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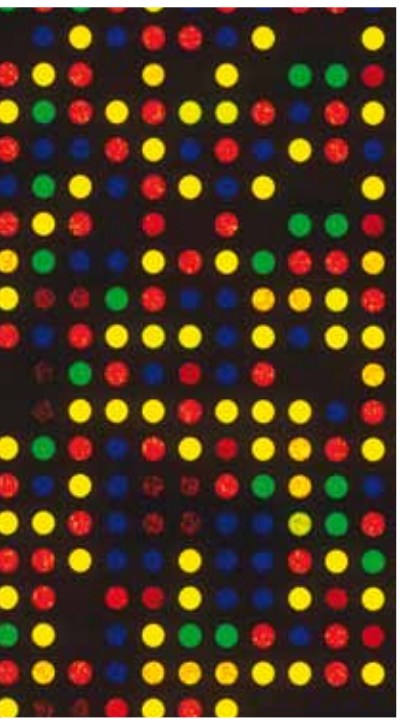
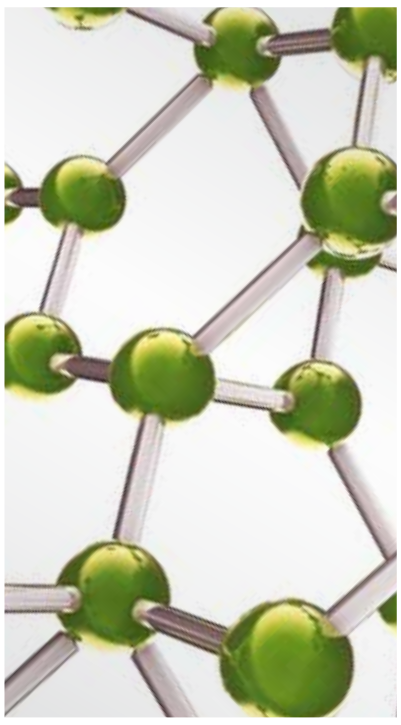
Herbal Medicines for Cardiovascular Diseases

Guest Editors: Xingjiang Xiong, Francesca Borrelli, Arthur de Sá Ferreira, Tabinda Ashfaq, and Bo Feng



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Editorial Board

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Editorial

Herbal Medicines for Cardiovascular Diseases

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The global burden of disease has driven a broad shift from communicable, maternal, neonatal, and nutritional causes to noncommunicable diseases. Cardiovascular diseases (CVDs) remain the most prevalent cause of human morbidity and mortality all over the world [1]. According to the survey by Global Burden of Disease Study, 29.6% of all deaths worldwide were caused by CVDs in 2010 [2]. It is estimated that the number of people that die from CVDs, mainly from heart disease and stroke, will increase to more than 24 million by 2030 [3]. Despite progress in molecular medicine and biology and translational scientific efforts on improvement of diagnostic and therapeutic strategies over the past 20 years, CVDs continue to be a major global health problem.

The use of herbal medicines, one of the main therapeutic approaches of complementary and alternative medicine (CAM), can be tracked back thousands of years ago in the East [4]. Currently, there is a recent resurgence of the use of herbal medicines in popularity among patients in the West and they were consumed by more than 15 million people in the US [5]. With increasing enhancement of people's awareness of self-care and concerning on the inevitable adverse effects of conventional medicine, herbal medicines are favored by people with CVDs all over the world for their unique advantages in preventing and curing diseases, rehabilitation, and health care [6]. There is growing evidence showing that many herbal medicines and their active ingredients contribute to the standard therapy for CVDs, for example, aspirin, digitalis, and reserpine [7].

Despite enormous interests in the medicinal uses by consumers, there is still a great deal of confusion and misunderstanding about their identification, effectiveness, pharmacology, toxicology, and herb-drug interaction to science world [8]. Therefore, the role of herbal medicines in CVDs still needs more scientific and clinical data proving their efficacy and safety. The special issue aims to summarize the current progress of promising herbal medicines and their extractions for various CVDs.

Altogether, we gathered 31 papers for publication, out of which 14 papers were accepted. The original research articles and reviews in this issue cover a wide range of topics, including coronary heart disease, hypertension, heart failure, dyslipidemia, and arrhythmia. Five papers addressed the clinical application and the mechanism of herbal medicines in the treatment of coronary heart disease. "*A multicentre randomized clinical trial on efficacy and safety of huxin formula in patients undergoing percutaneous coronary intervention*" provided evidence on *huxin* formula, an experienced Chinese medicine formula, for the treatment of patients undergoing percutaneous coronary intervention. "*Traditional formula, modern application: Chinese medicine formula sini tang improves early ventricular remodeling and cardiac function after myocardial infarction in rats*" evaluated the improvement of early ventricular remodeling and cardiac function in myocardial infarction in rats by *sini tang*, which is a traditional Chinese classical herbal formula first described by Zhongjing Zhang (150–219 A.D.). "*The comparative study on expression of SIRT1 signal transduction by xuefuzhuyu capsule*" tested the protective effect of *xuefuzhuyu* formula, another classical herbal formula in traditional Chinese medicine (TCM), on ischemic myocardial cells induced by

ischemia through SIRT1-mediated signal transduction pathway. "*Protective effects of shen-yuan-dan, a traditional Chinese medicine, against myocardial ischemia/reperfusion injury in vivo and in vitro*" investigated the effectiveness and mechanisms of *shen-yuan-dan*'s pharmacological postconditioning on myocardial ischemia/reperfusion injury by targeting the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway. "*Ligusticum wallichii extract inhibited the expression of IL-1β after AMI in rats*" addressed the effects of *Ligusticum wallichii* (chuanxiong) extract on IL-1*β*expression in myocardium and central nervous system after acute myocardial infarction.

Hypertension is an important public-health challenge worldwide and a major risk factor for stroke, myocardial infarction, vascular disease, and chronic kidney disease. Prevention, detection, treatment, and control of this condition should receive high priority. How about the role of TCM for managing hypertension? One review article "*Traditional Chinese medicine syndromes for essential hypertension: a literature analysis of 13,272 patients*" analyzed the diagnosis rules and common TCM syndromes of hypertension and recommended the corresponding Chinese herbal medicines and formulas. "*Chinese herbal medicine bushen qinggan formula for blood pressure variability and endothelial injury in hypertensive patients: a randomized controlled pilot clinical trial*" examined the therapeutic effects of *bushen qinggan* formula as adjunctive therapy for antihypertensive drugs on mean blood pressure, blood pressure variability, and endothelial function for hypertension.

One paper "*Yiqi huoxue recipe improves heart function through inhibiting apoptosis related to endoplasmic reticulum stress in myocardial infarction model of rats*" explored the mechanism of cardioprotective effects of *yiqi huoxue* formula in rats with myocardial infarction-induced heart failure by inhibiting endoplasmic reticulum stress response pathway.

Two papers discussed the cardiovascular protective effects of Hawthorn (*Crataegus oxyacantha*). "*Effect of Crataegus usage in cardiovascular disease prevention: an evidence-based approach*" reviewed the cardiovascular pharmacological properties of *Crataegus in vivo* and *in vitro*. "*Evaluation of a Crataegus-based multiherb formula for dyslipidemia: a randomized, double-blind, placebo-controlled clinical trial*" examined the effects of a multiherb formula containing *Crataegus* pinnatifida on plasma lipid and glucose levels in Chinese patients with dyslipidemia.

Finally, "*Yiqihuoxuejiedu formula inhibits vascular remodeling by reducing proliferation and secretion of adventitial fibroblast after balloon injury*" analyzed effects and mechanisms of the *yiqihuoxuejiedu* formula on inhibiting vascular remodeling, especially adventitial remodeling. "*Ganoderma lucidum polysaccharides reduce lipopolysaccharide-induced interleukin-1β expression in cultured smooth muscle cells and in thoracic aortas in mice*" examined the effects of an extract of *Ganoderma lucidum* (Reishi) polysaccharides on interleukin-1*β* expression by human aortic smooth muscle cells (HASMCs) and the underlying mechanism. A review article "*Aspirin resistance and promoting blood circulation and removing blood stasis: current situation and prospectives*" provided insight into the relationship between aspirin resistance and blood stasis syndrome and explored the therapeutic role of Chinese herbal medicines with promoting blood circulation and removing blood stasis for this condition.

Recently, a great progress has been made focusing on the effectiveness and safety of herbal medicines in patients with CVDs. Some RCTs and systematic reviews provided strong evidence for clinical usage. The special issue presented the updated knowledge of partial herbal medicines for CVDs, which unraveled a complex posttranscriptional gene-regulating machinery and paved the evidence-based way.

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Tabinda Ashfaq
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Research Article

Ligusticum wallichii Extract Inhibited the Expression of IL-1β after AMI in Rats

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This study investigated the effects of *Ligusticum wallichii* on IL-1β expression in myocardium and central nervous system after AMI. AMI rat was administrated with *Ligusticum wallichii* extract. A series of assays were used to detect the effects of *Ligusticum wallichii* extract on infarct size, left ventricular ejection fraction, expression of TLR-4, NF-κB, and IL-1β in myocardium, IL-1β expression in serum and hypothalamus, and NPY expression in hypothalamus. We observed that *Ligusticum wallichii* extract improved the left ventricular ejection fraction and reduced infarct area enlargement after AMI, by inhibiting the expression of IL-1β in myocardium, serum, and hypothalamus. *Ligusticum wallichii* extract reduced the expression of IL-1β in myocardium by regulating TLR4-NF-κB signaling pathway and inhibited IL-1β in hypothalamus by regulating NPY mRNA expression.

1. Introduction

Myocardial infarction (MI) is a major cause of death globally; worldwide studies have indicated that MI is characterized by an intense inflammatory response within the myocardium. IL-1β is considered a key inflammatory mediator after acute myocardial infarction. IL-1β has been demonstrated to be significantly related to infarction and left ventricular function after MI [1, 2], and inhibited IL-1β expression could prevent heart failure after MI [3].

There are substantial evidences implicating that central nervous system- (CNS-) mediated mechanism involved the regulation of cardiac function after MI. Recent studies show that, following myocardial infarction elicited by coronary artery occlusion, there is an increase in IL-1β levels in the hypothalamus within 24 h after myocardial infarct. It has also elevated at the time when heart failure is established, approximately 6–8 weeks after a myocardial infarct in the rat [4]. The IL-1β elevated both in the circulation and in the hypothalamus after MI, but the relationship between them is not clear.

Ligusticum wallichii (ChuanXiong) is a Chinese medicinal herb that has been used orally with other herbs for heart [5] and brain diseases [6, 7] for thousands of years. Previous studies have indicated the possible link between the plant and heart disease. *L. wallichii* could improve blood fluidity [8] and inhibits endothelial cell damage [9] and vascular smooth muscle cell proliferation [10]. Current research shows that proinflammatory cytokines are modulators of cardiovascular function by a variety of mechanisms. *L. wallichii* was found to be related to anti-inflammatory activity by reducing serum TNF-α, IL-6 and IL-8 levels [11]. *L. wallichii* also inhibited production of TNF-α and IL-1β activated by cerebral ischemia [12, 13].

An emerging area in cardiovascular research is the apparent significance of inflammation mechanisms. Many recent studies have suggested that anti-inflammation radicals may be important participants in a wide array of cardiac conditions, and several clinical trials evaluating the use of anti-inflammation as therapeutics either have already been conducted or are underway. The present study was designed

to investigate the effects of *Ligusticum wallichii* extract on anti-inflammation activities in AMI rats.

2. Materials and Methods

2.1. Materials. The reagents used in the study were purchased as follows: pentobarbital sodium from Sigma, America; ELISA kits for IL-1β from Jiancheng Biotech, Nanjing, China; NBT from Light Biotech, Beijing, China; mice anti-rat toll-like receptor 4 (TLR4) and mice anti-rat TNF receptor associated with factor-6 (TRAF-6) from Boster Biotech, Wuhan, China; mice anti-rat NF-κB p65 from Santa Cruz, America; IL-1β monoclonal antibodies from Sigma, America; SP kits and DAB kits from ZhongShanJinqiao Biotech, Beijing, China; prestained protein ladder 10–170 kDa from Thermo Scientific, America; *Ligusticum wallichii* from First Teaching Hospital of Tianjin university of TCM, lot number Y20050124; aspirin from Bayer, National License Medical Number J20080078.

2.2. Preparation of Ligusticum wallichii Extract. A batch of 63 g of the *Ligusticum wallichii* was soaked in 1000 mL deionized water for 0.5 h and continuously extracted using deionized water at boiling point for 1 h; the extracts were collected. The herbs were then soaked in 800 mL deionized water extracted for another 1 h. Then, all these extracts were mixed, filtered, concentrated, dried, and weighed.

2.3. Animals, AMI Induction, and Treatment. Sprague-Dawley rats (180 ± 50 g, 8–10 weeks) were obtained from the experimental animal center of Military Medical Science Academy, Tianjin, China. They were housed in groups (4 per cage) with free access to a regular diet and clean drinking water. All the experimental procedures described below adhered strictly to the guidelines set forth by the National Science and Technology Commission of China and approved by the institutional ethics committee.

For AMI induction, rats were anesthetized with an intraperitoneal dose of 0.2 mL 2% pentobarbital under intubated mechanical ventilation at 80 breaths per minute. Thoracotomy was performed through the fourth intercostal space and the left anterior descending coronary artery was identified and ligated with 6.0 prolene suture in the middle portion. A few minutes after ligation, pallor and akinesia were seen in the anterior wall and apical left ventricular area. The interface between the pale and normal areas was defined as "infarction border zone." The presence of infarction zone and ST segment elevation were considered as criteria of successful induction of AMI. A group of animals ($n = 10$) were left untreated controls (sham group). The animals that were successfully induced to develop AMI were randomized to AMI group and received PBS (10 mg/Kg/d, $n = 10$), *Ligusticum wallichii* group (10 mg/Kg/d, $n = 10$) and aspirin group (10 mg/Kg/d, $n = 10$), respectively.

2.4. Left Ventricular Ejection Fraction Evaluation. Echocardiographic examinations were performed 7 days after surgery, with the Philips Sonos 5500 ultrasound system (HP, Germany). The frequency of the probe was set at 17.5 MHz, sampling frequency in M-mode at 1000/s, and scanning speed 50–100 mm/s. The probes were placed on precordium and the detection was carried out from the section of ventricular bands. Left ventricular anterior wall (LVAW) thickness, left ventricular end-diastolic diameters (LVEDD), and left ventricular end-systolic diameters (LVESD) were measured. Based on these measurements, left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), and left ventricular ejection fraction (LVEF) were calculated as follows:

$$\begin{aligned} \text{LVEDV} &= \frac{7.0 \times \text{LVEDD}^3}{(2.4 + \text{LVEDD})}, \\ \text{LVESV} &= \frac{7.0 \times \text{LVESD}^3}{(2.4 + \text{LVESD})}, \\ \text{LVEF} &= \frac{\text{LVEDV} - \text{LVESV}}{\text{LVEDV}} \times 100\%. \end{aligned} \tag{1}$$

2.5. Infarct Area Size Assessment. Areas identified as infarct, nonischemic based on NBT staining were measured by computerized video planimetry and from these measurements infarct size (MIS) was calculated as a percentage of the region at risk [14]. MIS = ischemia area weight/ventricular weight × 100%.

2.6. Expression of TLR4, TRAF-6, NF-κB, and IL-1β in Myocardium by Immunohistochemical Staining. Sections were deparaffinized by a standard method, cut, fixed in 100% acetone, and stored at −20°C. The mouse anti-rat TLR4, TNF receptor associated to factor-6 (TRAF-6), NF-κB and IL-1β polyclonal antibody were used for the identification of TLR4, TRAF-6, NF-κB, and IL-1β positive cells. Sections were washed twice in Tris HCl and pH 7.6 and briefly in buffer containing 1% polymerized bovine albumin.

The sections were incubated for 1 hour at room temperature with the primary antibodies diluted 1 : 100 for TLR4, 1 : 150 for TRAF-6, 1 : 25 for NF-κB, and 1 : 200 for IL-1β. And then the secondary antibody is biotinylated and the label is peroxidase conjugated streptavidin. Control sections were treated with the same procedure except they were incubated without the specific primary antibodies. And then diaminobenzidine (DAB) staining was performed. Positive signals were quantified using the Image Pro-Plus 5.1 software. The number of cells positive for TLR4, TRAF-6, NF-κB, or IL-1β was counted in 10 high power fields (×400).

2.7. Measurement of IL-1β in Serum by ELISA. The cytokine measurements were performed according to the manufacturer's instructions for the ELISA kit (BD Biosciences, USA).

2.8. Measurement of IL-1β and NPY in Myocardium and Hypothalamus by Real-Time Quantitative RT-PCR Analysis. Total RNA was extracted using a commercial RNAprep pure kit (number DP430, Tiangen, China). Total RNA concentration was determined from spectrophotometric optical

density measurement (260 and 280 nm). For each sample tested, the ratio between the spectrophotometric readings at 260 nm and 280 nm (OD260/OD280) was used to provide an estimate of the purity of the nucleic acid, and the ratio in all samples ranged between 1.8 and 2.0.

Total RNA was reverse transcribed at 46°C for 60 min, 70°C for 5 min, and 5°C for 5 min using the PrimeScript RT master mix kit (number DRR036, TaKaRa, Japan) and then cDNA was stored at −20°C. Quantitative PCR reactions were carried out with the SYBR Premix Ex TaqTM II kit (number DRR081, TaKaRa, Japan) in a thermocycler (Applied Biosystems 7500 Real-Time PCR System, USA). Amplification conditions included 95°C for 10 s followed by 39 cycles of 95°C for 5 s and 60°C for 20 s. The forward IL-1β primer was 5′-TGTGATGTTCCCATTAGAC-3′, and the reverse IL-1β primer was 5′-AATACCACTTGTTGGCTTA-3′ (131 bp product). The forward GAPDHP primer was 5′-CTGATGCCTCCATGTTTGTG-3′; the reverse GAPDHP was 5′-GGATGCAGGGATGATGTTCT-3′ (254 bp product). The forward NPY primer was 5′-CTGACCCTCGCTCTA-TCC-3′, and the reverse NPY primer was 5′-GGTCTT-CAAGCCTTGTTCT-3′ (247 bp product). The forward GAPDHP primer was 5′-CTGATGCCTCCATGTTTGTG-3′; the reverse GAPDHP was 5′-GGATGCAGGGATGAT-GTTCT-3′ (254 bp product).

Amplification specificity was verified by the melting curve following the manufacturer's instructions and 1.5% agarose gel electrophoresis. The data were normalized to the Ct value of the internal housekeeping gene beta-actin and the relative mRNA level in the untreated group was used as calibrator. Fold change of the target gene mRNA expression was calculated using the 2-ΔΔCT method.

2.9. Statistical Analysis. Values of all experiments were represented as mean ± SD. Statistical significance was assessed by one-way ANOVA followed by LSD post hoc multiple comparisons. The level of significance was set at $P < 0.05$.

3. Result

3.1. Ligusticum wallichii Extract Improved the Left Ventricular Ejection Fraction (LVEF). The left ventricular ejection fraction was assessed by echocardiograph (Figure 1). The LVEF was significantly decreased in the AMI group when compared with the sham group ($P < 0.01$). However, that alternation was reduced ($P < 0.05$) by feeding LW and aspirin. But there were no remarkable differences between LW group and aspirin group.

3.2. Ligusticum wallichii Extract Reduced Infarct Area Size. The infarct area was assessed by NBT staining (Figure 2). NBT stain showed that the color of myocardium in the control group was dark blue, but the infarct area of AMI model group was hoar. One week after infarction, a large infarcted area with a collapsed and pale left ventricular wall was seen under the ligated silk. Compared to the sham group, larger infarcted area was detected accompanying global enlargement of the heart in the AMI group. *Ligusticum wallichii* and aspirin treatment reduced infarct area enlargement.

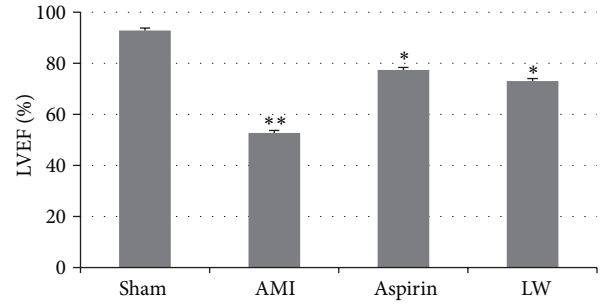

Figure 1: Left ventricular ejection fraction was assessed by echocardiograph. The LVEF was significantly decreased in the AMI group when compared with the sham group ($^{**}P < 0.01$). *Ligusticum wallichii* (10 mg/Kg/d body weight) and aspirin (10 mg/Kg/d body weight) could improve the LVEF of AMI rat versus AMI group ($^{*}P < 0.05$). But there were no remarkable differences between LW group and aspirin group.

3.3. Ligusticum wallichii Extract Inhibited the Expression of IL-1β in Myocardium, Serum, and Hypothalamus. IL-1β immunohistochemical staining could be detected to the cells of rat myocardium after AMI (Figures 3(a) and 3(b)). The IL-1β protein was predominantly expressed in the cytoplasm. Few cells positive for IL-1β-like immunoreaction were observed in the sham group. Intensive IL-1β-like immunostaining was present in myocardium after AMI. Significant changes of IL-1β protein expression were observed in the LW group and aspirin group.

The serum IL-1β level was assessed by ELISA kit (Figure 3(c)). The serum IL-1β level was elevated 1W after AMI. LW and aspirin inhibited the IL-1β overexpression in serum, but there were no remarkable differences between LW group and aspirin group.

The hypothalamus IL-1β mRNA expression was assessed by quantitative reverse transcription-PCR (Figure 3(d)). The hypothalamus IL-1β mRNA expression was elevated 1W after AMI. LW and aspirin inhibited IL-1β mRNA expression in hypothalamus, but there were no remarkable differences between LW group and aspirin group.

3.4. Ligusticum wallichii Extract Inhibited the Expression of TLR4, TRAF-6, and NF-κB in Myocardium. TLR4 (Figures 4(a) and 4(b)) and TRAF-6 (Figures 4(c), 4(d)) immunohistochemical staining could be detected to the cells of rat myocardium after AMI. The TLR4 and TRAF-6 protein was predominantly expressed in the cytoplasm. Few cells positive for TLR4-like and immunoreaction were observed in the sham group. Intensive TLR4-like and TRAF-6-like immunostaining was present in myocardium after AMI. Significant changes of TLR4 and TRAF-6 protein expression were observed in the *Ligusticum wallichii* group and aspirin group.

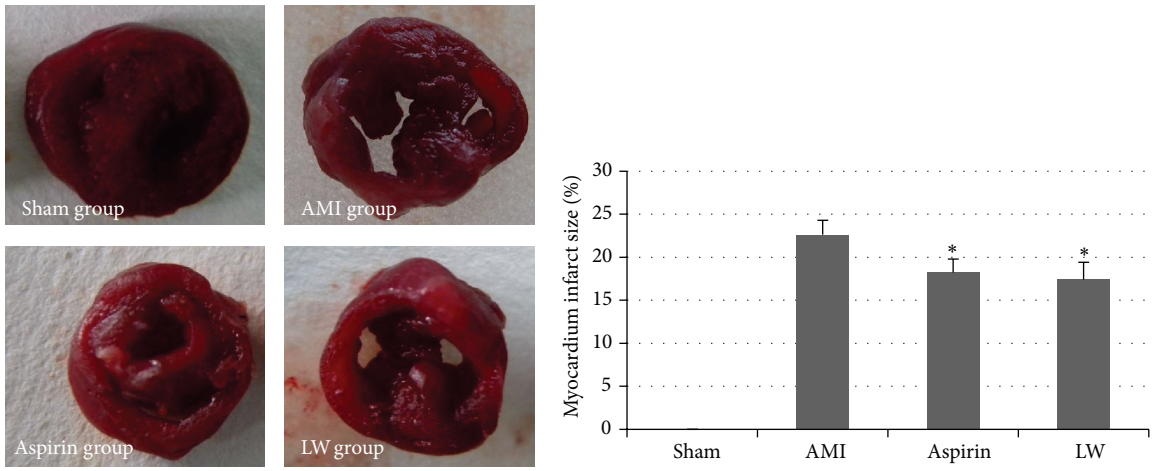

Figure 2: Representative images for infarcted hearts at one week time point after AMI. (a) NBT staining of infarcted area. The color of myocardium in the control group was dark blue, but the infarct area was hoar. (b) *Ligusticum wallichii* (10 mg/Kg/d body weight) and aspirin (10 mg/Kg/d body weight) reduced the MIS versus AMI group ($^{*}P < 0.05$). But there were no remarkable differences between LW group and aspirin group.

NF-κB immunohistochemical staining could be detected to the cells of rat myocardium after AMI (Figures 4(e) and 4(f)). In sham group, the NF-κB protein was predominantly expressed in the cytoplasm. However, after AMI, expression was observed both in the cytoplasm and nucleus. Few cells positive for NF-κB-like immunoreaction were observed in the sham group. Intensive NF-κB-like immunostaining was present in myocardium after AMI. Significant changes of NF-κB protein expression were observed in the *Ligusticum wallichii* group and aspirin group.

3.5. Ligusticum wallichii Extract Inhibited NPY mRNA Expression in Hypothalamus. The hypothalamus NPY mRNA expression was assessed by quantitative reverse transcription-PCR (Figure 5). The hypothalamus NPY mRNA expression was elevated 1W after AMI. LW and aspirin inhibited NPY mRNA expression in hypothalamus, but there were no remarkable differences between LW group and aspirin group.

4. Discussion

The objective of our study was to investigate the effects of *Ligusticum wallichii* extract on anti-inflammation activities after AMI. We found that *Ligusticum wallichii* extract (10 mg/Kg/d body weight) could improve the left ventricular ejection fraction and reduced infarct area enlargement. *Ligusticum wallichii* extract also inhibited the expression of IL-1β in myocardium, serum, and hypothalamus. Previous studies have indicated that MI is characterized by an intense inflammatory response. IL-1β is considered a key inflammatory mediator after acute myocardial infarction. IL-1β has been demonstrated to be significantly related to infarction and left ventricular function after MI [2]. Recent studies show that, following myocardial infarction elicited by coronary artery occlusion, there is an increase in IL-1β levels in the hypothalamus within 24 h after myocardial infarct [15]. And inhibited brain IL-1β synthesis could reduce infarction and improve left ventricular function [16], this suggests that inhibiting IL-1β expression in brain could improve heart function.

Our results identify that *Ligusticum wallichii* extract (10 mg/Kg/d body weight) could inhibit the expression of TLR4 and NF-κB in myocardium after AMI. Toll-like receptors (TLRs) play an important role in the regulation of innate immune and inflammatory responses by recognition of pathogen associated molecular patterns (PAMPs) that are not present in the host [17]. TLR4 is a member of the TLRs that have natural pattern recognition. The function of TLR4 is to mediate transmembrane signaling transduction in which TLR4 could serve as a bridge that links innate immunity and vascular inflammation. TLR4 widely recognizes specific pathogen-associated molecular patterns, such as couples signal transduction pathways to activate inflammatory cells, which results in a series of inflammatory responses and leads to the synthesis and release of cytokines and inflammatory mediators. In TLR4-deficient mice, this vascular proinflammatory gene cannot be expressed, regardless of the extent of obesity, dyslipidemia, or high fat intake [18].

