRNAs were depicted in red by Search & Color Pathway in KEGG.

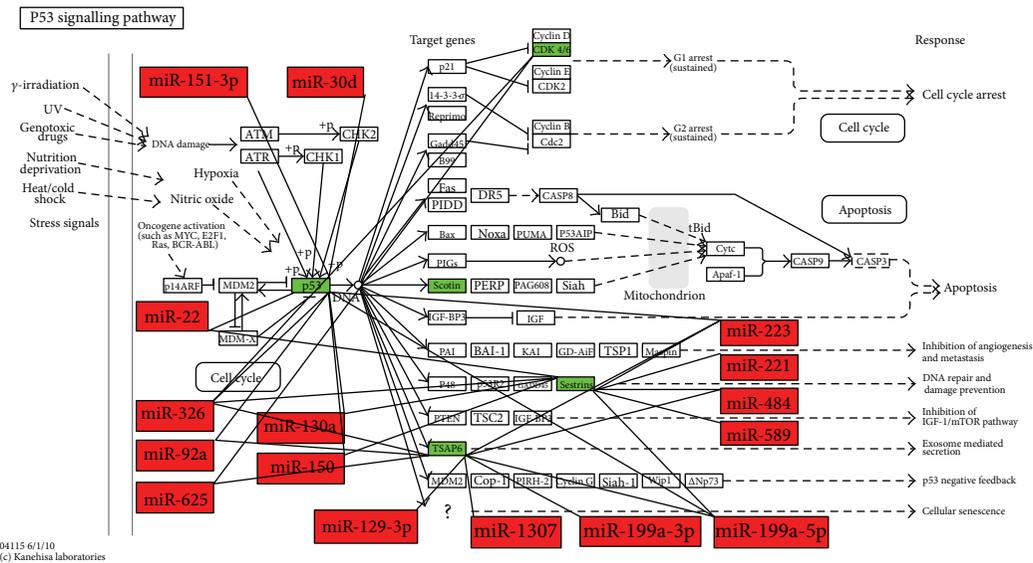
to occur in advanced plaques at higher levels, comparing both patients with stable angina and control subjects [43, 44]. The apoptosis of vascular smooth muscle cells (VSMCs) and macrophages altered plaque composition and made it prone to disruption and acute luminal thrombosis [45, 46]. CASP8, an important executor of apoptosis [47], was upregulated in NOD-like receptor signaling pathway. The upregulation of CASP8 indicated active apoptosis in BSS of UA. RIPK2 was critical for both the innate and adaptive immune pathways [48]. However, there were fewer studies about the role of RIPK2 in UA. Our study showed that RIPK2 may contribute to the apoptosis in BSS of UA by activating CASP8. This indicated the innate and adaptive immunity may regulate the apoptosis in BSS of UA. Except RIPK2, CXCL1, and IL1B were also key upregulated genes in the NOD-like receptor signaling pathway. Studies showed that CXCL1 could support arrest of human monocytic cell lines and primary monocytes under flow conditions and may play an important role in monocyte recruitment to atherosclerotic lesions [49]. IL-1B was a proinflammatory cytokine with pleiotropic effects implicated in the various stages of atherosclerosis [50, 51]. Studies showed that IL-1B was at higher levels in UA patients, comparing both stable angina ones and control subjects in peripheral blood [52, 53]. Both CXCL1 and IL1B were important proinflammatory

mediators, and they promoted the inflammation. Inflammation can regulate the integrity of the interstitial collagen of the plaque's fibrous cap and be responsible for plaque rupture and triggering UA [54]. The upregulation of CXCL1 and IL1B may promote inflammation in BSS of UA. In the apoptosis pathway, the effect of upregulation of IL1B and CASP8 was discussed. IL1A, IL1B, and IL1RAP all belonged to the IL-1 family. IL-1 was proinflammatory and destabilized the proteinaceous scaffold of the cap by inducing up-regulation of matrix metalloproteinases [55]. The upregulation of IL-1 and IL1RAP may promote the inflammation in BSS of UA. IRAK3 was a kinase-deficient member of the TLR/IRAK family that was an important negative regulator of TLR signaling and regulated innate immune homeostasis [56]. The latest research showed that IRAK3 was a key inhibitor of TLR2/NF-κB mediated chronic inflammation [57]. Although the role of IRAK3 in UA was not defined, our study showed that the upregulation of IL-1 may activate IRAK3 to regulate innate immunity and inhibit inflammation. PRKAR2B was one of the regulatory subunits bound to cAMP. The activation of cAMP/PKA pathway induced the phosphorylation and inactivation of BAD, a proapoptotic protein [58]. BIRC2 and BIRC3 were members of the inhibitor of apoptosis protein (IAP) family [59]. BCL2 was an antideath factor, and it inhibited the apoptosis of VSMCs and macrophages in advanced





(a) Antigen processing and presentation pathway



(b) P53 signaling pathway

FIGURE 5: The regulation of upregulated miRNAs on the downregulated mRNAs in antigen processing and presentation pathway and p53 signaling pathway. Upregulated miRNAs were depicted in red and downregulated mRNAs were depicted in green on the maps.

were members of natural killer (NK) cell lectin-like receptors subfamily C. KIR3DS1 and KIR2DS3 were members of natural killer (NK) cell immunoglobulin-like receptors. These NK cell receptors played an important role in regulation of the immune response [78]. NK cells were identified in human and mouse atherosclerotic lesions and infiltrated the vessel wall and promoted atherosclerotic lesion development [79]. The downregulation of these NK cell receptors may

indicate the suppression of immune response mediated by NK cells in BBS of UA. In the p53 signaling pathway, TP53 (p53) responded to diverse cellular stresses to regulate target genes that induced cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism [80]. The increased TP53 expression was related with the enlargement of necrotic cores, plaque rupture, and clinical manifestations of carotid plaques. Concomitant increases of TP53 level may lead to

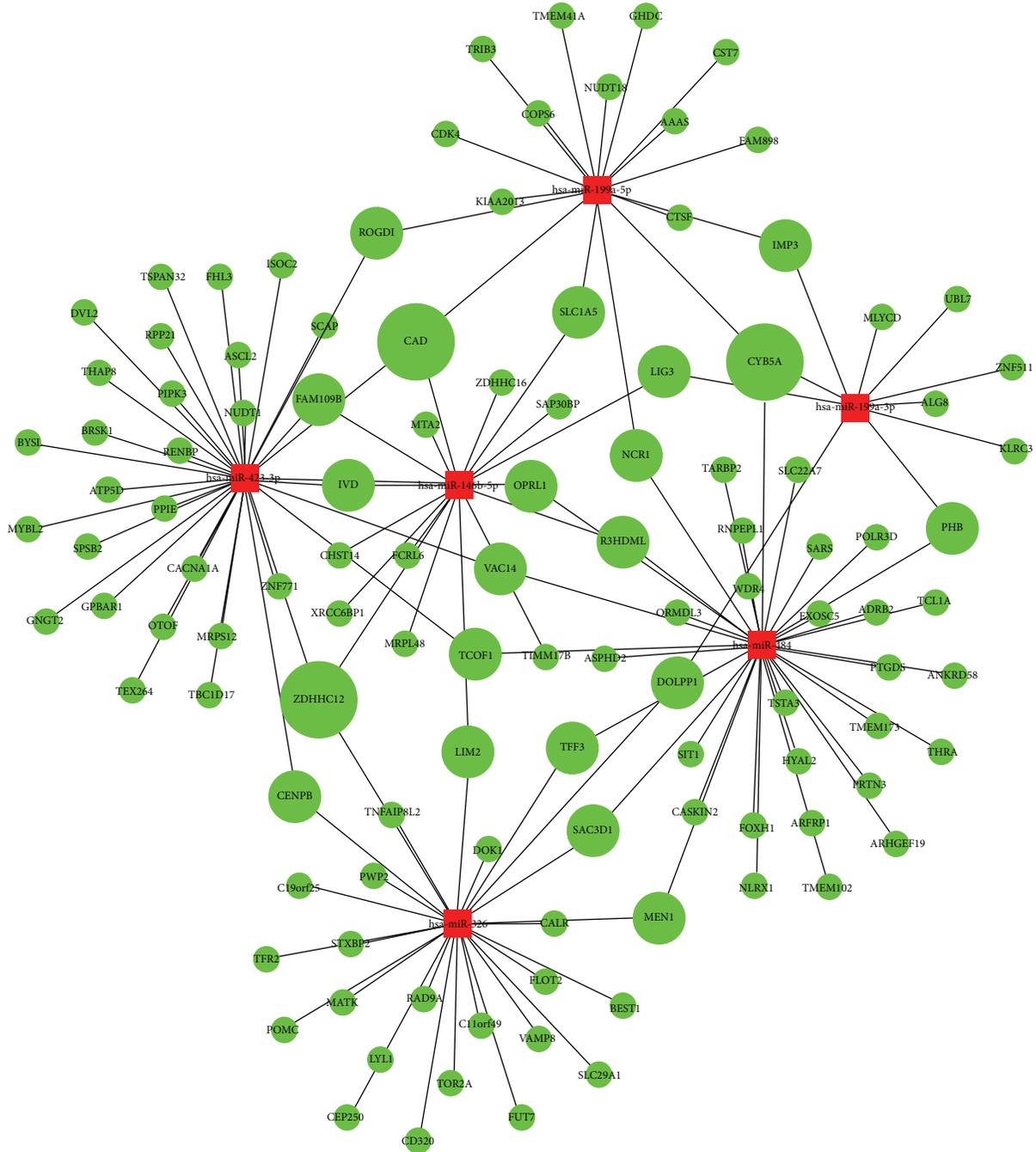


FIGURE 6: The miRNA/mRNA interactive network. Round represented mRNAs and rectangular represented miRNAs. Red color indicated upregulation and green color downregulation, respectively. Sizes of the mRNAs were according to their degrees.

the apoptosis and atheroma progression in patients with carotid atherosclerosis [81]. The downregulation of TP53 may inhibit the apoptosis in BSS of UA. CDK4 was a catalytic subunit of the cyclin-dependent kinase. In proliferating cells, the activation of CDK4 was necessary for cell cycle progression [82]. Proliferation of VSMCs and macrophages was observed in atherosclerotic plaques [83]. There was evidence that oxLDL-stimulated VSMC proliferation was associated with significant increases in the expression of CDK4 [84].

The downregulation of CDK4 may inhibit cell proliferation in BSS of UA. STEAP3 regulated apoptosis and the cell cycle [85]. SHISA5 encoded a protein named Scotin. Scotin induced apoptosis by causing cell cycle arrest [86]. SESN2 played a role in the regulation of cell growth and survival [87]. There was still lack of research about the function of STEAP3, SHISA5, and SESN2 in UA. Whether these genes involved in the regulation of apoptosis of BSS in UA was worth further exploring.

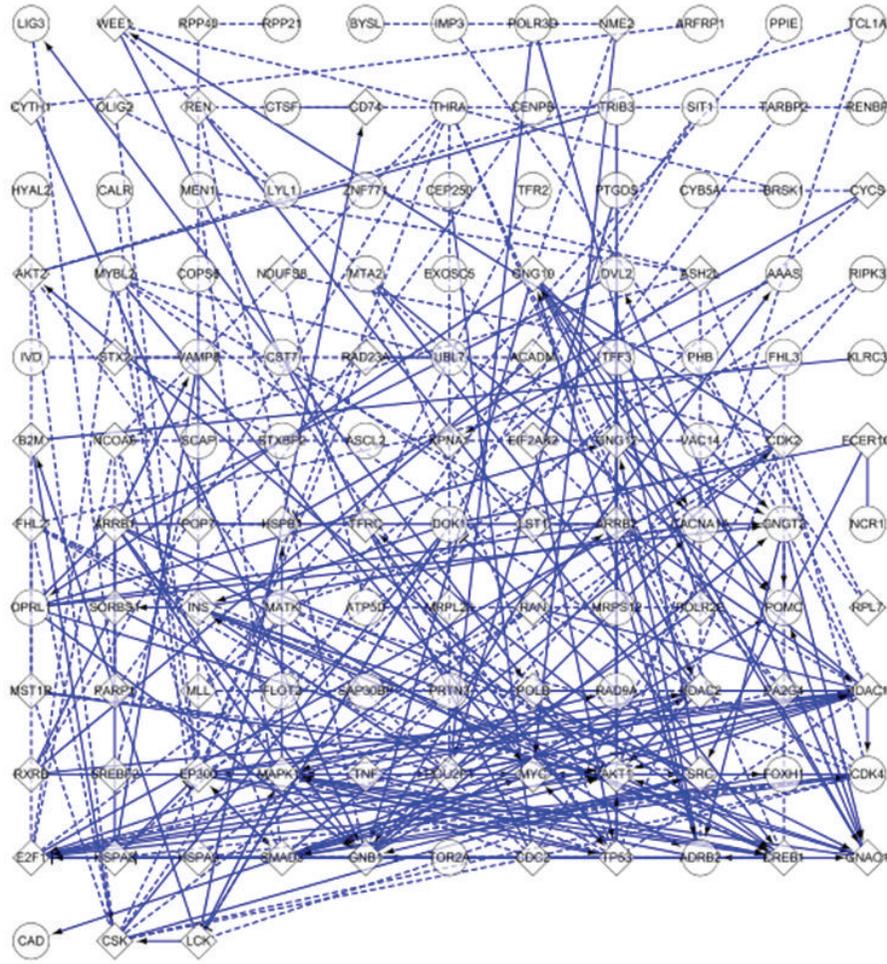


FIGURE 7: The PPIs network of downregulated mRNAs of the miRNA/mRNA interactive network. Rectangular represented downregulated mRNAs and round represented added interactive mRNAs. The interactions between two mRNAs extracted from pathways were shown as solid lines while those predicted interactions were shown as dashed lines. Extracted interactions involved in activation, expression regulation, or catalysis were shown with an arrowhead on the end of the line, while interactions involved in inhibition were shown with a “T” bar.

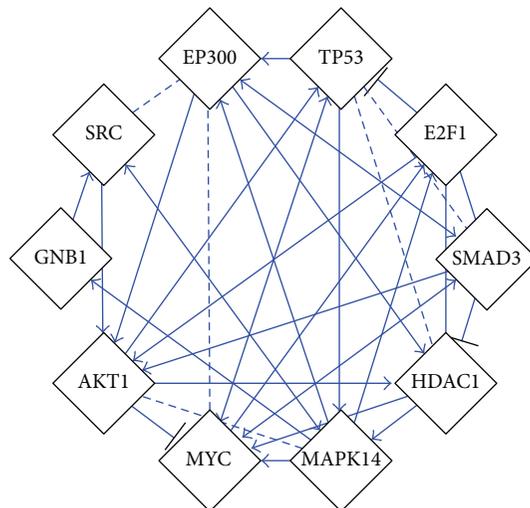


FIGURE 8: Hubs of the PPIs network.

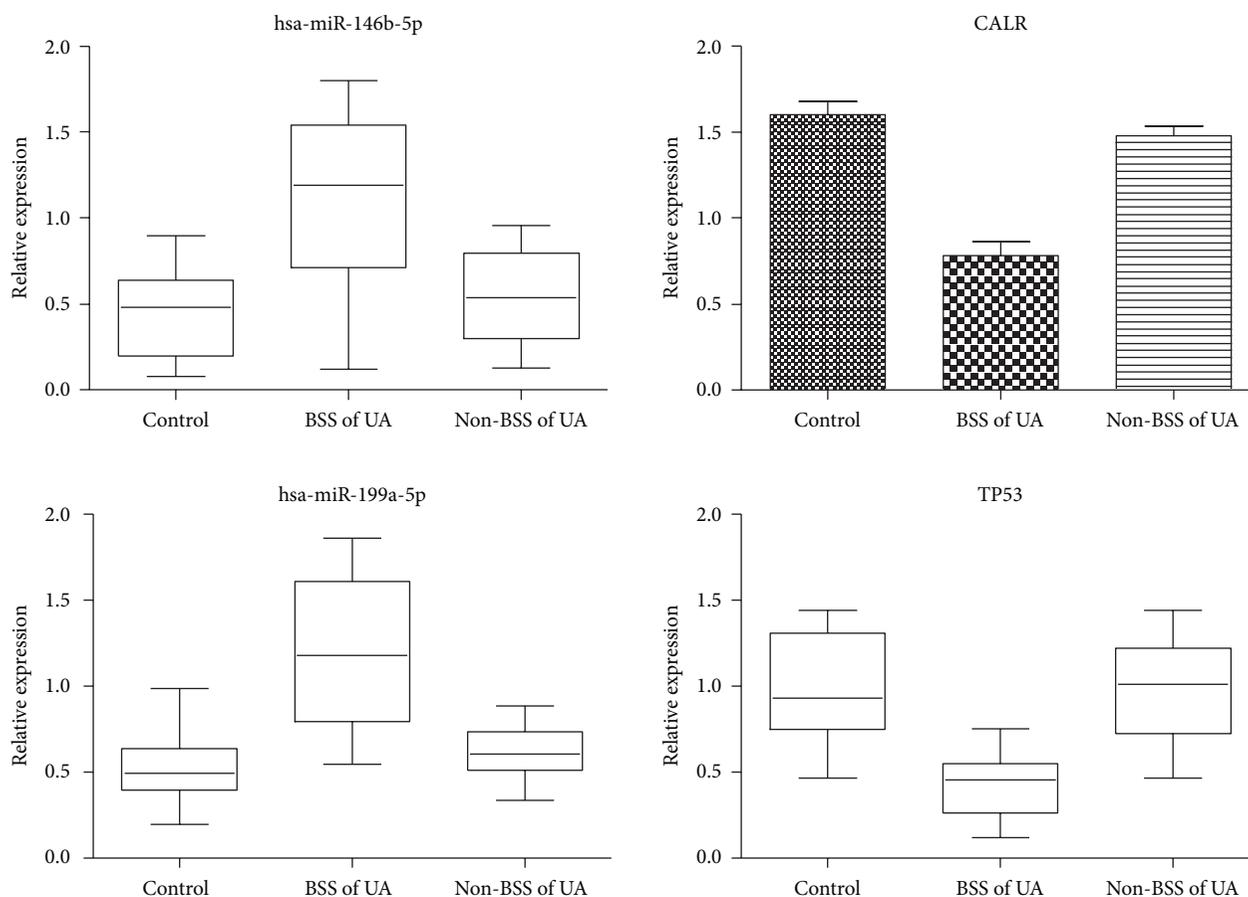


FIGURE 9: Validation of differentially expressed miRNAs and mRNA among UA patients with BSS, UA patients with non-BSS, and healthy control by RT-PCR analysis.

Putting the functional analysis of pathways together, most of the upregulated genes in BSS of UA were implicated in inflammation, apoptosis, and innate and adaptive immunity, and most of the downregulated genes in BSS of UA were implicated in apoptosis, angiogenesis, inflammation, immune response, and cell cycle arrest. The inflammation and immune response in BSS of UA was in accordance with previous reports demonstrating inflammatory and immune related genes were related to BSS of CAD by microarray analysis [88]. In addition, the analysis showed that apoptosis was involved in BSS of UA and some genes related with the regulation of cell cycle may regulate apoptosis in BSS of UA. Apoptosis added a new layer for the complex pathophysiology of BSS of UA. Interestingly, we found that some genes of the active innate and adaptive immunity may regulate inflammation and apoptosis in BSS of UA, such as RIPK2 and IRAK3. The deregulation and imbalance of apoptosis, inflammation and immunity could be the key biomedical mechanisms in BSS of UA.

**4.2. miRNA Expression Data.** Compared with healthy controls, the miRNA expression of BSS of UA was mainly upregulated. Considering the negative regulation of miRNA on gene, it indicated that miRNAs may mainly play an

inhibitory role in BSS of UA. Among the upregulated miRNAs, miR-126, miR-129, miR-146, miR-150, miR-151a-3p, miR-199a-5p, miR-221, miR-223, miR-30d, miR-326 and miR-92a were reported to be upregulated in PBMCs of CAD patients compared with healthy controls [89–91]. There were compelling evidences that some of these upregulated miRNAs played fundamental roles in the development and progression of UA. miR-126 was an endothelial cell-restricted microRNA and enhanced the proangiogenic actions of VEGF and FGF and promoted blood vessel formation [92]. miR-130a downregulated the antiangiogenic homeobox proteins GAX and HoxA5 [93]. The proangiogenic properties of miR-126 and miR-130a suggested that their upregulation may enhance angiogenesis in BSS of UA. In human blood cells, miR-150 was selectively packaged into microvesicles (MVs) and actively secreted. Secreted monocytic miR-150 enhanced targeted endothelial cell migration. MVs isolated from the plasma of patients with atherosclerosis contained higher levels of miR-150, and they more effectively promoted endothelial cell migration than MVs from healthy donors [94]. The upregulation of miR-150 may promote the endothelial cell migration in BSS of UA. miR-221 was necessary for VSMC proliferation and vascular neointimal lesion formation [95]. The upregulation of miR-221

may promote VSMC proliferation in BSS of UA. miR-92a was highly expressed in endothelial cells and regulated angiogenic functions of endothelial cells. Forced overexpression of miR-92a in endothelial cells blocked angiogenesis *in vitro* and *in vivo* [96]. Upregulation of miR-92a may hinder angiogenesis in BSS of UA.