Nuclear factor (NF)-κB is downstream of the signaling pathway activating IL-1β; NF-κB pathway plays an important role in TLR4-mediated inflammatory regulation [19]. It is an essential transcription factor that regulates inflammatory responses through modulation of the expression of various proinflammatory mediators, including cytokines and NO. NF-κB is also a primary regulator of genes that are involved in the production of proinflammatory cytokines and enzymes involved in the process of inflammation [20, 21]. TLR4 was upregulated in response to IL-1β. IL-1β activates NF-κB

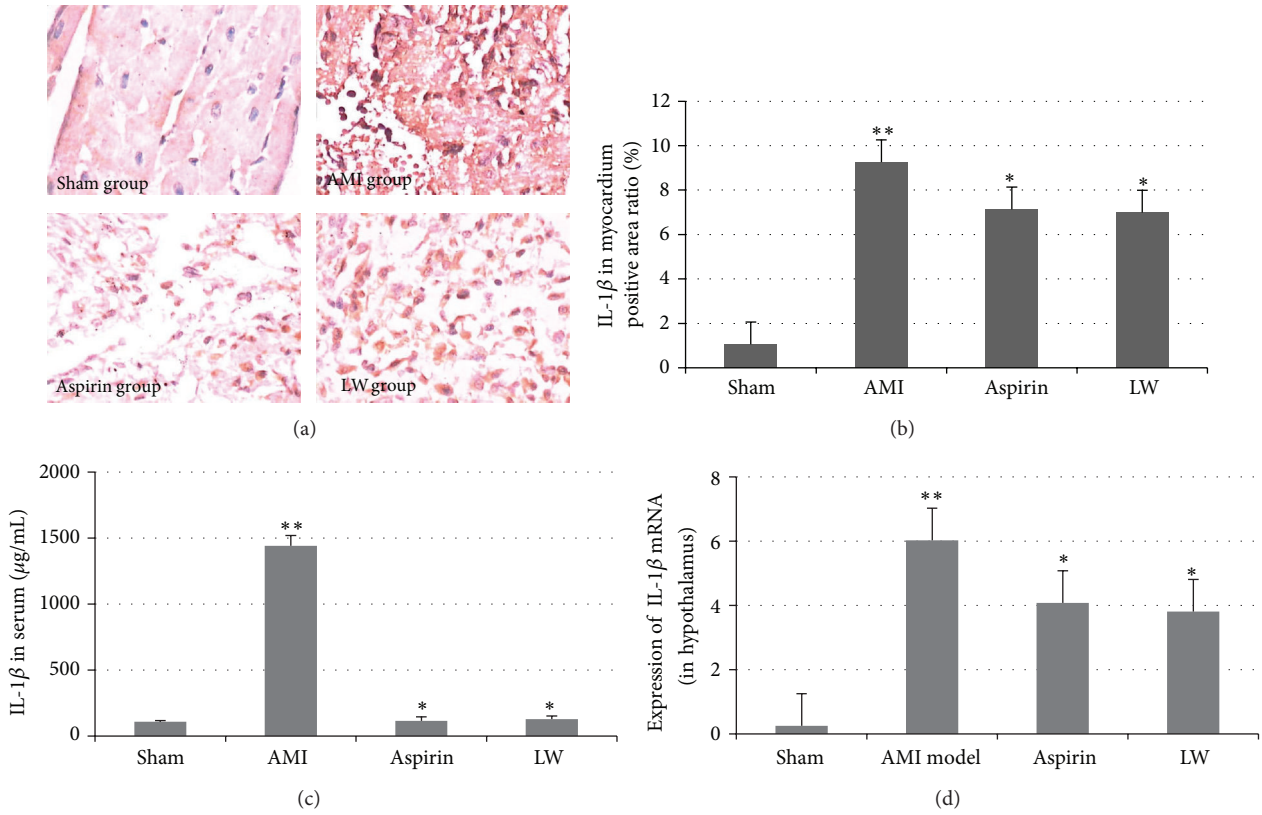

FIGURE 3: *Ligusticum wallichii* extract inhibited the expression of IL-1β. (a) Representative micrographs were taken at a magnification of ×400. The brown areas were IL-1β positive cell; the brown areas were significantly increased in the AMI model group. *Ligusticum wallichii* and aspirin decreased the brown area. (b) In the sham group, the immunoreactive staining occurred less in the cytoplasm. The number of IL-1β-like immunoreactive cells increased significantly in the myocardium after AMI when compared with the sham group ($^{**}P < 0.01$). *Ligusticum wallichii* (10 mg/Kg/d body weight) and aspirin (10 mg/Kg/d body weight) both inhibited IL-1β protein expression versus AMI group ($^{*}P < 0.05$), but there were no remarkable differences between LW group and aspirin group. (c) Serum IL-1β level was assessed by ELISA kit. The serum IL-1β level was significantly upregulated 1w after AMI when compared with the sham group ($^{**}P < 0.01$). *Ligusticum wallichii* (10 mg/Kg/d body weight) and aspirin (10 mg/Kg/d body weight) both inhibited the IL-1β overexpression after AMI versus AMI model ($^{*}P < 0.05$), but there were no remarkable differences between LW group and aspirin group. (d) Effect of *Ligusticum wallichii* on the IL-1β mRNA expression in the hypothalamus of AMI rats. Quantitative RT-PCR was performed. The IL-1β mRNA expression was significantly upregulated 1w after AMI versus sham group ($^{**}P < 0.01$). *Ligusticum wallichii* and aspirin inhibited the hypothalamus IL-1β mRNA expression versus AMI group ($^{*}P < 0.05$), but there were no remarkable differences between LW group and aspirin group.

resulting in transcriptional activation of a wide variety of genes such as inflammatory mediators.

Tumor necrosis factor (TNF) receptor associated factors (TRAFs) play important roles in intracellular signal transduction of many receptor families such as the IL-1 receptors (IL-1R) [22]. They could lead to activation of transcription factors such as NF-κB and inflammatory responses. Remarkably, TRAF6 is uniquely pleiotropic in participating in the signal transduction of many receptor systems while TRAF2, TRAF3, and TRAF5 appear to signal only within the TNF receptor superfamily [23]. TRAF-6 could be active by TLR4 and then activate the inhibitor of κB (IκB) kinase (IKK), leading ultimately to activation of NF-κB [24]. Levels of TRAF-6 are related to inflammation in CAD patients [25].

Neuropeptide Y (NPY) is one of the most abundant neuropeptides present in the human peripheral and central nervous systems [26]. It acts as a neurotransmitter, regulating various autonomic and endocrine functions [27]. Specifically, NPY-containing neurons are present in the paraventricular nucleus (PVN) of the hypothalamus, the ventrolateral medulla (VLM), the NTS, and the sympathetic fibres that innervate blood vessels [28]. NPY played a significant role in central cardiovascular regulation [29, 30]. More recently, genome wide association studies have linked NPY to human coronary artery disease (CAD): SNPs in the NPY gene correlated to CAD in humans and even more so in early onset patients [31].

In recent years, NPY has been also described to play a pivotal role in the immune system; NPY can increase the IL-1β secretion [32]. And NPY receptors are present on the surface of various leukocyte subgroups modulating the release of different cytokines. NPY Y1 receptor signaling could prevent NF-κB activation triggered by IL-1β [33]. In our study, *Ligusticum wallichii* extract inhibited the expression of

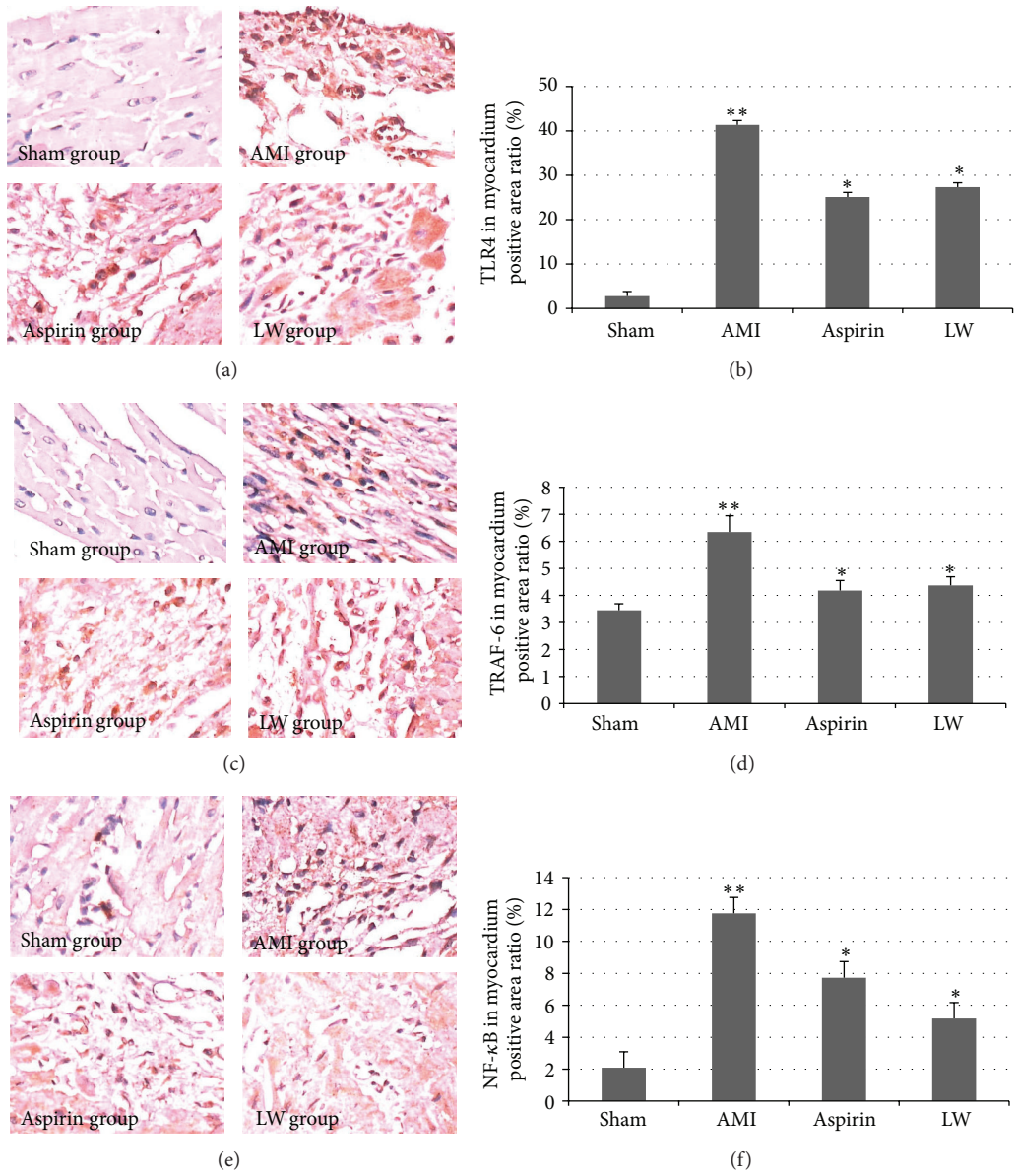

FIGURE 4: *Ligusticum wallichii* extract inhibited the expression of TLR4, TRAF-6, NF-κB in myocardium. (a) Representative micrographs were taken at a magnification of ×400. The brown areas were TLR4 positive cell; the brown areas were significantly increased in the AMI model group. *Ligusticum wallichii* and aspirin decreased the brown area. (b) In the sham group, the immunoreactive staining occurred less in the cytoplasm. The number of TLR4-like immunoreactive cells increased significantly in the myocardium after AMI when compared with the sham group ($^{**}P < 0.01$). *Ligusticum wallichii* (10 mg/Kg/d body weight) and aspirin (10 mg/Kg/d body weight) both inhibited TLR4 protein expression versus AMI group ($^{*}P < 0.05$), but there were no remarkable differences between LW group and aspirin group. (c) The brown areas were TRAF-6 positive cell; the brown areas were significantly increased in the AMI model group. *Ligusticum wallichii* and aspirin decreased the brown area. (d) In the sham group, the immunoreactive staining occurred less in the cytoplasm. The number of TRAF-6-like immunoreactive cells increased significantly in the myocardium after AMI when compared with the sham group ($^{**}P < 0.01$). *Ligusticum wallichii* and aspirin both inhibited TRAF-6 protein expression versus AMI group ($^{*}P < 0.05$), but there were no remarkable differences between LW group and aspirin group. (e) The brown areas were NF-κB positive cell; the brown areas were significantly increased in the AMI model group. *Ligusticum wallichii* and aspirin decreased the brown area. (f) In the sham group, the immunoreactive staining occurred less in the cytoplasm. The number of NF-κB-like immunoreactive cells increased significantly in the cytoplasm and nucleus after AMI. *Ligusticum wallichii* and aspirin both inhibited NF-κB protein expression versus AMI group ($^{*}P < 0.05$).

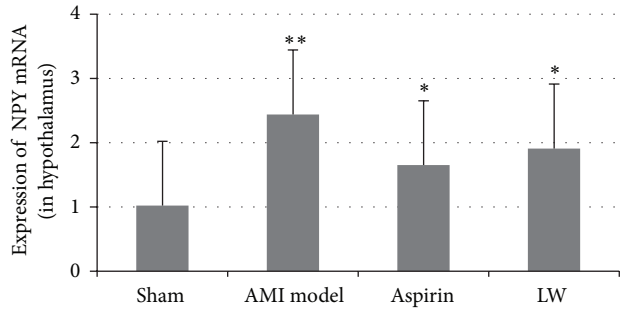

Figure 5: Effect of *Ligusticum wallichii* on the NPY mRNA expression in the hypothalamus of AMI rats. Quantitative RT-PCR was performed. The NPY mRNA expression was significantly upregulated 1w after AMI versus sham group ($^{**}P < 0.01$). *Ligusticum wallichii* and aspirin inhibited the hypothalamus NPY mRNA expression versus AMI group ($^{*}P < 0.05$), but there were no remarkable differences between LW group and aspirin group.

NPY in hypothalamus, suggesting that it could reduce the IL-1β level in hypothalamus after AMI by inhibiting NPY mRNA expression.

5. Conclusion

Ligusticum wallichii extract improved the left ventricular ejection fraction and reduced infarct area enlargement after AMI, by inhibiting the expression of IL-1β in myocardium, serum, and hypothalamus. *Ligusticum wallichii* extract reduced the expression of IL-1β in myocardium by regulating TLR4-NF-κB signaling pathway and inhibited the expression of IL-1β in hypothalamus by regulating NPY mRNA expression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Comparative Study on Expression of SIRT1 Signal Transduction by Xuefuzhuyu Capsule

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The Xuefuzhuyu capsule (XFZY) is widely used for the treatment of ischemic heart disease (IHD) in China. We previously demonstrated that XFZY could reduce apoptosis in Sprague-Dawley rat cardiomyocytes with the similar effect of resveratrol (Res) Hori et al. (2013), although its molecular mechanism underlying this protective effect is still unclear. Silent information regulator of transcription 1 (SIRT1) had been demonstrated to be responsible for cardioprotection against ischemia-reperfusion injury via long-term transcriptionally regulatory mechanism Braunersreuther and Jaquet (2012). Therefore, in the present study, we aimed to test if XFZY might contribute to the protection of ischemic myocardial cells induced by ischemia through SIRT1-mediated signal transduction pathway by using electron micrograph, RT-PCR assay, and western-blot test. All the result showed that the target genes of SIRT1 pathway including P53, NF-kB, FOXO1, FOXO3, and FOXO4 were significantly downregulated to SIRT1, suggesting that apoptosis pathway might transcriptionally be regulated to SIRT1. In addition, the expression level of SIRT1 was significantly increased by XFZ, it might prove that SIRT1 is the target of XFZY working on ischemia heart disease. Our findings supported that XFZY might function to protect myocardial cells and reduce myocardial injury though SIRT1 signaling pathway and has the same pharmacological effect with Res.

1. Introduction

Ischemic heart disease (IHD) is one of the most serious human disorders leading to long-term reduced mobility and high mortality. It induces irreversible myocardial damage despite relieving the myocardial ischemia, which in turn leads to cardiac remodeling characterized by dilation of the left ventricle (LV) and reduced contractility. In this disorder, ischemia reperfusion had been found to be a major cause of myocyte necrosis and apoptosis [1], and ischemia/reperfusion (I/R) injury remains the major cause of heart failure and arrhythmia. Although many interventions alleviating the extent of myocardial injury in animal models of I/R have been tested in patients, thus far none of them have exhibited definitive advantages over the control, suggesting that a novel mechanism of intervention is needed [2].

Elucidating the mechanisms mediating aging and controlling the lifespan of organisms is an important theme in modern biology, and it is widely accepted that the myocardial oxygen delivery that is insufficient in relation to myocardial oxygen demand is a prominent factor in triggering the events that ultimately result in cardiomyocyte death [3–5]. A recent finding suggested that cardiac SIRT1 is significantly upregulated in response to oxidative stress and because of that it can prolong the lifespan of cardiac myocyte and recover cardiac myocyte function [6]. Sirtuin 1 (SirT1) belongs to the sirtuin family of nicotinamide adenine dinucleotide NAD-dependent protein deacetylases, which are involved in a variety of cellular functions such as gene silencing, heterochromatin formation, cell survival, metabolism, and development [7–9] and its activation is considered beneficial for metabolic, neurodegenerative, and inflammatory diseases and to augment longevity, as well as protective effectiveness during ischemia/reperfusion processes and neurotic generation [10].

A variety of studies had demonstrated that Chinese medicine has specific protective effects against ischemia episodes in heart and brain. Zhu et al. investigated that icariin (ICA) could protect brain from ischemic injury by increasing expression level of SIRT1 and PGC-1a during ICA's

neuroprotection against ischemia [11]. Other studies had also shown that Chinese herbs or traditional medicine monomers are cardioprotective against ischemia, but related mechanism is still unclear. However, few studies focused on the role of Chinese herbal compound for myocardial ischemia [12, 13]. Resveratrol (Res) is known to improve treatment outcome after ischemic episodes in heart and require SIRT1 to mediate ischemic protection to increase lifespan [14]. The Xuefuzhuyu capsule (XFZY) is widely used for the treatment of ischemic heart disease (IHD) in China with the protection of cardiomyocytes from injury by ischemia, but the molecular mechanism underlying this protective effect is still unclear.

In this study, we observed morphological changes of ischemic myocardium of Sprague-Dawley rats by electron micrograph. To investigate the action mechanism of XFZY on antiapoptosis in IHD and to identify a promising strategy for the treatment of ischemia-induced injury, we also examined the expression levels of SIRT1 as well as its target genes and the protein in ischemic myocardium by RT-PCR assay and Western-blot analysis.

2. Materials and Methods

2.1. Experimental Materials

2.1.1. Animals. Adult male Sprague-Dawley rats (200 ± 5 g) were from the Experimental Animal Center at the Xiyuan Hospital China Academy of Chinese Medical Sciences. Five rats were kept in each cage, and the rats were conditioned for one week at room temperature (23 ± 1°C), with a constant humidity of 55 ± 5%, under a cycle of 12 h of light/dark, and had free access to food and tap water.

2.1.2. Drugs. The Xuefuzhuyu capsules (XFZY) were purchased from Tianjin Hong Ren Tang Pharmaceutical Co., Ltd. The drug compositions are *Rehmannia*, Peach kernel, Safflower, Chinese Angelica, Licorice, Radix Paeoniae Rubra, bellflower, Fructus aurantii, *Bupleurum*, Chuanxiong, and *Achyranthes bidentata*. The powder of resveratrol (Res) with 100 mg/bottle was purchased from Sigma Biotech Company.

2.1.3. Instruments. The instruments used were RM-6000-type eight polygraph (NIHON KOHDEN XDH-3B), ECG machine (Shanghai Medical Electronic Instrument Factory), DH-150 animal-artificial respiration machine (Zhejiang University Medical Instrument Factory), 7900HT quantitative PCR instrument (Applied Bio systems Inc.), Gel image analyzer Image Master VDS (United States Pharmacia Biotech), High-speed refrigerated centrifuge (Germany BACKMAN Company), UV spectrophotometer (Germany BACKMAN the DU640-type), and Electrophoresis instrument (Beijing sixty-one Instrument CYY-III-5 type).

2.2. Experimental Methods

2.2.1. Grouping Method. Sprague-Dawley rats were screened by electrocardiogram (ECG) before experiment. The normal ones were divided randomly into 6 groups: normal group, sham-operated group, ischemia group as negative control, XFZY-treated group, Res-treated group, and L-NAME group, with 10 of rats in each group (Figure 1). Except for normal and sham-operated groups, the rest of rats were prepared for the model of myocardial ischemia. Sham-operated rats treated with sham operation.

2.2.2. Animal Experimental Model. We ligate the left coronary artery of rats; the basic approach is to perform a left thoracotomy and secure a ligature around the intramyocardial portion of the artery that lies just ventral to the left atrium. Both male and female rats have undergone coronary artery ligation, and while the most common species is adult Sprague-Dawley rats, our laboratory has also infarcted Fischer-344 and Brown Norway Fischer-344 cross rats. The approach used in our laboratory is as follows. After induction of anesthesia with acepromazine maleate 50 mg/kg, xylazine 5 mg/kg, and ketamine He1 50 mg/kg intraperitoneally, a left anterior thoracotomy is performed under sterile conditions. The heart is expressed through the incision and a 7-0 synthetic ligature is secured snugly around the proximal left anterior coronary artery. The lungs are inflated to reduce the pneumothorax, and the muscle layer and skin are closed separately. Postoperative analgesia is provided with acetaminophen (67 rag/L) in the drinking water. An acute survival rate of approximately 50% is generally achieved. Other variations on this basic approach are to use endotracheal intubation with ventilator support so as to have more time to perform the ligation and to treat rats with perioperative lidocaine to decrease the incidence of ventricular tachycardia and fibrillation.

2.2.3. Administration. After modeling, rats in XFZY groups were treated orally with 30 mg/kg of XFZY 1/d. Rats in normal group, sham-operated group, and ischemia group were treated with equal volume of saline, 1/d. Rats in Res group were treated with 10 mg/kg of Res by sublingual intravenous administration, 1/d for 10 days. Rats in L-NAME group were intraperitoneal injected before modeling and on the day of the experiment, 2 mg of L-NAME for each 1/d.

2.2.4. Specimen Collection for Further Assays. After intraperitoneal anesthesia by 10% chloral hydrate, each rat heart exposed for thoracotomy was infused with 200–300 mL of saline and electron microscopy fixative (1% paraformaldehyde and 2.5% glutaraldehyde), respectively, from the left ventricular rapidly, and was sustained for 30–60 minutes until the upper limbs, neck, and lower extremity of the rat were stiff. For electron micrograph, the left ventricular anterior myocardial above the ligature was cut into 1 × 1 × 1 mm pieces. Subsequently, specimens were prefixed with 2.5% of glutaraldehyde and fixed with 1% of osmium tetroxide, followed by dehydrated with acetone. For RT-PCR assay, the myocardial above the ligature was cut into 4 mm thick pieces.

2.2.5. Quantitative Real-Time QRT-PCR Assay. L-NAME was purchased from Alexis Company. Trizol was purchased from Invitrogen, Revert Aid TM First Strand cDNA Synthesis Kit was purchased from Fermentas and SYBR Green PCR Master Mix was purchased from Applied Biosystems.

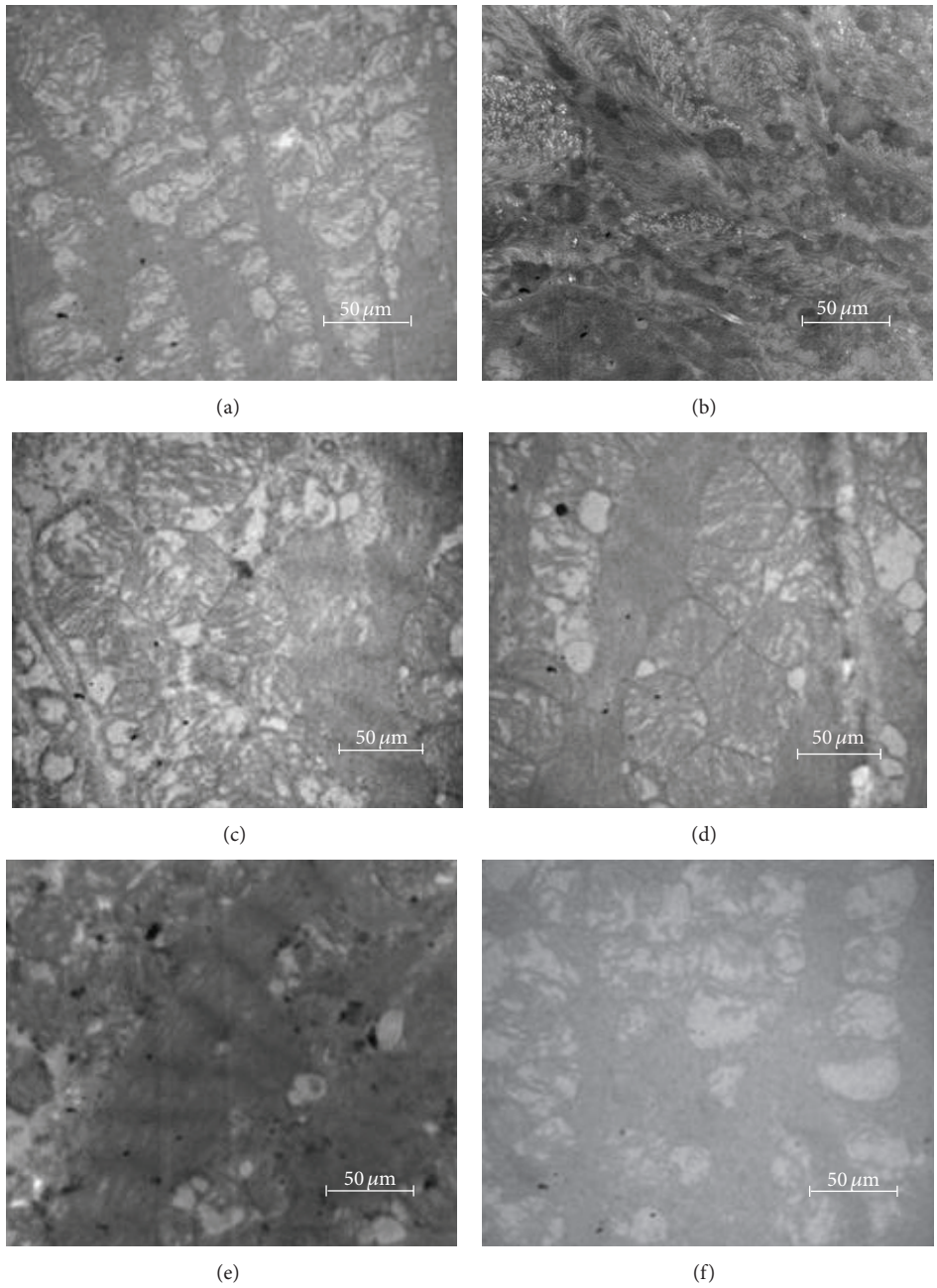

Figure 1: (a) Normal group. (b) Ischemia group. (c) Sham-operated group. (d) Res group. (e) XFZY group. (f) L-NAME group.

Primers were designed using Primer Premier 5.0 software and their sequence was as follows: Bcl-2 (GenBank Accession number L14688).

Real-time PCR was performed using a LightCycler (Roche Diagnostics, Indianapolis, IN, USA) and individual PCRs were carried out in 96-well optical reaction plates according to the manufacturer's instructions. Briefly, the PCR was carried out in a 25 μL final volume containing the following: 12.5 μL of 2x SYBR Green I master mix, 0.5 μL of 10 μM each primer, 2 μL of 1 μg/μL cDNA template, and 9.5 μL of 0.1% diethylpyrocarbonate-treated water. The PCR conditions were as follows: initial denaturation at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 59°C for 15 s, and extension at 72°C for 15 s. Fluorescent product was measured by a single acquisition mode at 72°C after each cycle. The quantification of the target gene expression was conducted according to a published method [12]. Briefly, standard curves for target genes and the housekeeping gene were constructed using their DNA isolated using a DNA extraction kit with serial dilutions (10-fold dilution). The standard curve samples were included in each PCR. Standards for both target and internal DNA were defined to contain an arbitrary starting concentration because no primary calibrators exist. Hence, all calculated concentrations are relative to the concentration of the standard. Negative controls (replacement of cDNA with water)

Table 1

Target gene	The primer sequences	Amplification length (bp)
P53	Upstream: 5′-GCAGTTCCTCTTCCTGCAGTACTC-3′; Downstream: 5′-AAC CAGACCTCAGGCGGCTCATAG-3′	241
NF-κB	Upstream: 5′-CGATCTGTTTCCCCT CATCT-3′ Downstream: 5′-ATTGGGTGCGTCTTAGTGGT-3′	175
FOX01	Upstream: 5′-AACCAGCTCAAAT GCTAGTACCATC-3′ Downstream: 5′-CAGAAGGTTCTCCATGTTTTTCT GGA-3′	198
FOX03	Upstream: 5′-TACACGGCTTGCTTACGG-3′ Downstream: 5′-GGG TTC AGA ACG AAG GGA CT-3′;	423
FOX04	Upstream: 5-TCATCAGCCAGGCCATTGAA-3′ Downstream: 5′-TGCTGTGCAAAGACAGGTTGTG-3′;	174
Sirt1-2	Upstream: 5′-TTTCAGAACCACCAAAGCG-3′ Downstream: 5′-TCCCACAGGAAACAGAAACC-3′	206
β-Actin	Upstream: 5-GAG ACC TTC AAC ACC CCA GCC-3 Downstream: 5-AAT GTC ACG CAC GAT TTC CC-3	263

were run with each set of reactions. To distinguish the specific PCR products from nonspecific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 15 s followed by a gradual increase in temperature to 95°C for 50 s. The signal acquisition was set at "continuous" mode for the detection. GAPDH mRNA level was used as an internal quantitative control, and the level of each target gene transcript was normalized on the basis of GAPDH mRNA content.

The relative expression level was determined by $2^{-\Delta\Delta Ct}$ method according to the following formula: ΔCt (target gene) = target gene Ct − actin Ct; $\Delta\Delta Ct = \Delta Ct$ (target gene) $-\Delta Ct$ (standard value); the copy of the target gene is $2^{-\Delta\Delta Ct}$.

RNA was determined by ultraviolet spectrophotometer at $\lambda 260\,nm/\lambda 280\,nm$ with the range between 1.8 and 2.0. The melting curve of PCR amplification product was substantially a single temperature peak without distortion.

2.2.6. Western-Blot Analysis. After treatment, cells were washed twice with ice-cold PBS and then lysed on ice in extraction buffer containing 50 mMTris-base (pH 7.4), 100 mM NaCl, 1% NP-40, 10 mM EDTA, 20 mM NaF, 1 mM PMSF, 3 mM Na_3VO_4, and protease inhibitors. The homogenates were centrifuged at 12,000 g for 15 min at 4°C. Supernatant was separated and stored at −80°C until use. Protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After being blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, transferred membranes were incubated overnight at 4°C with different primary antibodies (Table 2). After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 5000) in TBST with 3% nonfat milk for 1 h at room temperature. After repeated washes, membranes were reacted with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 3 min and visualized with X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and the optical density of the bands was determined using Optiquant software (Packard Instruments). The expression levels of SIRT1, P53, FOXO family, and NF-κB of treated cultures were compared with those of untreated control cultures. Normalization of results was ensured by running parallel Western blots with β-actin.

Table 2: The information of primary antibodies.

Antibody	Company	Species	Dilution factor
Sirt1	Epitomics	Rabbit	1 : 2500
FOXO1	Epitomics	Rabbit	1 : 1000
TP53	Epitomics	Rabbit	1 : 2000
FOXO3	Epitomics	Rabbit	1 : 2000
FOXO4	Epitomics	Rabbit	1 : 2000
NF-kB	Epitomics	Rabbit	1 : 2000
GAPDH	Sigma	Goat	1 : 8000
Anti-rabbit	Sigma	Goat	1 : 8000

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Guang' anmen Hospital, China Academy of Chinese Medical Sciences, Beijing, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication number 85-23, revised 1985).

3. Results

3.1. The XFZY Treatments Restored Injury of Myocardial Ischemia in the Rat Models with Electron Micrograph. In both ischemia and L-NAME groups, there was almost no complete

structure of muscle fiber observed and the myocardial cells were replaced by certain amount of clutter collagen fibers, mitochondrial swelling, vacuolization and cristae disorganized. The nuclear membrane was broken with uneven matrix, vacuolization, widened week gap, and fuzzy structure of intercalated disc.

The results in both normal and Res groups showed that the myocardial cells arranged in neat rows and the gap was clear with a small amount of collagen fibers. The sarcomere was clear with normal structures of intercalated disc and mitochondrial showing membrane integrity. Nuclear membrane and organelles were intact with no inflammatory cell infiltration observed.

In XFZY group, local myofilament was broken with the clear structure of sarcomere, while length of sarcomere was uniform and swelling of mitochondria was mild with the neat structure of crest. The nuclear matrix was mildly cavitative and nucleoli were visible with normal structure of intercalated disc, showing similar images to that of Res group.

3.2. The Impact of Interventions of XFZY on SIRT1 Pathway in RT-PCR Assay. Compared with the normal group, the expression levels of SIRT1 had significant difference in ischemia group and L-NAME group. The expression levels of P53, (NF)-kappa B, FOXO1, FOXO3, and FOXO4, as SIRT1 target genes (Table 1), were significantly different with ischemia group and L-NAME group too. XFZY was upregulated with the SIRT1, while downregulated with P53, (NF)-kappa B, FOXO1, FOXO3, and FOXO4. Compared with the ischemia group, the expression levels of SIRT1 had significant difference compared with normal group, sham-operated group, XFZY group, and Res group, while the expression levels of P53, (NF)-kappa B, FOXO1, FOXO3, and FOXO4 were significantly different with ischemia group and L-NAME group. The expression of SIRT1, P53, (NF)-kappa B, FOXO1, FOXO3, FOXO4, and L-NAME in XFZY group were very similar with Res group and that might be the reason for why XFZY could treat IHD. (See Figure 2 and Tables 3, 4, 5, 6, 7, and 8).

3.3. The Impact of Interventions of XFZY on SIRT1 Pathway in Western-Blot Analysis. (See Figures 3 and 4).

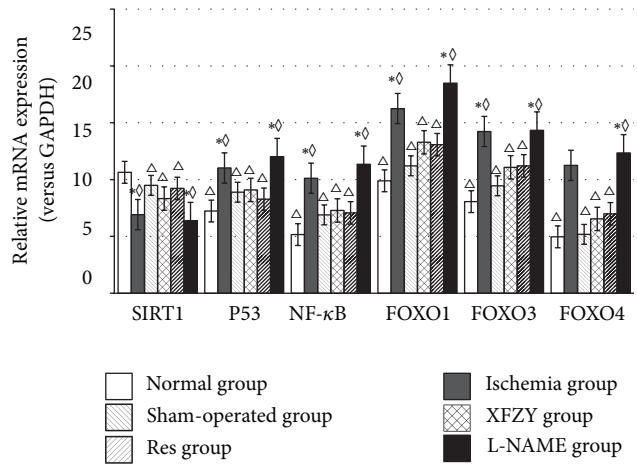

Figure 2

Table 3: Expression of SIRT1 by quantitative real-time RT-PCR.

Group	n	SIRT1
Normal group	10	$10.64 \pm 2.01^{\triangle}$
Ischemia group	10	$6.92 \pm 1.21^{*\lozenge}$
Sham-operated group	10	$9.49 \pm 1.31^{\triangle}$
XFZY group	10	$8.33 \pm 2.14^{\triangle}$
Res group	10	$9.22 \pm 2.29^{\triangle}$
L-NAME group	10	$6.38 \pm 2.93^{*\lozenge}$

Compared with the normal group, $^{*}P < 0.05$; compared with the ischemia group, $^{\triangle}P < 0.05$; compared with the XFZY group, $^{\lozenge}P < 0.05$.

Table 4: Expression of P53 by real-time PCR.

Group	n	P53
Normal group	10	$7.23 \pm 1.31^{\triangle}$
Ischemia group	10	$11.02 \pm 1.78^{*\lozenge}$
Sham-operated group	10	$8.89 \pm 2.63^{\triangle}$
XFZY group	10	$9.09 \pm 1.65^{\triangle}$
Res group	10	$8.27 \pm 4.29^{\triangle}$
L-NAME group	10	$12.01 \pm 2.18^{*\lozenge}$

Compared with the normal group, $^{*}P < 0.05$; compared with the ischemia group, $^{\triangle}P < 0.05$; compared with the XFZY group, $^{\lozenge}P < 0.05$.

4. Discussion

In this study, we used Chinese medicine XFZY to treat IHD model rats, observed morphological changes of ischemic myocardium by electron micrograph, and examined the mRNA expression levels of SIRT1 and its target genes by RT-PCR assay, and then we examined the relative protein by western-blot analysis. Among those 6 groups, Res is well known to improve the outcome after ischemic episodes and requires SIRT1 to mediate ischemic protection to increase lifespan [14]; therefore, we used Res as a drug positive control to investigate the action mechanism of XFZY. We also designed L-NAME group as the negative control group.

The results from electron micrograph in normal and Res groups apparently showed the best image of myocardium that the cells arranged in neat rows, the gap was clear with a small amount of collagen fibers, sarcomere was clear; structure of intercalated disc and mitochondrial were normal. In the ischemia and L-NAME group, the damages of myocardium with almost no complete structures of muscle fiber were shown. The image in the L-XFZY group was even worse than the ischemia group with local myofilament broken. Abbatea and so forth had observed ischemic myocardium of New Zealand rabbits by electron microscopy and found that the changes of mitochondria and myofibrillar were highly correlated with myocardial cell apoptosis [15]. Others also drew similar conclusions by researches on characterization of cardiomyocyte apoptosis in ischemic heart disease, and the changes of mitochondrial were most obvious [16, 17].

Table 5: Expression of nf-κB by real-time PCR.

Group	n	nf-κB
Normal group	10	$5.16 \pm 2.23^{\triangle}$
Ischemia group	10	$10.12 \pm 1.89^{*\lozenge}$
Sham-operated group	10	$6.89 \pm 2.15^{\triangle}$
XFZY group	10	$7.28 \pm 1.35^{\triangle}$
Res group	10	$7.07 \pm 1.29^{\triangle}$
L-NAME group	10	$11.34 \pm 2.09^{*\lozenge}$

Compared with the normal group, $^{*}P < 0.05$; compared with the ischemia group, $^{\triangle}P < 0.05$; compared with the XFZY group, $^{\lozenge}P < 0.05$.

Table 6: Expression of FOXO1 by real-time PCR.

Group	n	FOXO1
Normal group	10	$9.89 \pm 1.83^{\triangle}$
Ischemia group	10	$16.25 \pm 2.07^{*\lozenge}$
Sham-operated group	10	$11.21 \pm 1.59^{\triangle}$
XFZY group	10	$13.28 \pm 1.35^{\triangle}$
Res group	10	$13.07 \pm 1.09^{\triangle}$
L-NAME group	10	$18.48 \pm 2.79^{*\lozenge}$

Compared with the normal group, $^{*}P < 0.05$; compared with the ischemia group, $^{\triangle}P < 0.05$; compared with the XFZY group, $^{\lozenge}P < 0.05$.

Table 7: Expression of FOXO3 by real-time PCR.

Group	n	FOXO3
Normal group	10	$8.06 \pm 1.32^{\triangle}$
Ischemia group	10	$14.23 \pm 2.09^{*\lozenge}$
Sham-operated group	10	$9.45 \pm 1.15^{\triangle}$
XFZY group	10	$11.08 \pm 0.97^{\triangle}$
Res group	10	$11.21 \pm 0.67^{\triangle}$
L-NAME group	10	$14.34 \pm 2.13^{*\lozenge}$

Compared with the normal group, $^{*}P < 0.05$; compared with the ischemia group, $^{\triangle}P < 0.05$; compared with the XFZY group, $^{\lozenge}P < 0.05$.

Table 8: Expression of FOXO4 by real-time PCR.

Group	n	FOXO4
Normal group	10	$4.96 \pm 1.13^{\triangle}$
Ischemia group	10	$11.25 \pm 0.89^{*\lozenge}$
Sham-operated group	10	$5.18 \pm 1.45^{\triangle}$
XFZY group	10	$6.54 \pm 1.65^{\triangle}$
Res group	10	$6.99 \pm 1.19^{\triangle}$
L-NAME group	10	$12.34 \pm 1.09^{*\lozenge}$

Compared with the normal group, $^{*}P < 0.05$; compared with the ischemia group, $^{\triangle}P < 0.05$; compared with the XFZY group, $^{\lozenge}P < 0.05$.

Previous study proved that SIRT1 activation elicited resistance to oxidative stress via regulation of transcription factors and coactivators such as FOXO 1, 3, and 4, Hif-2a, and NF-κB [17, 18]. P53 is an important factor regulated with myocardial apoptosis, whose role is mainly through the activation of the renin-angiotensin system. Lots of researches supported that when myocardial ischemia happened, SIRT1 could reduce activity of P53 through deacetylation and

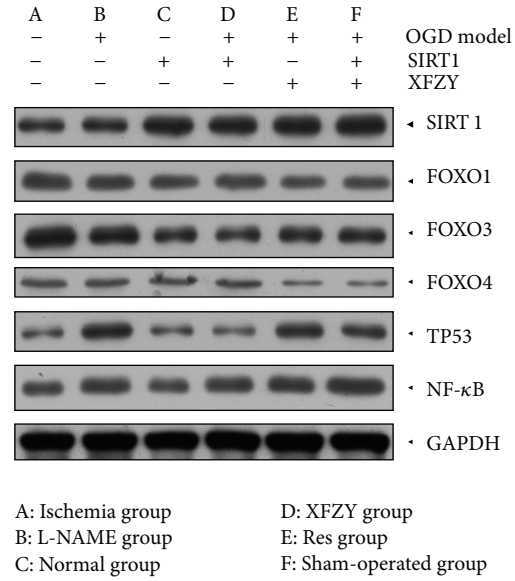

Figure 3

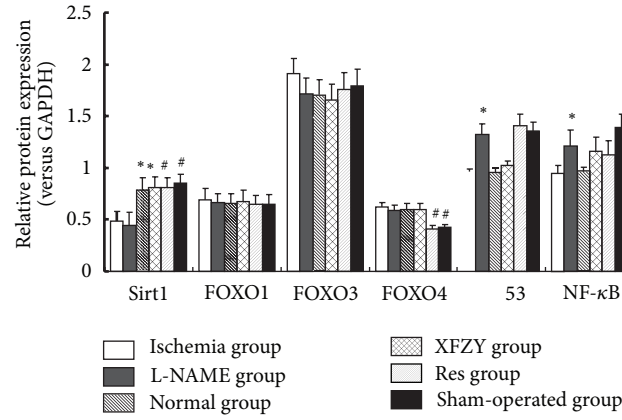

Figure 4

suppress the cardiomyocyte apoptosis [19–21]. NF-κB is a heterodimeric protein, controlling the expression of the cell survival gene. Wang and so forth revealed that SIRT1 inhibits transcription of RelA/p65 subunit by deacetylation of NF-κB and reduces the generation of oxygen free radicals so as to inhibit the main pathological factors which promote atherosclerosis and cardiovascular disease [22]. FOXO family is a kind of important acting factor of SIRT1, and it was generally believed that SIRT1 could promote activity of FOXO1 to control cellular oxidative stress response by making FOXO1 deacetylation [23]. Recent research found that SIRT1 has a similar role of FOXO3 and FOXO4 [24]. The study on signaling pathway of ischemic heart disease showed that the SIRT1-FOXO pathway is a major signaling pathway for inhibition of myocardial apoptosis [25]. Also, our research findings verified those previous studies.

With the in-depth research on pathogenesis of ischemic heart disease, to protect undead ischemic myocardial cells is becoming the most valuable therapeutic strategy which

should be taken in preventing myocardial apoptosis. SIRT1 plays an important role in a number of human physiological and pathological processes, including chronic inflammation, cancer, diabetes, and longevity, especially in ischemic injury [25–29]. As a regulatory protein deacetylase, SIRT1 has certain cardioprotection including activation of endothelial nitric oxide synthase, insulin receptor signalling, heart embryonic development, and autophagy [30, 31]; therefore, in this study we chose SIRT1 as a key point. Although we initially validated the impact of interventions of XFZY on SIRT1 signal transduction pathway, the mechanism of its action needs to be further elucidated through the SITR1 pathway by using both *in vitro* and *in vivo* models in our future studies.

In summary, XFZY had shown the bioactivities of antimyocardial apoptosis and cardioprotection in this study. The mechanism of this drug action might be through the impact of SIRT1-mediated signal transduction pathway on cardiomyocytes of ischemic heart disease, which provides us with a promising insight for the intervention of TCM on IHD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Chinese Herbal Medicine Bushen Qinggan Formula for Blood Pressure Variability and Endothelial Injury in Hypertensive Patients: A Randomized Controlled Pilot Clinical Trial

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Background. Blood pressure variability (BPV) independent of average blood pressure is related to cardiovascular damage. Meanwhile, BPV is also associated with measures of endothelial injury. Decoction, a traditional used form of Traditional Chinese Medicine (TCM), is inconvenient to prepare, carry, and store. Dispensing granules is now developing as an alternative to decoction, but the evidence supporting its clinical efficacy the same as decoction remains unclear. *Objective.* To examine the therapeutic effects on mean blood pressure (MBP), blood pressure variability, and endothelial function by giving Bushen Qinggan Formula, a compound Chinese Herbal Medicine and also to evaluate the difference in efficacy between decoction and granule. *Methods.* A total of 150 patients with hypertension were enrolled and randomly assigned to receive the placebo, Bushen Qinggan decoction, or Bushen Qinggan granule in addition to the standard medications (amlodipine-5 mg/d) for the treatment of essential hypertension (EH). The outcome was the reduction in the MBP and BPV and also included changes in the endothelial markers including endothelin-1 (ET-1) and nitric oxide (NO) after 8 weeks of treatment. *Results.* Compared with the control group, the Bushen Qinggan decoction and granule groups had significant improvement ($P < 0.01$) in BPV and endothelial founction. The level of BPV and endothelial function between decoction and granule group had no significant difference ($P > 0.05$). *Conclusion.* Based on the standard treatment, Bushen Qinggan Formula further improved BPV and endothelial function. The efficacy of Bushen Qinggan decoction and granule is similar in improving BPV and endothelial function. However, no significant antihypertensive effects could be demonstrated.

1. Introduction

In developed countries, during one's lifetime, the risk of becoming hypertensive exceeds 90% [1]. Now, data from national surveys show that hypertension is also very common in China, almost 1 in 5 adults diagnosed with high blood pressure (BP) [2]. The morbidity of hypertension has been increasing in China for decades; however, the prevalence, awareness, management, and control of hypertension from the current study are still much lower than those reported by the NHANES (National Health and Nutrition Examination surveys), 1999–2010 [3]. BP is a continuous, consistent, and independent risk factor for cardiovascular disease and stroke [4]. Stroke is also the major complication of hypertension in the Chinese population [2]. Two-thirds of strokes and half of coronary disease can be attributed to nonoptimum BP [5]; therefore, the final purpose of controlling of hypertension is to reduce the cases of events [6]. A cost-effective approach should be put in practice to achieve these goals in resource-limited settings in China.

Hypertension is one of the most prevalent vascular risk factor; the mechanisms contributing to the increased end-organ damages and vascular events risk as yet are unclear [7]. Nevertheless, it has been suggested that increased variability of blood flow induces the augmented mechanical stress. The pressure placed on the blood vessels may induce the endothelial dysfunction [8]. Blood pressure variability (BPV) plays a vital role in the progression of end-organ

damage and in triggering of vascular events. Two recent studies, Pressioni Arteriose Monitorate E Loro Associazioni (PAMELA) and the Ohasama, both reported that measures of BPV within a 24-hour period were independent predictors of cardiovascular mortality [9, 10]. In addition, antihypertensive drugs, which decrease both BPV and mean blood pressure, more effectively reduce the risk of target organ damage [11]. Meanwhile, BPV is also associated with measures of endothelial injury and endothelial function [12].

When considering that impaired endothelial function has been strongly associated with cardiovascular morbidity and mortality, the previously observed associations between high BPV and increased cardiovascular risk could be mediated via the impairment of endothelial function. Our preliminary study shows that Chinese herbal medicine "Bushen Qinggan Formula" improved endothelial injury in patients with high blood pressure [13]. The purpose of this study, therefore, was to investigate that whether Bushen Qinggan Formula improves both endothelial function and ambulatory BPV.

Decoction, a traditional and commonly used form of Traditional Chinese Medicine (TCM), is prepared by decocting different kinds of medicinal herbs together [14]. While the benefits of decoction are apparent, it is more beneficial for absorption and having higher bioavailability, and the compliance of the patient also needs to be assessed. Most hypertensive patients need lifelong medication. Similarly, Chinese medicine treatment can take a very long time. The preparation of decoction is so completed that a part of patients failed to sustain the course of treatment. That is why it is very difficult for patients to decoct TCM daily until a significant effect can be seen. Granules are much more convenient; they only need to dissolve in boiling water. If granules really can replace decoction, the compliance of patients definitely will be improved.

Dispensing granule is the granule of individual herb. Dispensing granules now are developing as an alternative to decoction, prescribed by traditional medicine practitioners in China, Japan, Korea, Singapore, even United States, and some European countries. The popularization of the dispensing granules may be because that one of the most obvious inconveniences of TCM is the preparation. Therefore, this research group decided to compare the effect of granule and decoction.

Chinese herbal formulas are known to have an advantage with regard to prevent and treat target organ damage (TOD) [15]. Researches also show that Chinese medicine has some protective function, including both improvement of BPV and endothelial function [16, 17]. Bushen Qinggan Formula is frequently used in clinical practice for treating hypertension, which is composed of *Gastrodia elata* (tian ma, TM) 30 g, *Uncaria* (gouteng, GT) 30 g, *Eucommia* bark (du zhong, DZ) 30 g, radix *Scutellariae* (huang qin, HQ) 15 g, and bitter butyl tea (kudingcha, KDC) 15 g. In Traditional Chinese Medicine (TCM) theory, most hypertensive patients show unbalance of Yin and Yang. There is a therapeutic strategy, namely, using "reinforcing" and "reducing" herbs together to balance Yin/Yang [18], and this strategy is used in the present study.

On the basis of previous, we hypothesize that BPV plays a role in the development of endothelial injury and leads to cardiovascular disease finally. Therefore, the aim of this clinical study is (1) to evaluate if the efficacy of Bushen Qinggan granule for blood pressure variability and endothelial injury in patients with hypertension is equivalent to that of the decoction and (2) to evaluate the difference in efficacy between the TCM formula and placebo.

2. Patients and Methods

This study was designed as a randomized, placebo-controlled study based on standard therapy and parallel groups. It was conducted in accordance with the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of Xiyuan Hospital of China Academy of Chinese Medical Sciences (2010XL016).

2.1. Patients. The target enrollment was 150 patients from the Cardiovascular Disease Clinic at the Xiyuan Hospital, the teaching hospital of China Academy of Chinese Medical Sciences. The enrollment criteria consisted of patients aged from 18 to 75 years and clinical findings of essential hypertension (EH) for at least 3 months prior to screening. Both men and women were included. Level 1 and level 2 hypertension was diagnosed in accordance with Chinese guidelines published in 2005 and 2010 for the management of hypertension [2, 19]. To be included in this trial, patients had to (1) have a 24-hour ambulatory blood pressure monitor (24 h ABPM): 24 h average blood pressure needs to ≥130/80 mm Hg, average blood pressure ≥135/85 mmHg during waking hours, or average blood pressure ≥120/70 mm Hg during sleeping hours, or (2) systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mm Hg, which was based on the average of 3 times seated BP readings or more office visits. Patients should have never used blood pressure medication or more than 3 times measured in one week reached the diagnostic criteria after elution. All included patients had signed written informed consent. Patients were excluded with secondary hypertension or hypertensive crisis. In addition, patients were excluded if they had uncorrected valvular heart disease, severe cardiac dysfunction, unstable angina, myocardial infarction, or stroke within half a year, had severe primary hepatic or renal disease, or had severe mental disorders or other uncontrolled systemic diseases. Finally, patients were excluded if females were pregnant or lactating, were known or suspected to be allergic to the study drugs, or had join other new drug clinical trials in recent 3 months.

From January 2010 to May 2012, participants were recruited from outpatients or via mailed brochures. Patients were evaluated based on physical examinations, laboratory screening, and ambulatory blood pressure monitor. Eligible patients were randomly assigned to 3 groups in a 1 : 1 : 1 ratio, who would have receive Bushen Qinggan granule, Bushen Qinggan decoction, or placebo in addition to a standard medication. A total of 150 patients who met the selection criteria were recruited. This study is designed as a pilot study and allowing for a 20% dropout rate. Participants will be assigned with a 1 : 1 : 1 allocation ratio according to a randomization list generated with an SAS software package.

The allocation will be concealed in sequentially numbered, opaque, sealed envelopes. Each participant received an envelope upon recruitment sequence. The random allocation envelops will be opened only after the participant has satisfied all selection criteria and completed baseline assessments. The researchers will know the allocated group but the participants, outcome assessors, and statisticians will not. The dosage used in this study was one bag of Bushen Qinggan granule, decoction, or placebo 2 times daily. Patients attended follow-up appointments at the secondary, fourth, 6th, and 8th weeks of treatment. At each visit, patients were asked about the occurrence of any clinical event or adverse effect.

The study period lasted 8 weeks.

2.1.1. Basic Interventions. The participants of all three groups used amlodipine 5 mg per day (Batch no. H10950224).

2.1.2. Granule and Decoction Group. The Bushen Qinggan formula was taken orally by patients in both groups. The Bushen Qinggan formula was produced in the form of granules and decoction by Sichuan New Green Pharmaceutical Technology Development Co., LTD (Batch no. 100718206). Both granule and decoction were composed of Gastrodia elata (tianma, TM) 30 g, *Uncaria* (gouteng, GT) 30 g, *Eucommia* bark (du zhong, DZ) 30 g, radix *Scutellariae* (huang qin, HQ) 15 g, and bitter butyl tea (kudingcha, KDC) 15 g. Granules were dissolved in boiling water and drunk while still warm.

2.1.3. Control Group. Placebo was taken by patients in the control group. The appearance, taste, and smelling of the placebo granules were made up to be extremely similar to the Bushen Qinggan granules. Instruction for usage was the same as Bushen Qinggan granules.

2.2. Endpoints. All patients were evaluated at baseline and weeks 2, 4, 6, and 8. The following clinical and laboratory variables were assessed at the first and last visit.

2.2.1. Primary Endpoints. The primary endpoints were mean blood pressure and BPV, both measured by ambulatory blood pressure monitoring. We recorded 24-hour ambulatory blood pressure monitoring with 9027-ABP (SP(a) celabs Medica, USA). The cuff was programmed to inflate every 30 minutes between 6 a.m. and 10 p.m. (daytime) and every 60 minutes between 10 p.m. and 6 a.m. (night-time). The blood pressure data were edited by blood pressure data analysis software and processed by experienced analysts. 24-hour ambulatory blood pressure monitoring data were recorded at baseline and 8 weeks. There was no limit on activities during the monitoring.

Blood pressure variability (BPV) was defined as the standard deviation of mean blood pressure depending on its time period.

According to above definition, blood pressure data were collected both daytime (d) and nighttime (n) including SBP, DBP, SBP variability (SBPv), DBP variability (DBPv), SBP deviation (SBPd), and DBP deviation (DBPd) over 24 h.

2.2.2. Secondary Endpoints. After at least 8 h of fasting, we collected venous blood from all participants at 8:00 a.m., for the test of endothelium related factors: endothelin-1 (ET-1, measured by nonequilibrium radioimmunoassay method; radiation immunoassay kits are provided by Beijing Huaying Biotechnology Research Institute; radioimmunoassay instrument is r-911 automatic counting device, produced by China University of Science and Technology Industrial Corporation), and nitric oxide (NO, is measured by colorimetric method, and the kit is provided by Beijing Huaying Biotechnology Research Institute; instrument manufacturer is Japan's Hitachi 7160 automatic biochemical analyzer).

2.2.3. Assessment of Adverse Reactions. At each visit, patients were asked if there were any adverse effects. When an adverse event appeared, the timing relative to the administration of the drugs was noted. Blood cell count, electrolytes, serum creatinine, liver function test, urinalysis, and electrocardiogram results were recorded before and after treatment.

2.3. Statistical Analyses. Analyses were performed using SPSS for Windows, version 14.0 (SPSS, Chicago, IL). Data were described as mean ± standard deviation. Proportions were used to express dichotomous variables. One-way analysis of variance (ANOVA) test was used to compare the difference between three groups. Consistent with the requirement for ANOVA, all data were checked for variance homogeneity and for normal distribution. Data that were not normally distributed were analysed using Kruskal-Wallis H test. The paired t-test was used to compare data before and after treatment. All data were checked for normal distribution. Data that were not normally distributed were analysed using Wilcoxon rank sum test. Chi-square test was performed for dichotomous variables. For dropout and withdrawal patients, perprotocol (PP) analysis was adopted, considering one of the main purposes is to prove the equivalence of granule and decoction. P value of less than 0.05 was considered significant.

3. Results

A total of 137 patients (91.3%) completed the 8-week study, with premature termination occurring in 5 patients in the placebo group, 6 patients in the granule group, and 2 patients in the decoction group (see Figure 1).

3.1. Baseline Characteristics. There were 45 patients enrolled in the granule group (31 men and 14 women), and the average age was 49.93 ± 3.49 years old. There were 47 patients enrolled in the decoction group (33 men and 14 women), and the average age was 47.58 ± 5.02 years old. The control group had 45 cases (29 men and 16 women) with an average age of 48.34 ± 4.25 years old. The baseline characteristics were comparable and statistically insignificant among three groups (see Table 1).

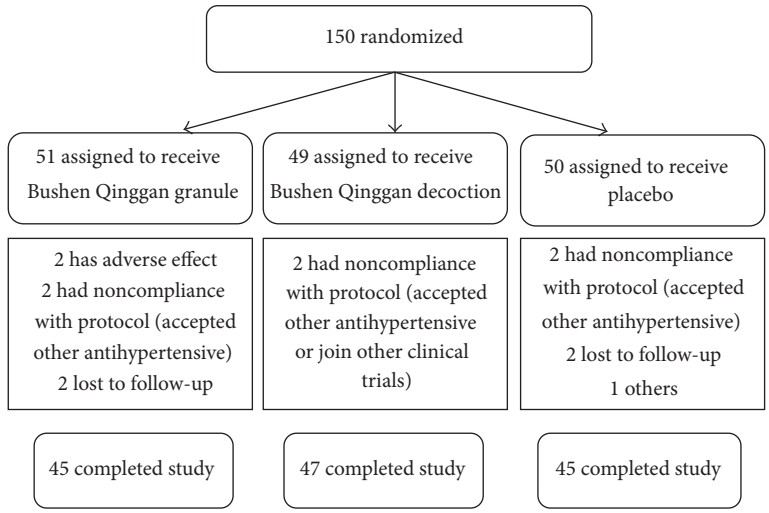

Figure 1: Flow diagram of patients in each group throughout the study.

Table 1: Baseline characteristics of three groups.

Variables	Granule group ($n = 45$)	Decoction group ($n = 47$)	Control group ($n = 45$)	P value
Age, y	49.93 ± 3.49	47.58 ± 5.02	48.34 ± 4.25	0.071
Men/women	31/14	33/14	29/16	0.382
Body mass index, kg/m^2	26.75 ± 4.03	25.19 ± 4.87	25.46 ± 3.43	0.082
Stage of hypertension (cases)				
Stage I	20	23	22	0.242
Stage II	25	24	23	0.242
Previous cardiovascular diseases (cases)	8	6	7	0.448
Dyslipidemia, (cases)	9	11	8	0.455
Heart rates, bpm	75.66 ± 8.28	76.23 ± 8.99	75.94 ± 8.47	0.084

3.2. Comparison of 24 h ABPM. Prior to interventions, there was no statistically significant difference in the blood pressure parameters measured by 24 h ABPM among three groups.

3.2.1. Changes in Blood Pressure at Different Intervals before and after Treatment. In the control and two Bushen Qinggan groups, there was significant difference in blood pressure at different intervals before and after treatment ($P < 0.01$ or $P < 0.05$). There was significant decrease ($P < 0.05$) in the level of dSBP in the decoction group compared with the other groups (see Table 2).

3.2.2. Changes in BPV at Different Intervals before and after Treatment. A favorable effect of Bushen Qinggan Formula was observed on control of BPV level after 8 weeks of treatment; both groups showed a significant decrease in BPV levels from baseline, but treatment with Bushen Qinggan Formula led to a significantly greater reduction than did the placebo There were significant decreases ($P < 0.05$) in the levels of 24-hSBPd, dSBPd, nSBPd, 24-hDBPv, 24-hDBPd, dDBPd, and nDBPd in the decoction and granule group compared with the control group; no significant differences were between granule and decoction group ($P > 0.05$) (see Table 3).

3.3. Changes in Endothelial Function. Compared with control group, granule and decoction group decreased ET-1 and elevated NO/ET-1 ($P < 0.05$); no significant differences were between granule and decoction group ($P > 0.05$) (see Table 4).

4. Discussion

Most scientific literature about comparison between granule and decoction focused on animal and cell experiments, including the animal experiment showing that Bushen Qinggan Formula improved NO in SHR (spontaneously hypertensive rat) [20]. According to the result, we suppose that Bushen Qinggan Formula trends to protect endothelial function of hypertension patients. Therefore, this study examined the integrative treatment with conventional drug therapy and Chinese herbal formula of Bushen Qinggan in hypertension patients. Compared with antihypertensive drugs alone, the integrative treatment exhibited better results in reducing BPV. At the same time, it effectively protected the function

Table 2: Changes in blood pressure at different intervals before and after treatment in three groups (mean ± standard deviation, mmHg).