**4.3. Integrated miRNA/mRNA Network Analysis.** Among up-regulated miRNAs, miR-146b-5p, miR-199a-3p, miR-199a-5p, miR-326, miR-423-3p, and miR-484 were found to be key deregulated miRNAs in miRNA/mRNA network. MAPK14, AKT1, EP300, HDAC1, TP53, E2F1, SMAD3, GNB1, MYC, and SRC were found to be hubs of the PPIs network of downregulated target genes of the 6 miRNAs. Among the hubs, EP300, HDAC1, TP53, E2F1, SMAD3, and MYC were involved in the regulation of cell cycle [80, 97–101]. AKT1 and TP53 were involved in the regulation of apoptosis [80, 102]. MAPK14, AKT1, and SRC were involved in the regulation of angiogenesis [103–105]. It indicated that downregulated target genes of the 6 miRNAs were mainly functioned in the regulation of cell cycle, apoptosis, and angiogenesis. This result was partly consistent with the pathway analysis of downregulated genes. Interestingly, TP53 was confirmed by both pathway and network analysis to be the key downregulated target genes.

Compared the network analysis with pathway analysis, we chose miR-146b-5p and miR-199a-5p and their target genes for further analysis for 2 reasons. First, miR-146b-5p and miR-199a-5p were proved to be key upregulated miRNAs in both pathway and network analysis. Second, CALR and TP53 were the key downregulated genes in BSS of UA, while CALR was the target gene of miR-146a-5p and TP53 was the target gene of miR-199a-5p. Although there was lack of research about the role of miR-146b-5p in UA, miR-146b was demonstrated to be rapidly induced in human monocytes in response to a variety of microbial components and proinflammatory cytokines [106]. It was reported that miR-146b decreased the expression of TNF $\alpha$ , IL-1B, and IL-6 in THP-1 monocytes [106, 107]. Other studies showed that IL-1 receptor signaling initiated both miR-146b upregulation and cytokine secretion, and that miR-146b was expressed in response to rising inflammatory cytokine levels and suppressed IL-6 and IL-8 secretion in primary human fibroblasts [108]. Moreover, miR-146b was induced by the potent proresolving mediator RvD1 in human macrophages in the resolution of inflammation and decreased protein levels of proinflammatory IL-8 and RANTES [109]. Furthermore, a recent study showed that miR-146b-5p, decreased in monocytes during obesity, was a major mediator of the anti-inflammatory action of globular adiponectin [110]. Collectively, all these studies implied that miR-146b-5p was induced in the inflammation and played an anti-inflammatory role in innate immunity. According to the pathway analysis, CALR could be the target gene of miR-146b-5p and upregulation of miR-146b-5p may induce the downregulation of CALR. According to functional analysis, both upregulation of miR-146b-5p and downregulation of CALR could attenuate the inflammation, and it implied that miR-146b-5p may attenuate inflammation by targeted

repression of CALR in BSS of UA. Moreover, if CALR was confirmed to be the target gene of miR-146b-5p, miR-146b-5p may also function to inhibit apoptosis and promote angiogenesis by suppressing CALR in BSS of UA. miR-199a was reported to be acutely downregulated in cardiomyocytes in hypoxia and replenishing miR-199a during hypoxia reduced apoptosis [111]. According to the pathway analysis, TP53 could be the target gene of miR-199a-5p and upregulation of miR-199a-5p may induce the downregulation of TP53. According to functional analysis, both upregulation of miR-199a-5p and downregulation of TP53 could inhibit apoptosis, and it implied that miR-199a-5p may inhibit apoptosis by targeted repression of TP53 in BSS of UA.

Based on functional enrichment analysis of upregulated genes, there was active inflammation, apoptosis, and immune response in BSS of UA. Intriguingly, upregulation of miR-146b-5p and miR-199a-5p may attenuate inflammation and apoptosis in BSS of UA. Analysis of miR-146b-5p and miR-199a-5p expression unveiled a pattern of induction in response to inflammation and apoptosis in BSS of UA. The expression pattern of miR-146b-5p and miR-199a-5p in BSS of UA compared with healthy control were more like consequences than causes. Such a pattern was partly supported by the studies about the role of miR-146b in inflammation. There were evidences that pro-inflammatory cytokines could induce the upregulation of miR-146b which in return attenuate inflammation as part of a negative feedback regulation loop [106]. In addition, the miR-146b regulatory circuit just fine-tuned inflammation related signaling, rather than totally abrogating the signal [106]. Whether apoptosis induced the upregulation of miR-199a-5p and miR-199a-5p functioned as a negative feedback regulation loop to inhibit apoptosis was worth further exploring.

The present study revealed that microRNAs and mRNAs of PBMCs might be used as biomarkers for BSS of UA patients. Compared with healthy controls, levels of miR-146b-5p and miR-199a-5p were significantly higher, while levels of CALR and TP53 were significantly lower in BSS of UA patients. These miRNAs and mRNAs were closely related with BSS of UA patients. They might serve as biomarkers for distinguishing BSS of UA patients from healthy controls. To confirm this result, miR-146b-5p and miR-199a-5p and their downregulated target genes (CALR and TP53) were selected to validate the expression level in an independent cohort of 30 BSS of UA patients, 30 non-BSS of UA patients and 15 healthy controls by qRT-PCR. Compared with healthy controls and non-BSS of UA patients, significant upregulation of miR-146b-5p and miR-199a-5p and downregulation of CALR and TP53 were proven. It confirmed the robustness of the expression pattern of miR-146b-5p, miR-199a-5p, CALR and TP53 in BSS of UA patients.

However, research limitations existed in our study. First, the sample size was small, and clinical studies with larger cohorts of BSS of UA patients and healthy controls were definitely required to extensively evaluate the miRNAs and mRNAs as practical biomarkers in comparison with the diagnostic criteria and scale of BSS, as well as the false-positive rate. Second, there was lack of validation by Western blot. Because TCM syndrome differentiation was based on

a collection of multiple symptoms and signs, the related biomedical mechanisms were likely quite complex. Thus, various miRNAs, genes, and intricate interactions were contained in the results, which made short time validation of results difficult. Nonetheless, the present study laid the groundwork for future efforts to identify and develop miR-146b-5p, miR-199a-5p, CALR and TP53 as a novel class of blood-based biomarkers for BSS of UA patients. According to these results, larger cohort investigations were designed to validate the diagnostic sensitivity and specificity of miR-146b-5p and miR-199a-5p as biomarkers. In addition, future studies will be performed to clarify the pathophysiological role of circulating miRNAs during pathogenesis of BSS of UA patients.

## 5. Conclusions

In general, the present study revealed that miR-146b-5p, miR-199a-5p, CALR, and TP53, which were related to the negative feedback regulation loop to attenuate inflammation and apoptosis, were significant biomarkers of BSS of UA patients. The systems biology-based miRNA and mRNA expression biomarkers for BSS of UA may be useful for further stratification of UA patients when making decisions on treatments.

## Authors' Contribution

Jie Wang and Gui Yu contributed equally to this paper.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Acknowledgments

The current work was supported by the National Natural Science Foundation Project of China (no. 81173116). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

## References

- [1] R. S. Wright, J. L. Anderson, C. D. Adams et al., "2011 ACCF/AHA focused update of the guidelines for the management of patients with unstable angina/ Non-ST-elevation myocardial infarction (updating the 2007 guideline): a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines," *Circulation*, vol. 123, no. 18, pp. 2022–2060, 2011.
- [2] E. Braunwald, "Unstable angina and non-ST elevation myocardial infarction," *American Journal of Respiratory and Critical Care Medicine*, vol. 185, no. 9, pp. 924–932, 2012.
- [3] N. Kuzniatsova, E. Shantsila, A. Blann, and G. Y. H. Lip, "A contemporary viewpoint on 'aspirin resistance,'" *Annals of Medicine*, vol. 44, no. 8, pp. 773–783, 2012.
- [4] B. A. Golomb, M. A. Evans, J. E. Dimsdale, and H. L. White, "Effects of statins on energy and fatigue with exertion: results from a randomized controlled trial," *Archives of Internal Medicine*, vol. 172, no. 15, pp. 1180–1182, 2012.
- [5] Y. C. Zhang, B. J. Lu, M. H. Zhao, Y. Z. Rong, and R. M. Chen, "Effect of Shengmai injection on vascular endothelial and heart functions in patients with coronary heart disease complicated with diabetes mellitus," *Chinese Journal of Integrative Medicine*, vol. 14, no. 4, pp. 281–285, 2008.
- [6] F. Qin and X. Huang, "Guanxin II for the management of coronary heart disease," *Chinese Journal of Integrative Medicine*, vol. 15, no. 6, pp. 472–476, 2009.
- [7] Y. R. Jiang, Y. Miao, L. Yang et al., "Effect of chinese herbal drug-containing serum for activating-blood and dispelling-toxin on ox-LDL-induced inflammatory factors' expression in endothelial cells," *Chinese Journal of Integrative Medicine*, vol. 18, no. 1, pp. 30–33, 2012.
- [8] H. Y. Liu, W. Wang, D. Z. Shi et al., "Protective effect of Chinese herbs for supplementing qi, nourishing yin and activating blood circulation on heart function of patients with acute coronary syndrome after percutaneous coronary intervention," *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 423–430, 2012.
- [9] M. Xue, H. J. Yin, C. F. Wu et al., "Effect of Chinese drugs for activating blood circulation and detoxifying on indices of thrombosis, inflammatory reaction, and tissue damage in a rabbit model of toxin-heat and blood stasis syndrome," *Chinese Journal of Integrative Medicine*, vol. 19, no. 1, pp. 42–47, 2013.
- [10] J. H. Zhang, H. C. Shang, X. M. Gao et al., "Compound salvia droplet pill, a traditional Chinese medicine, for the treatment of unstable angina pectoris: a systematic review," *Medical Science Monitor*, vol. 14, no. 1, pp. RA1–RA7, 2008.
- [11] X. Qiu, A. Miles, X. Jiang, X. Sun, and N. Yang, "Sulfotanshinone sodium injection for unstable angina pectoris: a systematic review of randomized controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 203043, 17 pages, 2012.
- [12] A. S. Ferreira and A. J. Lopes, "Chinese medicine pattern differentiation and its implications for clinical practice," *Chinese Journal of Integrative Medicine*, vol. 17, no. 11, pp. 818–823, 2011.
- [13] A. P. Lu, Z. X. Bian, and K. J. Chen, "Bridging the traditional Chinese medicine pattern classification and biomedical disease diagnosis with systems biology," *Chinese Journal of Integrative Medicine*, vol. 18, no. 12, pp. 883–890, 2012.
- [14] A. P. Lu and K. J. Chen, "Chinese medicine pattern diagnosis could lead to innovation in medical sciences," *Chinese Journal of Integrative Medicine*, vol. 17, no. 11, pp. 811–817, 2011.
- [15] K. W. Yao, Q. Y. He, F. Teng et al., "Logistic regression analysis of syndrome essential factors in patients with unstable angina pectoris," *Journal of Traditional Chinese Medicine*, vol. 31, no. 4, pp. 273–276, 2011.
- [16] K. J. Chen, "Blood stasis syndrome and its treatment with activating blood circulation to remove blood stasis therapy," *Chinese Journal of Integrative Medicine*, vol. 18, no. 12, pp. 891–896, 2012.
- [17] Y. Liu, H. J. Yin, D. Shi et al., "Chinese herb and formulas for promoting blood circulation and removing blood stasis and antiplatelet therapies," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 184503, 8 pages, 2012.
- [18] M. Xue, K. J. Chen, and H. J. Yin, "Relationship between platelet activation related factors and polymorphism of related genes in patients with coronary heart disease of blood-stasis syndrome," *Chinese Journal of Integrative Medicine*, vol. 14, no. 4, pp. 267–273, 2008.

- [19] Y. Feng, H. Xu, D. Qu, F. Zheng, D. Z. Shi, and K. J. Chen, "Study on the tongue manifestations for the blood-stasis and toxin syndrome in the stable patients of coronary heart disease," *Chinese Journal of Integrative Medicine*, vol. 17, no. 5, pp. 333–338, 2011.
- [20] Y. Liu, H. J. Yin, and K. J. Chen, "Research on the correlation between platelet gelsolin and blood-stasis syndrome of coronary heart disease," *Chinese Journal of Integrative Medicine*, vol. 17, no. 8, pp. 587–592, 2011.
- [21] K. W. Yao, F. Y. Chu, and J. Wang, "A clinical epidemiological study of the quantitative diagnosis scale of blood stasis syndrome," *Chinese Journal of Integrative Medicine*, vol. 17, no. 3, pp. 200–204, 2011.
- [22] J. Wang, K. J. Chen, W. L. Weng et al., "Study on the diagnostic criteria of blood stasis syndrome," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 8, no. 10, pp. 585–589, 1988.
- [23] F. Y. Chu, J. Wang, K. W. Yao, and Z. Z. Li, "Effect of Xuefu Zhuyu Capsule on the symptoms and signs and health-related quality of life in the unstable angina patients with blood-stasis syndrome after percutaneous coronary intervention: a randomized controlled trial," *Chinese Journal of Integrative Medicine*, vol. 16, no. 5, pp. 399–405, 2010.
- [24] Y. F. Liu, H. M. Yu, C. Zhang et al., "Effects of Quyu xiaoban capsules on clinical outcomes and platelet activation and aggregation in patients with unstable angina pectoris," *Journal of Alternative and Complementary Medicine*, vol. 13, no. 5, pp. 571–576, 2007.
- [25] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.
- [26] E. van Rooij and E. N. Olson, "MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles," *Nature Reviews Drug Discovery*, vol. 11, no. 11, pp. 860–872, 2012.
- [27] E. E. Creemers, A. J. Tijssen, and Y. M. Pinto, "Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?" *Circulation Research*, vol. 110, no. 3, pp. 483–495, 2012.
- [28] M. J. L. de Hoon, S. Imoto, J. Nolan, and S. Miyano, "Open source clustering software," *Bioinformatics*, vol. 20, no. 9, pp. 1453–1454, 2004.
- [29] A. J. Saldanha, "Java treeview—extensible visualization of microarray data," *Bioinformatics*, vol. 20, no. 17, pp. 3246–3248, 2004.
- [30] D. W. Huang, B. T. Sherman, and R. A. Lempicki, "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources," *Nature Protocols*, vol. 4, no. 1, pp. 44–57, 2009.
- [31] M. Kanehisa, S. Goto, Y. Sato, M. Furumichi, and M. Tanabe, "KEGG for integration and interpretation of large-scale molecular data sets," *Nucleic Acids Research*, vol. 40, no. 1, pp. D109–D114, 2012.
- [32] M. Maragkakis, M. Reczko, V. A. Simossis et al., "DIANA-microT web server: elucidating microRNA functions through target prediction," *Nucleic Acids Research*, vol. 37, no. 2, pp. W273–W276, 2009.
- [33] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.
- [34] C. Laczny, P. Leidinger, J. Haas et al., "miRTrail—a comprehensive webserver for analyzing gene and miRNA patterns to enhance the understanding of regulatory mechanisms in diseases," *BMC Bioinformatics*, vol. 12, no. 1, p. 36, 2012.
- [35] J. Kuentzer, T. Blum, A. Gerasch et al., "BN++—a biological information system," *Journal of Integrative Bioinformatics*, vol. 3, no. 2, p. 34, 2006.
- [36] J. de Las Rivas and C. Fontanillo, "Protein-protein interaction networks: unraveling the wiring of molecular machines within the cell," *Briefings in Functional Genomics*, vol. 11, no. 6, pp. 489–496, 2012.
- [37] G. Wu, X. Feng, and L. Stein, "A human functional protein interaction network and its application to cancer data analysis," *Genome Biology*, vol. 11, no. 5, article R53, 2010.
- [38] M. E. Smoot, K. Ono, J. Ruscheinski, P. Wang, and T. Ideker, "Cytoscape 2.8: new features for data integration and network visualization," *Bioinformatics*, vol. 27, no. 3, pp. 431–432, 2011.
- [39] A. L. Barabási and Z. N. Oltvai, "Network biology: understanding the cell's functional organization," *Nature Reviews Genetics*, vol. 5, no. 2, pp. 101–113, 2004.
- [40] C. Y. Lin, C. H. Chin, H. H. Wu, S. H. Chen, C. W. Ho, and M. T. Ko, "Hubba: hub objects analyzer—a framework of interactome hubs identification for network biology," *Nucleic Acids Research*, vol. 36, pp. W438–W443, 2008.
- [41] T. Nolan, R. E. Hands, and S. A. Bustin, "Quantification of mRNA using real-time RT-PCR," *Nature Protocols*, vol. 1, no. 3, pp. 1559–1582, 2006.
- [42] N. Inohara, L. del Peso, T. Koseki, S. Chen, and G. Núñez, "RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis," *Journal of Biological Chemistry*, vol. 273, no. 20, pp. 12296–12300, 1998.
- [43] Y. J. Geng and P. Libby, "Evidence for apoptosis in advanced human atheroma: colocalization with interleukin-1 $\beta$ -converting enzyme," *The American Journal of Pathology*, vol. 147, no. 2, pp. 251–266, 1995.
- [44] F. Chen, P. Eriksson, T. Kimura, I. Herzfeld, and G. Valen, "Apoptosis and angiogenesis are induced in the unstable coronary atherosclerotic plaque," *Coronary Artery Disease*, vol. 16, no. 3, pp. 191–197, 2005.
- [45] M. C. H. Clarke, N. Figg, J. J. Maguire et al., "Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis," *Nature Medicine*, vol. 12, no. 9, pp. 1075–1080, 2006.
- [46] I. Tabas, "Macrophage death and defective inflammation resolution in atherosclerosis," *Nature Reviews Immunology*, vol. 10, no. 1, pp. 36–46, 2010.
- [47] T. J. Fan, L. H. Han, R. S. Cong, and J. Liang, "Caspase family proteases and apoptosis," *Acta Biochimica et Biophysica Sinica*, vol. 37, no. 11, pp. 719–727, 2005.
- [48] J. G. Magalhaes, J. Lee, K. Geddes, S. Rubino, D. J. Philpott, and S. E. Girardin, "Essential role of Rip2 in the modulation of innate and adaptive immunity triggered by Nod1 and Nod2 ligands," *European Journal of Immunology*, vol. 41, no. 5, pp. 1445–1455, 2011.
- [49] Y. Huo, C. Weber, S. B. Forlow et al., "The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium," *Journal of Clinical Investigation*, vol. 108, no. 9, pp. 1307–1314, 2001.
- [50] H. Kirii, T. Niwa, Y. Yamada et al., "Lack of interleukin-1 $\beta$  decreases the severity of atherosclerosis in apoE-deficient mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 4, pp. 656–660, 2003.
- [51] A. Qamar and D. J. Rader, "Effect of interleukin 1 $\beta$  inhibition in cardiovascular disease," *Current Opinion in Lipidology*, vol. 23, no. 6, pp. 548–553, 2012.