Index	Granule group ($n = 45$)		Decoction group ($n = 47$)		Control group ($n = 45$)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
24-hSBP	136.93 ± 12.49	129.22 ± 9.71*	138.55 ± 11.02	127.57 ± 12.15**	135.06 ± 13.25	130.37 ± 14.16*
dSBP	147.75 ± 9.03	132.44 ± 10.55**	144.19 ± 11.87	129.23 ± 12.28$^{**\triangle}$	146.46 ± 7.43	133.35 ± 15.24*
nSBP	127.66 ± 14.59	122.93 ± 12.91*	125.49 ± 10.96	120.89 ± 13.31*	127.60 ± 12.41	122.40 ± 13.01*
24-hDBP	84.22 ± 9.89	77.60 ± 10.55*	83.36 ± 10.69	78.38 ± 9.58*	84.08 ± 8.92	79.84 ± 10.85*
dDBP	86.48 ± 9.83	79.75 ± 11.12*	85.49 ± 11.32	80.79 ± 10.56*	86.44 ± 9.04	81.86 ± 11.40*
nDBP	78.04 ± 10.70	71.13 ± 10.53*	75.85 ± 10.43	73.17 ± 10.37*	77.37 ± 9.58	72.82 ± 9.93*

$^{*}P < 0.05$, $^{**}P < 0.01$, versus before treatment; $^{\triangle}P < 0.05$, versus control group. 24-hSBP: 24-hour systolic blood pressure; 24-hDBP: 24-hour diastolic blood pressure; dSBP: daytime systolic blood pressure; dDBP: daytime diastolic blood pressure; nSBP: night-time systolic blood pressure; nDBP: night-time diastolic blood pressure.

Table 3: Changes in blood pressure variability at different intervals before and after treatment in three groups (mean ± standard deviation, mmHg).

Index	Granule group ($n = 45$)		Decoction group ($n = 47$)		Control group ($n = 45$)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
24-hSBPd	15.68 ± 3.50	11.84 ± 1.93$^{**\triangle}$	15.08 ± 4.89	11.57 ± 2.53$^{**\triangle}$	15.41 ± 3.91	13.31 ± 3.03**
24-hSBPv	11.43 ± 2.93	10.34 ± 2.19	10.60 ± 6.45	9.94 ± 2.12	11.02 ± 2.38	10.18 ± 2.28
dSBPd	13.17 ± 3.15	9.97 ± 2.34$^{**\triangle}$	12.66 ± 3.20	9.96 ± 1.66$^{**\triangle}$	13.68 ± 3.62	11.57 ± 2.74*
dSBPv	8.92 ± 1.96	7.65 ± 2.55*	9.16 ± 2.18	6.83 ± 4.83**	9.33 ± 2.57	7.19 ± 3.69**
nSBPd	11.68 ± 3.81	8.22 ± 2.42$^{*\triangle}$	11.04 ± 3.40	8.21 ± 2.51$^{*\triangle}$	12.42 ± 4.64	10.24 ± 4.27*
nSBPv	8.92 ± 3.43	6.81 ± 2.60*	8.76 ± 2.56	8.11 ± 3.57*	9.03 ± 4.02	7.59 ± 4.46
24-hDBPd	10.53 ± 1.74	8.51 ± 1.54$^{**\triangle}$	10.02 ± 2.63	8.50 ± 1.46$^{**\triangle}$	11.26 ± 2.95	9.77 ± 1.69**
24-hDBPv	12.78 ± 2.25	11.06 ± 2.09$^{**\triangle}$	12.15 ± 3.25	10.24 ± 6.35$^{**\triangle}$	13.20 ± 2.94	12.39 ± 2.28
dDBPd	8.84 ± 2.37	6.66 ± 1.53$^{**\triangle}$	8.55 ± 2.29	6.89 ± 1.38$^{*\triangle}$	9.33 ± 2.86	7.68 ± 1.75**
dDBPv	10.35 ± 2.76	8.36 ± 2.83**	10.58 ± 3.26	7.86 ± 4.94**	10.63 ± 3.05	8.25 ± 3.58**
nDBPd	8.95 ± 3.06	6.57 ± 1.87$^{**\triangle}$	8.98 ± 2.87	6.79 ± 2.21$^{**\triangle}$	9.66 ± 3.46	8.11 ± 3.20*
nDBPv	11.35 ± 4.64	8.94 ± 3.39**	10.32 ± 6.94	7.19 ± 5.29*	10.98 ± 5.47	10.15 ± 5.41

$^{*}P < 0.05$, $^{**}P < 0.01$, versus before treatment; $^{\triangle}P < 0.05$, versus control group. 24-hSBPd: 24-hour systolic blood pressure deviation; 24-hSBPv: 24-hour systolic blood pressure variability; dSBPd: daytime systolic blood pressure deviation; dSBPv: daytime systolic blood pressure variability; nSBPd: night-time systolic blood pressure deviation; nSBPv: night-time systolic blood pressure variability; 24-hDBPd: 24-hour diastolic blood pressure deviation; 24-hDBPv: 24-hour diastolic blood pressure variability; dDBPd: daytime diastolic blood pressure deviation; dDBPv: daytime diastolic blood pressure variability; nDBPd: night-time diastolic blood pressure deviation; nDBPv: night-time diastolic blood pressure variability.

of endothelium. Bushen Qinggan Formula exerts multiple actions on hypertension patients and may therefore be an effective and efficient tool in the management of hypertension.

TCM practitioners treat patients based on certain syndromes of patients, that is, "Syndrome Differentiation and Treatment" or "Correspondence of Prescription and Syndrome." Bushen Qinggan Formula is also guided by this concept. Use of the combination of 5 herbal medicines in Bushen Qinggan formulation for the antihypertensive effects was a common practice according to the experts in TCM. The use of Bushen Qinggan formulation highlights the combination of syndrome and disease which coincides with the concept and treatment of modern medicine [21].

A lot of clinical evidence shows that CM (Chinese Medicine) was effective for hypertension in clinical use [22], also possessing the advantage of the whole body regulation and TOD protection [23]. In recent years, pharmacologic studies also have confirmed that phenolic compounds and indole alkaloids contained in Gouteng have the antihypertensive, free radical scavenging, and antiexcitotoxic effects [24]. Huang qin [25] and tian ma [26] also have effects of being anti-inflammatory and antipyretic and may attribute to the protection of vascular endothelium. Furthermore, the vascular endothelium protective function also was approved in our research. Vascular health may influence the pathogenesis of cardiovascular disease, in part, through influences on BPV.

Currently, TCM granules are commonly used in the clinic, but the role and efficacy comparing with traditional decoction are still controversial. For this research, the efficacy of two different forms of Bushen Qinggan Formula is similar in improving BPV and endothelial function. This study is designed as a pilot clinical trial. From its results, we find the trends of equivalent conversion for the two formulations. However, the approximate effect of decoction and granules is noteworthy and deserving further study, in order to make more patients willing to accept TCM treatment.

Table 4: Changes inendothelial function before and after treatment in three groups (mean ± standard deviation).

Index	Granule group ($n = 45$)		Decoction group ($n = 47$)		Control group ($n = 45$)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
NO (μmol/L)	56.61 ± 15.30	62.63 ± 15.51	56.77 ± 9.60	62.81 ± 8.69	57.86 ± 13.77	58.98 ± 14.47
ET-1 (pg/mL)	66.26 ± 9.20	$59.97 \pm 6.21^{*\triangle}$	65.05 ± 13.77	$59.91 \pm 10.41^{*\triangle}$	64.37 ± 8.88	64.71 ± 9.35
NO/ET	0.87 ± 0.30	$1.05 \pm 0.28^{**\triangle}$	0.95 ± 0.52	$1.08 \pm 0.24^{**\triangle}$	0.92 ± 0.28	0.93 ± 0.27

$^{*}P < 0.05$, $^{**}P < 0.01$, versus before treatment; $^{\triangle}P < 0.05$, versus control group.

Hypertension in three groups has no statistical difference; however, it is well controlled at the end of the study maybe because of the application of antihypertensive. These results suggest that blood pressure changes may not be the main function of the Chinese herb formula.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Yiqihuoxuejiedu Formula Inhibits Vascular Remodeling by Reducing Proliferation and Secretion of Adventitial Fibroblast after Balloon Injury

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Vascular remodeling occurs in atherosclerosis, hypertension, and restenosis after percutaneous coronary intervention. Adventitial remodeling may be a potential therapeutic target. Yiqihuoxuejiedu formula uses therapeutic principles from Chinese medicine to supplement Qi, activate blood circulation, and resolve toxin and it has been shown to inhibit vascular stenosis. To investigate effects and mechanisms of the formula on inhibiting vascular remodeling, especially adventitial remodeling, rats with a balloon injury to their common carotid artery were used and were treated for 7 or 28 days after injury. The adventitial area and α-SMA expression increased at 7 days after injury, which indicated activation and proliferation of adventitial fibroblasts. Yiqihuoxuejiedu formula reduced the adventitial areas at 7 days, attenuated the neointima and vessel wall area, stenosis percent, and α-SMA expression in the neointima, and reduced collagen content and type I/III collagen ratio in the adventitia at 28 days. Yiqihuoxuejiedu formula had more positive effects than Captopril in reducing intimal proliferation and diminishing stenosis, although Captopril lowered neointimal α-SMA expression and reduced the collagen content at 28 days. Yiqihuoxuejiedu formula has inhibitory effects on positive and negative remodeling by reducing adventitial and neointimal proliferation, reducing content, and elevating adventitial compliance.

1. Introduction

Vascular remodeling occurs in a variety of diseases such as atherosclerosis, hypertension, and restenosis after PCI. Although these diseases have different causes, they ultimately lead to common pathological changes such as vascular remodeling, which results from and causes pathology in these diseases. Vascular remodeling results from activation, proliferation and migration of cells in the vessel wall, and extracellular matrix synthesis and degradation. Vascular smooth muscle cells (VSMC) in the media have been considered the core of vascular remodeling [1]. However, more and more evidence indicates that "passive" adventitial fibroblasts (AFs) change into "active" myofibroblasts (MFs), which contribute to the arterial remodeling process [2]. Adventitial fibroblasts acquire a migratory phenotype and populate the damaged tissue after stretch injury resulting from a balloon angioplasty [3]. These myofibroblasts produce a thickened and rigid adventitia rich in collagen fibers and further result in a reduction of the vessel lumen size. Thus, adventitia remodeling plays a more important role than previously thought in atheroma formation and restenosis [4]. Inhibiting activation, proliferation, migration, and secretion of adventitial fibroblasts is a new target for the prevention and treatment of vascular remodeling [5].

MF was first discovered in wound healing through electron microscopy. The evidence indicates that MF plays a pivotal role in tissue repair and remodeling and is also a key player in pathological conditions such as hypertrophic scars and organ fibrosis. Professor Jiazhen Liao, a famous cardiovascular disease expert on integrated Chinese and western medicine from China's first generation, gets his

inspiration from the surgical wound repair and thinks that the process of vascular remodeling after balloon injury, including thrombosis, inflammatory cell infiltration, and local tissue excessive repair that eventually leads to restenosis, is similar to the pathology of surgical wound repair. He used the wound repair idea and Chinese medicine therapeutic principles of supplementing Qi, activating blood circulation, and detoxifying to develop Yiqihuoxuejiedu formula. The formula is composed of Radix Astragali, Radix Salviae Miltiorrhizae, Flos Lonicerae, Cortex Moutan, and others. Clinical and basic researches, conducted for over 10 years, indicate that the Yiqihuoxuejiedu formula significantly inhibits vascular hyperplasia, lowers blood lipids [6], and reduces neointimal collagen content [7]. In addition, the formula serum promotes VSMC secretion of nitric oxide (NO) and/or reduces the decomposition of NO [8] and inhibits VSMC proliferation and lipid peroxidation injury. A clinical study finds that the formula significantly reduced the probability and cumulative risk of overall cardiovascular events in patients with coronary artery disease (CHD) who underwent stent implantation. The beneficial effect presents at about six months after PCI [9].

According to 2011's PCI guideline [10], secondary prevention after PCI includes antihypertensive therapy with β receptor blockers and angiotensin converting enzyme inhibitors (ACEI), lipid-lowering therapy with statins, and antiplatelet/anticoagulant therapy with aspirin and clopidogrel. Of these drugs, ACEI, one of the most important drugs that inhibit cardiovascular remodeling, diminishes the development of atherosclerotic lesions and restenosis after angioplasty through suppressing the generation of angiotensin II (Ang II), which promotes cellular migration, proliferation, and hypertrophy [11]. In this study, ACEI serves as a positive control. Although western medicine therapies have obtained some effects, risks remain and new drugs that prevent vascular remodeling are required. Yiqihuoxuejiedu formula is associated with surgical wound and reduces neointimal hyperplasia after balloon injury. Thus, we hypothesize that Yiqihuoxuejiedu formula inhibits vascular remodeling by reducing adventitial fibroblasts function.

2. Materials and Methods

2.1. Animals. Normal male Sprague-Dawley (SD) rats were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing, China. SD rats were raised in a specific pathogen free environment at a room temperature of 22°C to 24°C, 40%–50% relative humidity, and a 12-hour light/dark cycle. Procedures were performed in accordance with the National Institute of Health's Guide for the Use and Care of Laboratory Animals and were approved by the Committee on Animal Care and Use of the Dongzhimen Hospital. SD rats weighing 380–450 g ($n = 80$) were chosen for a carotid artery model after balloon injury.

2.2. Animal Model of Common Carotid Artery Injury. A rat model of the common carotid artery after balloon injury was established to evaluate the vascular remodeling. Sodium pentobarbital 1% (40 mg/kg) was intraperitoneally injected to anaesthetize the rats. The left common carotid artery was isolated through a midline cervical incision to expose a 3 cm segment of the artery from the bifurcation and a 2F Fogarty balloon catheter (diameter of balloon 2 mm and length 20 mm, Baxter Company) was introduced through the left external carotid artery and advanced 4 cm towards the thoracic aorta with the left internal carotid artery blocked. The balloon was inflated with isotonic Na chloride (NS) at 0.5 atm to 0.6 atm to distend the artery and was then pulled back to the bifurcation with constant rotation. This procedure was repeated three times to ensure endothelial denudation and consistent vascular injury. After removing the catheter, the external carotid artery was ligated, the blood flow in the internal carotid artery was restored, the wound closed, and animals were allowed to recover. The external carotid artery of sham operated animal was ligated and common carotid artery was not exposed to balloon injury. Rats were sacrificed and both carotid arteries were collected at 7 or 28 days after balloon injury.

2.3. Medications and Grouping. Yiqihuoxuejiedu formula was composed of ingredients such as *Astragalus membranaceus* (Fisch) Bge. var. *mongholicus* (Bge.) Hsiao., *Salvia miltiorrhiza* Bge., *Lonicera japonica* Thunb., and *Paeonia suffruticosa* Andr. The formula was produced by the Chinese Herbal Company of Beijing University of Chinese Medicine (Beijing, China) and the final concentration is 1.456 g crude drug/mL.

After balloon injury, rats were randomly divided into 3 groups of 10 rats each: the model group, Captopril group, and Yiqihuoxuejiedu formula group. The sham operated group served as a control. The dosages of Captopril and Yiqihuoxuejiedu formula, which are based on the clinical daily dosages for adult humans with a dose conversion coefficient, were the following: Yiqihuoxuejiedu formula group: 13.368 g/kg/d (corresponding with 12 times the clinical dosages), Captopril group: 12.857 mg/kg/d (12 times clinical dosages). In addition, Acenterine (17.143 mg/kg/d, 6 times) and Pravastatin sodium (1.714 mg/kg/d, 12 times) were used for basic medicine for the Captopril and Yiqihuoxuejiedu groups. Sham operated and model groups received distilled water, 10 mL/kg/d. Medicine was dissolved in distilled water and rats were administered the medicine using a gastric lavage once daily for 7 or 28 days.

2.4. Histological and Immunohistochemistry Staining. At 7 or 28 days after balloon injury, animals were euthanized under terminal anaesthesia by exsanguination and retrograde aortic perfusion with 200 mL saline followed by 250 mL saline containing formalin (2% v/v) and glutaraldehyde (0.2% v/v) to fix the tissues before careful excision of the left common carotid artery to avoid any damage to the adventitial layer. The left common carotid arteries were fixed for 24 hours. The slices were stained with Sirius Red and Masson stains as well as haematoxylin and eosin following routine paraffin sections. The expression of smooth muscle α-actin was determined in sections incubated in 0.3% hydrogen peroxide for 15 min

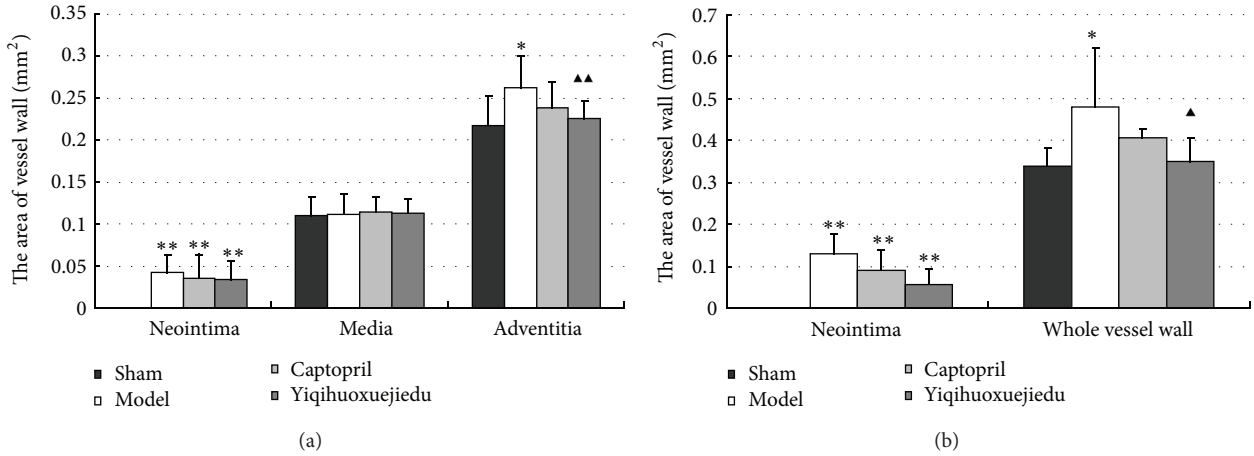

FIGURE 1: (a) Vessel wall area at 7 days after injury; (b) vessel wall area at 28 days after injury ($^{*}P < 0.05$ and $^{**}P < 0.01$ versus the sham group; $^{\blacktriangle}P < 0.05$ versus the model group).

before using a primary monoclonal α-SMA actin antibody (1 : 300, AlPha Sr-1, SANTA) and secondary goat anti-mouse IgG antibody-HRP multimer (DSGB-BIO Origene) and was visualized with diaminobenzidine substrate.

2.5. Histological and Immunohistochemistry Quantitative Measurements. The complete cross sections of the left common carotid artery were photographed with a low power lens following haematoxylin and eosin stains to measure and calculate remodeling index including the area of intima, tunica media, and adventitia, as well as stenosis percentage: [(left lumen area around the internal elastic lamina−left narrow lumen area)/left lumen area around the internal elastic lamina] $*$ 100%.

2.6. Data Processing Method. Three typical and discrete fields were photographed with high powered lens after immunohistochemical staining. α-SMA expression was determined in the intima, tunica media, and adventitia according to brown endochylema. Under high power lens of polarized light, the average optical density and percentage of type I and type III collagen-positive areas were evaluated in 3 representative areas for each compartment of the vessel wall using azure-blue and Sirius Red stained sections.

2.7. Statistical Analysis. Statistical analyses were performed using SPSS 11.0 statistics software. The measurement data were presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Dunnett's test was used to determine the differences among groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The Arterial Wall Area after Balloon Injury

3.1.1. Arterial Wall Area at 7 Days after Injury. At 7 days after injury, the areas of neointima and adventitia in the model group and two treatment groups were larger than those of the sham group ($P < 0.01$). There was no change in the medial compartment area among all groups. There was also no significant difference in the neointimal areas of the model and two treatment groups, although the level of two treatment groups decreased to some extent. The Yiqihuoxuejiedu formula reduced the adventitial areas compared with the model group ($P < 0.01$), but Captopril did not (Figures 1(a) and 2).

3.1.2. Arterial Wall Area at 28 Days after Injury. The areas of neointima and of the whole vessel wall were increased in the model group compared with the sham group ($P < 0.01$). The Yiqihuoxuejiedu-treated rats had significantly smaller areas of neointima and the whole vessel wall than did the model rats ($P < 0.05$; Figure 1(b)).

3.2. Arterial Stenosis Percentage at 28 Days after Injury. At 28 days after injury, the percent stenosis in the model group and two treatment groups increased compared with the sham group ($P < 0.01$ or $P < 0.05$). The Yiqihuoxuejiedu-treated rats had a lower percentage of stenosis occupied by plaque than did the model rats (Figure 3).

3.3. The Percentage of Positive Expression of α-SMA

3.3.1. The Percentage of Positive Expression of α-SMA at 7 Days. Positive α-SMA expression, indicated by brownish yellow or brown granulation in the cytoplasm, was only present in media and was not in the intima and adventitia of the sham group. At 7 days after vascular injury, there was α-SMA expression in the media and also in the neointima and adventitia of the model group and the two drug groups, which decreased significantly in the media but increased in the neointima and adventitia compared with the sham group ($P < 0.01$). There was no change in different compartments among the model group and the two drug groups although α-SMA expression in the two drug groups decreased (Figure 4(a)).

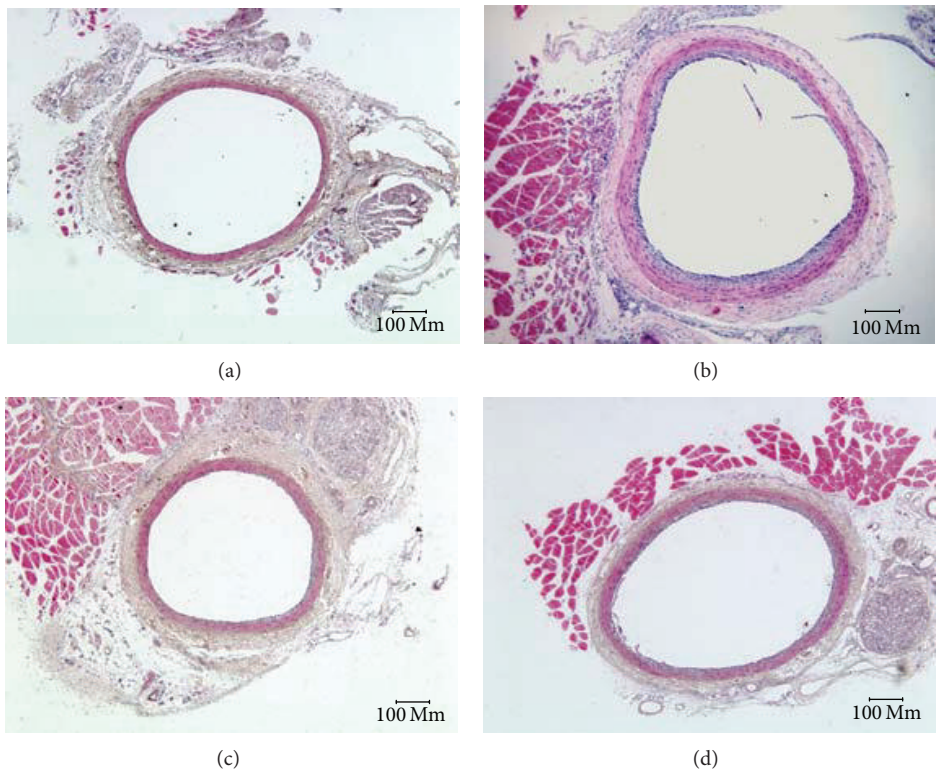

Figure 2: Left common carotid artery slices stained with HE at 7 days after injury. (a) Sham group, (b) model group, (c) Captopril group, and (d) Yiqihuoxuejiedu group.

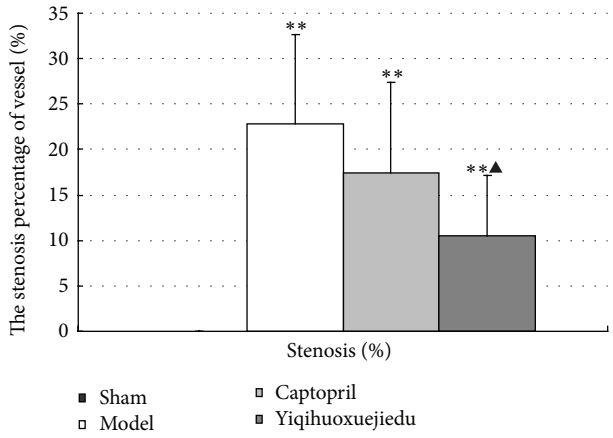

Figure 3: Percentage of arterial stenosis at 28 days after injury ($^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham group; $^{\blacktriangle}P < 0.05$ versus model group).

3.3.2. The Percentage of α-SMA Expression at 28 Days. Twenty-eight days after balloon injury, α-SMA expression was only seen in the media and there was no expression in the intima and adventitia of the sham group; while there was positive expression in the media and neointima, there was no expression in adventitia of the model and drug groups. The Yiqihuoxuejiedu and Captopril rats had lower α-SMA expression in the neointima than the model rats ($P < 0.01$; Figure 4(b)).

3.4. The Percent of Collagen at 28 Days after Injury. A large amount of collagen, observed using Masson staining, accumulated in the vessel wall of the model group compared with the sham group ($P < 0.01$). Collagen content of the two drug groups diminished significantly compared with the model group ($P < 0.01$). The Yiqihuoxuejiedu formula reduced collagen hyperplasia more than in the Captopril group ($P < 0.01$; Figure 5).

3.5. Ratio of Type I/Type III Collagen in the Adventitia at 28 Days after Injury. Rats of the model group obviously increased in the ratio of type I/type III collagen in the adventitia compared with rats in the sham group ($P < 0.01$). There was significant degradation in type I/III collagen in the adventitia of rats in the Yiqihuoxuejiedu group, but there was no difference in the Captopril group compared with the model group ($P < 0.01$). The ratio of types I/III collagen in the Yiqihuoxuejiedu group was lower than that of the Captopril group ($P < 0.01$; Figures 6 and 7).

4. Discussion

There are three types of pathological changes in vascular remodeling: vascular structure, cell biology, and function.

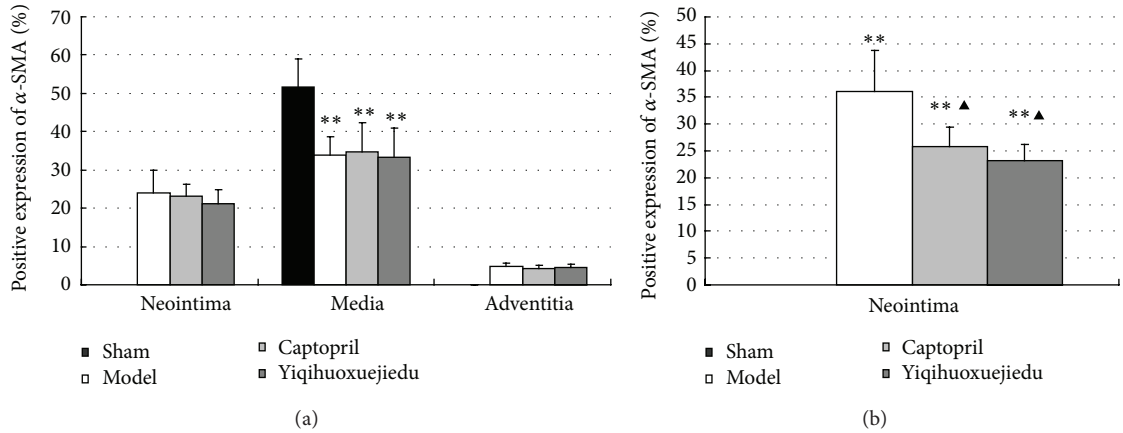

FIGURE 4: (a) α-SMA expression at 7 days after injury; (b) α-SMA expression at 28 days after injury ($^{**}P < 0.01$ versus sham group; $^{\blacktriangle}P < 0.05$ versus the model group).

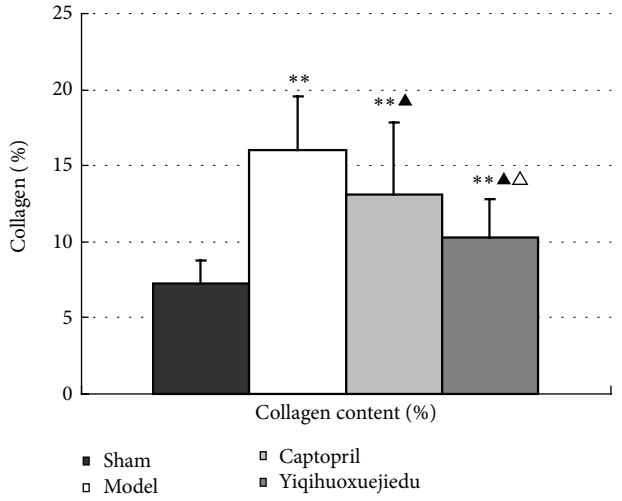

FIGURE 5: Percent collagen content percent in the vessel wall at 28 days after injury ($^{**}P < 0.01$ versus the sham group; $^{\blacktriangle}P < 0.05$ versus the model group; $^{\triangle}P < 0.05$ versus the Captopril group).

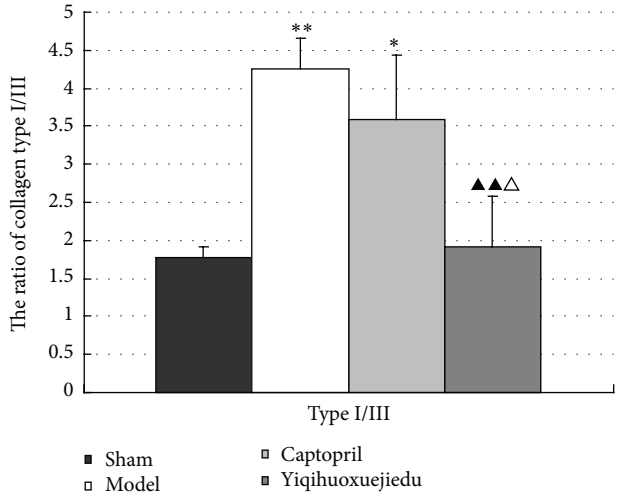

FIGURE 6: Ratio of type I/type III collagen in the adventitia at 28 days after injury. ($^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group; $^{\blacktriangle}P < 0.05$ versus model group; $^{\triangle}P < 0.05$ versus Captopril group).