- [52] A. Ozeren, M. Aydin, M. Tokac et al., "Levels of serum IL-1 $\beta$ , IL-2, IL-8 and tumor necrosis factor- $\alpha$  in patients with unstable angina pectoris," *Mediators of Inflammation*, vol. 12, no. 6, pp. 361–365, 2003.
- [53] A. D. Simon, S. Yazdani, W. Wang, A. Schwartz, and L. E. Rabbani, "Circulating levels of IL-1 $\beta$ , a prothrombotic cytokine, are elevated in unstable angina versus stable angina," *Journal of Thrombosis and Thrombolysis*, vol. 9, no. 3, pp. 217–222, 2000.
- [54] P. Libby, "What have we learned about the biology of atherosclerosis? The role of inflammation," *American Journal of Cardiology*, vol. 88, no. 7, pp. 3J–6J, 2001.
- [55] M. R. Alexander, C. W. Moehle, J. L. Johnson et al., "Genetic inactivation of IL-1 signaling enhances atherosclerotic plaque instability and reduces outward vessel remodeling in advanced atherosclerosis in mice," *The Journal of Clinical Investigation*, vol. 122, no. 1, pp. 70–79, 2012.
- [56] S. Janssens and R. Beyaert, "Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members," *Molecular Cell*, vol. 11, no. 2, pp. 293–302, 2003.
- [57] M. Hulsmans, B. Geeraert, D. de Keyzer et al., "Interleukin-1 receptor-associated kinase-3 is a key inhibitor of inflammation in obesity and metabolic syndrome," *PLoS One*, vol. 7, no. 1, Article ID e30414, 2012.
- [58] B. S. Skalhogg and K. Tasken, "Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and sub-cellular localization of subunits of PKA," *Frontiers in Bioscience*, vol. 5, pp. D678–D693, 2000.
- [59] Q. L. Deveraux and J. C. Reed, "IAP family proteins—suppressors of apoptosis," *Genes and Development*, vol. 13, no. 3, pp. 239–252, 1999.
- [60] O. Kutuk and H. Basaga, "Bcl-2 protein family: implications in vascular apoptosis and atherosclerosis," *Apoptosis*, vol. 11, no. 10, pp. 1661–1675, 2006.
- [61] T. Korn, E. Bettelli, W. Gao et al., "IL-21 initiates an alternative pathway to induce proinflammatory T H17 cells," *Nature*, vol. 448, no. 7152, pp. 484–487, 2007.
- [62] A. Zlotnik and O. Yoshie, "Chemokines: a new classification system and their role in immunity," *Immunity*, vol. 12, no. 2, pp. 121–127, 2000.
- [63] J. Hesselgesser, H. P. Ng, M. Liang et al., "Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor," *Journal of Biological Chemistry*, vol. 273, no. 25, pp. 15687–15692, 1998.
- [64] A. Valenzuela-Fernández, T. Planchenault, F. Baleux et al., "Leukocyte elastase negatively regulates stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4," *Journal of Biological Chemistry*, vol. 277, no. 18, pp. 15677–15689, 2002.
- [65] J. K. Damás, T. Waehre, A. Yndestad et al., "Stromal cell-derived factor-1 $\alpha$  in unstable angina: potential antiinflammatory and matrix-stabilizing effects," *Circulation*, vol. 106, no. 1, pp. 36–42, 2002.
- [66] D. F. Smith, E. Galkina, K. Ley, and Y. Huo, "GRO family chemokines are specialized for monocyte arrest from flow," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 289, no. 5, pp. H1976–H1984, 2005.
- [67] C. A. Power, R. B. Furness, C. Brawand, and T. N. C. Wells, "Cloning of a full-length cDNA encoding the neutrophil-activating peptide ENA-78 from human platelets," *Gene*, vol. 151, no. 1–2, pp. 333–334, 1994.
- [68] A. Yilmaz, B. Lipfert, I. Cicha et al., "Accumulation of immune cells and high expression of chemokines/chemokine receptors in the upstream shoulder of atherosclerotic carotid plaques," *Experimental and Molecular Pathology*, vol. 82, no. 3, pp. 245–255, 2007.
- [69] R. E. Eid, D. A. Rao, J. Zhou et al., "Interleukin-17 and interferon- $\gamma$  are produced concomitantly by human coronary artery-infiltrating T cells and act synergistically on vascular smooth muscle cells," *Circulation*, vol. 119, no. 10, pp. 1424–1432, 2009.
- [70] G. Liuzzo, A. N. Vallejo, S. L. Kopecky et al., "Molecular fingerprint of interferon- $\gamma$  signaling in unstable angina," *Circulation*, vol. 103, no. 11, pp. 1509–1514, 2001.
- [71] T. Nagata, H. Kai, R. Shibata, M. Koga, A. Yoshimura, and T. Imaizumi, "Oncostatin M, an interleukin-6 family cytokine, upregulates matrix metalloproteinase-9 through the mitogen-activated protein kinase kinase-extracellular signal-regulated kinase pathway in cultured smooth muscle cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 4, pp. 588–593, 2003.
- [72] M. Michalak, E. F. Corbett, N. Mesaeli, K. Nakamura, and M. Opas, "Calreticulin: one protein, one gene, many functions," *Biochemical Journal*, vol. 344, part 2, pp. 281–292, 1999.
- [73] S. Lim, W. Chang, B. K. Lee et al., "Enhanced calreticulin expression promotes calcium-dependent apoptosis in postnatal cardiomyocytes," *Molecules and Cells*, vol. 25, no. 3, pp. 390–396, 2008.
- [74] X. Liu, X. Wu, L. Cai, and S. Sun, "Calreticulin downregulation is associated with FGF-2-induced angiogenesis through calcineurin pathway in ischemic myocardium," *Shock*, vol. 29, no. 1, pp. 140–148, 2008.
- [75] S. J. Gardai, Y. Q. Xiao, M. Dickinson et al., "By binding SIRP $\alpha$  or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation," *Cell*, vol. 115, no. 1, pp. 13–23, 2003.
- [76] G. T. Nepom and H. Erlich, "MHC class-II molecules and autoimmunity," *Annual Review of Immunology*, vol. 9, pp. 493–525, 1991.
- [77] R. de Palma, F. del Galdo, G. Abbate et al., "Patients with acute coronary syndrome show oligoclonal T-cell recruitment within unstable plaque evidence for a local, intracoronary immunologic mechanism," *Circulation*, vol. 113, no. 5, pp. 640–646, 2006.
- [78] R. J. Boyton and D. M. Altmann, "Natural killer cells, killer immunoglobulin-like receptors and human leucocyte antigen class I in disease," *Clinical and Experimental Immunology*, vol. 149, no. 1, pp. 1–8, 2007.
- [79] S. C. Whitman, D. L. Rateri, S. J. Szilvassy, W. Yokoyama, and A. Daugherty, "Depletion of natural killer cell function decreases atherosclerosis in low-density lipoprotein receptor null mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 6, pp. 1049–1054, 2004.
- [80] A. V. Vaseva and U. M. Moll, "The mitochondrial p53 pathway," *Biochimica et Biophysica Acta*, vol. 1787, no. 5, pp. 414–420, 2009.
- [81] X. M. Yuan, E. Osman, S. Miah et al., "P53 expression in human carotid atheroma is significantly related to plaque instability and clinical manifestations," *Atherosclerosis*, vol. 210, no. 2, pp. 392–399, 2010.
- [82] L. Connell-Crowley, J. W. Harper, and D. W. Goodrich, "Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation," *Molecular Biology of the Cell*, vol. 8, no. 2, pp. 287–301, 1997.

- [83] D. Gordon, M. A. Reidy, E. P. Benditt, and S. M. Schwartz, "Cell proliferation in human coronary arteries," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 12, pp. 4600–4604, 1990.
- [84] M. E. Zettler, M. A. Prociuk, J. A. Austria, H. Massaeli, G. Zhong, and G. N. Pierce, "OxLDL stimulates cell proliferation through a general induction of cell cycle proteins," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 284, no. 2, pp. H644–H653, 2003.
- [85] B. J. Passer, V. Nancy-Portebois, N. Amzallag et al., "The p53-inducible TSAP6 gene product regulates apoptosis and the cell cycle and interacts with Nix and the Myt1 kinase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 5, pp. 2284–2289, 2003.
- [86] R. K. Gupta, R. Tripathi, B. J. Naidu, U. K. Srinivas, and L. S. Shashidhara, "Cell cycle regulation by the pro-apoptotic gene scotin," *Cell Cycle*, vol. 7, no. 15, pp. 2401–2408, 2008.
- [87] M. C. Maiuri, S. A. Malik, E. Morselli et al., "Stimulation of autophagy by the p53 target gene Sestrin2," *Cell Cycle*, vol. 8, no. 10, pp. 1571–1576, 2009.
- [88] X. J. Ma, H. J. Yin, and K. J. Chen, "Differential gene expression profiles in coronary heart disease patients of blood stasis syndrome in traditional Chinese medicine and clinical role of target gene," *Chinese Journal of Integrative Medicine*, vol. 15, no. 2, pp. 101–106, 2009.
- [89] S. Fichtlscherer, S. de Rosa, H. Fox et al., "Circulating microRNAs in patients with coronary artery disease," *Circulation Research*, vol. 107, no. 5, pp. 677–684, 2010.
- [90] C. Taurino, W. H. Miller, M. W. McBride et al., "Gene expression profiling in whole blood of patients with coronary artery disease," *Clinical Science*, vol. 119, part 8, pp. 335–343, 2010.
- [91] M. Hoekstra, C. A. C. van der Lans, B. Halvorsen et al., "The peripheral blood mononuclear cell microRNA signature of coronary artery disease," *Biochemical and Biophysical Research Communications*, vol. 394, no. 3, pp. 792–797, 2010.
- [92] S. Wang, A. B. Aurora, B. A. Johnson et al., "The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis," *Developmental Cell*, vol. 15, no. 2, pp. 261–271, 2008.
- [93] Y. Chen and D. H. Gorski, "Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5," *Blood*, vol. 111, no. 3, pp. 1217–1226, 2008.
- [94] Y. Zhang, D. Liu, X. Chen et al., "Secreted monocytic miR-150 enhances targeted endothelial cell migration," *Molecular Cell*, vol. 39, no. 1, pp. 133–144, 2010.
- [95] X. Liu, Y. Cheng, S. Zhang, Y. Lin, J. Yang, and C. Zhang, "A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia," *Circulation Research*, vol. 104, no. 4, pp. 476–486, 2009.
- [96] A. Bonauer, G. Carmona, M. Iwasaki et al., "MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice," *Science*, vol. 324, no. 5935, pp. 1710–1713, 2009.
- [97] A. W. Snowden, L. A. Anderson, G. A. Webster, and N. D. Perkins, "A novel transcriptional repression domain mediates p21<sup>WAF1/CIP1</sup> induction of p300 transactivation," *Molecular and Cellular Biology*, vol. 20, no. 8, pp. 2676–2686, 2000.
- [98] H. S. Zhang, M. Gavin, A. Dahiya et al., "Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and RB-hSWI/SNF," *Cell*, vol. 101, no. 1, pp. 79–89, 2000.
- [99] B. Ren, H. Cam, Y. Takahashi et al., "E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints," *Genes and Development*, vol. 16, no. 2, pp. 245–256, 2002.
- [100] L. Li, Y. Iwamoto, A. Berezovskaya, and V. A. Bousiotis, "A pathway regulated by cell cycle inhibitor p27Kip1 and checkpoint inhibitor Smad3 is involved in the induction of T cell tolerance," *Nature Immunology*, vol. 7, no. 11, pp. 1157–1165, 2006.
- [101] B. Amati, T. D. Littlewood, G. I. Evan, and H. Land, "The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max," *The EMBO Journal*, vol. 12, no. 13, pp. 5083–5087, 1993.
- [102] A. H. Kim, G. Khursigara, X. Sun, T. F. Franke, and M. V. Chao, "Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1," *Molecular and Cellular Biology*, vol. 21, no. 3, pp. 893–901, 2001.
- [103] K. Issbrücker, H. H. Marti, S. Hippenstiel et al., "p38 MAP kinase—a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability," *The FASEB Journal*, vol. 17, no. 2, pp. 262–264, 2003.
- [104] E. Ackah, J. Yu, S. Zoellner et al., "Akt1/protein kinase B $\alpha$  is critical for ischemic and VEGF-mediated angiogenesis," *Journal of Clinical Investigation*, vol. 115, no. 8, pp. 2119–2127, 2005.
- [105] B. P. Eliceiri, R. Paul, P. L. Schwartzberg, J. D. Hood, J. Leng, and D. A. Cheresh, "Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability," *Molecular Cell*, vol. 4, no. 6, pp. 915–924, 1999.
- [106] K. D. Taganov, M. P. Boldin, K. J. Chang, and D. Baltimore, "NF- $\kappa$ B-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 33, pp. 12481–12486, 2006.
- [107] L. A. O'Neill, F. J. Sheedy, and C. E. McCoy, "MicroRNAs: the fine-tuners of Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 11, no. 3, pp. 163–175, 2011.
- [108] D. Bhaumik, G. K. Scott, S. Schokrpur et al., "MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8," *Ageing*, vol. 1, no. 4, pp. 402–411, 2009.
- [109] A. Recchiuti, S. Krishnamoorthy, G. Fredman, N. Chiang, and C. N. Serhan, "MicroRNAs in resolution of acute inflammation: identification of novel resolvin D1-miRNA circuits," *The FASEB Journal*, vol. 25, no. 2, pp. 544–560, 2011.
- [110] M. Hulsmans, E. van Dooren, C. Mathieu, and P. Holvoet, "Decrease of miR-146b-5p in monocytes during obesity is associated with loss of the anti-inflammatory but not insulin signaling action of adiponectin," *PloS One*, vol. 7, no. 2, Article ID e32794, 2012.
- [111] S. Rane, M. He, D. Sayed et al., "Downregulation of MiR-199a derepresses hypoxia-inducible factor-1 $\alpha$  and sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes," *Circulation Research*, vol. 104, no. 7, pp. 879–886, 2009.

## Research Article

# Therapeutic Effects of Water Soluble Danshen Extracts on Atherosclerosis

Yoon Hee Cho,<sup>1</sup> Cheol Ryong Ku,<sup>2</sup> Zhen-Yu Hong,<sup>1</sup> Ji Hoe Heo,<sup>3</sup> Eun Hee Kim,<sup>3</sup>  
Dong Hoon Choi,<sup>4</sup> Dongkyu Kim,<sup>1</sup> Ae-Jung Kim,<sup>5</sup> Cheol Soon Lee,<sup>5</sup> Mankil Jung,<sup>6</sup>  
Hyun Chul Lee,<sup>2</sup> MiRan Seo,<sup>1</sup> and Eun Jig Lee<sup>2</sup>

<sup>1</sup> Severance Hospital Integrative Research Institute for Cerebral & Cardiovascular Diseases, Severance Hospital, Seoul 120-752, Republic of Korea

<sup>2</sup> Division of Endocrinology, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea

<sup>3</sup> Division of Neurology, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea

<sup>4</sup> Division of Cardiology, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea

<sup>5</sup> eGene, Inc., Lee Gil Ya Cancer and Diabetes Institute, Republic of Korea

<sup>6</sup> Department of Chemistry, Yonsei University, Seoul 120-752, Republic of Korea

Correspondence should be addressed to MiRan Seo; seo99@yuhs.ac and Eun Jig Lee; ejlee423@yuhs.ac

Received 14 September 2012; Revised 9 December 2012; Accepted 24 December 2012

Academic Editor: Keji Chen

Copyright © 2013 Yoon Hee Cho et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Danshen is a traditional Chinese medicine with many beneficial effects on cardiovascular diseases. The aim of this study was to evaluate the mechanisms responsible for the antiatherogenic effect of water soluble Danshen extracts (DEs). Rat vascular smooth muscle cells (VSMCs) and human umbilical vein endothelial cells (HUVECs) were treated with DE. To evaluate the effects of DE *in vivo*, carotid balloon injury and tail vein thrombosis were induced in Sprague-Dawley (SD) rats and iliac artery stent was induced in New Zealand white rabbits. The inhibitory action of DE on platelet aggregation was confirmed with an impedance aggregometer. DE inhibited the production of reactive oxygen species, and the migration and proliferation of platelet-derived growth factor-BB stimulated VSMCs. Furthermore, DE prevented inflammation and apoptosis in HUVECs. Both effects of DE were reconfirmed in both rat models. DE treatment attenuated platelet aggregation in both *in vivo* and *ex vivo* conditions. Pretreatment with DE prevented tail vein thrombosis, which is normally induced by  $\kappa$ -carrageenan injection. Lastly, DE-treated rabbits showed decreased in-stent restenosis of stented iliac arteries. These results suggest that water soluble DE modulates key atherogenic events in VSMCs, endothelial cells, and platelets in both *in vitro* and *in vivo* conditions.

## 1. Introduction

Danshen (*Salvia miltiorrhiza*) has been used for the treatment of cardiovascular and cerebrovascular diseases [1, 2]. Specific clinical uses include angina pectoris, hypercholesterolemia, and acute ischemic stroke. This traditional medicine is a popular drug in China, where it is used either on its own or mixed with other herbs [3]. Even in the United States, Danshen has been widely used in recent years [4, 5]. Specifically, the Fufang Danshen Dripping Pill has cleared American Phase II clinical trials in patients with chronic stable angina pectoris (<http://clinicaltrials.gov/>, no. NCT00797953). The beneficial effects of Danshen on cardiovascular diseases arise

from its ability to prevent atherosclerosis. Danshen, as a therapeutic agent for cardiovascular diseases, contributes to improved microcirculation, vasodilation, anticoagulation, and anti-inflammation [6, 7]. In addition to its effects on cardiovascular diseases, some studies have shown that Danshen may be useful in a diverse range of diseases including liver fibrosis, chronic renal failure, and acute pancreatitis [2].

Recently, several chemical compounds belonging to two specific classes were isolated from Danshen; caffeic acid-derived phenolic acids and tanshinones belonging to the diterpene quinone family [8]. According to the solubility, these compounds could be simply classified as either water-soluble agents or lipid-soluble agents. Most studies have

reported that the lipid-soluble components of Danshen extracts (DE), such as tanshinone IIA, have specific effects on cardiovascular disease [9].

Although the beneficial effects of Danshen on cardiovascular diseases have been reported in other studies, the underlying mechanisms of DE as well as the role of its water-soluble compounds have not yet been evaluated. With respect to the pharmacological and therapeutic profiles of Danshen in the vascular system, the aim of the present study was to evaluate the mechanisms involved in the antiatherogenic effects of DE, which contains an abundance of water-soluble compounds.

## 2. Materials and Methods

This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). Furthermore, approval was granted by the Institutional Animal Care and Use Committee of Yonsei University Health System (permit number 2010-0187). All animal studies were performed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care. In all animal models, animals were anesthetized with an intraperitoneal injection of Ketamine-Xylazine (100 mg:10 mg per kg of body weight) once every procedure. The adequacy of anesthesia in Sprague-Dawley (SD) was assessed by absence of reflexes prior to rapid cervical dislocation, and that in New Zealand white rabbits was confirmed by monitoring respiratory rate and ECG.

**2.1. Production of Danshen Extracts.** Dried and powdered roots of *Salvia miltiorrhiza* (200 g) were boiled with water (1600 mL) for 2 hours. The extracted solution was then filtered and solid deposits were removed. The extracted solution was then concentrated by heating with a rotary evaporator. Next, identical volumes of extracted solution and butanol were mixed in a separatory funnel. The upper solution of the mixture was then collected and concentrated with a rotary evaporator. The components of the collected DE were evaluated via ultra performance liquid chromatography-ultraviolet (UPLC-UV). Powdered DE was dissolved in distilled water containing 1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co., MO, USA) and diluted to final experimental concentrations.

**2.2. Primary Cell Isolation and Culture.** Rat vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of SD rats weighing between 200 to 250 g (Orient-Charles River Technology, Seoul, Korea), as described previously [10]. VSMCs between passages four and five were used in this study. Human umbilical vein endothelial cells (HUVECs) (Invitrogen Life Technology, CA, USA) were grown in endothelial cell growth medium-2 supplemented with 2% fetal bovine serum (FBS), 0.04% hydrocortisone, 0.4% human epidermal growth factor (hEGF)-B, 0.1% vascular endothelial growth factor (VEGF), 0.1% R3-insulin like growth factor (IGF)-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, and

0.1% heparin (Lonza, Basel, Switzerland) and synchronized by serum deprivation (0.1% FBS) for 2 h. HUVECs between passages 8 and 10 were used in this study. After synchronization, VSMCs and HUVECs were treated with DE for 24 h prior to stimulation with platelet-derived growth factor (PDGF)-BB (R&D Systems, MN, USA) and tumor necrosis factor (TNF)- $\alpha$  (Millipore, MA, USA). PDGF-BB (20 ng/mL) and TNF- $\alpha$  (10 ng/mL) were applied to both VSMCs and HUVECs for 24 h.

**2.3. Cell Proliferation Analysis, Wound Healing Assay, and Reactive Oxygen Species Assay.** Cell proliferation was assessed using a modified 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2 tetrazolium bromide (MTT) (Sigma-Aldrich Co.) assay following a standard protocol [11]. Wound healing assays were performed using 6-well plates. When cells reached 90% confluence, synchronized cells were pretreated with DE (100  $\mu$ g/mL) in serum-free medium for 24 h. After 24 h of PDGF-BB (20 ng/mL) stimulation, a single wound was created in the center of the cell monolayers by gentle removal of the attached cells with a sterile plastic pipette tip. After 24 h of incubation, the cells that migrated into the wounded area or protruded from the border of the wound were visualized and photographed under an inverted microscope. The effect of DE on intracellular reactive oxygen species (ROS) levels was examined as described previously [12]. Briefly, at 24 h after PDGF (20 ng/mL) following DE (100  $\mu$ g/mL) treatment, cells were incubated for 30 min at 37°C with CM-H<sub>2</sub>DCF-DA (Molecular Probes Inc., OR, USA).

**2.4. Propidium Iodide Staining for Cell Cycle Analysis.** At 24 h after PDGF (20 ng/mL) treatment following DE (100  $\mu$ g/mL) pretreatment, cells were harvested and washed with PBS, followed by fixation with 70% ethanol overnight at -20°C. After washing with 3% BSA/PBS twice, the cells were resuspended in PBS containing 50  $\mu$ g/mL propidium iodide and 10  $\mu$ g/mL RNase A for 30 min at room temperature. Samples were analysed for DNA content by flow cytometry. The cell-cycle phases were analysed using CELLQuest software (Becton Dickinson, San Jose, CA, USA).

**2.5. Immunoblotting.** Cell lysates were subsequently prepared and subjected to western blot analysis. Membranes were then immunoblotted with primary antibodies for phospho-Akt (Ser473) (Cell Signaling), nonmuscle heavy chain II-A (Abcam, MA, USA), manganese superoxide dismutase (MnSOD) (Abcam), lamin A (Santa Cruz, CA, USA), JNK (Cell Signaling Technologies, MA, USA), p65 (Cell Signaling Technologies), and hemoxygenase-1 (HO-1) (Santa Cruz). Peroxidase-conjugated anti-rabbit or anti-mouse antibodies were used as secondary antibodies (Thermo Fisher Scientific). Isolation of nuclear fractions was carried out by using a commercial kit (Cayman Chemical Item Number 10009277).

**2.6. Rat Model for Carotid Artery Balloon Injury.** The *in vivo* therapeutic effect of DE on neointimal hyperplasia was

investigated with a balloon injury animal model as previously described [13]. Male Sprague-Dawley rats (250–300 g; ORIENT-Charles River Technology) were separated into three groups, including vehicle treatment ( $n = 7$ ), DE (10 mg/kg) treatment ( $n = 7$ ), and DE (50 mg/kg) treatment ( $n = 7$ ). DE and vehicle were administered via oral sonde daily beginning two weeks before until four weeks after induction of balloon injury. To evaluate the effect of DE pretreatment on neointimal hyperplasia, SD rats were classified into three groups in separate studies, including vehicle treatment ( $n = 9$ ), DE treatment for four weeks after balloon injury ( $n = 9$ ), and DE pretreatment for two weeks followed by DE treatment for four weeks after balloon injury ( $n = 9$ ). Vehicle or DE (10 mg/kg/d) was administered daily. Four weeks after balloon injury, the morphometric analysis of common carotid arteries was performed by staining with hematoxylin and eosin. Images of each carotid artery section were analyzed by computerized morphometry (Scion Image Software), and luminal, intimal, and medial areas were then calculated for each arterial cross section. Neointimal formation was expressed as a percent computed as follows:  $[\text{intima}/(\text{media} + \text{intima})] \times 100 (\%)$ .

**2.7. Rat Model for Tail Vein Thrombosis.** To evaluate the effect of DE on thrombosis formation, thrombosis was induced via the tail veins of male SD rats (250–300 g) (Orient-Charles River Technology) classified into three groups, namely, vehicle (normal saline,  $n = 6$ ) or DE pretreatment for two weeks before the induction of tail vein thrombosis (10, 50 mg/kg, each  $n = 6$ ). DE was administered daily through oral gavage. Bleeding time was checked after pretreatment for two weeks as described previously [14]. Tail vein thrombosis was achieved with a modified Beckmeier's model as previously described [15]. The length of the thrombosis was evaluated 6, 24, 48, 96, and 168 hours after  $\kappa$ -carrageenan injection. Heparin (200 U (mL/Kg)<sup>-1</sup>) was injected i.p. as a positive control for antithrombotic agents 10 minutes after  $\kappa$ -carrageenan administration.

**2.8. Inhibitory Action on Rat Platelet Aggregation.** Platelet aggregation was evaluated using an impedance aggregometer (Chronolog model 700, Chronolog Corporation, Havertown, PA, USA) after *ex vivo* and *in vivo* treatment of DE. For *ex vivo* studies, whole blood (9 mL) was obtained from male SD rats (Orient-Charles River Technology) weighing between 250–300 g. Whole blood was collected in plastic syringes containing heparin (1.5%) (JW Pharmaceuticals, Seoul, Korea) to avoid premature aggregation. Single-use cuvettes containing a silicon-coated stirrer (Chronolog Corporation) (1200 rpm) were filled with 500  $\mu$ L physiological saline and 500  $\mu$ L prepared whole blood. After incubating for 15 minutes at 37°C, the aggregation of platelets in whole blood was initiated by adding ADP (20  $\mu$ M) (Sigma-Aldrich Co., MO, USA) as a stimulating agonist. Pretreatment of DE (0.2 mg/mL, 2.5 mg/mL, and 5.0 mg/mL) was performed 10 minutes prior to the initiation of aggregation. For *in vivo* studies, male SD rats (Orient-Charles River Technology) weighing between 200 to 250 g were classified into three

groups, namely, DE (10 mg/kg and 50 mg/kg) and vehicle treatments. In the DE treatment group (each group  $n = 4$ ), DE was administered daily via oral sonde for two weeks. After treatment with DE or normal saline, whole blood was obtained and analyzed with an impedance aggregometer.

**2.9. Rabbit Iliac Artery Stent Model.** The therapeutic effect of DE on thrombosis was investigated with an iliac artery stent insertion rabbit model. Briefly, the iliac artery stent was inserted as previously described [16]. Male New Zealand white rabbits ( $n = 3$ ) (Orient-Charles River Technology) weighing 3.5 to 4.0 kg were classified into two groups, namely, the normal saline group ( $n = 1$ ) and DE group ( $n = 2$ ). Each agent was injected subcutaneously twice a day from two weeks before to four weeks after the procedure. DE was dissolved in normal saline containing 1% DMSO and injected with a single 20 mg/kg dose. Aspirin was administered orally once a day at a dose of 100 mg/animal to all rabbits, beginning two days before the procedure and continued until sacrifice. Four weeks after the procedure, stented arteries were evaluated with intravascular optical coherence tomography (OCT). Animals were subsequently sacrificed and the stented segments of both iliac arteries were harvested. Using OCT and harvested arteries, the size of in-stent restenosis was calculated.

**2.10. Statistical Analyses.** Data were analyzed using Mann-Whitney tests. All statistical analyses were performed using SPSS (ver. 13.0 for Windows; SPSS, Inc., Chicago, IL, USA). All statistical tests were two-tailed, and  $P$  values <0.05 were considered significant.

### 3. Results

**3.1. Danshen Extract Contains an Abundance of Water-Soluble Components.** Twelve compounds were utilized as reference agents in UPLC-UV [17]. They included salvianic acid (danshensu), dihydroxybenzoic acid (protocatechuic acid), dihydroxybenzaldehyde (protocatechuic aldehyde), caffeic acid, rosmarinic acid, lithospermic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone I, cryptotanshinone, tanshinone I, and tanshinone IIA. Most of the compounds in DE were water soluble, whereas the number of solely liposoluble compounds was minimal (Table 1).

**3.2. Danshen Extract Has Antioxidant, Antimigratory and Antiproliferative Effects on PDGF-BB Stimulated VSMCs.** The effects of DE on ROS generation, migration, and proliferation of VSMCs were evaluated by PDGF-BB induction. According to the CM-H<sub>2</sub>DCF-DA emission results from FACS, DE most significantly decreased the amount of ROS at a concentration of 100  $\mu$ g/mL (Figure 1(a)). Further *in vitro* studies were conducted with a 100  $\mu$ g/mL concentration of DE, the levels of which increased MnSOD and HO-1 expression. Both are important molecules for the inhibition of VSMC proliferation and ROS production. The expression of HO-1 was increased by DE even after PDGF-BB treatment. On MnSOD, DE treatment prevented the decreased expression induced by

TABLE 1: Ultra performance liquid chromatography—ultraviolet assay of Danshen extracts.

No.	Reference compound	Amount in DE 1 mg ( $\mu\text{g}/\text{mg}$ )	Solubility
1	Salvianolic acid A (danshensuan A)	22.959	Water
2	Dihydroxybenzoic acid (Protocatechuic acid)	0.41	Water
3	Dihydroxybenzaldehyde (Protocatechuic aldehyde)	19.37	Water
4	Caffeic acid	4.34	Water
5	Rosmarinic acid	87.558	Water
6	Lithospermic acid	30.298	Water
7	Salvianolic acid B	133.932	Water
8	Salvianolic acid A	53.763	Water
9	Dihydrotanshinone I	<0.205	Lipid
10	Cryptotanshinone	<0.273	Lipid
11	Tanshinone I	<0.236	Lipid
12	Tanshinone IIA	<0.661	Lipid

PDGF-BB (Figure 1(b)). To evaluate changes in PDGF signaling after DE treatment, the expression of PI3K/Akt was analyzed. Although PDGF-BB (20 ng/mL, 24 h) stimulated the phosphorylation of Akt, this effect was blocked by DE treatment with a dose of 100  $\mu\text{g}/\text{mL}$  (Figure 1(c)).

According to migration studies with VSMCs, PDGF (20 ng/mL, 24 h) treatment resulted in wound-induced migration (Figure 1(d)). However, DE treatment significantly prevented PDGF induced wound migration. For MTT assays, quiescent cells were treated with DE (100  $\mu\text{g}/\text{mL}$  for 24 h) in the absence of PDGF-BB, and then stimulated with PDGF-BB (20 ng/mL, 24 h). Proliferation of VSMCs treated with PDGF-BB was 53% higher compared to that in PDGF-BB (–) control cells. DE inhibited PDGF-BB-induced VSMC proliferation in a dose-dependent manner, up to 65.7% inhibition at 100  $\mu\text{g}/\text{mL}$  of DE (Figure 1(e)). According to the cell cycle analysis, DE blocked the induction of S-phase entry, which normally occurs in PDGF-stimulated VSMCs (Figure 1(f)).

**3.3. Danshen Extract Exerts Anti-Inflammatory and Anti-apoptotic Effects on HUVECs.** To determine the effects of DE on endothelial cells, HUVECs with or without DE pretreatment for 24 h were incubated with TNF- $\alpha$  (10 ng/mL) for 24 hours. TNF- $\alpha$  alone increased the expression of VCAM-1 in HUVECs, whereas DE pretreatment significantly inhibited TNF- $\alpha$  induced VCAM-1 expression (Figure 2(a)). The effect of DE on VCAM-1 expression in the HUVECs correlated with that of balloon injured rat arteries. Although endothelium exhibited a significant increase in VCAM-1 expression after balloon injury, the expression of VCAM-1 in those of rats who had been given DE was significantly decreased (Figure 2(a)).

To determine the effect of DE on upstream signaling of VCAM-1, changes to the mitogen-activated protein kinase

(MAPK) and nuclear factor (NF)- $\kappa\text{B}$  pathways were evaluated. Briefly, cells were exposed to TNF- $\alpha$  (100 ng/mL) for 15 min with or without pretreatment with DE (100  $\mu\text{g}/\text{mL}$ ) for 24 h. With respect to TNF- $\alpha$ -induced MAPK activation, DE significantly inhibited the TNF- $\alpha$ -induced phosphorylation of JNK (Figure 2(b)). Furthermore, DE blocked the NF- $\kappa\text{B}$  pathway, which was confirmed by analyzing nuclear protein levels of NF- $\kappa\text{B}$  p65 (Figure 2(c)). Following TNF- $\alpha$  treatment on HUVECs, levels of cytosolic NF- $\kappa\text{B}$  were decreased, whereas increased nuclear expression was noted. However, DE apparently inhibited the increase of NF- $\kappa\text{B}$  p65 at nucleus with minimal change of cytosolic expression. In brief, DE inhibited TNF- $\alpha$ -activated MAPK and NF- $\kappa\text{B}$  signaling pathways in endothelial cells, which are known markers involved in the expression of VCAM-1. In addition, DE increased the expression of HO-1 in HUVECs, similar to VSMCs (Figure 2(d)).

**3.4. Danshen Extract Inhibits Balloon Injury-Induced Neointimal Hyperplasia.** Balloon injury-stimulated neointimal hyperplasia in the carotid artery of SD rats was explicitly compared to sham-operated rats. With respect to the dose of DE, the observed effects were significantly prevented by both doses of DE treatment, compared to the normal saline-treated group (58% and 37% in the DE 10 mg/kg and DE 50 mg/kg treated groups, respectively, both  $P < 0.001$ ) (Figures 3(a) and 3(b)). In aspect of the significance in DE pretreatment, both methods of DE treatment decreased neointimal hyperplasia to 42%, compared to the vehicle-treated group (55% and 42% in the DE 10 mg/kg only after the procedure, and DE 10 mg/kg both before and after procedure-treated groups, respectively, both  $P < 0.001$ ) (Figure 3(c)). Although there was not statistically significance, pretreatment with DE showed a more intense inhibitory effect on neointimal hyperplasia compared to the DE treatment group without pretreatment after the procedure (Figure 3(c)).

**3.5. Danshen Extract Serves As a Blood Thinner In Vivo and Ex Vivo.** DE treatment for two weeks resulted in a significantly prolonged bleeding time ( $P < 0.05$ ) (Figure 4(a)). For induction of thrombosis, Beckmeier's modified model was utilized, specifically. Rats pretreated with or without DE were observed over a period of seven days after  $\kappa$ -carrageenan injection, analyzing tail length and color. During the first 2 to 3 h after  $\kappa$ -carrageenan injection, the tail swelled and turned red. Next, the tail changed to an auburn color around 6 h after  $\kappa$ -carrageenan injection. Finally, 96 h after  $\kappa$ -carrageenan injection, the injured tails of the DE pretreatment group had recovered significantly compared to the vehicle pretreatment group ( $P < 0.05$ ). With respect to the formation of tail vein thrombosis, DE pretreatment produced the similar effect as heparin pretreatment (Figures 4(b) and 4(c)).

To evaluate the effect of DE on platelet activation, DE was administered using both *in vivo* and *ex vivo* models. In both models, DE inhibited the activation of platelets in a dose-dependent manner (Figures 4(d) and 4(e)). Interestingly, according to the *in vivo* study results, DE

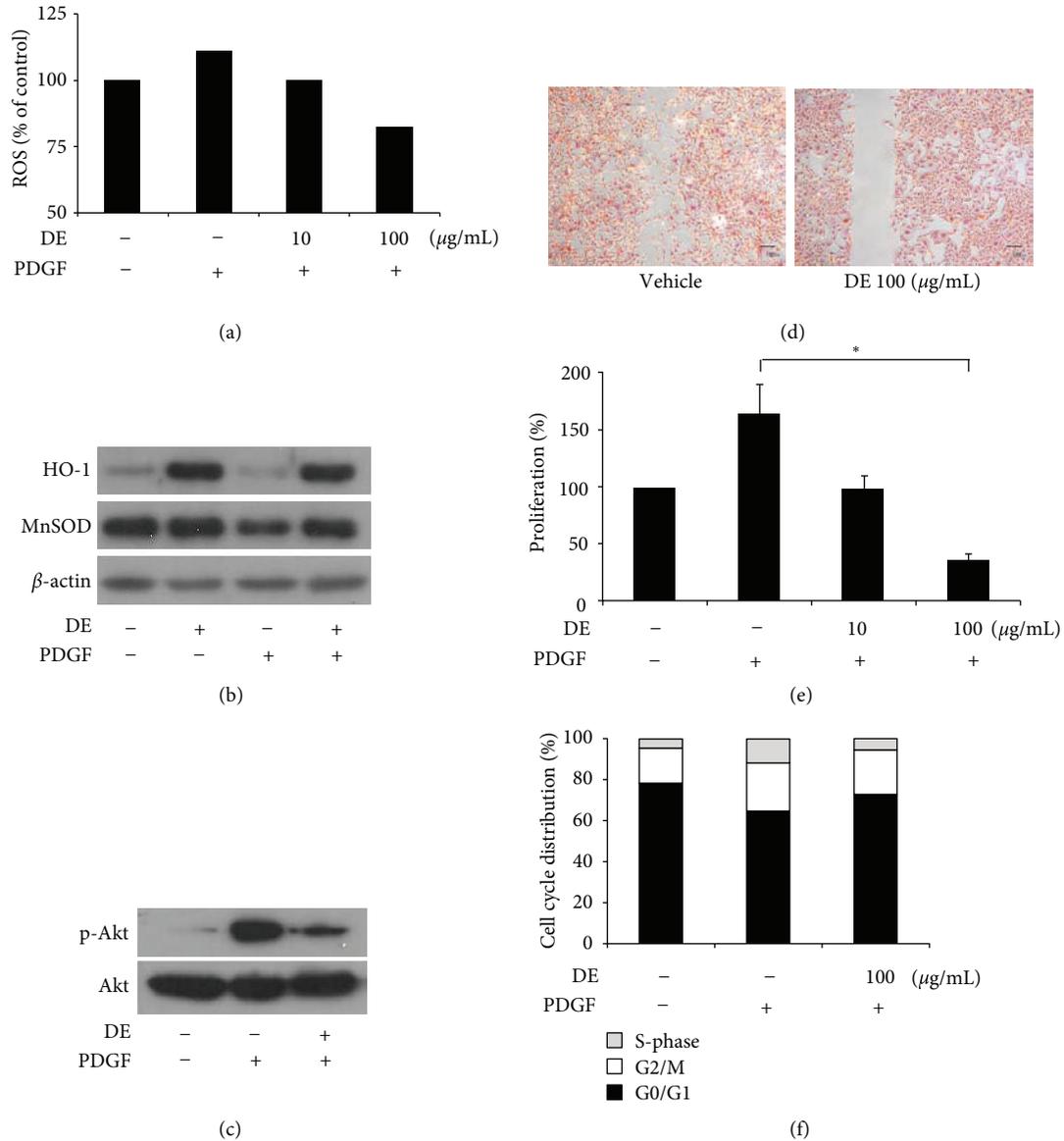


FIGURE 1: The antioxidant, antimigratory, and antiproliferative effects of Danshen extract on PDGF-BB-treated vascular smooth muscle cells. Platelet-derived growth factor (PDGF)-BB (20 ng/mL) was treated for 24 h after Danshen extracts (DE) treatment at each concentration for 24 h. (a) Fluorescence activated cell sorting for PDGF-BB-induced intracellular reactive oxygen species in vascular smooth muscle cells (VSMCs). (b) Western blotting for manganese superoxide dismutase and hemoxygenase-1. DE (100 μg/mL) treated for 24 h before PDGF treatment. (c) Western blotting for proteins on Akt signaling pathway after treatment DE (100 μg/mL) for 24 h. (d) The wound-healing experiment with or without DE (100 μg/mL, 24 h). (e) Proliferation assessed by the MTT cell proliferation assay. Relative proliferation activities were expressed using untreated control cells as a standard. Results are expressed as the mean ± standard error; \**P* < 0.05 versus vehicle treatment after PDGF-BB induction. (f) The effects of DE on cell cycle progression. DE: Danshen extract.

treatment induced early recovery from platelet aggregation, which was confirmed with an impedance aggregometer (Figure 4(e)).

**3.6. Danshen Extract Prevented In-Stent Restenosis.** Four weeks after stent insertion, the extent of in-stent restenosis was assessed in histological sections of stented rabbit iliac arteries. Compared with the levels in the control group treated with aspirin during procedure, DE treatment

combined with aspirin markedly attenuated the formation of in-stent restenosis (Figure 5(a)). Furthermore, OCT technology demonstrated that the area of restenosis in the DE-treated group was decreased to 30% compared with the control group (Figures 5(b) and 5(c)). The size of restenosis was analyzed at six points in each stented artery. In addition, minimal numbers of both red and white thrombi were found in the DE-treated group, whereas these cells were ubiquitously present in control cells.