Structural remodeling refers to transformation of vessel lumen and thickness or area of the vessel wall. Cell biology changes mean that cells in the vascular wall activate, proliferate, migrate, and secrete extracellular matrix (ECM), and the function variation refers to reduced vascular compliance and the reaction to vasoactive substances.

Vascular remodeling is also divided into positive remodeling and negative remodeling. The positive remodeling, also known as compensatory or expansion remodeling, has a compensatory vessel enlargement and relatively sustained changes to the lumen to maintain a constant flow despite an increased plaque burden [12]. Negative remodeling, also called decompensated or constrictive remodeling, causes a reduction in the vessel lumen size that contributes to main pathogenesis of atherosclerosis and restenosis after PCI [12]. The adventitia remodeling can result in either positive or negative remodeling or both. Evidence has specifically shown that the injured adventitia elicits intimal and medial pathological changes that are involved in the evolution of restructuring [4]. Similarly, endothelial damage can lead to pathological processes in the media and adventitia.

Various pathological stimuli (e.g., stretch injury from a balloon angioplasty or endothelial lesion) can induce adventitial fibroblasts differentiation into myofibroblasts (MF, a key player in wound healing and a slow, irreversible retraction [13]) in the adventitia. Differentiated MFs acquire an α-smooth muscle actin (α-SMA, which is not expressed in normal fibroblasts) phenotype, propagate, and migrate to the neointima, eventually becoming a part of the newly generated intima [14]. These MFs produce a thickened and rigid adventitia rich in collagen fibers leading to adventitia remodeling, consisting of both structural and functional reorganization [15]. In α-SMA expression, medial smooth muscle cells (SMC) are opposite to adventitial fibroblasts.

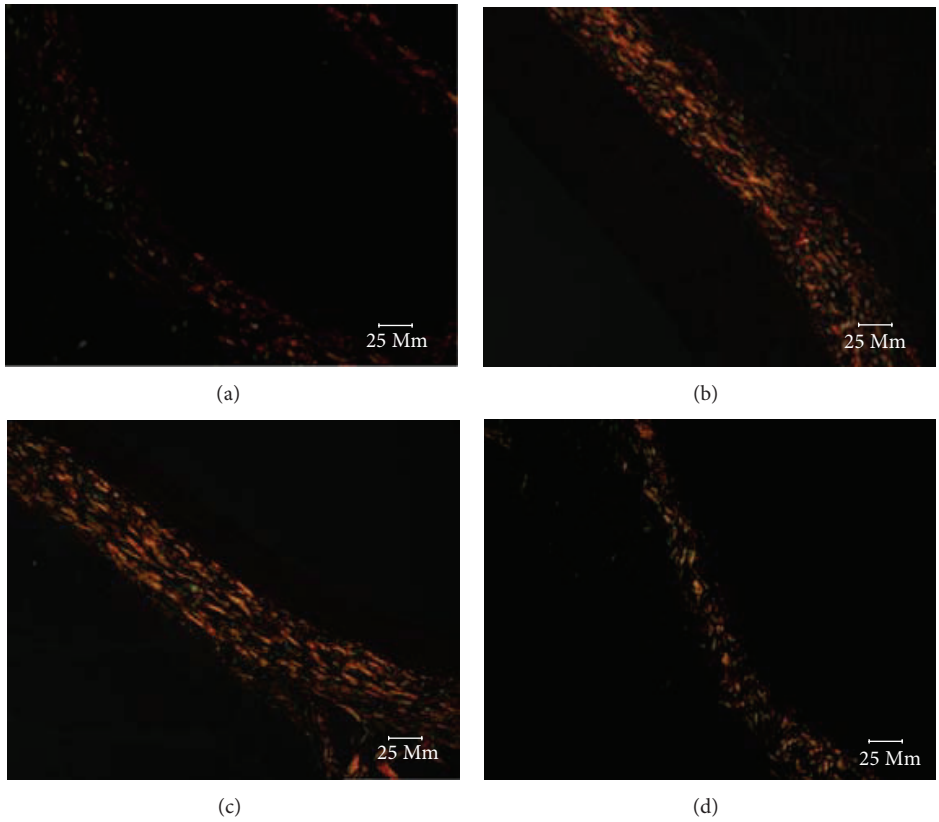

Figure 7: Representative images of adventitia at 28 days after injury (Sirius Red staining ×400). Type I collagen closely spaced and showed strong double refraction, yellow or red fibers. Type III collagen showed weak double refraction, greenish fine fibers. (a) Sham group, (b) model group, (c) Captopril group, and (d) Yiqihuoxuejiedu group.

SMC in the media has positive α-SMA expression in normal conditions, but it loses the α-SMA expression following activation of SMC. Therefore, the changes to the α-SMA expression in the vascular walls are one of the most important signs of activation in fibroblasts and smooth muscle cells. Vascular remodeling, especially adventitia remodeling, plays an important role in atherosclerosis, hypertension, and restenosis after PCI, and methods of improving vascular remodeling have been recently studied. Chinese medicine is a main part of complementary and alternative medicine and has gained better and more characteristic clinical effects [16]. Xiongshao capsules, which activate the blood circulation, are known to be effective in prevention and treatment of vascular remodeling and restenosis [17, 18]. Yiqihuoxuejiedu formula uses the therapeutic principles of supplementing Qi, activating blood circulation and detoxification based on the Qi blood-related theory of Chinese medicine, and it has effects on inhibiting vascular hyperplasia, reducing blood lipids, and attenuating collagen content in neointima [6, 7]. The formula contains a number of bioactive components. Of these components, *Astragalus* polysaccharide has various important bioactivities, such as immunomodulation, antioxidant, antitumor, antidiabetes, antiviral, hepatoprotection, anti-inflammation, antiatherosclerosis, hematopoiesis, and neuroprotection [19]. *Astragalus* membranaceus and *Astragalus* saponin can potently protect endothelium-dependent relaxation against the acute injury from homocysteic acid (HCA) through nitric oxide regulatory pathways [20]. Tanshinone IIA prevents cardiac fibroblast proliferation by interfering with the generation of reactive oxygen species (ROS) and involves the activation of the endothelial nitric oxide synthase-nitric oxide (eNOS-NO) pathway [21]. Tanshinone IIA can also inhibit H_2O_2 -induced collagen synthesis via attenuation of O_2^- -generation and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity [22]. Paeonol suppresses vascular smooth muscle cell proliferation in vivo and in vitro, which might be related to a decrease in lipid content, lipid peroxidation, and proinflammatory cytokine concentrations. Paeonol also regulates the proliferation periods of vascular smooth muscle cells through inhibiting the PCNA protein expression [23]. Chlorogenic acid has various pharmacologic actions, such as antioxidation, antibacterial, antiviral, antitumor, anti-inflammation, liver protective effect, and antiendotoxin [24]. Salviol IIA and paeonol inhibit adventitial fibroblast hyperplasia and collagen synthesis induced by ANG-II [25]. The above studies suggest that the components in Yiqihuoxuejiedu formula have many kinds of effects, especially on inhibiting fibrosis

and reducing cells proliferation and collagen content of vessel wall.

The vascular remodeling mechanism of the formula still requires more research. The present study focuses on the proliferation, activation, and secretion of adventitial fibroblasts following balloon injury and aims to provide experimental evidence on the adventitia. A rat model was established by injuring the common carotid artery, and rats were treated with Yiqihuoxuejiedu formula for 7 or 28 days. At 7 days after injury, the area and α-SMA-positive expression in the adventitia significantly increased compared with the sham group, which indicates that adventitial fibroblasts generated clear activation and proliferation. In the sham group, α-SMA is expressed in the media rather than in the intima or adventitia. However, in the model group, α-SMA has low expression in the media and high expression in the neointima and adventitia. Thus, both adventitial fibroblasts and medial smooth muscle cells are activated, and the former proliferated more than the latter, resulting in larger adventitial areas. At 28 days after injury, some indexes, that is, the area of neointima, percentage of the stenosis, the percentage of collagen content in the vessel wall, and the ratio of type I/type III collagen in the adventitia in the model group, were greater than those of the sham group. These showed that the intimal hyperplasia, vascular stenosis, and vascular stiffness increased, especially in the rigid adventitia that is rich in collagen fibers. Compared with the model group, Yiqihuoxuejiedu formula greatly reduced the adventitial areas but could not suppress α-SMA expression in the vessel walls at 7 days after balloon injury. With longer treatment, the formula further reduced neointima and vessel wall areas, diminished the percentage of stenosis, reduced α-SMA expression in the neointima, and lowered the collagen content and ratio of type I/type III collagen in the adventitia compared with those of the model group at 28 days. The Yiqihuoxuejiedu formula had better effects than did the Captopril. During the initial period (7 days), Yiqihuoxuejiedu formula inhibited positive (expansionary) remodeling by preventing adventitial hyperplasia, and, in the late stages (28 days), it improved negative (constrictive) remodeling by suppressing neointimal proliferation, reducing the collagen content of the vessel wall and elevating adventitial compliance. However, Yiqihuoxuejiedu formula did not inhibit activation of adventitial fibroblasts (positive α-SMA expression in the adventitia) at 7 days. Previous studies of formulas were mainly concerned about intimal thickening and collagen content in vascular wall [26]. Active Components from Chinese herbs had an effect on the proliferation and secretion of smooth muscle cells or adventitial fibroblasts culture in vitro [25]. This study focused on the adventitial remodeling in vivo, especially on adventitial proliferation and collagen changes after intimal damage, and these pathological processes were inhibited by Yiqihuoxuejiedu formula developed from the idea of the surgical wound repair.

In this study, Captopril did not reduce neointimal proliferation or diminish stenosis, although it reduced α-SMA expression in the neointima and lowered the collagen content at 28 days. These results might relate to the type of the animal model and mechanism of the angiotensin converting enzyme inhibitor (ACEI) used in this study. ACEI inhibits Ang I conversion to Ang II, which has effects such as constricting blood vessels, increasing blood pressure, and stimulating vascular cell wall proliferation. The level of renin and angiotensin in an injured artery model is not as high as the level in long-term hypertension, which has sustained high levels of Ang II. In addition, ACEI does not completely block effects of Ang II and Ang II still plays a role. This may explain why the effect of ACEI is not significant in this study.

Thus, Yiqihuoxuejiedu formula inhibits positive and negative remodeling by reducing hyperplasia in the adventitia in the early stages and suppresses intimal proliferation, reduces the vessel wall content, and elevates adventitial compliance in the later stages. Inhibiting proliferation and secretion of adventitial fibroblasts is characteristic of the formula. This study suggests new information and a new, complementary method for alternative treatment in the prevention of vascular remodeling, which contributes to further improving atherosclerosis, hypertension, and restenosis after PCI.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Ming-Jing Zhao, Ai-Ming Wu, Jie Wang, and Hong Chang contributed equally to this work.

Acknowledgment

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Research Article

A Multicentre Randomized Clinical Trial on Efficacy and Safety of Huxin Formula in Patients Undergoing Percutaneous Coronary Intervention

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Percutaneous coronary intervention (PCI) is widely used in clinical treatment of coronary artery disease. However, the effects of PCI on preventing restenosis after revascularization and improving the quality of life were not satisfying. Huxin Formula is formulated by modifying an experienced Chinese medicine formula and has been widely used in clinical practice due to its marked effects on coronary heart disease. A multicentre double-blind randomized controlled clinical trial was designed to evaluate the effects and safety of Huxin Formula in patients undergoing PCI. Our results showed that there was no significant difference between the two groups in main outcomes. For patients with ejection fraction (EF) >50%, score of the quality of life scale was higher in treatment group compared with control group. For patients with unstable angina, score of the quality of life scale in 360 days was significantly higher in treatment group compared with control group ($P < 0.05$). No obvious adverse reaction was found in the use of Huxin Formula. In conclusion, Huxin Formula, believed to be a safe treatment for patients after PCI, has benefits in improving the quality of life in patients with unstable angina though it failed to show superiority in primary and secondary outcomes.

1. Introduction

Coronary heart disease causes huge damage in human health and lives. Percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG), which greatly reduce the incidence of cardiovascular events and alleviate the symptoms, are widely used in clinical treatment since the development of modern medicine. However, the effects of both treatments on preventing restenosis after revascularization and improving the quality of life were not satisfying. Recent studies had showed that traditional Chinese medicine (TCM) has advantages in preventing restenosis after PCI and improving the quality of life, but these trials had limited internal validity due to poor design and small sample size.

Huxin Formula is formulated by modifying an experienced Chinese medicine formula which is created by a well-known Chinese doctor, Professor DENG Tie-tao, and has been widely used in clinical practice due to its marked effects on coronary heart disease.

In this trial, we evaluated the efficacy and safety of Huxin Formula in patients after PCI using a multicenter, double-blind, randomized, and placebo-controlled clinical trial.

2. Methods

2.1. Subjects. Participants were screened by investigators at 12 first-rate domestic hospitals in China, including Guangdong Provincial Hospital of Chinese Medicine, Guangdong Provincial People's Hospital, Xinjiang Hospital of Chinese Medicine, Jiangsu Provincial Hospital of Chinese Medicine, The No. 10 Hospital of Shanghai, Shanxi Provincial People's Hospital, Gansu Provincial People's Hospital, The First Affiliated Hospital of Sun Yat-sen University, The First Affiliated Hospital of Guangzhou University of TCM, Huaqiao Hospital

Table 1: Inclusion and exclusion criteria.

Inclusion criteria
(1) Age 40 to 75 without gender limitation
(2) Patients who meet the diagnostic criteria of unstable angina or myocardial infarction
(3) Patients who were diagnosed as "deficiency of heart-QI and phlegm stasis-bizu" according to traditional Chinese medicine (TCM)
(4) One week after PCI
Exclusion criteria
(1) Patients who have acute myocardial infarction or severe heart failure (cardiac function class IV according to the cardiac function standard of New York Heart Association and the left ventricular ejection fraction ≤30% by heart color Doppler)
(2) Patients who have severe liver and kidney dysfunction (serum alanine aminotransferase > three times the normal cap and/or plasma creatinine ≥442 umol/L)
(3) Patients who have cancer or active gastrointestinal bleeding
(4) Patients with mental illness
(5) Patients who were pregnant or breast-feeding
(6) Patients who have medical history of CABG and undergo PCI in vascular bridge
(7) Patients with stable angina

of Jinan University, Jiangmen Wuyi Hospital of TCM, and People's Hospital of Liuzhou. The study was conducted between April 2008 and December 2010. Patients were assessed according to ESC 2006 guidelines for management of patients with stable angina and ACC/AHA 2005 guidelines for management of patients with unstable angina/non-ST-elevation myocardial infarction. The inclusion and exclusion criteria are shown in Table 1. Patients were free to withdraw from the study at any time. Potential participants who were interested in this study received a complete explanation of the protocol and signed the consent form. The ethical approval for the study was granted by the Ethics Committee of Guangzhou University of Traditional Chinese Medicine.

2.2. Design, Randomization, and Allocation. A double-blind randomized controlled clinical trial was conducted by using stratified randomized method. Center is a stratified factor in the present trial. Eligible participants after PCI were randomly assigned into 2 groups: treatment group who received conventional treatment and Huxin Formula or control group who received conventional treatment and the placebo. A sample size calculation based on the incidence rate of cardiovascular events in previous studies determined that 289 patients were needed to reduce the incidence rate of cardiovascular events of 8.5% (power 0.85, significance level 0.05). With an estimated 15% dropout rate, we set the total sample size at 680. Randomized codes were performed with SAS6.12 (statistical software package UNIFORM(n)). Eligible patients at each center were assigned randomization numbers from Institute of Basic Medical and Clinical Sciences, China Academy of Chinese Medical Sciences, which is responsible for the randomization of this research and was external to the trial. Participants and investigators were masked to group assignment. The prepared drugs were dispensed in similar looking bags, and the nature of the drug was concealed by the providers. The random allocation sequence was concealed until the data collection for the entire study was completed.

2.3. Treatment Protocols. Patients in the treatment group were provided Huxin Formula (in the form of granules) prepared by Jiangyin Pharmaceutical Co., Ltd. (Jiangsu, China). Every 10 g Huxin Formula granules consisted of ginseng (10 g), Exocarpium Citri Rubrum (5 g), *Panax pseudoginseng* (8 g), *Pinellia ternata* (10 g), *Salvia miltiorrhiza* (10 g), and *Agastache* (10 g) as crude drug. Patients in the control group were given placebo granules prepared by the same supplier. Placebo granules were designed to taste, smell, and look similar to Huxin Formula. Placebo consisted of amylum, bitter principle, excipient, and so forth. Huxin Formula or placebo was used once a day (10 g QD) after fully dissolving in 300 mL of boiled water cooled to 70°C. Treatment started in one week after PCI and continued for 6 months (180 days). Then patients were followed up for another 6 months (180 days).

2.4. Outcomes. The baseline data, including gender, age, personal history, medical history, family history of cardiovascular disease, classification of cardiac function, and clinical classification of coronary heart disease, were assessed. Main cardiovascular events (death, nonfatal myocardial infarction, and repeat revascularization) were also observed and recorded. We assessed quality of life using two scales: (1) Seattle Angina Questionnaire (SAQ) and (2) scale of the life quality in integrative medicine for CAD (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/143064). The outcomes were assessed at 0, 90, 180, 270, and 360 days after treatment.

2.5. Safety Monitoring. To assess the safety of the 6-month treatment, routine blood and liver and renal function tests were conducted before randomization, 90 days and 180 days after treatment. During the trial, adverse events were observed in detail and documented using case report forms.

2.6. Statistical Analysis. Statistical analysis sets included intention-to-treat (ITT) sets, per-protocol (PP) sets, and safety analysis sets. Descriptive analysis and inferential analysis of clinical features before and after treatment were as follows.

(1) Measurement data: functional indexes are presented as mean ± standard deviation. Each group compared before and after treatment with paired t-test and Student's t-test was used to compare small sample data (including 95% confidence interval). Rank-sum test (Wilcoxon test) is used for nonnormal distribution or heterogeneity of variance. Analysis of covariance or stratified analysis is used when baseline is not neat.

(2) Enumeration data: the constituent ratio and rate of each index were calculated. Two-group comparisons were performed using the fourfold table χ^2 test (or accurate probability method) for the total effective rate and the $2 \times C$ table χ^2 test for the constituent ratio.

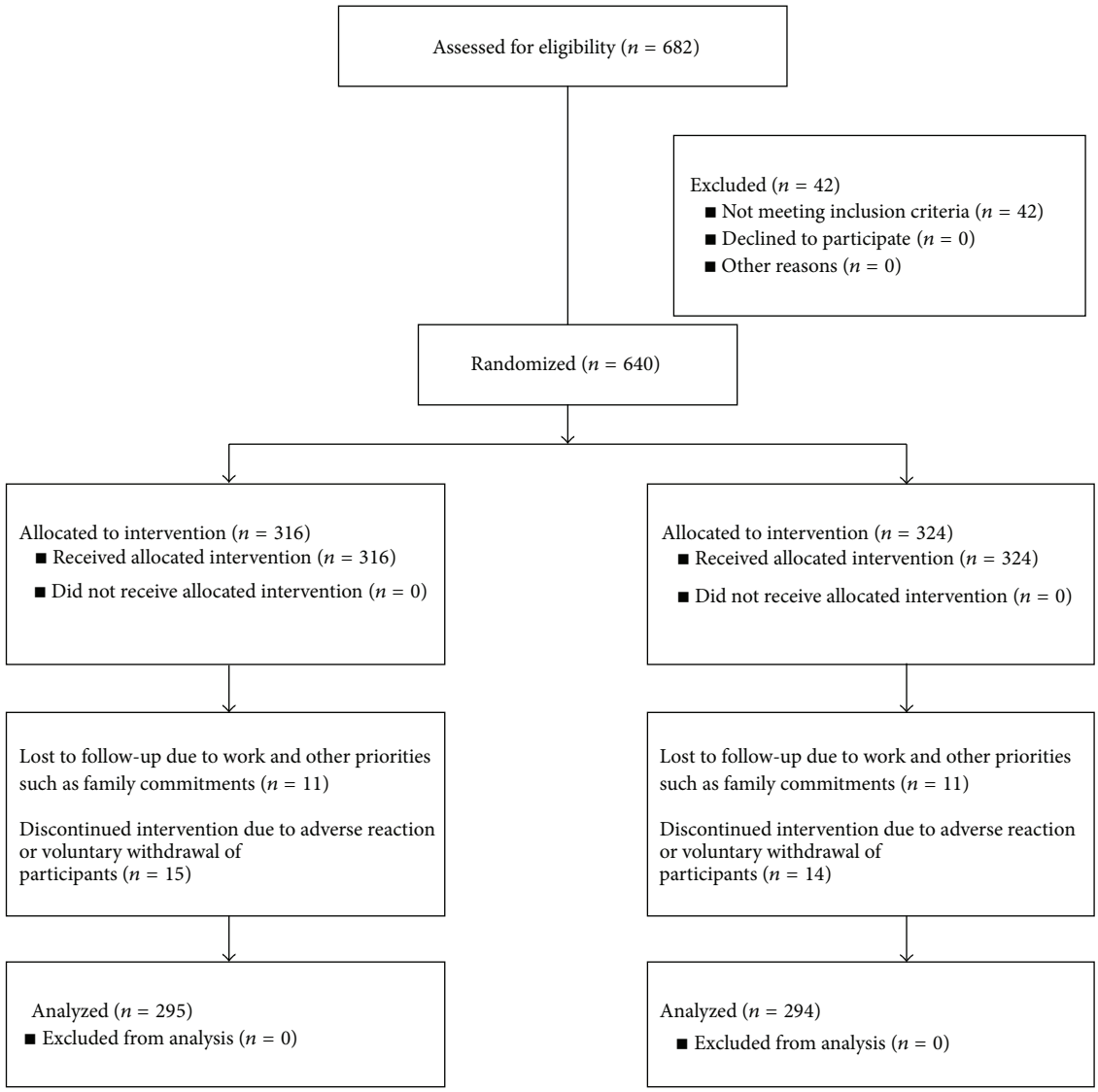

Figure 1: Consort 2010 flow diagram.

(3) Ranked data: two-group comparisons were performed using Mann-Whitney test.

(4) Life quality analysis: the general descriptive analysis is presented as mean ± standard deviation, maximum, minimum, and median.

(5) The analysis of the factors affecting curative effect: the logistic regression analysis was used to analyze the factors that may affect the judgment of curative effect, including age, duration, classification of coronary heart disease, survival quality assessment scale of integrated traditional Chinese and western medicine, PCI operator, and center.

(6) The Kaplan-Meier method was used to compare the cardiovascular event rates and median time. Influence factors of cardiovascular events were analyzed by using the Cox regression analysis.

(7) Baseline comparison test level $\alpha = 0.10$, and two-group effects comparison test level $\alpha = 0.05$.

Safety analysis of each case was mainly performed as descriptive statistics, including incidence and description of adverse events, the changes of laboratory testing results before and after test, and the relationship between abnormal changes and investigational drugs.

3. Results

3.1. Participants Flow. There were 682 patients with PCI treatment included in this research. 42 patients were eliminated, and 51 patients were withdrawn after 360 days. The flow of participants in the study is summarized in Figure 1.

Table 2: Baseline clinical data (n, %).

	Control group ($N = 316$)	Treatment group ($N = 324$)	P
Age ($\overline{x} \pm s$)	59.67 ± 9.33	60.91 ± 8.97	0.09
Gender			
Male	249 (78.8)	250 (77.2)	0.62
Female	67 (21.2)	74 (22.8)	
Height ($\overline{x} \pm s$)	166.34 + 7.14	166.04 + 7.37	0.60
Weight ($\overline{x} \pm s$)	66.56 ± 8.89	64.82 ± 9.53	0.02
Pulse ($\overline{x} \pm s$)	74.6 ± 11.93	75.56 ± 11.92	0.31
Breath ($\overline{x} \pm s$)	19.52 ± 2.03	19.41 ± 1.69	0.46
Heart rate ($\overline{x} \pm s$)	74.71 ± 12.01	75.75 ± 12.29	0.28
SBP ($\overline{x} \pm s$)	129.17 ± 19.29	130.30 ± 22.00	0.49
DBP ($\overline{x} \pm s$)	77.21 ± 11.44	77.89 ± 12.58	0.47
Combined disease			
Dyslipidemia	96 (30.4)	91 (28.1)	0.52
Hypertension	182 (57.6)	179 (55.2)	0.55
Diabetes	72 (22.8)	60 (18.5)	0.18
Stroke	16 (5.1)	13 (4.0)	0.52
Heart failure	10 (3.2)	12 (3.7)	0.71
Gastrointestinal disorder	31 (9.8)	26 (8.0)	0.43
Arrhythmia	18 (5.7)	18 (5.6)	0.94
Medical history			
Smoking	159 (50.3)	165 (50.9)	0.88
Drinking	50 (15.8)	52 (16.0)	0.94
Family history of CVD	41 (13.0)	49 (15.1)	0.43
Allergy	31 (9.8)	30 (9.3)	0.81
Clinical classification of CHD			
Unstable angina	168 (53.2)	193 (59.6)	0.10
Ml	148 (46.8)	131 (40.4)	
Cardiac functions			
I	117 (37.0)	122 (37.7)	
II	157 (49.7)	166 (51.2)	0.70
III	42 (13.3)	36 (11.1)	

CHD: coronary heart disease, CVD: cardiovascular disease, DBP: diastolic blood pressure, MI: myocardial infarction, and SBP: systolic blood pressure.

3.2. Baseline Data. The general characteristics of the patients are shown in Table 2. The comparisons between control group and treatment group were performed using the chi-square test for baseline classification data before treatment, including the demographic data (gender, ethnic, and cultural), the general situation (height, pulse, respiration, heart rate, systolic blood pressure, and diastolic blood pressure), personal history (history of smoking, drinking, and allergy and family history of cardiovascular disease), medical history (dyslipidemia, high blood pressure, diabetes, stroke, heart failure, and arrhythmia), classification of cardiac function, and clinical classification of coronary heart disease (unstable angina and myocardial infarction). There were no significant deviations ($P > 0.05$) between the two groups except the weight (66.56 versus 64.82).

3.3. Major Endpoints. There were no significant differences ($P > 0.05$) between control group and treatment group in major end points at 360-day follow-up (Table 3), including mortality, number of patients with nonfatal myocardial infarction, proportion of further revascularization, and rehospitalization caused by cardiovascular events.

3.4. Secondary Outcomes. There were no significant differences ($P > 0.05$) between control group and treatment group on secondary outcomes at all time points after PCI, including angina scale (frequency of attack, duration time, intensity of pain, and dose of nitroglycerin), SAQ (dimension of body activities limitation, angina pectoris stable state, heart attacks, disease knowledge, and treatment satisfaction), and the quality of life scale of integrative medicine.

3.5. Stratified Analysis

3.5.1. Stratified Analysis of EF before Treatment. As shown in Table 4, according to PCI per-protocol population and

Table 3: Major end points at 360 days.

Events		Control group (n = 295)	Treatment group (n = 294)	χ^2	P value
Death	Y	2 (0.7)	0 (0.0)	2.00	0.16
	N	293 (99.3)	294 (100.0)		
Nonfatal MI	Y	0 (0.0)	0 (0.0)	—	—
	N	295 (100.0)	294 (100.0)		
Repeat revascularization	Y	2 (0.7)	3 (1.0)	0.21	0.65
	N	293 (99.3)	291 (99.0)		
Readmission	Y	5 (1.7)	9 (3.1)	1.19	0.28
	N	290 (98.3)	285 (96.9)		

Values are n (%). MI: myocardial infarction.

Table 4: Score of the quality of life scale for patients with EF > 50% ($\overline{x} \pm s$).

Time	Group	n	$\overline{x} \pm s$	Z	P
0 day	Control group	244	71.80 ± 12.62	−0.24	0.81
	Treatment group	251	72.03 ± 12.18		
90 days	Control group	244	81.32 ± 9.592	−0.42	0.68
	Treatment group	251	81.79 ± 9.220		
180 days	Control group	244	82.84 ± 9.24	−0.67	0.51
	Treatment group	251	83.39 ± 8.58		
270 days	Control group	244	84.72 ± 8.77	−0.66	0.51
	Treatment group	251	85.26 ± 8.431		
360 days	Control group	244	86.29 ± 8.55	−1.04	0.30
	Treatment group	251	87.11 ± 7.70		

stratified analysis of EF before treatment, for patients with EF > 50%, score of the quality of life scale was higher but not statistically significant ($P > 0.05$) in treatment group compared with control group at all time points.

3.5.2. Stratified Analysis of the Type of Coronary Heart Disease before Treatment. As shown in Table 5, according to PCI per-protocol population and stratified analysis of the type of coronary heart disease before treatment, for patients with unstable angina, score of the quality of life scale in 360 days was significantly higher in treatment group compared with control group ($P < 0.05$). There were no significant deviations ($P > 0.05$) between the two groups at another four time points.

3.6. Safety Analysis. Incidence of adverse event and specific event in treatment group was similar to control group. No obvious adverse reaction was found in the use of Huxin Formula.

4. Discussion

Coronary artery disease (CAD) is a major cause of mortality and morbidity in developed countries [1, 2]. Before developing the technique of PCI, coronary artery bypass graft (CABG) had been the standard and the only revascularization procedure. With the development of medical imaging and heart catheterization techniques, the PCI, as an effective, safe, less disabling, and less expensive revascularization procedure, had become a more frequently used treatment than CABG for CAD in most western countries as well as in China. Growing evidence had showed that PCI can improve myocardial ischemia and reduce the risks of long-term adverse cardiovascular events [3, 4]. The results of a meta-analysis of 13 randomized controlled trials showed that PCI with drug-eluting stents reduces the risks of major adverse cardiac events, recurrent myocardial infarction, and reintervention [5].