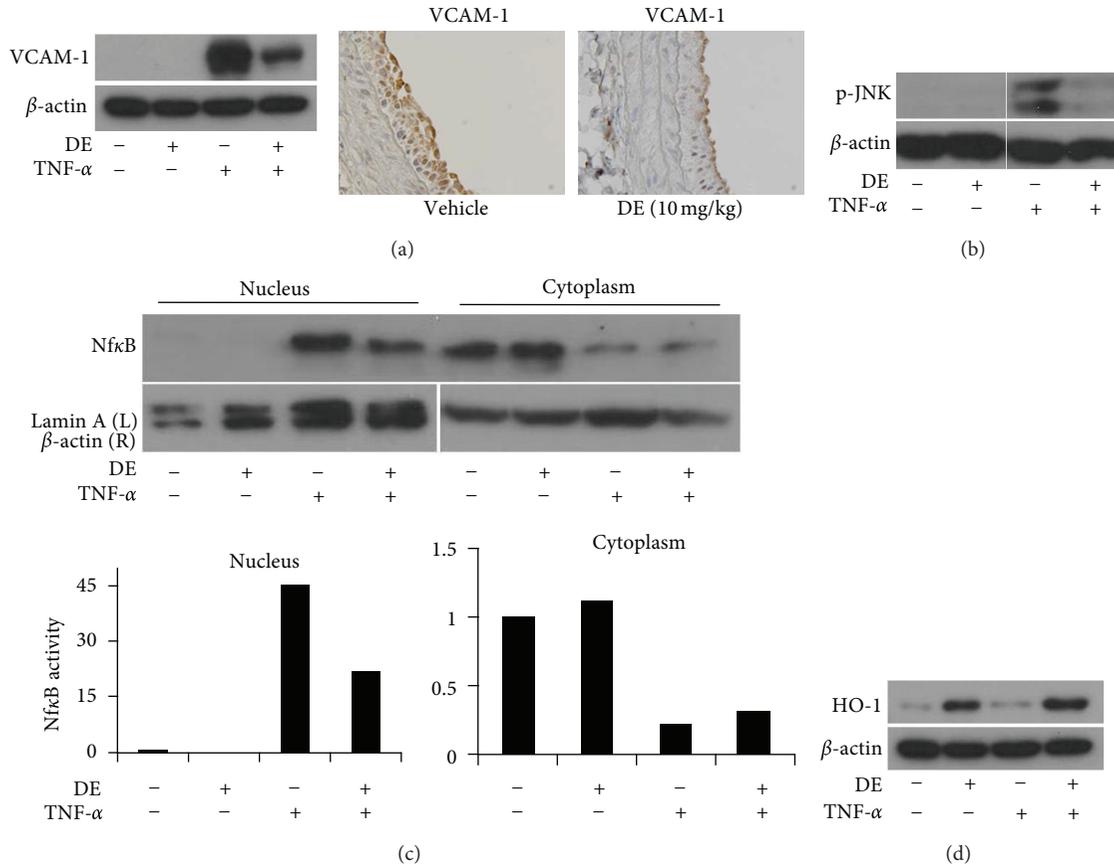


FIGURE 2: Anti-inflammatory and antiapoptotic effects of Danshen extract on human umbilical vein endothelial cells. (a) Western blotting (left panel) and immunohistochemical staining (right panel) for VCAM-1. Human umbilical vein endothelial cells (HUVECs) were pretreated with Danshen extract (DE) (100  $\mu$ g/mL) 24 h prior to exposure with TNF- $\alpha$  (10 ng/mL) for 24 h. Representative immunohistologic sections (200X) of VCAM-1 in balloon-injured rat carotid arteries were shown. (b)-(c) Western blotting for phosphorylated JNK and NF- $\kappa$ B p65. HUVECs were treated with TNF- $\alpha$  (100 ng/mL) for 15 min with or without pretreatment with DE (100  $\mu$ g/mL) for 24 h. (d) Western blotting for hemoxygenase-1 in both HUVECs with or without treatment of TNF- $\alpha$  (100 ng/mL) for 15 min. DE: Danshen extract.

#### 4. Discussion

Atherosclerosis is a multifactorial disease associated with various risk factors. The cellular pathogenesis of atherosclerosis is characterized by endothelial dysfunction, VSMC proliferation, and platelet aggregation. Importantly, these three factors play critical roles in the development of atherosclerosis and in the progress of cardiovascular disease [18, 19]. The mechanisms of aspirin and clopidogrel, which are two common medicines used in the treatment of atherosclerotic cardiovascular diseases, have various pathways affecting the vascular endothelium, VSMC, and platelets. However, these drugs have the potential for serious adverse reactions including hemorrhage and hematologic adverse reactions [20]. Furthermore, issues regarding resistance to aspirin and clopidogrel have led to the need for the development of newer agents.

Recently, several studies have reported that natural products such as herbal medicines have therapeutic effects on cardiovascular diseases. However, these treatments face limited acceptance by the clinical opinion makers due to the lack

of defined mechanisms involving their antiatherosclerotic effects. In this study we used three different animal models after analyzing the effects and mechanisms of DE *in vitro* with endothelial cells, VSMCs, and platelets.

The proliferation and migration of VSMCs to the intima leads to neointimal hyperplasia and this process is triggered by multiple factors. Cytokines and growth factors such as PDGF, which is produced by platelets, VSMCs, and endothelial cells in the injured vascular wall involved in this process. Abnormal proliferation of VSMCs is thought to play an important role in the pathogenesis of atherosclerosis and restenosis after stent insertion. Recent evidence indicates that increased intracellular level of ROS is one of the main mechanisms in proliferation and migration of VSMCs [21, 22]. MnSOD and HO-1 have the roles in the atherogenic effect of ROS. MnSOD is located in the mitochondrial matrix and protects the mitochondria against oxidative stress [23]. Together with MnSOD, HO-1 expression also attenuates PDGF-induced VSMC proliferation and ROS production [24]. In this study, treatment with DE led to decreased ROS production, followed by an increased abundance of MnSOD

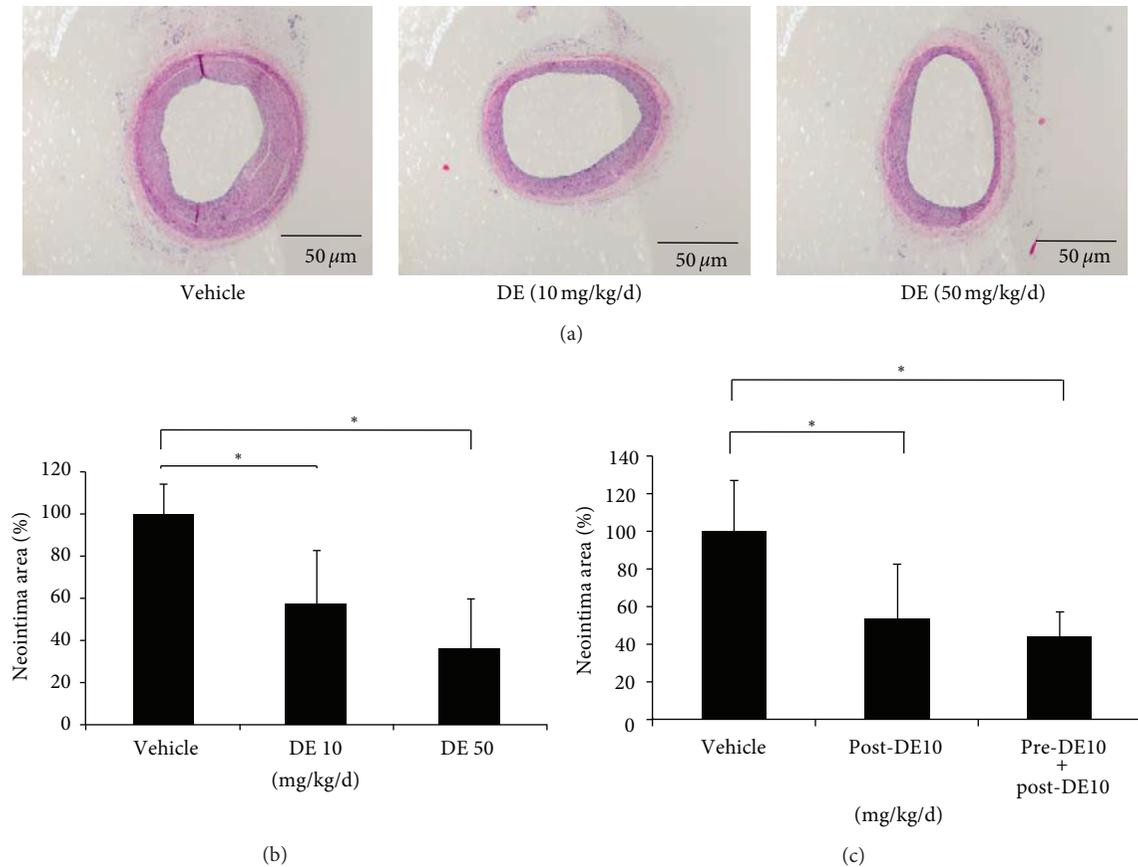


FIGURE 3: Effect of Danshen extract on neointimal formation after carotid artery balloon injury in rats. (a)-(b) Rats were orally administered one of two different concentrations of Danshen extract (DE) (10 and 50 mg/kg/d, both  $n = 7$ ; represented as DE 10 and DE 50, resp.) or vehicle ( $n = 7$ ) every day from 2 weeks before and to four weeks after balloon injury. (a) Representative histologic sections of neointimal formation. Left, vehicle treatment; Middle, DE (10 mg/kg/d) treatment; Right, DE (50 mg/kg/d) treatment. (b) Neointimal formation after balloon injury according to the doses of treated DE. (c) Neointimal formation after balloon injury according to the existence of DE pretreatment. To evaluate the effect of DE pretreatment on neointimal hyperplasia, rats were subjected to DE treatment (10 mg/kg/d) for one of two periods of time: four weeks after injury,  $n = 9$  (Post-DE 10); two weeks before injury until 4 weeks after injury,  $n = 9$  (Pre-DE10 + Post-DE10). The neointimal formation was calculated by  $[\text{intima}/(\text{media} + \text{intima})] \times 100$  (%). The bar indicates the standard error. \*  $P < 0.05$  for the indicated comparisons. DE: Danshen extract.

and HO-1 proteins. In aspect of VSMCs migration, PI3K-Akt pathway is important to activate the NADH oxidase, leading to ROS production. It is well known that PI3K/Akt is the main PDGF signaling pathway and that this pathway is linked to numerous cellular processes, including both proliferation and migration [25]. DE inhibited phosphorylation of Akt, resulting in the accompanying inhibition of VSMC migration. Furthermore, wound-induced migration was significantly prevented by DE. Briefly, DE inhibited proliferation and migration, decreased the production of ROS, and inhibited the PI3K-Akt pathway in VSMCs.

TNF- $\alpha$ , one of the major inflammatory cytokines, mediates systemic inflammation and immune responses by enhancing the expression of adhesion molecules and the secretion of inflammatory mediators in the vascular endothelium [26]. Accumulating evidence suggests that the induction of VCAM-1 or ICAM-1 by TNF- $\alpha$  in endothelium is mediated by the activation of MAPK and transcription factors such as NF- $\kappa$ B [13], leading to the pathogenesis

of atherosclerosis. Although TNF- $\alpha$  alone significantly increased the level of VCAM-1 expression, DE pretreatment clearly inhibited VCAM-1 expression in HUVECs. In the present study, the change of VCAM-1 expression was linked to effect of TNF- $\alpha$  in HUVECs as well as balloon-injured endothelium of rats after DE treatment. With respect to the upstream molecules of VCAM-1, MAPKs, which are a group of highly conserved serine/threonine kinases including JNK, participate in the vessel inflammatory reactions via integration of stimuli acting on cells, production of inflammatory mediators via the phosphorylation of downstream kinases, and the activation of transcription factors [27]. DE significantly inhibited TNF- $\alpha$ -induced phosphorylation of JNK in HUVECs. In this study, cytosolic NF- $\kappa$ B was decreased following increased expression of NF- $\kappa$ B protein in the nucleus after TNF- $\alpha$  treatment. However, treatment with DE apparently inhibited the NF- $\kappa$ B pathway by preventing translocation. Thus, our data suggested that DE inhibited TNF- $\alpha$ -induced activation of the JNK and NF- $\kappa$ B signaling

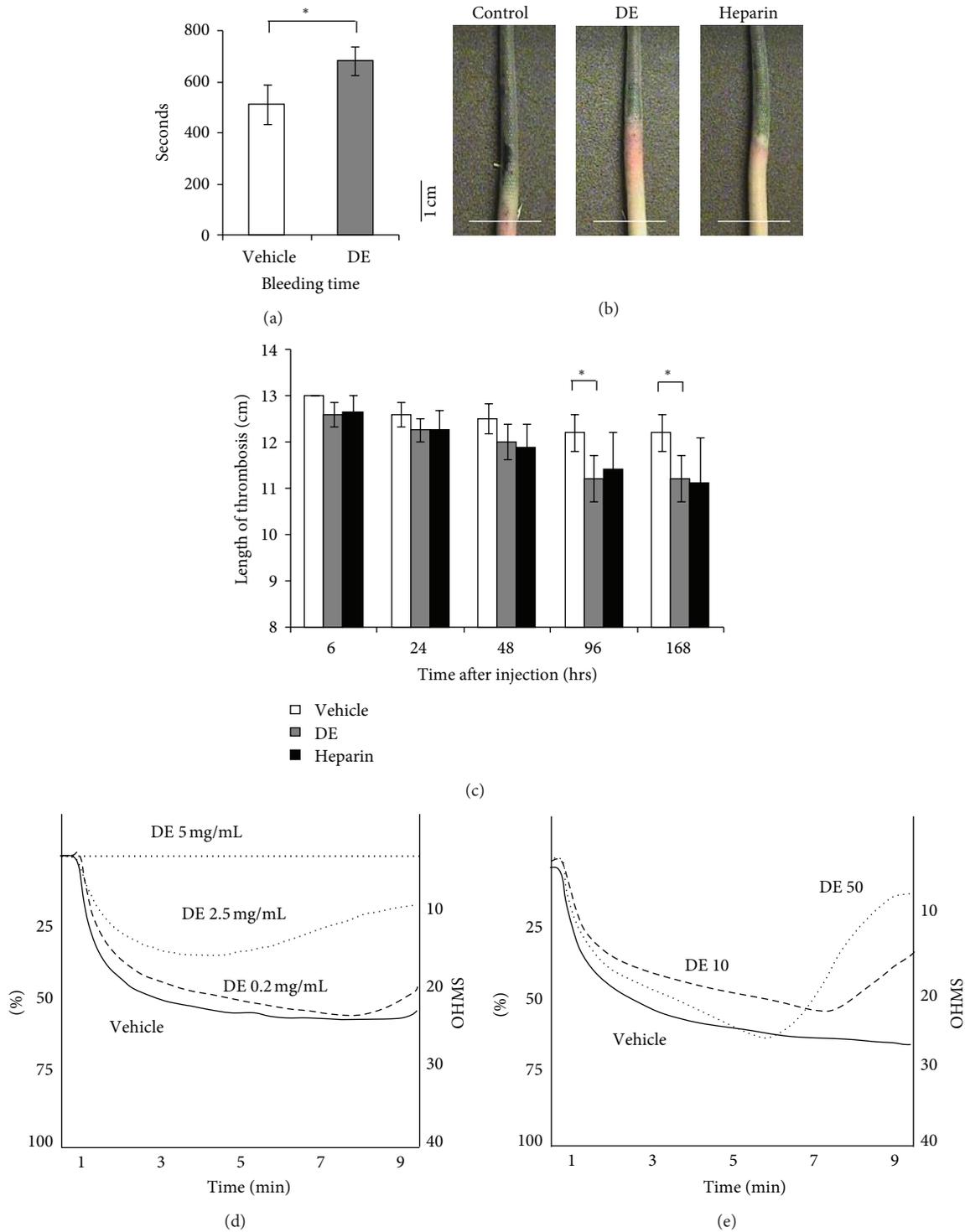


FIGURE 4: Blood thinning effect of Danshen extract. (a)–(c) Danshen extract (DE) pretreated at a dose of 10 mg/kg daily for 2 weeks via oral sonde ( $n = 6$  in each group). (a) Bleeding time in response to treatment with DE. (b) Gross changes in the tail vein 96 h after  $\kappa$ -carrageenan injection. The white bar indicates the position 13 cm from the tail tip. The black bar indicates the reference length. (c) The length of thrombosis after  $\kappa$ -carrageenan injection. In the tail vein thrombosis rat model, DE treatment significantly inhibited thrombosis formation at 96 h after  $\kappa$ -carrageenan injection. (d) Platelet aggregation after DE treatment in *ex vivo* condition. The impedance aggregometer revealed that DE prevented platelet aggregation in a dose-dependent manner *ex vivo*. (e) Platelet aggregation after DE treatment in *in vivo* condition. Whole blood was sampled after DE pretreatment. Danshen extract (DE) administered at a dose of 10 mg/kg/d (DE 10) or 50 mg/kg/d (DE 50) for 2 weeks via oral sonde ( $n = 4$  in each group). The platelet aggregation that was induced by ADP recovered more quickly with increasing doses of DE used in pretreatment. Graph of impedance aggregometer represented the mean value of each group. Results are expressed as the mean  $\pm$  standard error. \* $P < 0.05$  versus vehicle treatment. DE: Danshen extract.

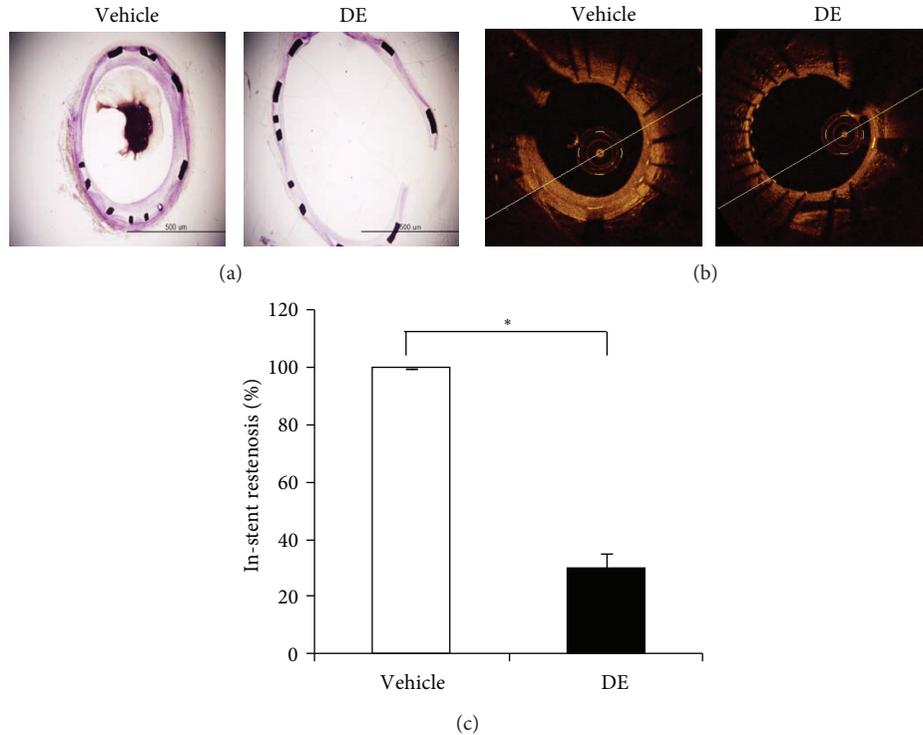


FIGURE 5: Effects of Danshen extract on in-stent restenosis. Rabbits were subcutaneously administered Danshen extract (DE; 20 mg/kg) twice a day from two weeks before to four weeks after the stent insertion. (a) Representative cross sections of stented iliac arteries in DE-treated and vehicle-treated rabbits 4 weeks after stent insertion. Sections were stained with hematoxylin and eosin; scale bar: 500  $\mu\text{m}$ . (b) Intravascular imaging for each iliac artery via intravascular optical coherence tomography (OCT). DE-treated rabbits showed significantly decreased in-stent restenosis. (c) Imaging analysis of in-stent restenosis, obtained from OCT. Results are expressed as the mean  $\pm$  standard error. \* $P < 0.05$  for the indicated comparisons. DE: Danshen extract.

pathways, which might contribute to the suppression of VCAM-1 in the endothelium.

Together with VSMCs and endothelial cells, thrombosis formation induced by platelets plays a major role in the development of atherosclerosis. In this study, we examined the antiplatelet and antithrombotic effects of DE. Platelet aggregation is a complex process that is mediated primarily through platelet adhesion at the injured site with the activation of endogenous agonists such as ADP, collagenase, and thrombin. These agonists stimulate platelet aggregation, resulting in thrombus formation through receptors on the platelet membrane [28]. In commercially popular agents, aspirin targets for the ADP. In this study, DE inhibited the ADP-induced aggregation of platelets in both *in vitro* and *in vivo* conditions. In the *in vivo* study with the impedance aggregometer, DE blocked the secondary activation of platelet aggregation in a manner similar to the effects of aspirin. Further study using multiple agonists for platelet activation could be helpful in understanding the antiplatelet effects of DE. The effect of DE on thrombus formation was studied using the rat model of tail vein thrombosis, a model that can be used to evaluate the effects of antithrombotic and thrombolytic agents [29]. Heparin treatment, which was used as a positive control agent, had similar effects on tail vein thrombosis as reported previously [29]. The antithrombotic effects of DE were similar to those of heparin. Furthermore,

antithrombotic effect of DE was reconfirmed in rabbit iliac artery stent model.

With results confirmed in both *in vitro* and *in vivo* rat models, the antiatherogenic effects of DE were analyzed in a rabbit animal model. The in-stent restenosis animal model is important for understanding thrombosis formation and vascular biology [30]. As seen with the rat models, DE had dramatic effects on the prevention of in-stent restenosis including vascular proliferation and thrombus formation in a rabbit model.

The progression of atherosclerosis consists of multiple processes including, oxidative stress, migration and proliferation of VSMC, inflammation and dysfunction of endothelial cells, and thrombosis formation. In this study, DE showed multiple therapeutic functions on each pathophysiology of atherosclerosis. Multifunctional roles of DE on pathogenesis of atherosclerosis could be explained by the multiple components constituting the extracts. The need for development of natural compounds as therapeutics might be explained with this reason. However, further studies should be advanced to evaluate the key mechanism of DE on atherosclerosis. The key mechanism could present the specified indication as therapeutics.

Several studies demonstrated the effect of each DE component on atherosclerosis, suggesting the possible mechanisms. Until now, most studies evaluating the

antiatherosclerotic effects of Danshen used lipid-soluble components and focused on it. In terms of the extract's water-soluble components, rosmarinic acid, lithospermic acid, ursolic acid, and salvianolic acid B have been reported to have antiatherogenic or antithrombotic effects [31, 32, 33]. Furthermore, we reported that protocatechuic aldehyde which is one of the main water-soluble components dissolved in DE inhibited migration and proliferation of VSMCs and intravascular thrombosis [34]. In aspect of molecular mechanisms, most of them had inhibitory actions on MMP-2/9, VACM-1, ICAM-1, and NF- $\kappa$ B. However, the protective action of the each component had been achieved with the relative high concentration compared with DE used in this study. DE used in this study was not comprised of a single component but rather a mixture of the components of the extracts. This included mostly water-soluble agents because of the method of extraction. DE used in this study showed more stronger effects compared with previously reported results for either single components or combinations of dual components at similar molecular concentrations [35]. This means that DE in this study had synergistic effects or at least additional effects on preventing atherosclerosis compared with that from each single component of danshen. The higher effects with relative lower concentration of each component are important to make it useful in developing new therapeutics. The results of this study suggest not only the medical usefulness of DE but also the need for evaluation of underlying mechanism on interaction between each component.

In conclusion, we have demonstrated that DE, with its prominent water-soluble components, exerts a protective effect against atherosclerosis by inhibiting multiple pathways associated with VSMCs, endothelial cells, and platelets. The antiatherogenic effects of DE were confirmed both *in vitro* and *in vivo*, including in rat and rabbit models. These results support the possibility of implementing water soluble DE as a therapeutic agent in the treatment of cardiovascular diseases. Further studies on the individual water-soluble components of DE are needed in order to evaluate the therapeutic effects of DE on cardiovascular diseases.

### Authors' Contribution

Y. H. Cho and C. R. Ku contributed equally to this work and should be considered co-first authors.

### Conflict of Interests

The authors declare that they have no conflict of interests.

### Acknowledgement

This work was supported by a Grant (A085136) from the Korea Healthcare R&D Project, Ministry for Health & Welfare Affairs, Republic of Korea.

### References

- [1] T. O. Cheng, "Cardiovascular effects of Danshen," *International Journal of Cardiology*, vol. 121, no. 1, pp. 9–22, 2007.
- [2] L. Zhou, Z. Zuo, and M. S. S. Chow, "Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use," *Journal of Clinical Pharmacology*, vol. 45, no. 12, pp. 1345–1359, 2005.
- [3] T. O. Cheng, "Warfarin danshen interaction," *Annals of Thoracic Surgery*, vol. 67, no. 3, p. 894, 1999.
- [4] T. O. Cheng, "Danshen: a popular chinese cardiac herbal drug," *Journal of the American College of Cardiology*, vol. 47, no. 7, pp. 1498–1500, 2006.
- [5] T. O. Cheng, "Danshen: what every cardiologist should know about this Chinese herbal drug," *International Journal of Cardiology*, vol. 110, no. 3, pp. 411–412, 2006.
- [6] J. Y. Han, J. Y. Fan, Y. Horie et al., "Ameliorating effects of compounds derived from *Salvia miltiorrhiza* root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion," *Pharmacology and Therapeutics*, vol. 117, no. 2, pp. 280–295, 2008.
- [7] A. Yagi, K. Fujimoto, K. Tanonaka, K. Hirai, and S. Takeo, "Possible active components of tan-shen (*Salvia miltiorrhiza*) for protection of the myocardium against ischemia-induced derangements," *Planta Medica*, vol. 55, no. 1, pp. 51–54, 1989.
- [8] J. Cao, L. W. Qi, J. Chen et al., "Application of liquid chromatography-electrospray ionization time-of-flight mass spectrometry for analysis and quality control of compound Danshen preparations," *Biomedical Chromatography*, vol. 23, no. 4, pp. 397–405, 2009.
- [9] M. S. Shiao, J. J. Chiu, B. W. Chang et al., "In search of antioxidants and anti-atherosclerotic agents from herbal medicines," *BioFactors*, vol. 34, no. 2, pp. 147–157, 2008.
- [10] S. Lee, H. J. Lim, H. Y. Park, K. S. Lee, J. H. Park, and Y. Jang, "Berberine inhibits rat vascular smooth muscle cell proliferation and migration *in vitro* and improves neointima formation after balloon injury *in vivo*. Berberine improves neointima formation in a rat model," *Atherosclerosis*, vol. 186, no. 1, pp. 29–37, 2006.
- [11] Z. Q. Yan and G. K. Hansson, "Overexpression of inducible nitric oxide synthase by neointimal smooth muscle cells," *Circulation Research*, vol. 82, no. 1, pp. 21–29, 1998.
- [12] M. Ohba, M. Shibamura, T. Kuroki, and K. Nose, "Production of hydrogen peroxide by transforming growth factor- $\beta$ 1 and its involvement in induction of egr-1 in mouse osteoblastic cells," *Journal of Cell Biology*, vol. 126, no. 4, pp. 1079–1088, 1994.
- [13] D. K. Jagadeesha, T. E. Lindley, J. DeLeon, R. V. Sharma, F. Miller, and R. C. Bhalla, "Tempol therapy attenuates medial smooth muscle cell apoptosis and neointima formation after balloon catheter injury in carotid artery of diabetic rats," *American Journal of Physiology*, vol. 289, no. 3, pp. H1047–H1053, 2005.
- [14] H. Nobukata, Y. Katsuki, T. Ishikawa, M. Inokuma, and Y. Shibutani, "Effect of dienogest on bleeding time, coagulation, fibrinolysis, and platelet aggregation in female rats," *Toxicology Letters*, vol. 104, no. 1-2, pp. 93–101, 1999.
- [15] M. Hagimori, S. Kamiya, Y. Yamaguchi, and M. Arakawa, "Improving frequency of thrombosis by altering blood flow in the carrageenan-induced rat tail thrombosis model," *Pharmacological Research*, vol. 60, no. 4, pp. 320–323, 2009.
- [16] H. S. Jang, H. Y. Nam, J. M. Kim et al., "Effects of curcumin for preventing restenosis in a hypercholesterolemic rabbit iliac artery stent model," *Catheterization and Cardiovascular Interventions*, vol. 74, no. 6, pp. 881–888, 2009.
- [17] J. Cao, Y. J. Wei, L. W. Qi et al., "Determination of fifteen bioactive components in *Radix et Rhizoma Salviae Miltiorrhizae* by

- high-performance liquid chromatography with ultraviolet and mass spectrometric detection,” *Biomedical Chromatography*, vol. 22, no. 2, pp. 164–172, 2008.
- [18] R. Ross, “The pathogenesis of atherosclerosis: a perspective for the 1990s,” *Nature*, vol. 362, no. 6423, pp. 801–809, 1993.
- [19] S. M. Schwartz, D. DeBlois, and E. R. M. O’Brien, “The intima: soil for atherosclerosis and restenosis,” *Circulation Research*, vol. 77, no. 3, pp. 445–465, 1995.
- [20] A. Kalyanasundaram and A. M. Lincoff, “Managing adverse effects and drug-drug interactions of antiplatelet agents,” *Nature Reviews Cardiology*, vol. 8, no. 10, pp. 592–600, 2011.
- [21] E. W. Raines, “PDGF and cardiovascular disease,” *Cytokine and Growth Factor Reviews*, vol. 15, no. 4, pp. 237–254, 2004.
- [22] J. Park, H. Ha, J. Seo et al., “Mycophenolic acid inhibits platelet-derived growth factor-induced reactive oxygen species and mitogen-activated protein kinase activation in rat vascular smooth muscle cells,” *American Journal of Transplantation*, vol. 4, no. 12, pp. 1982–1990, 2004.
- [23] D. Sivritas, M. U. Becher, T. Ebrahimian et al., “Antiproliferative effect of estrogen in vascular smooth muscle cells is mediated by Kruppel-like factor-4 and manganese superoxide dismutase,” *Basic Research in Cardiology*, vol. 106, no. 4, pp. 563–575, 2011.
- [24] J. E. Kim, J. Y. Sung, C. H. Woo et al., “Cilostazol inhibits vascular smooth muscle cell proliferation and reactive oxygen species production through activation of AMP-activated protein kinase induced by heme oxygenase-1,” *Korean Journal of Physiology & Pharmacology*, vol. 15, no. 4, pp. 203–210, 2011.
- [25] G. Gennaro, C. Ménard, S. É. Michaud, D. Deblois, and A. Rivard, “Inhibition of vascular smooth muscle cell proliferation and neointimal formation in injured arteries by a novel, oral mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor,” *Circulation*, vol. 110, no. 21, pp. 3367–3371, 2004.
- [26] J. S. Pober and R. S. Cotran, “Cytokines and endothelial cell biology,” *Physiological Reviews*, vol. 70, no. 2, pp. 427–451, 1990.
- [27] C. K. Wong, C. M. Tsang, W. K. Ip, and C. W. K. Lam, “Molecular mechanisms for the release of chemokines from human leukemic mast cell line (HMC)-1 cells activated by SCF and TNF- $\alpha$ : roles of ERK, p38 MAPK, and NF- $\kappa$ B,” *Allergy*, vol. 61, no. 3, pp. 289–297, 2006.
- [28] S. P. Jackson, W. S. Nesbitt, and S. Kulkarni, “Signaling events underlying thrombus formation,” *Journal of Thrombosis and Haemostasis*, vol. 1, no. 7, pp. 1602–1612, 2003.
- [29] M. Hagimori, S. Kamiya, Y. Yamaguchi, and M. Arakawa, “Improving frequency of thrombosis by altering blood flow in the carrageenan-induced rat tail thrombosis model,” *Pharmacological Research*, vol. 60, no. 4, pp. 320–323, 2009.
- [30] G. Nakazawa, A. V. Finn, E. Ladich et al., “Drug-eluting stent safety: Findings from preclinical studies,” *Expert Review of Cardiovascular Therapy*, vol. 6, no. 10, pp. 1379–1391, 2008.
- [31] L. Zhou, Z. Zuo, and M. S. S. Chow, “Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use,” *Journal of Clinical Pharmacology*, vol. 45, no. 12, pp. 1345–1359, 2005.
- [32] K. Steinkamp-Fenske, L. Bollinger, N. Völler et al., “Ursolic acid from the Chinese herb Danshen (*Salvia miltiorrhiza* L.) upregulates eNOS and downregulates Nox4 expression in human endothelial cells,” *Atherosclerosis*, vol. 195, no. 1, pp. e104–e111, 2007.
- [33] L. Chen, W. Y. Wang, and Y. P. Wang, “Inhibitory effects of lithospermic acid on proliferation and migration of rat vascular smooth muscle cells,” *Acta Pharmacologica Sinica*, vol. 30, no. 9, pp. 1245–1252, 2009.
- [34] C. Y. Moon, C. R. Ku, Y. H. Cho, and E. J. Lee, “Protocatechuic aldehyde inhibits migration and proliferation of vascular smooth muscle cells and intravascular thrombosis,” *Biochemical and Biophysical Research Communications*, vol. 423, no. 1, pp. 116–121, 2012.
- [35] H. Y. Fan, F. H. Fu, M. Y. Yang, H. Xu, A. H. Zhang, and K. Liu, “Antiplatelet and antithrombotic activities of salvianolic acid A,” *Thrombosis Research*, vol. 126, no. 1, pp. e17–e22, 2010.

## Research Article

# Banxia Baizhu Tianma Decoction for Essential Hypertension: A Systematic Review of Randomized Controlled Trials

Xingjiang Xiong,<sup>1</sup> Xiaochen Yang,<sup>1</sup> Wei Liu,<sup>1</sup> Bo Feng,<sup>1</sup> Jizheng Ma,<sup>2</sup>  
Xinliang Du,<sup>3</sup> Pengqian Wang,<sup>4</sup> Fuyong Chu,<sup>5</sup> Jun Li,<sup>1</sup> and Jie Wang<sup>1</sup>

<sup>1</sup> Department of Cardiology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beixiangge 5, Xicheng District, Beijing 100053, China

<sup>2</sup> Department of Gastroenterology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100053, China

<sup>3</sup> Graduate School, China Academy of Chinese Medical Sciences, Beijing 100700, China

<sup>4</sup> Department of Endocrinology, Traditional Chinese Medicine Hospital of Mentougou District, Beijing 102300, China

<sup>5</sup> Department of Cardiology, Traditional Chinese Medicine Hospital of Beijing, Beijing 100010, China

Correspondence should be addressed to Xingjiang Xiong, 5administration@163.com and Jie Wang, wangjie0103@yahoo.cn

Received 23 August 2012; Revised 16 November 2012; Accepted 22 November 2012

Academic Editor: MyeongSoo Lee

Copyright © 2012 Xingjiang Xiong et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Objectives.** To assess the current clinical evidence of Banxia Baizhu Tianma Decoction (BBTD) for essential hypertension (EH). **Search Strategy.** Electronic databases were searched until July 2012. **Inclusion Criteria.** We included randomized clinical trials testing BBTD against placebo, antihypertensive drugs, or combined with antihypertensive drugs against antihypertensive drugs. **Data Extraction and Analyses.** Study selection, data extraction, quality assessment, and data analyses were conducted according to Cochrane standards. **Results.** 16 randomized trials were included. Methodological quality of the included trials was evaluated as generally low. 2 trials compared prescriptions based on BBTD using alone with antihypertensive drugs. Meta-analysis showed no significant effect of modified BBTD compared with captopril in systolic blood pressure (MD:  $-0.75$  ( $-5.77, 4.27$ );  $P = 0.77$ ) and diastolic blood pressure (MD:  $-0.75$  ( $-2.89, 1.39$ );  $P = 0.49$ ). 14 trials compared the combination of BBTD or modified BBTD plus antihypertensive drugs with antihypertensive drugs. Meta-analysis showed there are significant beneficial effect on systolic blood pressure in the combination group compare to the antihypertensive drugs (MD:  $-4.33$  ( $-8.44, -0.22$ );  $P = 0.04$ ). The safety of BBTD is uncertain. **Conclusions.** There is encouraging evidence of BBTD for lowering SBP, but evidence remains weak. Rigorously designed trials are warranted to confirm these results.

## 1. Introduction

Hypertension is an increasingly important medical and public health issue, which could lead to severe complications [1]. High blood pressure is a major, independent risk factor for cardiovascular disease (CVD). The relationship between blood pressure (BP) and risk of CVD events is continuous, consistent, and independent of other risk factors. The higher the BP, the greater is the chance of heart attack, heart failure, stroke, and kidney diseases. The prevention and management of hypertension are major public health challenges. Much of hypertension, cardiovascular, and cerebrovascular diseases would be preventable if the rise in BP with age could be prevented or diminished [2].

Complementary and alternative medicine (CAM) is becoming increasingly popular and frequently used among patients with CVD [3–7]. Approximately 50% of US residents use some form of alternative medicine; 10% use it for their children [8]. Recent researches showed that CAM (also integrative medicine) could contribute to blood pressure control [9–12]. Chinese medicine (CM) [13, 14], including herbal medicine, acupuncture, moxibustion, and cupping *Tai chi* and *Qigong*, as one of the most important parts in CAM, is thought to be effective for the treatment of essential hypertension [15–18]. It has been considered as an effective adjunct treatment by either physicians or patients in China. More and more patients firstly select the combination

therapy, just CM combined with antihypertensive drugs, for better efficacy both in BP and clinical symptom such as headache, neck and shoulder pain, dizziness, and fatigue. For seeking the best evidence of CM in making decisions for hypertensive patients, an increasing number of systematic reviews (SR) and meta-analysis have been conducted to assess the efficiency of CM for hypertension [19–24]. It is found out that CM could contribute to lower BP smoothly, recover the circadian rhythm of BP, and improve symptoms and signs especially [25]. And the efficacy of CM for treating hypertension is suggested by a large number of published case series and randomized trials [26, 27], although some trials have demonstrated negative results [28, 29]. Mechanistic studies have demonstrated that the antihypertensive effect is related to activation of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) [30, 31], regulation of vascular endothelium function [26, 32], inhibiting proliferation of adventitial fibroblasts and collagen synthesis [33], inhibition of vascular smooth muscle cell proliferation [34], and so forth. A series of Chinese herbs have been authorized recommended by the Chinese government in Pharmacopoeia of the People's Republic of China (2010 edition).

Banxia baizhu tianma decoction (BBTD), containing eight commonly used herbs (*Pinellia ternata*, *atractylodes macrocephala*, *Gastrodia elata*, tangerine peel, poria cocos, *Glycyrrhiza*, ginger, and red jujube), is a classical Chinese herbal formula noted in *Medical Revelations* in Qing dynasty. It has been widely used to treat hypertension-related symptoms in clinical practice for centuries in China [25]. The most common symptoms include headache, dizziness, nausea, and vomiting, which belong to the liver yang hyperactivity and fluid retention syndrome [25]. The mechanism of the prescription maybe calming liver, suppressing liver yang hyperactivity, dissipating excessive fluid, and expelling phlegm according to the theory of TCM. Recently, modern researches showed that BBTD have potential effect of lowering BP *in vitro* and *in vivo* [25, 35–38]. Biochemically, BBTD also showed good effect in improving the mesenteric endothelial dysfunction and the hemodynamic parameters, inhibiting the expression of nitric oxide (NO) and interleukin-1 (IL-1), decreasing serum levels of total cholesterol (TC), triglycerides (TGs), and low-density lipoprotein-cholesterol (LDL-C), regulating rennin-angiotensin system (RAS), and improving the oxidative stress state, so as to lower the arterial pressure [35–38].

Currently, BBTD used alone or combined with antihypertensive drugs has been widely used as an alternative and effective method for the treatment of essential hypertension in China. And until now a number of clinical studies of BBTD reported the clinical effect ranging from case reports and case series to controlled observational studies and randomized clinical trials. However, there is no critically appraised evidence such as systematic reviews or meta analyses on potential benefits and harms of BBTD for essential hypertension to justify their clinical use and their recommendation. This paper aims to assess the current clinical evidence of BBTD for essential hypertension.

## 2. Methods

**2.1. Database and Search Strategies.** Literature searches were conducted in Chinese National Knowledge Infrastructure (CNKI), Chinese Scientific Journal Database (VIP), Chinese Biomedical Literature Database (CBM), PubMed, EMBASE, and the Cochrane Central Register of Controlled Trials (CENTRAL) in the Cochrane Library (July 2012). We also searched the reference list of retrieved papers. Databases in Chinese were searched to retrieve the maximum possible number of trials of BBTD for essential hypertension because BBTD is mainly used and researched in China. All of those searches ended on 3 July, 2012. Ongoing registered clinical trials were searched in the website of Chinese clinical trial registry (<http://www.chictr.org/>) and international clinical trial registry by US National Institutes of Health (<http://clinicaltrials.gov/>). The following search terms were used individually or combined: “hypertension,” “essential hypertension,” “banxia baizhu tianma decoction,” “clinical trial,” and “randomized controlled trial.” The bibliographies of included studies were searched for additional references.

**2.2. Inclusion Criteria.** All the parallel randomized controlled trials (RCTs) of all the prescriptions based on “banxia baizhu tianma decoction” compared with antihypertensive drugs in patients with hypertension were included. RCTs combined banxia baizhu tianma decoction with antihypertensive drugs compared with antihypertensive drugs and all the modified banxia baizhu tianma decoction were included as well. According to the principle of the similarity of traditional Chinese medicine (TCM) formula [39], the number of modified herbs should not be more than 4, so that to ensure the similarity is greater than or equal to 0.5. And the key herbs in the modified banxia baizhu tianma decoction should include *Pinellia ternata*, *atractylodes macrocephala*, *Gastrodia elata*, and poria cocos, according to the theory of TCM. There were no restrictions on population characteristics, language and publication type. The main outcome measure was blood pressure. Duplicated publications reporting the same groups of participants were excluded.

**2.3. Data Extraction and Quality Assessment.** Two authors conducted the literature searching (Xiong and Yang), study selection (Xiong and Wang), and data extraction (Xiong and Li) independently. The extracted data included authors, title of study, year of publication, study size, age and sex of the participants, details of methodological information, name and component of Chinese herbs, treatment process, details of the control interventions, outcomes, and adverse effects for each study. Disagreement was resolved by discussion and reached consensus through a third party (J. Wang).