In recent years, other than biochemical endpoints, quality of life (QoL) is considered to be an important indicator of health outcome in CAD [6] and the most common indication for PCI [7]. Seattle Angina Questionnaire (SAQ) was the most commonly used instrument to capture comprehensive and sensitive changes in QoL of the cardiac patients [8–10]. Besides SAQ, scale of the life quality in integrative medicine for CAD, which was established for the first time, was used in the present research. The internal reliability of this scale was supported by the values of Cronbach's α that exceeded 0.7 for all the subscales. Scale of the life quality in integrative medicine for CAD was used to collect the mental and social functioning information that SAQ might have missed.

Although the role of PCI in chronic stable angina is well established as it alleviates ischemic symptoms and improves quality of life [11, 12], its benefit in general health status of patients with unstable angina and non-ST-elevation

Table 5: Score of the quality of life scale for patients with unstable angina ($\overline{x} \pm s$).

Time	Group	n	$\overline{x} \pm s$	Z	P
0 day	Control group	168	69.61 ± 11.27	−0.32	0.75
	Treatment group	193	69.97 ± 10.65		
90 days	Control group	168	80.75 ± 8.94	−0.53	0.59
	Treatment group	193	81.09 ± 8.86		
180 days	Control group	168	82.44 ± 9.03	−1.06	0.29
	Treatment group	193	83.33 ± 8.11		
270 days	Control group	168	84.78 ± 8.18	−0.72	0.47
	Treatment group	193	85.23 ± 8.25		
360 days	Control group	168	85.70 ± 8.08	−2.51	0.01[#]
	Treatment group	193	87.62 ± 7.52		

[#] Significantly different versus control group ($P < 0.05$).

myocardial infarction (UA/NSTEMI) would not be sufficient. It is reported that poor QoL is highly prevalent in elderly patients undergoing PCI [13]. At 30 days after PCI, general health-related quality of life (HRQoL) was significantly lower (0.86 ± 0.21 versus 0.89 ± 0.17, $P = 0.001$) after adjusting baseline characteristics ($P < 0.001$) [14]. Therefore, in addition to angina-specific therapy, comprehensive supportive care would be needed to improve the quality of life, which might improve long-term clinical outcome especially in patients after PCI.

According to the 2007 ACCF/AHA guidelines for the management of patients with unstable angina/non-ST-elevation myocardial infarction [15], clopidogrel is recommend to be used for at least one year. Lipid-lowering therapy with the use of statin has benefits in blocking the progression of coronary artery plaque and further preventing cardiovascular events and death [16]. However, effects of these treatments on relieving the symptoms and improving quality of life are not satisfying.

It is reported that some kinds of herbal medicine, such as Xuefu Zhuyu Capsule [17], exhibit better efficacy on HRQoL in patients with unstable angina after PCI. However, the application of TCM in the post-PCI patients still lacks support from rigorously designed clinical trial. Huxin Formula is formulated by modifying an experienced Chinese medicine formula which is created by a well-known Chinese doctor, Professor DENG Tie-tao, and has been widely used in clinical practice because of its marked effects on coronary heart disease [18, 19]. Our earlier clinical study in 55 patients after CABG had showed that modified Huxin Formula can promote rehabilitation of the CABG patients, improving clinical symptoms and quality of life [20]. However, the effects of the formula in patients undergoing PCI were unknown. Therefore, the present study was designed to evaluate the efficacy and safety of Huxin Formula in patients after PCI.

Our results showed that, for patients with unstable angina, score of the quality of life scale in 360 days was significantly higher in treatment group (87.62 ± 7.52) compared with control group (85.70 ± 8.08) ($P < 0.05$). This result suggested that Huxin Formula may improve the quality of life of patients after PCI compared to the placebo. There are 22 patients with the event of MACE (2 deaths, 6 repeat revascularizations, and 14 readmissions caused by cardiovascular events) in this research. The rate of one-year MACE event is only 3.23%, slightly lower than 5–8% that has been suggested in foreign related reports. There was no statistical difference between the two groups in other outcomes. Limited sample size and the relatively short observational period may be the two reasons for the negative results. Therefore, further studies with larger sample sizes and longer observational period are warranted on the base of these precious experiences.

5. Conclusion

In this research, the results showed that Huxin Formula is a safe treatment for patients after PCI. Though Huxin Formula failed to show superiority in primary and secondary outcomes, it has benefits in improving the quality of life in patients with unstable angina.

Abbreviations

ACS: Acute coronary syndrome
BMS: Bare metal stents
CABG: Coronary artery bypass grafting
CAD: Coronary artery disease
CHD: Coronary heart disease
DES: Drug-eluting stents
EF: Ejection fraction
ITT: Intention-to-treat
MACE: Major adverse cardiovascular events
NSTEMI: Non-ST-elevation myocardial infarction
PCI: Percutaneous coronary intervention
PES: Paclitaxel-eluting stents
PP: Per-protocol
REVERSAL: Reversal of atherosclerosis with aggressive lipid lowering
SAQ: Seattle Angina Questionnaire
SES: Sirolimus-eluting stents
TCM: Traditional Chinese medicine
UA: Unstable angina.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Traditional Formula, Modern Application: Chinese Medicine Formula Sini Tang Improves Early Ventricular Remodeling and Cardiac Function after Myocardial Infarction in Rats

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Sini Tang (SNT) is a traditional Chinese herbal formula consisting of four different herbs: the root of *Aconitum carmichaelii*, the bark of *Cinnamomum cassia*, the rhizome of *Zingiber officinale*, and the root of *Glycyrrhiza uralensis*. This study aims to evaluate the improvement of early ventricular remodeling and cardiac function in myocardial infarction (MI) rats by SNT. A MI model was established by ligation of the left anterior descending coronary artery. Following treatment for 4 weeks, ultrasonic echocardiography was performed. Myocardial histopathological changes were observed using haematoxylin and eosin staining. Collagens (type I and type III), transforming growth factor-β1 (TGF-β1), and Toll-like receptors (TLR-2 and TLR-4) were measured in plasma, serum, and myocardial tissue. SNT treatment decreased the infarct size, the left ventricular cavity area/heart cavity area ratio, and the left ventricle dimension at end systole and increased the left ventricular ejection fraction. SNT reduced the levels of TLR-2 and TLR-4 in myocardial tissue significantly and decreased the collagens content in serum and in myocardial tissue. SNT could partially reduce the level of TGF-β1 in serum and in myocardial tissue. Our data suggest that the Chinese medicine formula SNT has the potential to improve early ventricular remodeling and cardiac function after MI.

1. Introduction

Myocardial infarction (MI) can be a killing disease. It is one of the major causes of heart failure (HF). Every sixth man and every seventh woman in Europe died from MI [1]. Recovery from MI is characterized by stages of initial inflammation, angiogenesis, fibroblast proliferation, and collagen deposition, followed by scar formation in the maturation [2] and remodeling phase leading to infarct area expansion and dilatation of the heart by left ventricle (LV) remodeling, and ultimately develops into chronic HF [3–5]. The current medical treatment of MI includes antithrombotic therapy, beta-blockers, lipid-lowering therapy, nitrates, calcium antagonists, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers [1]. Still, there is an urgent need for novel additional therapeutic compounds supporting either conservative pharmacological treatment or replacing it by newly identified pharmaceuticals.

Traditional Chinese medicine (TCM) has been used in China for centuries for treatment of cardiac disease and is now attracting interest in Western countries as a source of alternative or complementary therapies due to its reputed effectiveness, low cost, and relative absence of side effects. Previous studies provided scientific evidence to support the

use of Chinese herbal medicine (CHM) for treating MI and HF [6–11].

We used a popular traditional Chinese herbal formula, "Sini Tang" (SNT), first described by Zhang (150–219 A.D.) in his book "Treatise on cold-induced diseases," a medical collection from ancient China for this study [12]. In terms of TCM, SNT was described as a remedy acting on the heart and has the essential effect of recuperating the patients from collapse. It was used to treat the syndrome of displaying cold extremities, cold sweating, vomiting, and lethargy, which corresponds with clinical symptoms of MI and HF in Western medicine [13–16]. SNT used for this study consists of four Chinese medicinal herbs: the processed root of *Aconitum carmichaelii* Debeaux (aconite), the bark of *Cinnamomum cassia* (L.) J. Presl. (cinnamon), the rhizome of *Zingiber officinale* Roscoe (ginger), and the processed root of *Glycyrrhiza uralensis* Fisch. ex DC. (licorice) [17]. The SNT decoction has been used to improve blood circulation, remove blood stasis, and treat myocardial damage in Chinese clinics [2, 8, 13]. However, there is still a lack of further clinical evidence and definitive mechanisms of action to demonstrate the role of SNT in cardiovascular diseases. The purpose of this study was to evaluate the improvement of early ventricular remodeling after MI by SNT using a well-established experimental rat model of MI [18–20]. We analyzed cardiac tissue structure and function and associated pathophysiological indicators together with a number of potential biomarkers, that is, collagens (type I and type III), transforming growth factor-β1 (TGF-β), and Toll-like receptors (TLR-2 and TLR-4), which could play an important role in cardiac remodeling and wound repair after MI [21–25].

2. Materials and Methods

2.1. Ethics Statement. All animal experiments were approved by the Administrative Committee of Experimental Animal Care and Use of Xiyuan Hospital, China Academy of Chinese Medical Sciences (CACMS, permit number: CACMS/20100322), and conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals [26] and the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines [27].

2.2. Drugs and Reagents. Fosinopril sodium (FS) (10 mg/tablet) was supplied by Sino-American Shanghai Squibb Pharmaceutical Co., Ltd., TGF-β1, collagen type I (col-I) and collagen type III (col-III), and TLR-2 and TLR-4 kits were provided by R&D systems, USA. Coomassie blue protein assay kit was offered by Nanjing Science and Technology Co., Ltd. The Two-Step Immunohistochemical Detection Kit was produced by Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.

2.3. SNT Preparation. SNT (0.5 g/g extract/crude drug, batch number 20100820) containing *Glycyrrhiza uralensis*, *Aconitum carmichaelii*, *Zingiber officinale,* and *Cinnamomum cassia* (8 : 6 : 3 : 1) was prepared at the Pharmacy Department of Xiyuan Hospital, China Academy of Traditional Chinese Medicine (Beijing, China). According to WHO General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine, a sufficient number of dose levels should be used in rodents to determine the approximate lethal dose [28]. Therefore, we designed our study using two doses of SNT for MI rats, a low dose of 4.5 g/kg and a high dose of 13.5 g/kg. Herbs were soaked in drinking water (500 mL) for one hour in a clay pot at room temperature and then cooked to boiling. The decoction was performed twice by cooking gently for 30 min. Two extracts were combined, filtered, and stored at room temperature before administration.

Table 1: The five experimental groups. Drugs diluted with distilled water were administered orally once a day for 30 days starting two days after induction of AMI.

Group	*N*	Operation	Oral administration
Sham	10	Sham	Drinking water
Model	10	AMI	Drinking water
FS	10	AMI	Fosinopril sodium (0.9 mg/kg)
SNT-LD	10	AMI	Low dose of SNT (4.5 g/kg)
SNT-HD	10	AMI	High dose of SNT (13.5 g/kg)

2.4. Animals. Ninety (50% male and 50% female) Sprague-Dawley (SD) rats weighing 190 ± 10 g were provided by the Vital Laboratory Animal Technology Company, Beijing, China. Animals were acclimatized with a 12/12 hours light/dark cycle at a controlled room temperature of 23–25°C and a humidity of 50–70% and allowed free access to foods and water for seven days before use.

2.5. Animal Model of MI. Acute myocardial infarction (AMI) was induced in rats by left anterior descending artery (LAD) ligation. The surgical procedures were performed using the well-established technique [19, 29]. The rats were anaesthetized by intraperitoneal (i.p.) injection of a 1% solution of sodium pentobarbital (50 mg/kg) and placed in a supine position on a table. The preoperative recording was performed by a two-lead electrocardiogram (ECG, General Meditech Inc., Shenzhen, China). After disinfection of local skin, the chest was opened to expose the heart. The LV, aorta, and left atrium were made visible for suture placement. A 3-0 suture was placed in the anterior myocardium to occlude the left anterior descending artery (LAD). The heart was returned to its original position. The rats in the control group were sham operated with thoracotomy and cardiac exposure but without coronary artery ligation. The sternum and skin incision was closed with 7-0 sutures. Additional two-lead ECG recordings were made postoperatively. Successful ligation was confirmed by ST segment elevation in postoperative ECG, compared with preoperative ones. To prevent infection rats were given penicillin (40.000 units) by i.p. injection after operation for 3 days. Sixty surviving infarct rats were randomly divided into five groups (Table 1). The oral administration of the drugs began two days after the AMI-induction. SNT (4.5 and 13.5 g/kg) and FS (0.9 mg/kg), an angiotensin converting

enzyme (ACE) inhibitor used as a positive control [30, 31], were diluted with distilled drinking water and administered orally in a volume of 10 mL/kg body weight once every morning for 4 weeks.

2.6. Echocardiography Assessment. Transthoracic echocardiographic studies were performed 30 days after surgery according to the method of Yin et al. [19]. Ten rats from each group were anesthetized by i.p. injection of ethyl carbamate (6 mL/kg). Rats extremities were fixed to four electrocardiography leads on the table. After cleaning the rat chest the cardiac short axis (papillary level), left ventricle end-diastolic dimension (LVDd), and left ventricle end-systolic dimension (LVDs) were measured using an ATL HDI-5000 Diagnostic Ultrasound System (Philips Ultrasound Inc., China). The values were calculated on an average of three cycles. The ejection fraction (EF) was calculated from the left ventricle end-diastolic volume (LVEDV) and the left ventricle end-systolic volume (LVESV) as EF% = [(LVEDV − LVESV)/LVEDV] × 100. The echocardiographic analysis and the data calculation were performed using a single-blind method [32, 33].

2.7. Histopathology: Blood Sampling and Tissue Collection. After echocardiographic measurements, the anesthetized rats were sacrificed. Blood samples were taken with a heparinized syringe from the LV cavity and centrifuged at 3000 rpm for 15 min. Plasma and sera were conserved at −80°C for further analysis. The hearts were removed from the chest, excised, and weighed for determination of the heart weight/body weight (HW/BW) ratio. Six hearts randomly assigned of each group were stored in 10% neutral formalin solution for hematoxylin eosin (H&E) staining and immunohistochemistry measurements. One heart of each group was stored at −80°C for ELISA.

2.8. Measurement of Myocardial Infarct Size (IS). The pathological slice was placed under natural light and photographed in microdistance using a Canon IXUS 90IS digital camera. The microscopic color image processing system (DpxView Pro, Korea) was used to calculate the left ventricular IS (%, myocardial infarction area/left ventricular area × 100) by an investigator who was blinded to the identity of the pathological slice as described by Takagawa et al. [34].

2.9. Detection of Collagens and TGF-β1. Five of the frozen heart tissue samples of each group were randomly picked. The heart sections were weighed and 300 mg of each sample were cut into small pieces and suspended in 1 mL of saline solution. After homogenization on ice, samples were centrifuged and the supernatants were stored at −80°C for analysis. Col-I and col-III and TGF-β1 were measured by ELISA in serum and heart tissue samples according to the manufacturer's instructions [35, 36]. The total protein content of each tissue sample was measured with the BCA (bicinchoninic acid) Protein Assay Kit.

2.10. Immunohistochemistry of TLRs in Myocardial Tissue. Immunohistochemical staining for TLRs in myocardial tissue was performed using Power Vision Two-Step Histostaining Reagent (Golden Bridge International Inc., Beijing, China) as described by the manufacturer. Myocardial sections (5 μm thick) were fixed in 10% neutral buffered formalin for 18 hours and then deparaffinized and rehydrated. The tissue sections were incubated in 3% H_2O_2 for 10 min and in endogenous peroxidase for 10 min, respectively. After washing twice in PBS buffer, the tissue sections were incubated in 10% normal goat serum with anti-TLR-2 (1 : 200) and anti-TLR-4 (1 : 800) antibodies at 37°C for 1 hour, followed by incubation with a biotinylated secondary goat anti-mouse IgG for 30 min at room temperature. The detection was performed using the DAB Liquid System (Golden Bridge International Inc., Beijing, China). Expression of Toll-like receptors TLR-2 and TLR-4 in the myocardial tissue was determined as integrated optical density (IOD) values using an image analysis system (Imagepro-Plus 6.0, Media Cybernetics, USA).

2.11. Statistical Analysis. All results were tested on normal distribution by aid of One-Sample Kolmogorov-Smirnov Test. Data were tabulated and presented as the mean ± standard deviation, and the significance of changes was assessed with one-way repeated measures analysis of variance (ANOVA). Bonferroni Holm test was followed for multiple comparisons. One-way ANOVA Tukey HSD test was used for pairwise multiple comparisons. A value of $P < 0.05$ was considered statistically significant. Data were analyzed using the Statistical Package for the Social Sciences (version 17, SPSS Software, SPSS Inc., Chicago, USA).

3. Results

3.1. SNT Treatment Reduced Infarct Size and Left Ventricular Cavity Area/Heart Cavity Area. The infarct size (IS) obtained using the midline length measurement is shown in Table 2. The IS values from the FS, SNT-LD, and SNT-HD groups ($23.91 \pm 7.99\ mm^2$, $P < 0.01$, $31.25 \pm 10.68\ mm^2$ and $27.81 \pm 10.33\ mm^2$, $P < 0.05$) were significantly smaller than from the model group ($38.04 \pm 8.35\ mm^2$). As shown in Figures 1(a)–1(e) and Table 2, pathological H&E staining showed that the left ventricular cavity area of the model group after AMI was significantly increased by approximately 22.3% compared to the sham group ($P < 0.01$). The ventricular cavity area/heart cavity area (LVAC/HCA) ratios of the FS, SNT-HD, and SNT-LD groups (15.2%, $P < 0.01$; 18.5% and 17.3%, $P < 0.05$, resp.) were decreased significantly compared to the model group. The heart weight/body weight (HW/BW) ratio in the model group was significantly increased compared with the sham group (0.37 ± 0.07 mg/g versus 0.31 ± 0.01 mg/g, $P < 0.01$). The HW/BW ratios in the FS group (0.36 ± 0.08 mg/g) and the SNT groups (0.35 ± 0.08 mg/g and 0.36 ± 0.07 mg/g) were decreased compared with the model group, but not significantly.

3.2. SNT Treatment Decreased LVDd and LVDs and Improved the Cardiac Function by Increasing the EF. Four weeks after MI, ultrasound echocardiography showed a significant increase of the left ventricular dimension at end diastole

Table 2: Echocardiographic parameters—overview of ventricular remodeling effects.

	Sham	Model	FS	SNT-LD	SNT-HD	Remodeling effects (versus model)
LVDd (mm)	3.29 ± 0.81	6.24 ± 0.72	4.45 ± 1.28**	5.37 ± 1.78	4.87 ± 1.47	↓
LVDs (mm)	0.91 ± 0.20	6.44 ± 1.59**	3.81 ± 1.21$^{**\blacktriangle\blacktriangle}$	4.95 ± 1.95**	3.74 ± 1.47$^{**\blacktriangle\blacktriangle}$	↓
EF (%)	93.32 ± 2.94	55.48 ± 12.89**	78.03 ± 10.70$^{*\blacktriangle\blacktriangle}$	69.69 ± 13.91$^{*\blacktriangle}$	77.83 ± 12.32$^{**\blacktriangle\blacktriangle}$	↑
IS (mm^2)		38.04 ± 8.35	23.91 ± 7.99$^{\blacktriangle\blacktriangle}$	31.25 ± 10.68	27.81 ± 4.91$^{\blacktriangle}$	↓
HW/BW (mg/g)	0.31 ± 0.01	0.37 ± 0.07	0.36 ± 0.08	0.35 ± 0.08	0.36 ± 0.07	↓
LVAC/HCA (%)	10.82 ± 3.01	22.30 ± 3.92**	15.17 ± 6.54$^{\blacktriangle\blacktriangle}$	18.48 ± 3.96*	15.25 ± 2.78$^{\blacktriangle}$	↓

$^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus model group.

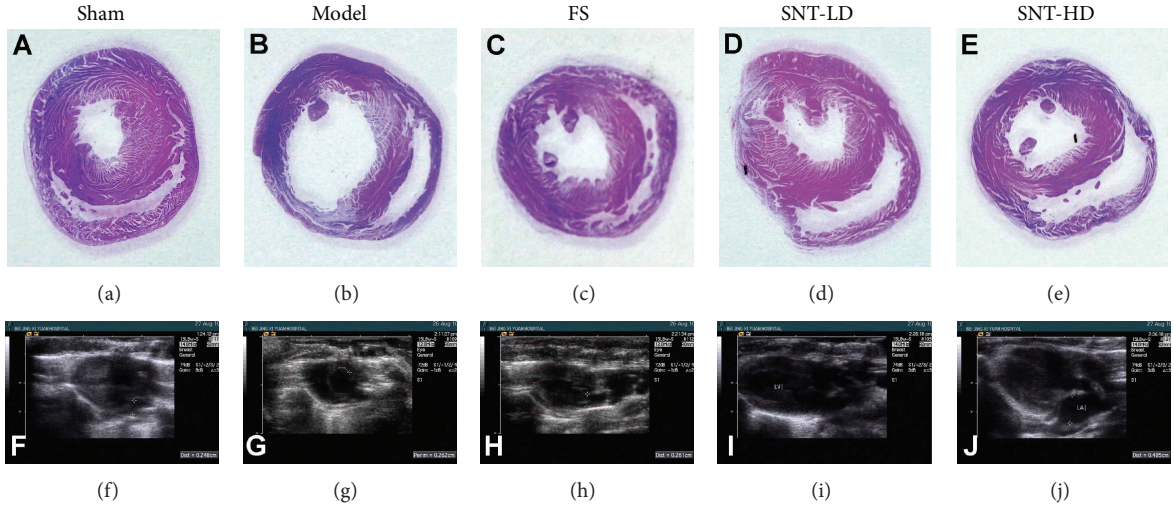

Figure 1: Histological and echocardiographic images. (a–e) Haematoxylin and eosin staining of midventricular cross-sections. (f–j) Two-dimensional-guided M-mode echocardiographic images of the left ventricle.

(LVDd) and the left ventricular dimension at end systole (LVDs) in the model group (6.24 ± 0.72 mm versus 3.29 ± 0.81 and 6.44 ± 1.59 mm versus 0.91 ± 0.20, $P < 0.05$) compared to the sham group. The FS and SNT treatment groups exhibited significantly decreased LVDs versus the sham group (FS: 3.81 ± 1.21 mm, SNT-LD: 4.95 ± 1.95 mm, and SNT-HD: 3.74 ± 1.47 mm, $P < 0.01$). The FS treatment group exhibited significantly decreased LVDd (4.45 ± 1.28 mm versus 3.29 ± 0.81 mm, $P < 0.01$). The SNT treatment groups also showed decreased LVDd versus the sham group (SNT-LD: 5.37 ± 1.78 mm and SNT-HD: 4.87 ± 1.47 mm, $P > 0.05$), but not significantly. The left ventricular ejection fraction (EF) was significantly lower in the model group compared to the sham group (55.48 ± 12.89% versus 93.32 ± 2.94%, $P < 0.01$). The FS and SNT treatment groups showed an improved left ventricular function of the EF compared to the sham (FS: 78.03 ± 10.70%, SNT-LD: 69.69 ± 13.9%, $P < 0.05$, and SNT-HD: 77.83 ± 12.32%, $P < 0.01$) and model groups (FS and SNT-HD: $P < 0.01$, SNT-LD: $P < 0.05$). SNT treatment improved the cardiac function by increasing the EF by 23.63% (difference between sham group: 93.32% and SNT-LD: 69.69%), (Table 2 and Figures 1(f)–1(j) and 2(c)–2(e)).

3.3. SNT Treatment Decreased the Expression Levels of TLR-2 and TLR-4 in Myocardial Tissues. Four weeks after MI immunohistochemical staining for TLRs in myocardial tissue was performed using the Power Vision Two-Step Histostaining method. In tissues of the sham group, only a small amount of brown granules was visible indicating basal expression of TLR-2 and TLR-4 proteins. In contrast, expression of TLRs is much stronger as shown by darker and larger granules in the tissue of the model group (indicated with arrowheads in Figures 3(b) and 3(g)). The FS and both SNT treatment groups showed reduced expression of TLR-2 and TLR-4 compared to the model group (arrows in Figures 3(c) to 3(e) and 3(h) to 3(j)).

TLR-2 and TLR-4 expressions in the myocardial tissue were determined using an Imagepro-Plus Media Cybernetics system shown in Table 3 and Figure 4(d) as integrated optical density (IOD) values. In comparison to the sham group, TLR-2 and TLR-4 expressions were increased significantly in the model group ($P < 0.01$) as well as in the FS and SNT treatment groups (model: 4254.60 ± 413.66, FS: 3381.00 ± 432.17, SNT-LD: 3646.60 ± 362.37, and SNT-HD: 3316.60 ± 439.06 versus 896.40 ± 89.18 for TLR-2 expression and model:

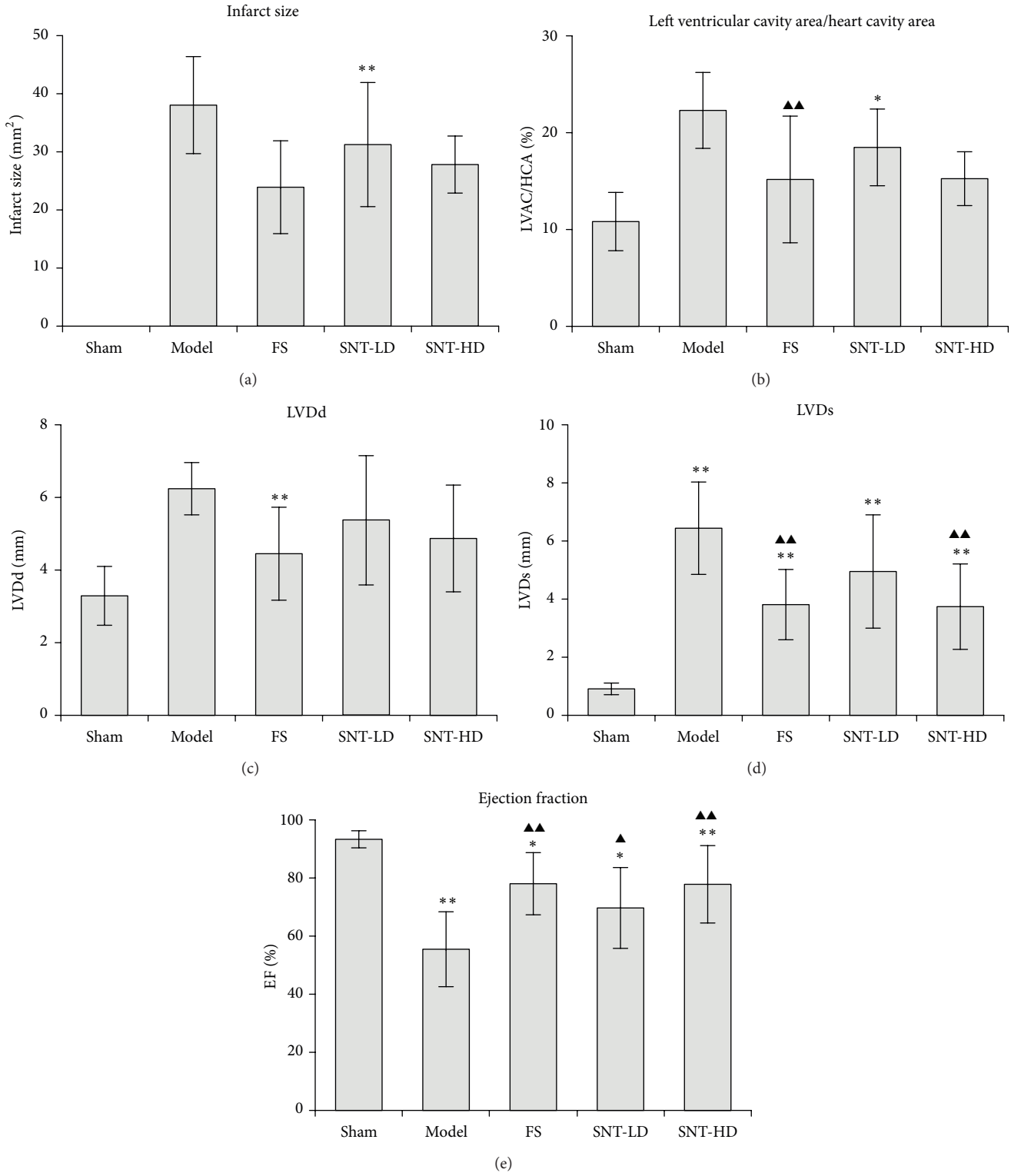

Figure 2: Echocardiographic parameters. (a) Infarct size results, (b) echocardiographic measurements of left ventricular dimension at end diastole (LVDd), (c) left ventricular dimension at end systole (LVDs), (d) left ventricular ejection fraction (EF), and (e) left ventricular cavity area/heart cavity area ratios (LVAC/HCA).