The methodological quality of trials was assessed independently using criteria from the Cochrane Handbook for Systematic Review of Interventions, Version 5.1.0 (Xiong and Yang) [56]. The items included random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete

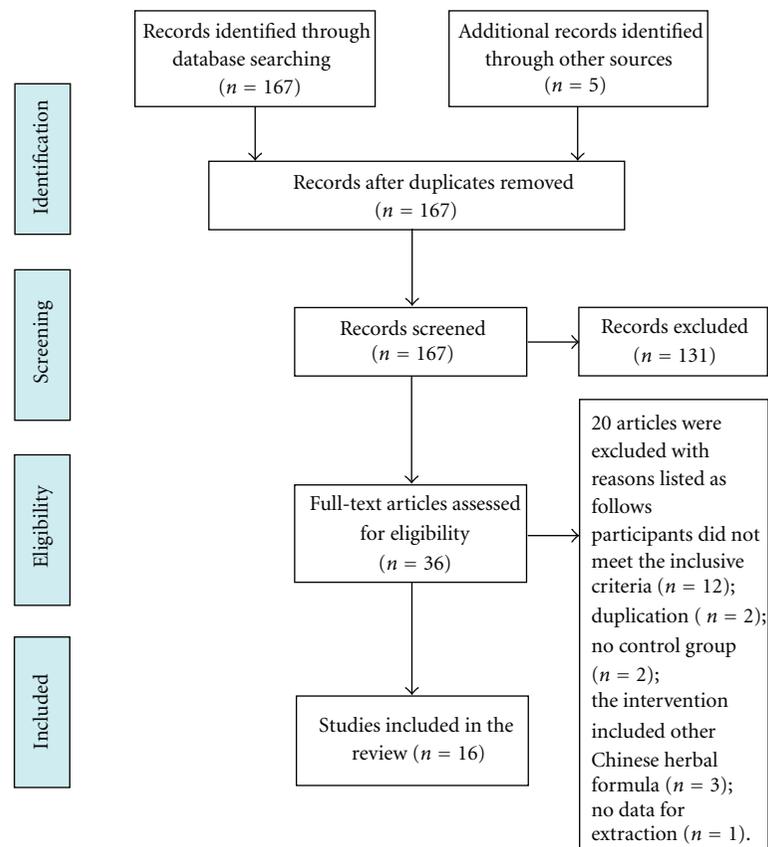


FIGURE 1: PRISMA 2009 flow diagram.

outcome data (attrition bias), selective reporting (reporting bias), and other bias. The quality of all the included trials was categorized to low/unclear/high risk of bias (“yes” for a low of bias, “no” for a high risk of bias, “unclear” otherwise). Then trials were categorized into three levels: low risk of bias (all the items were in low risk of bias), high risk of bias (at least one item was in high risk of bias), unclear risk of bias (at least one item was in unclear).

**2.4. Data Synthesis.** RevMan 5.1 software provided by the Cochrane Collaboration was used for data analyses. Continuous outcome will be presented as mean difference (MD) and its 95% CI. Heterogeneity was recognized significant when  $I^2 \geq 50\%$ . Fixed-effects model was used if there is no significant heterogeneity of the data; random-effects model was used if significant heterogeneity existed ( $50\% < I^2 < 85\%$ ). Publication bias would be explored by funnel plot analysis if sufficient studies were found.

### 3. Result

**3.1. Description of Included Trials.** A flow chart depicted the search process and study selection (as shown in Figure 1). After primary searches from the databases, 167 articles were screened. After reading the titles and abstracts, 131 articles of them were excluded. Full texts of 36 articles were

retrieved, and 20 articles were excluded with reasons listed as follows: participants did not meet the inclusive criteria ( $n = 12$ ), duplication ( $n = 2$ ), no control group ( $n = 2$ ), the intervention included other Chinese herbal formula ( $n = 3$ ), and no data for extraction ( $n = 1$ ). In the end, 16 RCTs [40–55] were included. All the RCTs were conducted in China and published in Chinese. The characteristics of included trials were listed in Table 1.

1424 patients with essential hypertension were included. There was a wide variation in the age of subjects (19–78 years). Sixteen (16) trials specified five diagnostic criteria of hypertension, five trials [40, 41, 45, 50, 52] used Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005), five trials [43, 44, 49, 53, 55] used 1999 WHO-ISH guidelines for the management of hypertension (1999 WHO-ISH GMH), two trials [46, 47] used China Guidelines on Prevention and Management of High Blood Pressure-2004 (CGPMHBP-2004), one trial [51] used Chinese Guidelines for the Management of Hypertension-1999 (CGMH-1999), one trial [51] used the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-VI), and two trials [42, 48] only demonstrated patients with essential hypertension. Sixteen (16) trials specified three diagnostic criteria of abundant phlegm-dampness syndrome in TCM, nine trials [41, 45, 46, 49–53, 55] used Guidelines of Clinical Research of New Drugs of Traditional Chinese

TABLE 1: Characteristics and methodological quality of included studies.

Study ID	Sample	Diagnosis standard	Intervention	Control	Course (week)	Outcome measure
Zheng, 2011 [40]	60	CGMH-2005	Modified BBTD (600 mL/d <sup>#</sup> )	Enalapril (10 mg qd)	3	BP; adverse effect
Xiong, 2010 [41]	60	CGMH-2005; GCRNDTCM	Modified BBTD (250 mL/d <sup>#</sup> ) plus L-amlodipine (2.5 mg qd)	L-amlodipine (2.5 mg qd)	4	BP
Chen et al., 2005 [42]	70	Hypertension diagnostic criteria (unclear); CDTDSS	Modified BBTD (240 mL/d <sup>#</sup> ) plus nitrendipine (no detailed information)	Nitrendipine (no detailed information)	8	BP
Wang, 2001 [43]	100	1999 WHO-ISH GMH	Modified BBTD (1 dose/d <sup>#</sup> ) plus captopril (25–37.5 mg tid)	Captopril (25–37.5 mg tid)	4	BP
Che et al., 2011 [44]	60	1999 WHO-ISH GMH; TCM diagnostic criteria (unclear)	Modified BBTD (400 mL/d <sup>#</sup> ) plus nifedipine controlled release tablet (10 mg bid)	Nifedipine controlled release tablet (10 mg bid)	4	BP; adverse effect
Jin, 2011 [45]	60	CGMH-2005; GCRNDTCM	BBTD plus antihypertensive drugs (400 mL/d <sup>#</sup> )	Antihypertensive drugs (no detailed information)	6	BP; adverse effect
Chen, 2007 [46]	120	CGPMHBP-2004; GCRNDTCM	Modified BBTD (1 dose/d <sup>#</sup> ) plus losartan (50 mg qd)	Losartan (50 mg qd)	12	BP
Guo, 2009 [47]	94	CGPMHBP-2004; CIM	Modified BBTD (400–500 mL/d <sup>#</sup> ) plus compound reserpine-triamterene tablets (1 pill, qd)	Compound reserpine-triamterene tablets (1 pill, qd)	4	BP
Li, 2011 [48]	139	Hypertension and TCM diagnostic criteria (unclear)	Modified BBTD (450 mL/d <sup>#</sup> ) plus felodipine sustained release tablets (1 pill, bid)/levamlodipine besylate tablets (1 pill, qd)	Felodipine sustained release tablets (1 pill, bid)/levamlodipine besylate tablets (1 pill, qd)	12	BP
Guo et al., 2006 [49]	116	1999 WHO-ISH GMH; GCRNDTCM	Modified BBTD (250 mL/d <sup>#</sup> ) plus levamlodipine besylate tablets (5 mg qd)	Levamlodipine besylate tablets (5 mg qd)	2	BP
Zhou, 2008 [50]	102	CGMH-2005; GCRNDTCM	Modified BBTD (400 mL/d <sup>#</sup> ) plus nifedipine sustained release tablets (10 mg bid)	Nifedipine sustained release tablets (10 mg bid)	4	BP; adverse effect
Wu et al., 2007 [51]	87	CGMH-1999; GCRNDTCM	Modified BBTD (1 dose/d <sup>#</sup> ) plus antihypertensive drugs (no detailed information)	Antihypertensive drugs (no detailed information)	8	BP
Lei and Lin, 2009 [52]	114	CGMH-2005; GCRNDTCM	BBTD (400 mL/d <sup>#</sup> ) plus benazepril (10 mg qd)	Benazepril (10 mg qd)	4	BP
Liu et al., 2007 [53]	80	1999 WHO-ISH GMH; GCRNDTCM	Modified BBTD (300 mL/d <sup>#</sup> )	Captopril (12.5 mg bid)	4	BP
Zhang, 2002 [54]	80	JNC-VI	Modified BBTD (1 dose/d <sup>#</sup> ) plus felodipine (5 mg qd)/hydrochlorothiazide (12.5 mg bid)	Felodipine (5 mg qd)/hydrochlorothiazide (12.5 mg bid)	1	BP
Wang, 2005 [55]	82	1999 WHO-ISH GMH; GCRNDTCM	Modified BBTD (1 dose/d <sup>#</sup> ) plus captopril (no detailed information)	Captopril (no detailed information)	4	BP

TABLE 2: Composition of formula.

Study ID	Formula	Composition of formula
Zheng, 2011 [40]	Modified BBTD	<i>Pinellia ternate</i> 9 g, <i>atractylodes macrocephala</i> 15 g, <i>Gastrodia elata</i> 10 g, tangerine peel 10 g, <i>poria cocos</i> 10 g, <i>Glycyrrhiza</i> 4 g, ginger 2 pieces, red jujube 5, grass leaved sweetflag 10 g, <i>ligusticum chuanxiong hort</i> 9 g, <i>alisma orientalis</i> 15 g, and <i>Grifola umbellata</i> 10 g
Xiong, 2010 [41]	Modified BBTD	<i>Pinellia ternate</i> 12 g, <i>atractylodes macrocephala</i> 15 g, <i>Gastrodia elata</i> 15 g, tangerine peel 12 g, <i>poria cocos</i> 12 g, <i>alisma orientalis</i> 15 g, plantain seed 15 g, bamboo bark 9 g, villous <i>amomum fruit</i> 3 g, <i>Pinellia pedatisecta</i> Schott 12 g, grass leaved sweetflag 15 g, ginger 9 g, red jujube 5, and <i>Glycyrrhiza</i> 6 g
Chen et al., 2005 [42]	Modified BBTD	<i>Pinellia ternate</i> 6 g, <i>Gastrodia elata</i> 9 g, <i>atractylodes macrocephala</i> 9 g, <i>poria cocos</i> 12 g, tangerine peel 12 g, <i>Pinellia pedatisecta</i> Schott 12 g, <i>fructus aurantii</i> 12 g, <i>Glycyrrhiza</i> 6 g
Wang, 2001 [43]	Modified BBTD	<i>Pinellia ternate</i> 15 g, <i>atractylodes macrocephala</i> 12 g, <i>Gastrodia elata</i> 15 g, tangerine peel 12 g, <i>poria cocos</i> 12 g, <i>alisma orientalis</i> 15 g, <i>Uncaria</i> 15 g (put in later), abalone shell 15 g (decocting first), ginger 15 g, jujube 5, and <i>Glycyrrhiza</i> 6 g
Che et al., 2011 [44]	Modified BBTD	<i>Pinellia ternate</i> 15 g, <i>atractylodes macrocephala</i> 25 g, <i>Gastrodia elata</i> 10 g, tangerine peel 10 g, <i>poria cocos</i> 10 g, kudzu root 10 g, <i>Sophora flower</i> 15 g, cassia seed 10 g, hawthorn 15 g, and <i>Glycyrrhiza</i> 5 g
Jin, 2011 [45]	BBTD	<i>Pinellia ternate</i> 10 g, <i>atractylodes macrocephala</i> 10 g, <i>Gastrodia elata</i> 10 g, tangerine peel 10 g, <i>poria cocos</i> 15 g, <i>Glycyrrhiza</i> 5 g, ginger 10 g, and jujube 10 g
Chen, 2007 [46]	Modified BBTD	<i>Pinellia ternate</i> 9 g, <i>atractylodes macrocephala</i> 12 g, <i>Gastrodia elata</i> 6 g, tangerine peel 10 g, <i>poria cocos</i> 15 g, <i>alisma orientalis</i> 10 g, hawthorn 10 g, cassia seed 15 g, grass leaved sweetflag 6 g, <i>ligusticum chuanxiong hort</i> 6 g, <i>Salvia miltiorrhiza</i> 12 g, and <i>Glycyrrhiza</i> 5 g
Guo, 2009 [47]	Modified BBTD	<i>Pinellia ternate</i> 12 g, <i>atractylodes macrocephala</i> 15 g, <i>Gastrodia elata</i> 10 g, tangerine peel 9 g, <i>poria cocos</i> 10 g, <i>ligusticum chuanxiong hort</i> 10 g, officinal magnolia bark 6–10 g, chrysoidine 9 g, grass leaved sweetflag 10 g, <i>curcuma longa</i> 10 g, ginger 3 pieces, and jujube 3
Li, 2011 [48]	Modified BBTD	<i>Pinellia ternate</i> 10 g, <i>atractylodes macrocephala</i> 10 g, <i>Gastrodia elata</i> 10 g, tangerine peel 12 g, <i>poria cocos</i> 15 g, <i>citrus aurantium</i> 10 g, bamboo bark 10 g, and <i>Glycyrrhiza</i> 6 g
Guo et al., 2006 [49]	Modified BBTD	<i>Pinellia ternate</i> 18 g, <i>atractylodes macrocephala</i> 12 g, <i>Gastrodia elata</i> 18 g, tangerine peel 12 g, <i>poria cocos</i> 15 g, grass leaved sweetflag 15 g, <i>Eucommia ulmoides Oliv.</i> 15 g, <i>Prunella vulgaris</i> 12 g, <i>Glycyrrhiza</i> 6 g, and jujube 5
Zhou, 2008 [50]	Modified BBTD	<i>Pinellia ternate</i> 12 g, <i>atractylodes macrocephala</i> 12 g, <i>Gastrodia elata</i> 6 g, tangerine peel 9 g, <i>poria cocos</i> 12 g, bamboo bark 9 g, <i>Glycyrrhiza</i> 6 g, villous <i>amomum fruit</i> 3 g, ginger 3 g, and jujube 5
Wu et al., 2007 [51]	Modified BBTD	<i>Pinellia ternate</i> 10 g, <i>atractylodes macrocephala</i> 10 g, <i>Gastrodia elata</i> 10 g, tangerine peel 10 g, <i>poria cocos</i> 15 g, bamboo bark 10 g, <i>Coix lacryma-jobi</i> 20 g, <i>Glycyrrhiza</i> 3 g, and ginger 3 pieces
Lei and Lin, 2009 [52]	BBTD	<i>Pinellia ternate</i> 12 g, <i>atractylodes macrocephala</i> 12 g, <i>Gastrodia elata</i> 15 g, tangerine peel 9 g, <i>poria cocos</i> 12 g, <i>Glycyrrhiza</i> 6 g, ginger 3 g, and jujube 5
Liu et al., 2007 [53]	Modified BBTD	<i>Pinellia ternate</i> 9 g, <i>atractylodes macrocephala</i> 15 g, <i>Gastrodia elata</i> 6 g, tangerine peel 6 g, <i>poria cocos</i> 6 g, <i>Glycyrrhiza</i> 5 g, <i>angelica sinensis</i> 10 g, white peony root 10 g, lotus leaf 15 g, and <i>alisma orientalis</i> 15 g
Zhang, 2002 [54]	Modified BBTD	<i>Pinellia ternate</i> 15 g, <i>atractylodes macrocephala</i> 15 g, <i>Gastrodia elata</i> 12 g, tangerine peel 12 g, <i>poria cocos</i> 12 g, <i>Glycyrrhiza</i> 10 g, plantain seed 15 g, and <i>Loranthus parasiticus</i> 15 g
Wang, 2005 [55]	Modified BBTD	<i>Pinellia ternate</i> 10 g, <i>atractylodes macrocephala</i> 15 g, <i>Gastrodia elata</i> 15 g, tangerine peel 15 g, <i>poria cocos</i> 30 g, hawthorn 15 g, and grass leaved sweetflag 15 g

Medicine (GCRNDTCM), one trial [42] used Convention of Diagnosis and Treatment of Disease and Syndrome in Shanghai (CDTDSS), one trial [47] used Chinese internal medicine (CIM), two trials [44, 48] only demonstrated patients with abundant phlegm-dampness syndrome in TCM, and three trials [40, 43, 54] did not report any TCM diagnostic criteria.

Interventions included all the prescriptions based on “banxia baizhu tianma decoction” alone, or combined with

antihypertensive drugs. The controls included antihypertensive drugs alone. Two trials investigated the prescriptions based on “banxia baizhu tianma decoction” used alone [40, 53] versus antihypertensive drugs, and the rest fourteen trials [41–52, 54, 55] compared the prescriptions based on “banxia baizhu tianma decoction” plus antihypertensive drugs versus antihypertensive drugs.

The total treatment duration ranged from 7 days to 3 months. The variable prescriptions are presented in Table 1.

TABLE 3: Quality assessment of included randomized controlled trials.

Included trials	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other sources of bias	Risk of bias
Zheng, 2011 [40]	Table of random number	Unclear	Unclear	Unclear	No	No	Unclear	Unclear
Xiong, 2010 [41]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Chen et al., 2005 [42]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Wang, 2001 [43]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Che et al., 2011 [44]	Table of random number	Unclear	Unclear	Unclear	No	No	Unclear	Unclear
Jin, 2011 [45]	Unclear	Unclear	Unclear	Unclear	No	No	Unclear	High
Chen, 2007 [46]	Table of random number	Unclear	Unclear	Unclear	Yes	No	Unclear	Unclear
Guo, 2009 [47]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Li, 2011 [48]	Drawing	Unclear	Unclear	Unclear	Yes	No	Unclear	Unclear
Guo et al., 2006 [49]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Zhou, 2008 [50]	Unclear	Unclear	Unclear	Unclear	No	No	Unclear	High
Wu et al., 2007 [51]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Lei and Lin, 2009 [52]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Liu et al., 2007 [53]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Zhang, 2002 [54]	Table of random number	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Wang, 2005 [55]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High

TABLE 4: Analyses of systolic blood pressure.

Trials	MD (95% CI)	P value
BBTD versus antihypertensive drugs		
Modified BBTD versus captopril	1 -0.75 (-5.77, 4.27)	0.77
<i>Meta-analysis</i>	1 -0.75 (-5.77, 4.27)	0.77
BBTD plus antihypertensive drugs versus antihypertensive drugs		
Modified BBTD plus L-amlodipine versus L-amlodipine	1 -0.13 (-4.93, 4.67)	0.96
Modified BBTD plus losartan versus losartan	1 -7.38 (-9.95, -4.81)	<0.00001
Modified BBTD plus antihypertensive drugs versus antihypertensive drugs	1 -4.31 (-8.39, -0.23)	0.04
<i>Meta-analysis</i>	3 -4.33 (-8.44, -0.22)	0.04

The different compositions of Chinese herbal formula BBTD are presented in Table 2. All of the 16 trials used the BP as the outcome measure. Adverse effect was described in detail.

**3.2. Methodological Quality of Included Trials.** The majority of the included trials were assessed to be of general poor methodological quality according to the predefined quality assessment criteria (Table 3). The randomized allocation of participants was mentioned in all trials; however, only 5 trials stated the methods for sequence generation including random number table [40, 44, 46, 54] and drawing [48]. Insufficient information was provided to judge whether or not it was conducted properly. Allocation concealment, blinding of participants and personnel, and blinding of outcome assessment were not mentioned in all trials. None of trials reported dropout or withdraw. None of trials had a pre-trial estimation of sample size. All the trials did not mention

followup. We tried to contact the author for further information; however, no information has been provided to date.

### 3.3. Effect of the Interventions

**3.3.1. “Banxia Baizhu Tianma Decoction” versus Antihypertensive Drugs (Western Medicine).** Two trials [40, 53] compared prescriptions based on BBTD used alone with antihypertensive drugs. A change in blood pressure was reported in all the two RCTs [40, 53]. One trial [53] showed the homogeneity in the consistency of the trial results. Thus, fixed-effects model should be used for statistical analysis. The meta-analysis showed no significant effect of modified BBTD compared with captopril alone in systolic blood pressure (MD: -0.75 (-5.77, 4.27);  $P = 0.77$ ) and diastolic blood pressure (MD: -0.75 (-2.89, 1.39);  $P = 0.49$ ) (Tables 4 and 5).

TABLE 5: Analyses of diastolic blood pressure.

Trials		MD (95% CI)	P value
BBTD versus antihypertensive drugs			
Modified BBTD versus captopril	1	-0.75 (-2.89, 1.39)	0.49
<i>Meta-analysis</i>	1	-0.75 (-2.89, 1.39)	0.49
BBTD plus antihypertensive drugs versus antihypertensive drugs			
Modified BBTD plus L-amlodipine versus L-amlodipine	1	1.55 (-2.39, 5.49)	0.44
Modified BBTD plus losartan versus losartan	1	-3.85 (-5.70, -2.00)	<0.0001
Modified BBTD plus antihypertensive drugs versus antihypertensive drugs	1	-1.24 (-4.04, 1.56)	0.39
<i>Meta-analysis</i>	3	-1.57 (-4.54, 1.40)	0.30

3.3.2. “*Banxia Baizhu Tianma Decoction*” Plus Antihypertensive Drugs versus Antihypertensive Drugs. Fourteen trials [41–52, 54, 55] compared the combination of BBTD or modified BBTD plus antihypertensive drugs with antihypertensive drugs. A change in blood pressure was reported in all the included RCTs.