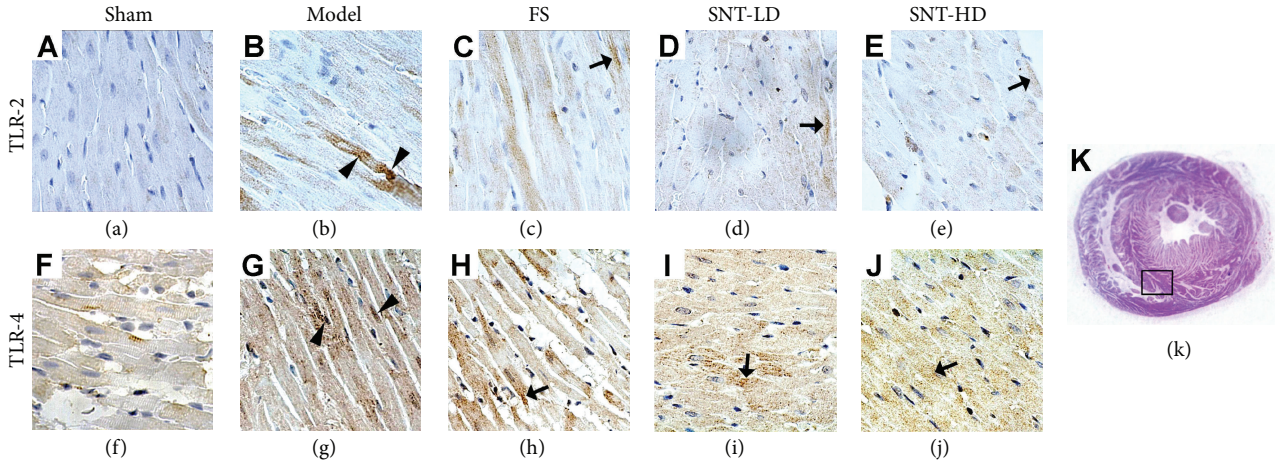

Figure 3: Immunohistochemical analysis of Toll-like receptors in myocardial tissue. TLR-2 (a–e) and TLR-4 (f–j) expression in early VR rats was observed in each experiment group after AMI using a 400x optical microscopy. Expression of TLRs is much stronger in the tissue of the model group in comparison to the sham group as shown by darker and lager granules (arrowheads in (b) and (g)). The FS and both SNT treatment groups showed reduced expression of TLR-2 and TLR-4 compared to the model group (arrows in (c), (d), (e), (h), (i), and (j)).

Table 3: Overview of TLR expression levels in myocardial tissue.

	Sham	Model	FS	SNT-LD	SNT-HD	Expression levels (versus model)
In myocardial tissue						
TLR-2 (IOD)	896.40 ± 89.18	4254.60 ± 413.66**	3381.00 ± 432.17**▲▲	3646.60 ± 362.37**	3316.60 ± 439.06**▲▲	↓
TLR-4 (IOD)	973.00 ± 95.13	5184.40 ± 566.30**	3470.60 ± 403.33**▲▲	4378.60 ± 748.49**	3732.20 ± 528.23**▲▲	↓

IOD: integral optical density. **$P < 0.01$ versus sham group; ▲▲$P < 0.01$ versus model group.

5184.40 ± 566.30, FS: 3470.60 ± 403.33, SNT-LD: 4378.60 ± 748.49, and SNT-HD: 3732.20 ± 528.23 versus 973.00 ± 95.13 for TLR-4 expression). In comparison to the model group, TLR-2 and TLR-4 expression were decreased in all other experimental groups and significantly lower in the FS and SNT-HD treatment groups ($P < 0.01$). The SNT-HD group showed higher expression levels for both TLR-2 and TLR-4 than the SNT-LD group in our experiments.

3.4. SNT Treatment Reduced the Levels of Collagens and TGF-β1. Collagen type I and type III and TGF-β1 were measured by ELISA using kits from R&D Systems, USA. In serum, the levels of col-I and col-III were increased significantly in the model group compared to the sham group (col-I: 4.11 ± 0.74 μg/L versus 2.74 ± 0.40 μg/L, col-III: 2.68 ± 0.43 μg/L versus 1.61 ± 0.27 μg/L, $P < 0.01$) 4 weeks after MI. The levels of col-I and col-III in the FS and SNT treatment groups were decreased significantly compared to the model group (col-I, FS: 2.67 ± 0.42 μg/L, SNT-LD: 2.43 ± 0.38 μg/L, and SNT-HD: 2.17 ± 0.21 μg/L versus 4.11 ± 0.74 μg/L, $P < 0.01$; col-III, FS: 2.00 ± 0.45 μg/L, SNT-LD: 2.02 ± 0.47 μg/L, and SNT-HD: 1.96 ± 0.47 μg/L versus 2.68 ± 0.43 μg/L, $P < 0.01$). The levels of TGF-β1 in serum did not show a significant difference between the model and SNT-LD groups. The levels of TGF-β1 were decreased in the FS group when compared to the sham group, but not significantly (Table 4 and Figures 4(a)–4(c)).

In myocardial tissue, the levels of collagen type I and type III in the model group were decreased compared to the sham group (col-I: 1.20 ± 0.15 μg/g versus 0.94 ± 0.16 μg/g, col-III: 0.45 ± 0.04 μg/g versus 0.28 ± 0.10 μg/g, $P < 0.05$). The levels of collagen type I and type III in the FS and SNT treatment groups were decreased compared to the model group (col-I, FS: 0.86 ± 0.15 μg/g, $P < 0.01$; SNT-LD: 1.03 ± 0.10 μg/g; SNT-HD: 0.89 ± 0.14 μg/g, $P < 0.01$, versus 1.20 ± 0.15 μg/g). The level of TGF-β1 in serum was increased in the model group compared with the sham group (6.48 ± 1.79 ng/g versus 5.99 ± 2.17 ng/g). The levels of TGF-β1 were decreased in the FS and SNT groups when compared with sham and model groups, but not significantly (Table 4 and Figures 4(a)–4(c)). SNT reduced collagen matrix accumulation in the serum and the myocardial tissue following MI, which is associated with a significant improvement in systolic function (Table 2).

4. Discussion

Traditional Chinese medicine (TCM) is becoming an increasingly popular form of alternative or complementary medicine in Europe not only due to its reputed effectiveness, low cost, and relative absence of side effects but also due to the personalized therapy urgently needed in many countries [37, 38]. In recent years, an increasing number of studies provide scientific evidence to support the use of TCM for

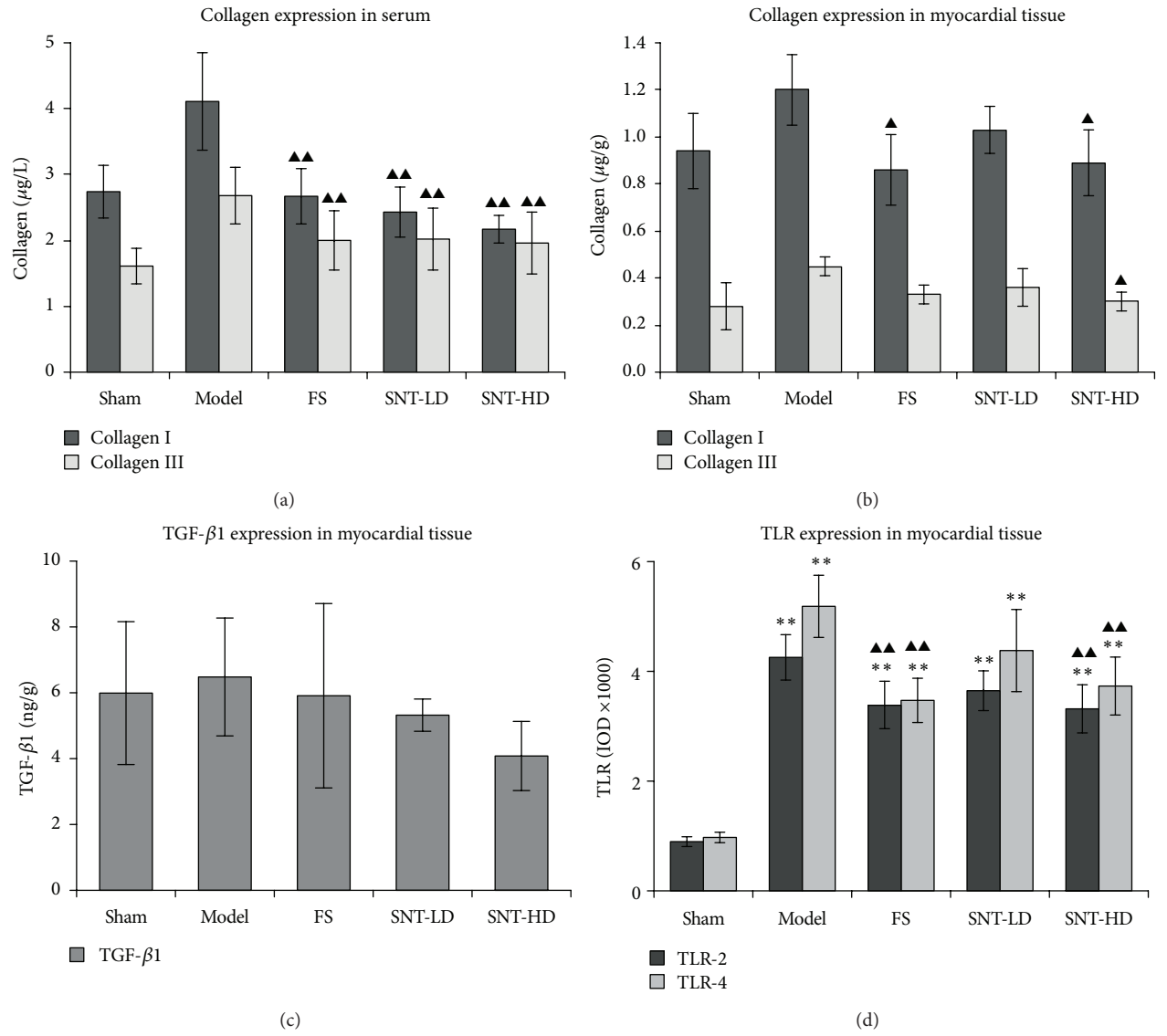

Figure 4: Expression levels of type I and type III collagens. Collagen I and collagen III levels in serum (a) and myocardial tissue (b), TGF-β1 expression levels in myocardial tissue (c), and TLR expression levels in myocardial tissue (d).

Table 4: Overview of collagen expression levels in serum and in myocardial tissue.

	Sham	Model	FS	SNT-LD	SNT-HD	Expression levels (versus model)
In serum						
Collagen I (μg/L)	2.74 ± 0.40	4.11 ± 0.74**	2.67 ± 0.42$^{\blacktriangle\blacktriangle}$	2.43 ± 0.38$^{\blacktriangle\blacktriangle}$	2.17 ± 0.21$^{\blacktriangle\blacktriangle}$	↓
Collagen III (μg/L)	1.61 ± 0.27	2.68 ± 0.43**	2.00 ± 0.45$^{\blacktriangle\blacktriangle}$	2.02 ± 0.47$^{\blacktriangle\blacktriangle}$	1.96 ± 0.47$^{\blacktriangle\blacktriangle}$	↓
TGF-β1 (ng/L)	24.95 ± 4.31	23.91 ± 5.76	22.84 ± 5.07	23.75 ± 8.78	25.61 ± 7.98	
In myocardial tissue						
Collagen I (μg/g)	0.94 ± 0.16	1.20 ± 0.15	0.86 ± 0.15$^{\blacktriangle}$	1.03 ± 0.10	0.89 ± 0.14$^{\blacktriangle}$	↓
Collagen III (μg/g)	0.28 ± 0.10	0.45 ± 0.04*	0.33 ± 0.04	0.36 ± 0.08	0.30 ± 0.04$^{\blacktriangle}$	↓
TGF-β1 (ng/g)	5.99 ± 2.17	6.48 ± 1.79	5.91 ± 2.80	5.32 ± 0.49	4.08 ± 1.05	↓

$^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus model group.

preventing and treating cardiovascular diseases [6–11] including SNT [15, 16]. Nevertheless, the role of TCM in cardiovascular diseases still requires further experimental evidence [39].

The Chinese herbal medicine SNT is a "cold-extremities" decoction, the representative formula of preventing and treating "cold syncope due to deficiency of the heart function" according to TCM theory. The present study indicated that SNT could play a role in treating MI caused by deficiency of the heart function.

Left ventricular remodeling is the process by which ventricular size, shape, and function are regulated by different mechanical, neurohormonal, and genetic factors. Remodeling may be physiological and adaptive during normal growth or pathological due to MI, cardiomyopathy, hypertension, or valvular heart disease [40]. After MI, the ventricle undergoes a progressive physiological and anatomical transformation. Progressive left ventricular dilation and eccentric hypertrophy, infarct scar thinning, and, ultimately, an alteration of the left ventricular geometry from a prolate ellipse to a spherical globe characterize this transformation. The structural changes in the left ventricle (infarct zone and noninfarct zone) are governed by cellular and molecular mechanisms in a pathological metamorphosis [22]. More recent concepts also include the genomic expression resulting in molecular, cellular, and interstitial changes of morphology and structure [5, 41]. In this study, we observed the morphology of the myocardial tissue with visible MI in the model and the control groups with an expansion in the total LV cavity area, an increase in thickness of the interventricular septum and free wall, and a decrease in thickness of the infarct area. The SNT treatment groups showed reduced changes in the pathological structure. Pathological H&E staining showed that the left ventricular cavity area of the model group after AMI was significantly increased by approximately 22.3% compared to the sham group ($P < 0.01$). The ventricular cavity area/heart cavity area ratios of the FS, SNT-HD, and SNT-LD treatment groups were decreased significantly by 15.2% ($P < 0.01$), 18.5% ($P < 0.01$), and 17.3% ($P < 0.01$), respectively, compared to the model group. The IS values from the FS, SNT-LD, and SNT-HD groups ($23.91 \pm 7.99\,mm^2$, $31.25 \pm 10.68\,mm^2$, and $27.81 \pm 10.33\,mm^2$) were significantly smaller than from the model group ($38.04 \pm 8.35\,mm^2$). SNT could decrease LVDs significantly (SNT-LD: 4.95 ± 1.95 mm versus the sham group, $P < 0.05$, and SNT-HD: 3.74 ± 1.47 mm versus the sham group, $P < 0.01$). SNT could also decrease LVDd (SNT-LD: 5.37 ± 1.78 mm and SNT-HD: 4.87 ± 1.47 mm versus the sham group, $P > 0.05$), but not significantly.

Echocardiography is the key diagnostic tool and was performed to assess LV function and volumes, valvular function, and extent of myocardial damage and to detect mechanical complications [42]. The results of our echocardiographic evaluation indicated that the rats in the SNT treated groups had a thinner wall in the infarct zone, an increased ventricular cavity area, extended circumference, wall motion abnormality, and decreased left ventricular function. The left ventricular ejection fraction (LVEF) was significantly decreased in all groups compared with the sham group ($P < 0.05$). The SNT groups showed an improved left ventricular function of the EF% (24%, $P < 0.05$) compared with the model group.

Surrounding the myocytes, the extracellular matrix (ECM) is a dynamic complex composed of structural components: collagen, especially type I and type III, and fibroblasts. Collagen type I, a fibrillar collagen which provides tensile strength, and collagen type III, an elastic collagen, are most abundant in the cardiac ECM [22]. Collagen is important in maintaining structural integrity after AMI [43]. Studies indicated that infarct expansion is associated with damage to the myocardial connective tissue matrix, including the apparent loss of collagen struts. Collagen synthesis is controlled by TGF-β, a member of the transforming growth factor beta superfamily of cytokines, which plays an important role in fibrosis-related cardiovascular disorders, postangioplasty restenosis, and postinfarct ventricular remodeling, all of which can lead to heart failure [44]. TGF-β1 serum levels are connected with prognostic values for ventricular remodeling and hypertrophy [23, 45] and LV function [24]. Our results showed that the SNT treatment groups had decreased levels of the collagens in sera and in myocardial tissues. There were no significant differences between TGF-β1 levels in serum in all experimental groups compared to the sham group. We could show that the mean level of TGF-β1 in myocardial tissue was lower in the SNT-HD groups than in the model group, but no significant difference was observed. These data indicated that SNT had a positive influence on the col-I and col-III regulation and therefore had a direct effect on the fibrosis formation and provided structural integrity after AMI. SNT reduced collagen matrix accumulation in the serum and the myocardial tissue, which is associated with a significant improvement in systolic function.

TLRs belong to a group of type I transmembrane receptors with endogenous and exogenous ligand binding ability to stimulate innate and adaptive immune responses by inducing the immune and inflammatory cytokines IL-6, TNF-alpha, and other genes. TLRs are expressed differentially in immune cells and nonimmune cells, such as cardiomyocytes and endothelial cells in the heart that are involved in cardiac stress reactions [46]. TLR-2 signaling is involved in myocardial ischemia/reperfusion injury [21] and in coronary artery endothelial dysfunction with impaired vessel relaxation induced by transient ischemia [47]. TLR-4 plays an important role in mediating immune cells infiltration, cytokine production, and complement activation during ischemia/reperfusion [48]. TLR-4 deficiency improves left ventricular function and attenuates pathophysiological key mechanisms in cardiomyopathy [25]. We could show that the expressions of TLR-2 and TLR-4 were decreased significantly in both SNT treatment groups compared to the sham and the model groups. These findings indicate again that SNT treatment could improve the early left ventricular function after MI.

The emerging understanding of the extracellular matrix and the various active molecules within it, such as the matrix metalloproteinases (MMPs), elicits new appreciation for their role in cardiac remodeling and as possible future therapeutic

targets. With further understanding of the complex interaction between MMPs and their temporal activation in the postinfarction heart, pharmacological or transgenic inhibition of MMPs or activation of TIMPs may still prove to be a viable therapeutic option to minimize cardiac remodeling [22]. This should be one of the next steps of our investigation on SNT by omics technologies.

5. Conclusions

In conclusion, the present study demonstrates that the Chinese medicine formula SNT has the potential to improve early ventricular remodeling and cardiac function after MI in rats. SNT reduced the left ventricle end-systolic dimension, increased the ejection fraction, and improved the left ventricular function. These effects may be related to the downregulation of Toll-like receptors and collagen deposition. The new insight underlying cardiac remodeling will enable us to develop more successful therapies including the traditional Chinese herbal medicine SNT in modern applications in the future.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Jiangang Liu and Karoline Peter contributed equally to this work.

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Research Article

Yiqi Huoxue Recipe Improves Heart Function through Inhibiting Apoptosis Related to Endoplasmic Reticulum Stress in Myocardial Infarction Model of Rats

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Objective. To explore the mechanism of cardioprotective effects of Chinese medicine, Yiqi Huoxue recipe, in rats with myocardial infarction- (MI-) induced heart failure. *Methods*. Male Sprague-Dawley rats underwent left anterior descending artery (LAD) ligation or sham operation. The surviving MI rats were divided randomly into three groups: MI (5 mL/kg/d NS by gavage), MI + Metoprolol Tartrate (MT) (12 mg/kg/d MT by gavage), and MI + Yiqi Huoxue (5 mL/kg recipe by gavage). And the sham operation rats were given 5 mL/kg/d normal saline. Treatments were given on the day following surgery for 4 weeks. Then rats were detected for heart structure and function by transthoracic echocardiography. Apoptosis in heart tissues was detected by TUNEL staining. To determine whether the endoplasmic reticulum (ER) stress response pathway is included in the cardioprotective function of the recipe, ER stress related proteins such as GRP78 and caspase-12 were examined. *Results*. Yiqi Huoxue recipe attenuated heart function injury, reversed histopathological damage, alleviated myocardial apoptosis and inhibited ER stress in MI rats. *Conclusion*. All the results suggest that Yiqi Huoxue recipe improves the injured heart function maybe through inhibition of ER stress response pathway, which is a promising target in therapy for heart failure.

1. Introduction

Heart failure is a syndrome with high mortality and morbidity where cardiac function is inadequate to meet metabolic needs of the body. Heart failure is now one of the greatest public health problems [1]. Thus, the development of novel treatments for patients with cardiovascular diseases remains a major research priority. The most common cause of heart failure is coronary artery disease (CAD) and CAD related myocardial infarction (MI). Multiple factors are involved in the mechanism of heart failure progression, such as the activity of sympathetic nervous system. During the last few years there has been increasing evidence from human and animal models suggesting that apoptosis could be a key modulator especially in the transition from compensatory hypertrophy to heart failure [2, 3]. It is well known that the death receptor and mitochondrial apoptotic pathways are the two main apoptotic pathways leading to cardiomyocytes death in heart failure. More and more researches have confirmed that endoplasmic reticulum (ER) is a primary target in various acute disorders and has been suggested as the third subcellular compartment implicated in apoptotic pathway [4]. Recent studies have revealed that ER stress and ER related apoptosis were important mechanisms included in heart failure progression [5, 6].

Oxidative stress, hypoxia, and enhanced protein synthesis found in failing hearts potentially enhance ER stress in myocardium [5]. Okada and colleagues revealed that cardiac expressions of ER chaperones-GRP78 (glucose-regulated protein 78) and CHOP (CCAAT/enhancer-binding protein

(C/EBP) homologous protein) were significantly increased at 1 and 4 weeks after transverse aortic constriction which induced cardiac hypertrophy and failure, respectively [6]. The endoplasmic reticulum, as the primary target in various disorders, determines the outcome of hypertrophic cardiomyocytes for restoring homeostasis or resulting in heart failure, through integrating the nucleus, mitochondria, and Golgi apparatus subcellular organelles reaction [7]. The adaptive and proapoptotic pathways of ER stress response play fundamental roles in the development and progression of cardiovascular diseases, including heart failure, ischemic heart diseases, and atherosclerosis. Thus, therapeutic interventions to regulate the gene expression of the target molecules for ER stress response and reduce ER stress should be the promising strategies for cardiovascular diseases treatment [8].

In Chinese medicine system, qi deficiency and blood stasis were the main pathogenesis of heart failure. Based on this theory Yiqi Huoxue method is widely used in clinical practice. Yiqi Huoxue recipe had been confirmed to be useful in treatment for heart failure in lab and clinical studies [9, 10]. It was reported that Yiqi Huoxue recipe improved heart function remarkably in patients with congestive heart failure, who were treated with the traditional Chinese medicine, on the bases of conventional therapy [10]. However, the mechanism of this effect was discussed little. In our study, we used the Yiqi Huoxue recipe (13 portions of *Astragalus*, 6 portions of *Angelica*, and 10 portion of *Ginseng*) as therapeutic drugs and the myocardial infarction (MI) rats as animal model, to study the molecular mechanisms of the cardioprotective effect of Yiqi Huoxue recipe in the MI rat model.

2. Materials and Methods

2.1. Chemicals and Drugs. Trizol was purchased from Invitrogen (CA, USA). Reverse transcription system (A3500) was purchased from Promega (WI, USA). The ab21685-100 antibody against GRP78 and the antibody against caspase-12 (Ab62484-100) were purchased from Abcam (Cambridge, UK). Antibody against GAPDH (Cs 2118) was provided from Cell Signaling Technology (Boston, US). The terminal deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) kit was obtained from Wuhan Boster Biological Technology Ltd. (Wuhan, China). Other chemicals and reagents were of analytical grade.

Preparation of Yiqi Huoxue Decoction. Yiqi Huoxue Decoction consists of Astragalus, *Angelica*, and *Ginseng* in a 13 : 6 : 10 ratios. Slices of the herbs were provided by pharmaceutical preparation section, Dongzhimen hospital, Beijing, China. The medicinal herbs were extracted twice. Based on the ratios, Slices of those herbs were first boiled together in 6x volume of water for 0.5 h, and then the residue from first extraction was boiled in 8x volume of water for 25 min. Finally, the filtered solutions were combined and concentrated into the resulting aqueous extracts containing 1.2 g/mL raw herbs. Metoprolol Tartrate (MT) was purchased from AstraZeneca Company (Jiangsu, China).

2.2. Animals. Male Sprague-Dawley rats, weighing 190–210 g, were obtained from the laboratory of the Academy of Medical Sciences, Beijing, China (certificate number SCXK (Beijing) 2009–0007). The animals were fed by a standard laboratory diet and given free access to tap water. The cages were kept in a room with controlled temperature (22 ± 1°C), relative humidity (65–70%), and day/night cycle (12 : 12 light/dark). All animal procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Chinese Medicine, Beijing, China.

2.3. Myocardial Infarction. Rats were anesthetized with intraperitoneal sodium pentobarbital (Inactin Byk-Gulden, 50 mg/kg body weight) and then were artificially ventilated with a small animal volume-control ventilator (Harvard Apparatus; Holliston, MA) with a tidal volume of 1 mL at a rate of 100 cycles/min. Thoracotomy was then performed at the left third intercostal space; the heart was exposed via a small retractor. A 5-0 suture was placed in the anterior myocardium to occlude the left anterior descending artery (LAD). The thorax and the skin incision were closed with 2-0 sutures. The endotracheal tube was gently retracted after spontaneous breathing was restored. Sham operated animals were subjected to similar surgery, except that no ligature was placed [11]. Successful ligation was confirmed by ST segment elevation in postoperative ECG, compared with preoperative ones. All animals were given penicillin after the operation for three days to prevent infection. The surviving MI rats were divided randomly into three groups: MI (5 mL/kg/d normal saline (NS) by gavage), MI + MT (12 mg/kg/d MT by gavage), and MI + Yiqi Huoxue (5 mL/kg recipe by gavage). And the rats with sham operation were given 5 mL/kg/d NS by gavage. Treatments were given on the day following surgery once a day for 4 weeks.

2.4. Echocardiography. Four weeks after LAD ligation, transthoracic echocardiography was performed using an AloCa5000 system (Sino-Japanese joint) equipped with a 7–15 MHz real-time microvisualization scan head probe. Briefly, each rat was anesthetized with sodium pentobarbital (Inactin Byk-Gulden, 50 mg/kg body weight) and the chest was shaved. Then, echocardiographic parameters (left ventricular end-diastolic volume (LVDd), left ventricular end-systolic volume (LVDs), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS)) were measured.

2.5. Real-Time. RT-PCR Total RNA was extracted by TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). For real-time PCR, reverse transcription was performed using 1 μg RNA, AMV reverse transcriptase, and oligo $(dT)_{15}$. PCR reactions were performed with the use of SYBR green master mix (ABI, Hercules, CA). The specific primer pairs are as follows: GRP78 forward 5′-CCACCAGGATGCAGACATTG-3′ and reward 5′-AGGGCCTCCACTTCCATAGA-3′ (100 bp); Caspase-12 forward

5′-CACTGCTGATACAGATGAGG-3′ and reward 5′-CCACTCTTGCCTACCTTCC-3′ (138 bp); GAPDH forward 5′-AGTTCAACGGCACAGTCAAG-3′ and reward 5′-TACTCAGCACCAGCATCACC-3′ (118 bp). Amplification was followed by melting curve analysis to verify the accuracy of the amplicon.

A negative control without cDNA was run with every PCR to assess the specificity of the reaction. PCR efficiency was between 90 and 110% for all primer sets. Analysis of data was performed through MxPro-Mx3000P software. Data were calculated as the change in cycle threshold (ΔC_T) for the target gene relative to the ΔC_T for GAPDH (control gene) according to the procedures of Muller's et al. [12].

2.6. Western Blot. Heart tissues were homogenized on ice in RIPA buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 min at 4°C, the supernatant was used for Western blot analysis. Protein concentration was measured by BCA method (Bradford, 1976). 40–100 μg proteins were loaded for Western blot analysis. The protein extract was transferred in sample buffer, loaded on 10% SDS-polyacrylamide gel, and blotted onto a nitrocellulose membrane with a wet blotting system. After being blocked for 1 h in Tris-buffered saline/Tween 20 (TBST) with 5% nonfat milk, the membranes were incubated with primary antibodies in blocking buffer (1 : 1000) at 4°C overnight. Then the membranes were incubated with peroxidase conjugated secondary antibody at a 1 : 6000 dilution at room temperature for 1 h. After being rinsed with TBST for 3 times, the proteins were detected by autoradiography on enhanced chemiluminescence (Super ECL Plus; Applygen Technologies, Beijing, China). Semiquantifications were performed with densitometric analysis using NIH ImageJ software.

2.7. Detection of Apoptosis in Heart Tissue. TUNEL stain was used to detect the apoptosis in heart tissues. Briefly, sections were deparaffinized and rehydrated and then pretreated with 20 μg/mL proteinase K (37°C, 10 min). The sections were then incubated with TdT reaction mixture in a humidified chamber (37°C, 2 h), washed with PBS and blocked for 30 min at room temperature, and then incubated with anti-digoxigenin antibody at 37°C for 30 min. Subsequently, slides were incubated with streptavidin-biotin-peroxidase for 30 min. 3,3-Diaminobenzidine tetrahydrochloride was then added. Sections were counterstained with Mayer's hematoxylin. For negative staining controls, the TdT reaction mixture was omitted. All sections were then dehydrated and coverslipped. The percentage of apoptotic cells was determined; labeled nuclei were counted; and the results were expressed as percentage apoptotic nuclei of total nuclei in the heart tissue.

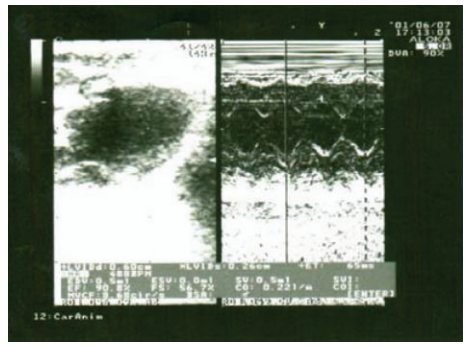
2.8. Statistical Analysis. Data are expressed as mean ± S.D. Statistical differences were evaluated by one-way ANOVA and then Newman-Student-Keuls test. A value of $P < 0.05$ was considered statistically significant.

3. Results

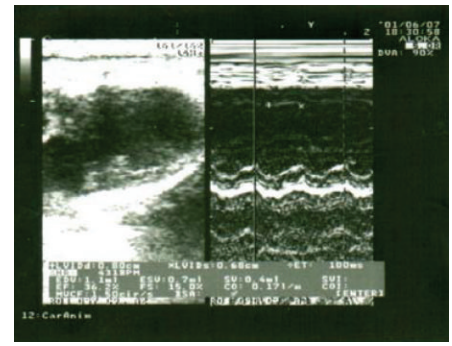
3.1. Yiqi Huoxue Recipe Attenuates Heart Function Injury and the Heart to Body Weight Ratio Increase in Myocardial Infarcted Rats. To determine the effect of the Yiqi Huoxue recipe on treatment of MI-induced heart failure in vivo, we induced MI in rats and detected the cardiac function by echocardiography assay and after sacrificing the rats we also measured the heart to body weight ratio. The result showed that a significant increase in the volume of left ventricle was demonstrated by the increase of LVDd and LVDs after 4 weeks of infarction (Figure 1(e)), which is attributed to the left ventricular dilatation. However, MT or Yiqi Huoxue recipe treatment had no significant effect on the MI-induced dilation. The result also demonstrated that the LVEF and LVFS were decreased significantly 4 weeks after MI surgery. Treatment with MT or Yiqi Huoxue recipe improved the cardiac function by increase in LVEF by 21% or 16.9% and in LVFS by 50.3% or 40.8%, respectively, compared with MI group (Figure 1(e)). However, MT or Yiqi Huoxue recipe showed no difference in the LVEF and LVFS improvements ($P > 0.05$). The heart to body weight ratio was increased in MI group rats compared with that in sham group rats ($P < 0.05$). The treatment with MT or Yiqi Huoxue recipe was observed to attenuate the increase in the heart to body weight ratio at 4 weeks after myocardial infarction surgery ($P < 0.05$) and showed no difference ($P > 0.05$) (Figure 1(e)).