*Systolic Blood Pressure (SBP)*. The 3 independent trials [41, 46, 51] did not show homogeneity in the trial results, chi-square = 7.18, ( $P = 0.03$ );  $I^2 = 72\%$ . Thus, random-effects model should be used for statistical analysis. The meta-analysis showed that there are significant beneficial effect on the combination group compared to the antihypertensive drugs used alone (MD: -4.33 (-8.44, -0.22);  $P = 0.04$ ) (Table 4).

*Diastolic Blood Pressure (DBP)*. Three trials [41, 46, 51] did not show homogeneity in the trial results, chi-square = 6.87, ( $P = 0.03$ );  $I^2 = 71\%$ . Thus, random-effects model should be used for statistical analysis. The meta-analysis showed that there are no significant beneficial effect on the combination group compare to the antihypertensive drugs used alone (MD: -1.57 (-4.54, 1.40);  $P = 0.30$ ) (Table 5).

3.4. *Publication Bias*. The number of trials was too small to conduct any sufficient additional analysis of publication bias.

3.5. *Adverse Effect*. Four out of sixteen trials mentioned the adverse effect [40, 44, 45, 50]. Four trials reported five specific symptoms including headache, distending feeling in head, palpitations, drowsiness, and fatigue. Among them, no adverse events were found in two trials [44, 45]. One trial reported adverse effect in enalapril group including headache, palpitations, drowsiness, and fatigue [40]. One trial mentioned adverse effect both in modified BBTD plus nifedipine sustained release tablets group and nifedipine sustained release tablets group including distending feeling in head [50].

## 4. Discussion

Based on the paper and meta-analyses of the outcome on either SBP or DBP, BBTD may have positive effects for lowering BP. BBTD as an adjunctive treatment to antihypertensive

drugs significantly lowered SBP in patients with hypertension. However, according to potential publication bias and low-quality trials, available data are not adequate to draw a definite conclusion of BBTD for essential hypertension. And the positive findings should be interpreted conservatively.

Several limitations should be considered before accepting the findings of this paper. First, the quality of the included RCTs is generally low. Sixteen trials included in this paper had risk of bias in terms of design, reporting, and methodology. They provided only inadequate reporting of study design, allocation sequence, allocation concealment, blinding, intention to treat analysis, and dropouts account in the majority of trials. Randomization was mentioned but without further details, which do not allow a proper judgment of the conduct of the trials. All the trials did not describe the blinding in details. It directly led to performance bias and detection bias due to patients and researchers being aware of the therapeutic interventions for the subjective outcome measures. All the sixteen RCTs prohibited us to perform meaningful sensitivity analysis. All the included trials were not multicenter, large-scale RCTs. If poorly designed, all the trials would show larger differences compared with well designed trials.

Second, all the sixteen trials did not report the adverse effect of banxia baizhu tianma decoction. Therefore, a conclusion about the safety of BBTD cannot be made clearly. In China, it is widely believed that it is safe to use herbal medicines for various diseases. With more and more reports of adverse effects of Chinese herbal medicines, the safety of Chinese herbs and formulae needs to be monitored rigorously and reported appropriately in the future clinical trials.

Third, Vickers et al. demonstrated that some countries, for example, China, generate virtually no “negative” studies at all [57]. In other words, publication and other biases may play an important role. We only identified and included trials published in Chinese after conducting comprehensive searches. Most of the trials are small sample with positive findings. We tried to avoid language bias and location bias, but we cannot exclude potential publication bias.

Fourth, it is pointed out that, lacking Chinese medicine (CM) pattern criteria (also called syndrome or zheng) become the key issue both for RCT and clinical practice [58–60]. For example, receiving CM or conventional therapies in patients with the same disease respectively, conventional treatment tends to produce a better curative effect than CM

[61–64]. This should be the major reason why the RCTs failed to evaluate the real efficacy of CM. In this systematic review, three out of the sixteen trials [40, 43, 54] did not report the TCM diagnostic criteria. Two trials [44, 48] reported the TCM diagnostic criteria but without further details. Therefore, further clinical trials should be conducted with clear TCM diagnostic criteria.

In conclusion, there is some encouraging evidence of BBTD for lowering SBP, but the evidence remains weak due to poor methodological quality of including studies. Rigorously designed trials seem to be warranted to confirm the results.

## Conflict of Interests

All authors declare that they have no conflict of interests.

## Authors' Contribution

X. Xiong, X. Yang, W. Liu, J. Ma, X. Du, P. Wang, F. Chu, and J. Li contributed equally to this paper.

## Acknowledgments

The current work was partially supported by the National Basic Research Program of China (973 Program, no. 2003CB517103) and the National Natural Science Foundation Project of China (no. 90209011). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

## References

- [1] A. V. Chobanian, G. L. Bakris, H. R. Black et al., "Seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure," *Hypertension*, vol. 42, no. 6, pp. 1206–1252, 2003.
- [2] T. Krause, K. Lovibond, M. Caulfield, T. McCormack, B. Williams, and Guideline Development Group, "Management of hypertension: summary of NICE guidance," *British Medical Journal*, vol. 343, Article ID d4891, 2011.
- [3] K. J. Chen, K. K. Hui, M. S. Lee, and H. Xu, "The potential benefit of complementary/alternative medicine in cardiovascular diseases," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 125029, 1 pages, 2012.
- [4] D. J. Su and L. F. Li, "Trends in the use of complementary and alternative medicine in the United States: 2002–2007," *Journal of Health Care For the Poor and Underserved*, vol. 22, pp. 295–309, 2011.
- [5] C. Hawk, H. Ndetan, and M. W. Evans, "Potential role of complementary and alternative health care providers in chronic disease prevention and health promotion: an analysis of National Health Interview Survey data," *Preventive Medicine*, vol. 54, pp. 18–22, 2012.
- [6] H. Xu and K. Chen, "Integrative medicine: the experience from China," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 1, pp. 3–7, 2008.
- [7] H. Xu and K. J. Chen, "Integrating traditional medicine with biomedicine towards a patient-centered healthcare system," *Chinese Journal of Integrative Medicine*, vol. 17, no. 2, pp. 83–84, 2011.
- [8] C. S. Moyer, "Weighing alternative remedies," February, 2012, *amednews.com*, <http://www.ama-assn.org/amednews/2012/02/20/prsa0220.htm>.
- [9] A. B. Luiz, I. Cordovil, J. Barbosa Filho, and A. S. A. Ferreira, "Zangfu zheng (patterns) are associated with clinical manifestations of zang shang (target-organ damage) in arterial hypertension," *Chinese Medicine*, vol. 6, p. 23, 2011.
- [10] R. Nahas, "Complementary and alternative medicine approaches to blood pressure reduction: an evidence-based review," *Canadian Family Physician*, vol. 54, no. 11, pp. 1529–1533, 2008.
- [11] E. Ernst, "Complementary/alternative medicine for hypertension: a mini-review," *Wiener Medizinische Wochenschrift*, vol. 123, pp. 386–391, 2005.
- [12] J. Wang and X. J. Xiong, "Current situation and perspectives of clinical study in integrative medicine in China," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 268542, 11 pages, 2012.
- [13] J. Wang, P. Q. Wang, and X. J. Xiong, "Current situation and re-understanding of syndrome and formula syndrome in Chinese medicine," *Internal Medicine*. In press.
- [14] H. Xu and K. J. Chen, "Making evidence-based decisions in the clinical practice of integrative medicine," *Chinese Journal of Integrative Medicine*, vol. 16, no. 6, pp. 483–485, 2010.
- [15] K. J. Chen and H. Xu, "The integration of traditional Chinese medicine and Western medicine," *European Review*, vol. 11, no. 2, pp. 225–235, 2003.
- [16] H. Xu and K. J. Chen, "Complementary and alternative medicine: is it possible to be mainstream?" *Chinese Journal of Integrative Medicine*, vol. 18, no. 6, pp. 403–404, 2012.
- [17] M. S. Lee, H. J. Lim, and M. S. Lee, "Impact of qigong exercise on self-efficacy and other cognitive perceptual variables in patients with essential hypertension," *Journal of Alternative and Complementary Medicine*, vol. 10, no. 4, pp. 675–680, 2004.
- [18] M. S. Lee, M. S. Lee, E. S. Choi, and H. T. Chung, "Effects of Qigong on blood pressure, blood pressure determinants and ventilatory function in middle-aged patients with essential hypertension," *American Journal of Chinese Medicine*, vol. 31, no. 3, pp. 489–497, 2003.
- [19] M. S. Lee, M. H. Pittler, R. E. Taylor-Piliae, and E. Ernst, "Tai chi for cardiovascular disease and its risk factors: a systematic review," *Journal of Hypertension*, vol. 25, no. 9, pp. 1974–1975, 2007.
- [20] J. I. Kim, J. Y. Choi, H. Lee, M. S. Lee, and E. Ernst, "Moxibustion for hypertension: a systematic review," *BMC Cardiovascular Disorders*, vol. 10, article 33, 2010.
- [21] M. S. Lee, T. Y. Choi, B. C. Shin, J. I. Kim, and S. S. Nam, "Cupping for hypertension: a systematic review," *Clinical and Experimental Hypertension*, vol. 32, no. 7, pp. 423–425, 2010.
- [22] M. S. Lee, M. H. Pittler, R. Guo, and E. Ernst, "Qigong for hypertension: a systematic review of randomized clinical trials," *Journal of Hypertension*, vol. 25, no. 8, pp. 1525–1532, 2007.
- [23] M. S. Lee, E. N. Lee, J. I. Kim, and E. Ernst, "Tai chi for lowering resting blood pressure in the elderly: a systematic review," *Journal of Evaluation in Clinical Practice*, vol. 16, no. 4, pp. 818–824, 2010.
- [24] J. Wang, K. W. Yao, X. C. Yang et al., "Chinese patent medicine liu wei di huang wan combined with antihypertensive drugs, a new integrative medicine therapy, for the treatment of essential hypertension: a systematic review of randomized

- controlled trials," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 714805, 7 pages, 2012.
- [25] J. Wang and X. J. Xiong, "Control strategy on hypertension in Chinese medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 284847, 6 pages, 2012.
- [26] G. W. Zhong, M. J. Chen, Y. H. Luo et al., "Effect of Chinese herbal medicine for calming Gan and suppressing hyperactive yang on arterial elasticity function and circadian rhythm of blood pressure in patients with essential hypertension," *Chinese Journal of Integrative Medicine*, vol. 17, no. 6, pp. 414–420, 2011.
- [27] H. Li, L. T. Liu, W. M. Zhao et al., "Effect of traditional and integrative regimens on quality of life and early renal impairment in elderly patients with isolated systolic hypertension," *Chinese Journal of Integrative Medicine*, vol. 16, no. 3, pp. 216–221, 2010.
- [28] E. A. Macklin, P. M. Wayne, L. A. Kalish et al., "Stop Hypertension with the Acupuncture Research Program (SHARP): results of a randomized, controlled clinical trial," *Hypertension*, vol. 48, no. 5, pp. 838–845, 2006.
- [29] J. J. Li, Z. L. Lu, W. R. Kou et al., "Long-term effects of xuezhikang on blood pressure in hypertensive patients with previous myocardial infarction: data from the Chinese coronary secondary prevention study (CCSPS)," *Clinical and Experimental Hypertension*, vol. 32, no. 8, pp. 491–498, 2010.
- [30] D. D. Kim, F. A. Sanchez, R. G. Duran, T. Kanetaka, and W. N. Duran, "Endothelial nitric oxide synthase is a molecular vascular target for the Chinese herb *Danshen* in hypertension," *American Journal of Physiology*, vol. 292, pp. H2131–H2137, 2007.
- [31] D. D. Kim, A. M. Pica, R. G. Durán, and W. N. Durán, "Acupuncture reduces experimental renovascular hypertension through mechanisms involving nitric oxide synthases," *Microcirculation*, vol. 13, no. 7, pp. 577–585, 2006.
- [32] C. F. Ng, C. M. Koon, D. W. S. Cheung et al., "The anti-hypertensive effect of *Danshen* (*Salvia miltiorrhiza*) and *Gegen* (*Pueraria lobata*) formula in rats and its underlying mechanisms of vasorelaxation," *Journal of Ethnopharmacology*, vol. 137, pp. 1366–1372, 2011.
- [33] M. Ren, J. Zhang, B. Wang et al., "Qindan-capsule inhibits proliferation of adventitial fibroblasts and collagen synthesis," *Journal of Ethnopharmacology*, vol. 129, no. 1, pp. 53–58, 2010.
- [34] S. Wang, Y. Chen, D. He et al., "Inhibition of vascular smooth muscle cell proliferation by serum from rats treated orally with *Gastrodia* and *Uncaria* decoction, a traditional Chinese formulation," *Journal of Ethnopharmacology*, vol. 114, no. 3, pp. 458–462, 2007.
- [35] J. Y. Jiang, X. Z. Wang, S. S. Luo, X. Wang, K. Bian, and Y. Yan, "Effect of *Banxia baizhu tianma* decoction on the left ventricular hypertrophy of hypertrophied myocardium in spontaneously hypertensive rat," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 30, no. 10, pp. 1061–1066, 2010.
- [36] X. Z. Wang, J. Y. Jiang, S. S. Luo et al., "Effect of *Banxia baizhu tianma* decoction on the vascular endothelial function of spontaneous hypertensive rats," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 31, no. 6, pp. 811–815, 2011.
- [37] M. Q. Zhang, "Effect of modified *Banxia baizhu tianma* decoction on 38 cases of elderly patients with hypertension," *Shiyong Zhong Xi Yi Jie He Lin Chuang*, vol. 10, no. 2, pp. 19–20, 2010.
- [38] Q. F. Wu, M. X. Wen, and D. H. Lan, "Effects of *Banxia baizhu tianma* decoction on salt sensitivity and blood lipid in hypertensive patients with abundant phlegm-dampness syndrome," *Fujian Yi Yao Za Zhi*, vol. 29, no. 1, pp. 146–148, 2007.
- [39] Y. B. Li, M. Cui, Y. Yang et al., "Similarity of traditional Chinese medicine formula," *Zhong Hua Zhong Yi Yao Xue Kan*, vol. 30, no. 5, pp. 1096–1097, 2012.
- [40] M. J. Zheng, "Effect of *Banxia baizhu tianma* decoction on 30 patients with hypertension," *Zhongguo Zhong Yi Yao Xian Dai Yuan Cheng Jiao Yu*, vol. 9, no. 14, p. 33, 2011.
- [41] Y. W. Xiong, "Clinical effect of the modified *Banxia baizhu tianma* decoction combining western medicine on 60 patients with phlegm-dampness type primary hypertension," *Zhongguo Zhong Yi Yao Xian Dai Yuan Cheng Jiao Yu*, vol. 8, no. 13, pp. 67–68, 2010.
- [42] L. Q. Chen, H. F. Yu, and W. Z. Wang, "Report of hypertension treated with *Banxia baizhu tianma* decoction and western medicine," *Gansu Zhong Yi*, vol. 18, no. 2, pp. 1–3, 2005.
- [43] J. T. Wang, "Effects of modified *Banxia baizhu tianma* decoction and captopril on essential hypertension with abundant phlegm-dampness syndrome," *Zhongguo Zhong Yi Ji Zhen*, vol. 10, no. 6, p. 364, 2001.
- [44] Q. F. Che, L. J. He, and J. Y. Xu, "Clinical effect of the modified *Banxia baizhu tianma* decoction on homocysteine in essential hypertension with abundant phlegm-dampness syndrome," *Liaoning Zhong Yi Za Zhi*, vol. 1813–1814, no. 9, pp. 1–3, 2011.
- [45] Z. X. Jin, "Clinical effect of *Banxia baizhu tianma* decoction on essential hypertension with abundant phlegm-dampness syndrome and blood uric acid metabolism," *Xin Zhong Yi*, vol. 43, no. 11, pp. 5–6, 2011.
- [46] L. Q. Chen, "The effects of *Banxia baizhu tianma* decoction and *Zexie* Decoction on body mass index and depressurization of patient with hypertension type of accumulation of phlegm-damp in TCM," *Zhongguo Zhong Yi Ji Zhen*, vol. 16, no. 6, pp. 650–651, 2007.
- [47] X. H. Guo, "Clinical effect of the modified *Banxia baizhu tianma* decoction on 48 patients with phlegm-dampness type hypertension," *Hebei Zhong Yi*, vol. 31, no. 6, pp. 870–871, 2009.
- [48] B. H. Li, "Clinical effect of *Wen Dan* Decoction and *Banxia baizhu tianma* decoction on 73 patients with hypertension," *Zhong Xi Yi Jie He Xin Nao Xue Guan Bing Za Zhi*, vol. 9, no. 8, p. 910, 2011.
- [49] S. P. Guo, X. P. He, X. M. Lin, and H. Zhu, "Effect of the modified *Banxia baizhu tianma* decoction on 60 patients with phlegm-dampness type primary hypertension," *Shanxi Zhong Yi*, vol. 27, no. 7, pp. 797–798, 2006.
- [50] H. M. Zhou, "Clinical effect of the modified *Banxia baizhu tianma* decoction on hypertension with abundant phlegm-dampness syndrome," *Beijing Zhong Yi Yao*, vol. 27, no. 5, pp. 363–365, 2008.
- [51] Q. F. Wu, M. X. Wen, and D. H. Lan, "Effect of *Banxia baizhu tianma* decoction on insulin resistance and blood lipid in hypertensive patients with abundant phlegm-dampness syndrome," *Fujian Zhong Yi Xue Yuan Xue Bao*, vol. 17, no. 2, pp. 8–10, 2007.
- [52] Z. Y. Lei and X. Lin, "Effect of *Banxia baizhu tianma* decoction on blood pressure variability of menopausal patients with hypertension," *Xian Dai Zhong Xi Yi Ji He Za Zhi*, vol. 18, no. 5, pp. 499–500, 2009.
- [53] Y. P. Liu, M. L. Hu, H. T. Zhang, and Y. S. Shen, "Clinical effect of the modified *Banxia baizhu tianma* decoction on middle-aged primary hypertension," *Zhong Yi Yao Xin Xi*, vol. 24, no. 1, pp. 27–28, 2007.
- [54] Z. Z. Zhang, "Clinical effect of the modified *Banxia baizhu tianma* decoction on isolated systolic hypertension," *Liaoning Zhong Yi Za Zhi*, vol. 29, no. 1, p. 31, 2002.

- [55] S. X. Wang, "Clinical effect of treating hypertension from spleen," *Zhong Yi Yao Xue Kan*, vol. 23, no. 11, p. 2100, 2005.
- [56] J. P. T. Higgins and S. Green, *Cochrane Handbook For Systematic Reviews of Interventions, Version 5. 1. 0*, The Cochrane Collaboration, 2009.
- [57] A. Vickers, N. Goyal, R. Harland, and R. Rees, "Do certain countries produce only positive results? A systematic review of controlled trials," *Controlled Clinical Trials*, vol. 19, no. 2, pp. 159–166, 1998.
- [58] K. J. Chen, "Clinical service of Chinese medicine," *Chinese Journal of Integrative Medicine*, vol. 14, no. 3, pp. 163–164, 2008.
- [59] K. J. Chen and L. Z. Li, "Study of traditional Chinese medicine—which is after all the right way?" *Chinese Journal of Integrative Medicine*, vol. 11, no. 4, pp. 241–242, 2005.
- [60] K. J. Chen, "Where are we going?" *Chinese Journal of Integrative Medicine*, vol. 16, no. 2, pp. 100–101, 2010.
- [61] N. Robinson, "Integrative medicine—traditional Chinese medicine, a model?" *Chinese Journal of Integrative Medicine*, vol. 17, no. 1, pp. 21–25, 2011.
- [62] A. P. Lu and K. J. Chen, "Chinese medicine pattern diagnosis could lead to innovation in medical sciences," *Chinese Journal of Integrative Medicine*, vol. 17, no. 11, pp. 811–817, 2011.
- [63] A. P. Lu and K. J. Chen, "Integrative medicine in clinical practice: from pattern differentiation in traditional Chinese medicine to disease treatment," *Chinese Journal of Integrative Medicine*, vol. 15, no. 2, p. 152, 2009.
- [64] M. Y. Liu and K. J. Chen, "Convergence: the tradition and the modern," *Chinese Journal of Integrative Medicine*, vol. 18, no. 3, pp. 164–165, 2012.