3.2. Yiqi Huoxue Recipe Alleviates the Heart Tissue Histologic Injury in Myocardial Infarcted Rats. The microphotographs showed that in sham group myocardial fibers were arranged orderly, cytoplasmic staining was uniform, and nucleus boundaries were clear (Figure 2(a)). It was observed that in MI group the range of myocardial cells in heart tissue was in disorder and there is marked neutrophilic infiltration around the myocardial cells. Myocardiocyte disarrangement and fibrosis accretion were observed in MI group as well (Figure 2(b)). In rats group with 4 weeks of treatment with MT (Figure 2(c)) or Yiqi Huoxue recipe (Figure 2(d)) after MI surgery, myocardiocyte disarrangement, neutrophilic infiltration and fibrosis accretion were alleviated compared to rats with MI surgery only.

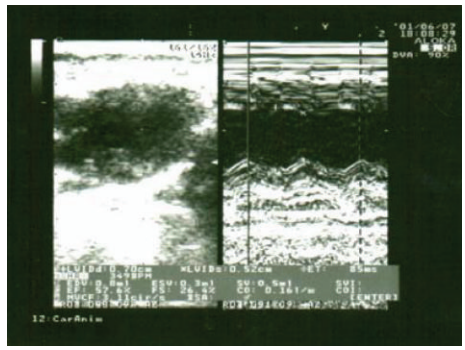
3.3. Yiqi Huoxue Recipe Attenuates Myocardial Apoptosis in MI Rats. Apoptosis has been suggested to be involved in the development of heart failure. To detect whether Yiqi Huoxue recipe protects heart function by apoptosis elimination, we performed the TUNEL assay. The results showed that LAD occlusion induced apoptosis in rats' heart tissue. As shown in Figure 3, at the border region, the ratio of TUNEL-positive myocardial cells to total myocardial cells was increased to 8.88%. In group treated with Yiqi Huoxue recipe or MT, the TUNEL-positive myocardial cell ratio was decreased to 4.12% or 4.52%, which was significantly lower than that in MI group ($P < 0.01$) and there was no difference between Yiqi Huoxue group and MT group.



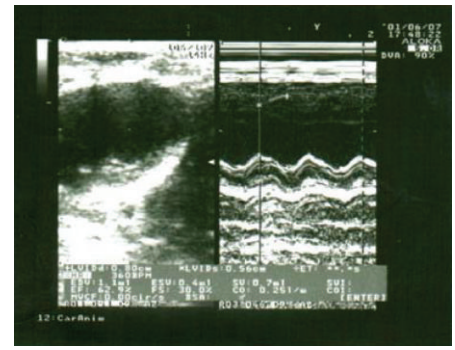
(a)



(b)



(c)

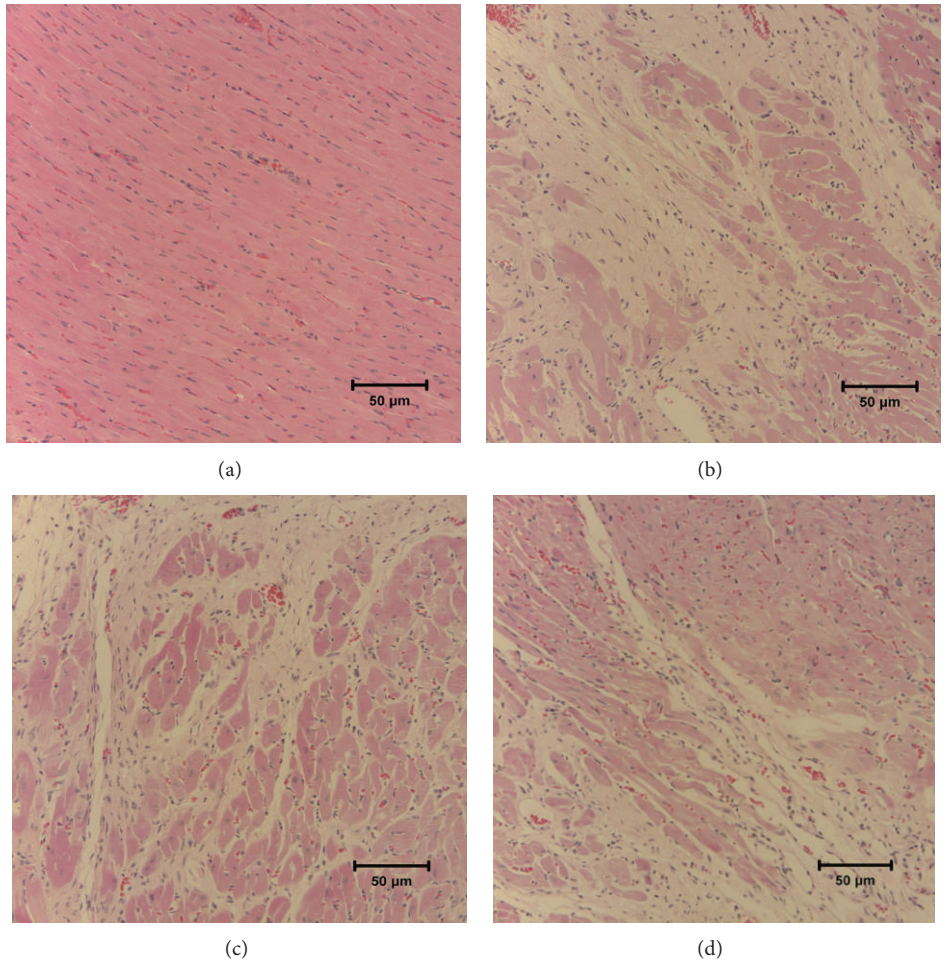


(d)

	Sham	MI	Metoprolol tartrate	Yiqi Huoxue recipe
Anatomic data	$n = 9$	$n = 9$	$n = 9$	$n = 9$
Body weight (kg)	342 ± 31	346 ± 20	352 ± 41	368 ± 32
Heart weight/body weight (g/kg)	3.15 ± 0.09	3.54 ± 0.68*	3.26 ± 0.20#	3.19 ± 0.36#
Echocardiographic data	$n = 9$	$n = 9$	$n = 9$	$n = 9$
LVDd (cm)	0.60 ± 0.09	0.79 ± 0.09**	0.77 ± 0.14**	0.82 ± 0.12**
LVDs (cm)	0.30 ± 0.07	0.61 ± 0.10**	0.59 ± 0.19**	0.62 ± 0.16**
LVEF (%)	86.6 ± 5.4	49.6 ± 12.2**	60.0 ± 11.9**#	58.8 ± 9.8**#
LVFS (%)	51.3 ± 6.5	19.1 ± 3.4**	28.7 ± 9.5**#	26.9 ± 7.3**#

(e)

Figure 1: Yiqi Huoxue recipe attenuates heart function injury and attenuates the heart to body weight ratio in myocardial infarcted rats model. Rats were operated with myocardial infarction (MI) surgery and after 4 weeks of treatment, echocardiography assay was performed ((a) sham, (b) MI, (c) MI + Metoprolol Tartrate, (d) MI + Yiqi Huoxue recipe, and (e) anatomic and echocardiographic data for rats 4 weeks after myocardial infarction with different treatment). And after sacrificing the rats, the heart to body weight ratio was also calculated. In the rat myocardial infarction (MI) model, the heart to body weight ratio is increased in the untreated MI group at 4 weeks after MI. Yiqi Huoxue recipe and MT treatment diminished the effect (e). As to the echocardiographic data, the LVEF and LVFS showed the same trend (e). Values are mean ± SD ($n = 9$), LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVDd, left ventricular end-diastolic volume; LVDs, left ventricular end-systolic volume. $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group. $^{\#}P < 0.05$ versus myocardial infarction group.



(a) (b)

(c) (d)

Figure 2: Microscopic observations (100x) of heart tissues. (a) Heart tissue of normal rat; (b) heart tissue of rat 4 weeks after MI; (c) heart tissue of rat treated with MT 4 weeks after MI; (d) heart tissue of rat treated with Yiqi Huoxue recipe 4 weeks after MI.

3.4. Endoplasmic Reticulum Stress in the Heart of MI Rats Was Inhibited by Yiqi Huoxue Recipe. Endoplasmic reticulum stress has been reported as an independent pathway leading to apoptosis and a new mechanism implicated in heart failure [5, 6]. To investigate the possible mechanism of heart protection of Yiqi Huoxue recipe, ERS related proteins such as GRP78 and caspase-12 were examined. Real-time PCR analysis of samples from heart tissue showed that GRP78 and caspase-12 mRNA levels were increased in MI rats hearts compared with the sham group (Figures 4(a) and 4(b)). When rats were treated with Yiqi Huoxue recipe or MT after LAD occlusion operation, GRP78 and caspase-12 mRNA levels in heart tissue were downregulated compared with those of MI rats without treatment (Figures 4(a) and 4(b)). Western blot showed that the protein levels of these two factors were increased in MI heart and Yiqi Huoxue recipe treatment can decrease the protein levels. And as to caspase-12, both the pro- and activated caspase-12 showed the same trend (Figures 4(c) and 4(d)).

4. Discussion

Heart failure, as the end point of most heart diseases, is a major public health problem with high morbidity and mortality, frequent hospitalizations, and a major cost burden on the community. Up to now, researches on the mechanisms and therapies of heart failure have made much progress [13, 14]. It is well known that activated neurohormonal system is one of the most important pathophysiologic factors. In particular, the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS) are the main reasons which contribute to the progression and pathophysiology of cardiac dysfunction. Thus, β-blockers, angiotensin-converting enzyme (ACE) inhibitors, aldosterone-receptor blockers, and angiotensin-receptor blockers (ARB) have all been found in major trials to confer morbidity and mortality benefits and thus are the mainstays of current treatment as recommended by guideline authorities. However, due to the complexity and severity of the symptom, the mortality

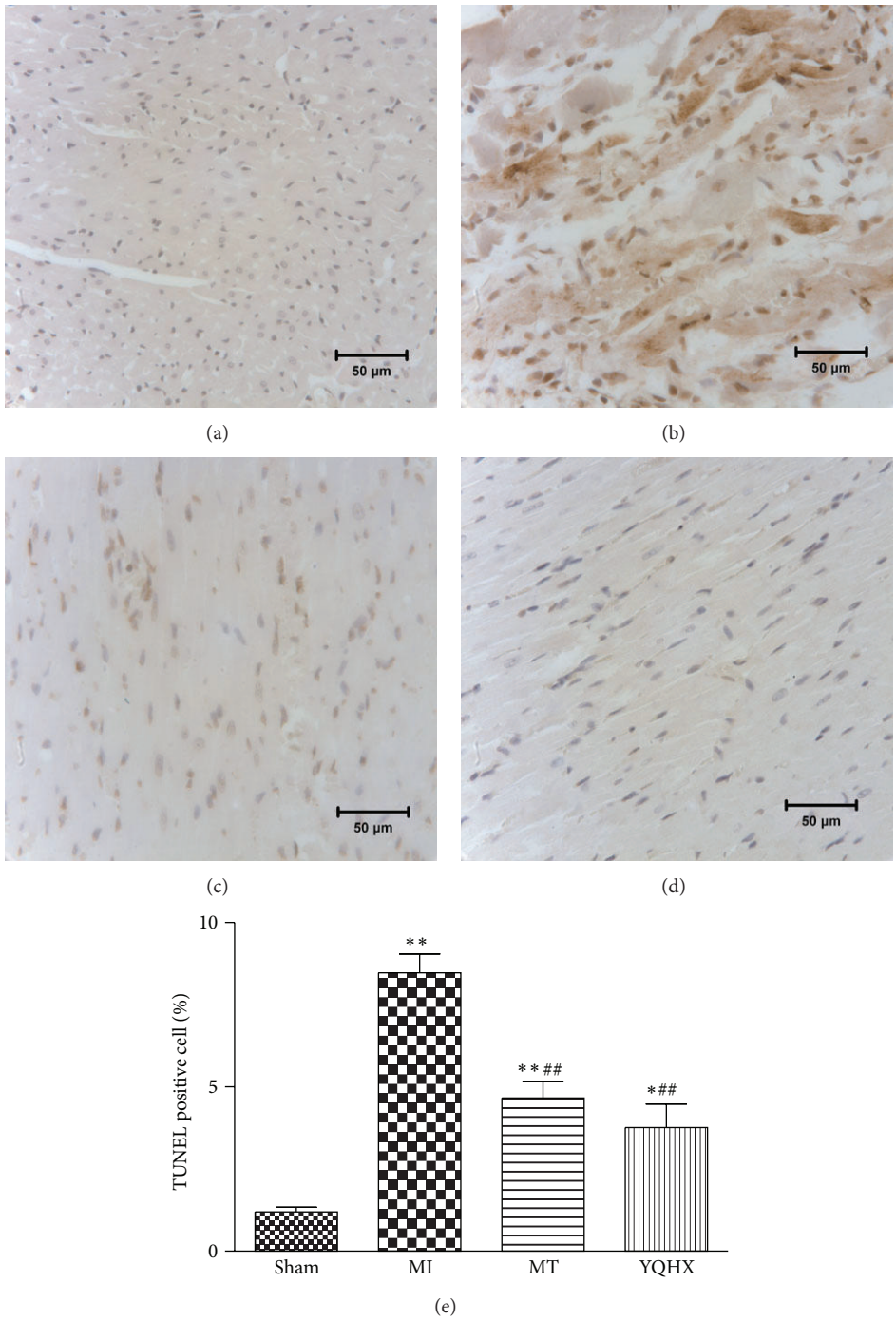


Figure 3: TUNEL staining of apoptosis in the border regions of heart sections from (a) sham rats, (b) MI rats, (c) MI + MT rats, and (d) MI + YQHX rats. Data are presented as the mean ± S.D. (e) Quantitative analysis of myocyte apoptosis in the border regions of rats after MI ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group. $^{\#\#}P < 0.01$ versus MI group.

remains unacceptably high at around 8–12% annually [15]. Therefore, novel and effective drugs that show few side-effects are needed [16, 17].

Traditional Chinese medicine has been used for a long time and formed a theory of diagnosis and treatment of heart failure [18–21]. Yiqi Huoxue recipe used in the current experiment is composed of *Astragalus*, *Angelica*, and *Ginseng*. *Astragalus*, the main ingredient of our recipe, is one of the most commonly used elements in traditional Chinese medicine for chronic heart failure in China. Modern pharmacological research has shown that *Astragalus* injection can enhance myocardial contractility, improve circulation, protect myocardial cells, and regulate immunity [22]. *Angelica*, another component of the recipe, contains organic acids, which has been proved to have the function of antimyocardial ischemia by stabilizing the cell member

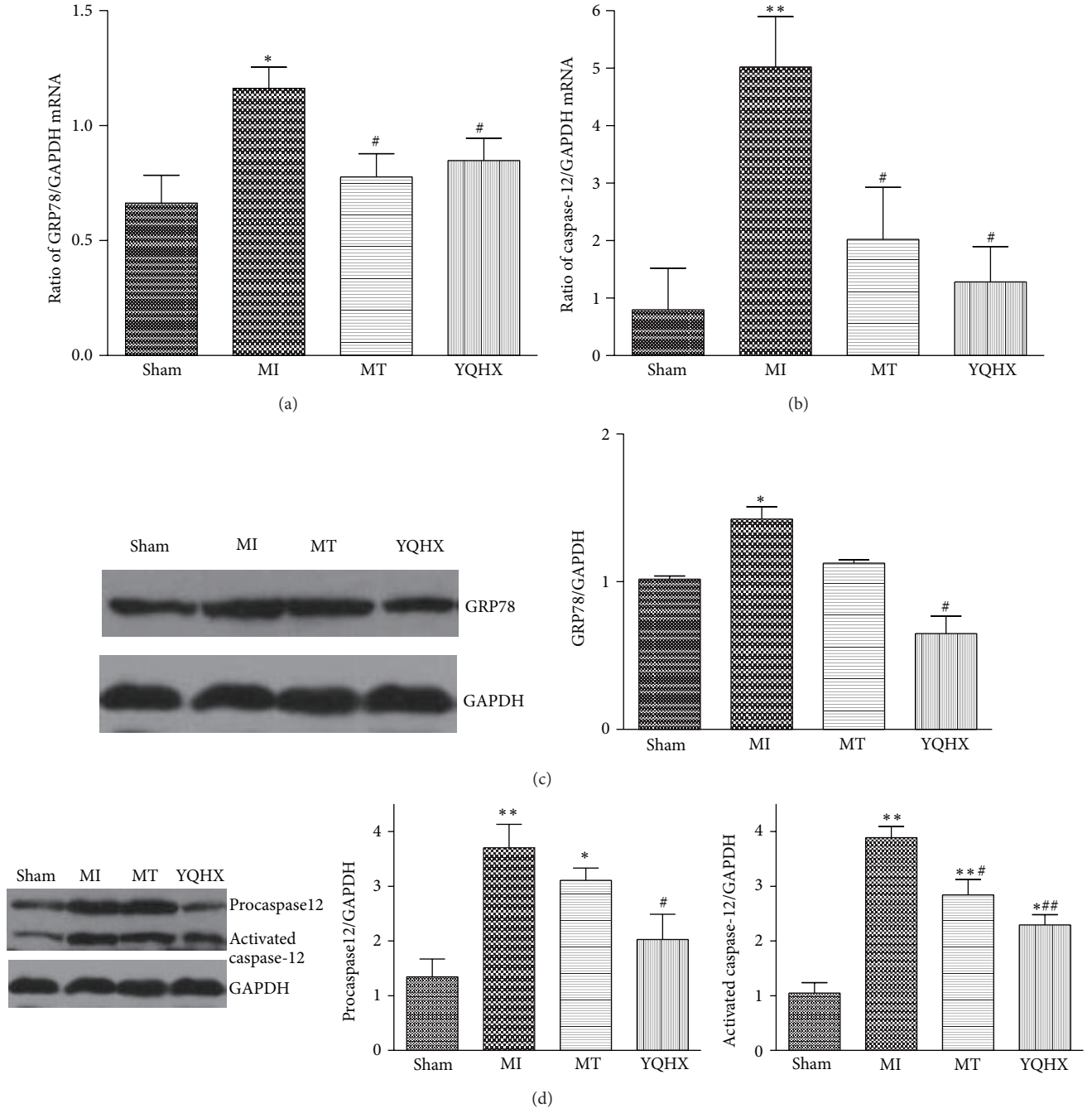


Figure 4: Expression of glucose response protein 78 (GRP78) and caspase-12 in mRNA level (a, b) and protein level (c, d) in heart tissues. Rats underwent MI surgery and after 4 weeks of treatment hearts were obtained. Heart tissues extracts were analyzed by immunoblotting by using antibodies against GRP78 and caspase-12. GAPDH was used as a loading control. Data are the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus myocardial infarction group.

system [23, 24]. The third component of recipe use in our study, *Ginseng*, is widely used in traditional Chinese medicine having cardiovascular benefits usually used in treatment of heart disease [25]. And emerging evidence also suggests that *Ginseng* attenuates myocardial hypertrophy, thus blunting the remodeling and heart failure processes [26].

In current study, we used recipe composed of the above three traditional Chinese herbs to deal with the heart failure development after LAD occlusion surgery in rat model, to explore the mechanisms underlying its protective effects.

The result demonstrated that rats developed heart failure 4 weeks after LAD occlusion surgery and the recipe or MT attenuated the heart function damage. Ratio of heart to body weight was increased in MI group and the increase was eliminated in recipe and MT groups. The recipe showed more effectiveness in the general condition of the model rats

compared with other groups. The rats' body weight in Chinese medicine group took advantage over that in MT group. Due to the individual difference of rats' response to Chinese medicine, the difference between MT and Chinese medicine group is not obvious after statistical analysis. However, the improvement tendency shown in Chinese medicine group suggests the Chinese medicine superiority in survival benefit, which is superior to the single-target therapy with western medicine. To discuss the apoptosis in the mechanism of heart protection of the recipe, rats underwent LAD occlusion surgery were sacrificed 4 weeks later and TUNEL assay was performed in the heart sections. The apoptosis was increased obviously in MI group and the recipe could inhibit the increase partially. These results suggest that, in our experiment, the recipe could improve the injured heart function through eliminating myocyte apoptosis. Since the ER stress response pathway is an emerging compartment involved in the apoptosis, we further detected the ER stress marker-GRP78 and ER stress-specific apoptosis inducing protein caspase-12. Results showed that GRP78 and caspase-12 were upregulated in heart tissue of MI group rats. Treatment with Yiqi Huoxue recipe significantly attenuated upregulated GRP78 protein expression and caspase-12 activation induced in hear failure. This suggests that a reduced ER stress response is involved in the protective effect of Yiqi Huoxue recipe against heart failure progression.

The ER is an organelle that has essential roles in multiple cellular processes including intracellular calcium homeostasis, protein secretion, and lipid biosynthesis [27]. Multiple disturbances in cellular redox regulation or calcium regulation caused by hypoxia, oxidants, glucose deprivation, or viral infection can cause accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response termed as the unfolded protein response (UPR) [28]. UPR induced by ER stress can lead to adaptation by expression of genes that are capable of enhancing the protein folding capacity of the ER and genes for ER-assisted degradation. If the adaptive mechanisms fail to compensate, the cell death is induced, typically by apoptosis. ER stress leads to apoptosis by mitochondria-dependent and -independent pathways. ER disturbances can lead to mitochondrial disruption and ER stress has been determined to signal apoptosis through a mitochondrial-dependent pathway. The mitochondrial-independent pathway is thought to occur through the initiator caspase-12 [29]. All the information suggests that ER-specific apoptosis pathway is an important apoptosis pathway [4]. Recent researches have shown the existence of spliced XBP1 (X-box-binding protein-1) and markedly increased GRP78 expression, suggesting that UPR activation is associated with the pathophysiology of heart failure [6, 30]. In the current study, we also detected increased GRP78 expression and caspase-12 in heart tissue of rats with MI-induced heart failure. And the result suggests that ER stress may be an important mechanism involved in Yiqi Huoxue recipe myocardial cells protection. The components of Yiqi Huoxue recipe such as *Astragalus*, *Angelica*, and *Ginseng* had been confirmed to have antioxidant function and can remove free radicals [31]. Reactive oxygen species or oxidative stress, as the main mechanism included in heart failure progression, has been reported to induce ER stress [32]. Taken together, all the information showed that reactive oxygen species may be a link from myocardial cells protection of Yiqi Huoxue recipe to ER stress inhibition in the treatment of heart failure. However, further study is required to investigate the mechanism of the Yiqi Huoxue recipe in protection of myocardial cells.

In conclusion, the current research demonstrated that Yiqi Huoxue recipe, composed of *Astragalus*, *Angelica*, and *Ginseng*, can improve the injured heart function in vivo. And it can inhibit the apoptosis and ER stress response pathway in MI-induced heart failure development. All the results suggest that Yiqi Huoxue recipe improves the injured heart function maybe through inhibition of ER stress response pathway and ER stress response pathway is a promising target in therapy for MI-induced heart failure development.

Abbreviations

ACE: Angiotensin-converting enzyme
ARB: Angiotensin-receptor blockers
CHOP: CCAAT/enhancer-binding protein homologous protein
CAD: Coronary artery disease
ER: Endoplasmic reticulum
GRP78: Glucose-regulated protein 78
LAD: Left anterior descending artery
LVEF: Left ventricular ejection fraction
LVDd: Left ventricular end-diastolic volume
LVDs: Left ventricular end-systolic volume
LVFS: Left ventricular fractional shortening
MT: Metoprolol Tartrate
MI: Myocardial infarction
NS: Normal saline
RAAS: Renin-angiotensin-aldosterone system
SNS: Sympathetic nervous system
UPR: Unfolded protein response
XBP1: X-box-binding protein-1.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Li-Xia Lou, Ai-Ming Wu, Dong-Mei Zhang, and Sheng-Xian Wu contributed equally to this work.

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Research Article

Evaluation of a *Crataegus*-Based Multiherb Formula for Dyslipidemia: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial

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Background. We for the first time examined the effects of a multiherb formula containing *Crataegus pinnatifida* (1 g daily), *Alisma orientalis*, *Stigma maydis*, *Ganoderma lucidum*, *Polygonum multiflorum*, and *Morus alba* on plasma lipid and glucose levels in Chinese patients with dyslipidemia. *Methods.* In this randomized, double-blind, placebo-controlled study, 42 patients were randomized at a ratio of 1 : 1 to receive the herbal formula or placebo for 12 weeks and 40 patients completed the study. Lipid profiles, glucose, glycated haemoglobin (HbA1c), and laboratory safety parameters were performed before and after treatment. *Results.* The difference in the changes in low-density lipoprotein cholesterol (LDL-C) levels between placebo and active treatment (−9%) was significantly ($P < 0.05$) better with active treatment. HbA1c levels significantly decreased by −3.9% in the active treatment group, but the change was not significantly different from that with placebo (−1.1%) ($P = 0.098$). There were no apparent adverse effects or changes in laboratory safety parameters with either treatment. *Conclusions.* The multiherb formula had mild beneficial effects on plasma LDL-C after 12-weeks treatment in subjects with dyslipidemia without any noticeable adverse effects.

1. Introduction

Herbal medicines have been used for thousands of years in China and other Eastern countries and have regained popularity in Western Countries in the last two decades [1, 2]. There is growing awareness of the place of dietary factors and herbal medicines in the prevention of cardiovascular disease (CVD) and the possibility of their use in treatment of CVD risk factors [3]. Herbal materials have also resulted in the development of many important conventional drugs, including digoxin and the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors or statins. More recently, some natural products were found to be effective in the treatment of hyperlipidaemia and diabetes in animal and in human studies [4–6]. Among 57 traditional Chinese medicine (TCM) formulas that have been approved by the China Food and Drug Administration (CFDA) to treat hyperlipidaemia in mainland China, hawthorn fruit (*Crataegus pinnatifida* Bge.), also known as Shan Zha, is the most popular TCM prescribed in more than 50% of the formulas, followed by *Polygonum multiflorum* (or Fo-ti root, known as He Shou Wu in China) (38%) and *Alisma orientalis* (Ze Xie) (33%) [6].

Crataegus products are widely used for the treatment of cardiac and circulatory disorders, particularly for angina, heart failure, and hyperlipidaemia as they are considered to have multiple cardiovascular protective effects (Figure 1) [7–9]. *Crataegus* leaves, flowers, and fruits contain varying amounts of a number of biologically active substances, such as oligomeric procyanidins, flavonoids, and triterpenes [10, 11]. Among these components, flavonoids and triterpenes, especially ursolic acid, have been reported as the main active constituents exerting hypolipidaemic effects [12]. Recent research indicated that the lipid-lowering effect of *Crataegus* may be related to the inhibitory effects of flavonoids on 3-hydroxy-3-methylglutaryl-coenzyme A reductase [10], downregulation of intestinal acyl-coA : cholesterol acyltransferase activity by the triterpenes [13], and activation of peroxisome proliferator activated receptor (PPAR) alpha in adipose tissue [14] or in liver [15]. Furthermore, *Crataegus* extract has been reported to have antioxidant and nitrite

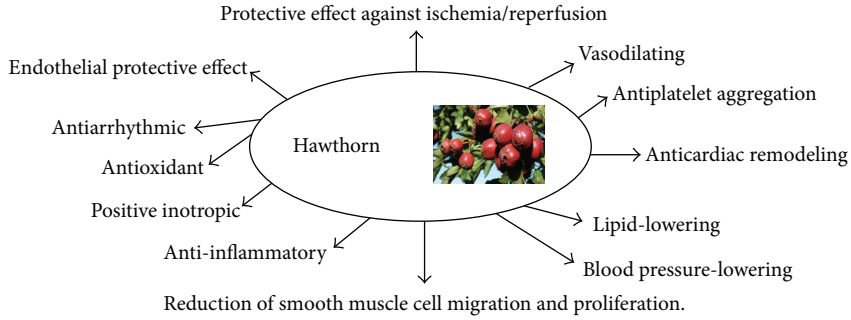

Figure 1: Multiple cardiovascular protective effects of *Crataegus*. Adapted from [9].

reductase activities and inhibits the formation of thromboxane to modify cardiovascular risk factors [16–18]. Although *Crataegus* has been found to decrease the plasma levels of low-density lipoprotein cholesterol (LDL-C), triglycerides, and glucose in various animal models [14, 19–22], limited data are available in humans.

Polygonum multiflorum and *Alisma orientalis* are also important components in TCM formulas for the treatment of hyperlipidaemia [6]. Stilbene glycosides extracted from the root of *Polygonum multiflorum* showed antagonistic effects on oxidation of lipoproteins and proliferation of coronary arterial smooth muscle cells and a decrease in their content of nitric oxide [23]. Some animal studies and an early uncontrolled clinical study suggested that *Polygonum multiflorum* has lipid-lowering effects which may be related to its regulatory effects on the genes involved in cholesterol synthesis and lipoprotein metabolism [6]. *Alisma orientalis* has been attributed with multiple pharmacological effects, for example, antidiabetic, antihepatitis, and antidiuretic, and it is utilized to treat hyperglycaemia, hyperlipidaemia, and nephritis and for neuroprotection in TCM [24]. It has been reported that triterpenes (alisol M 23-acetate and alisol A 23-acetate) from *Alisma orientalis* act as farnesoid X receptor agonists, which may be responsible for the antidiabetic and other therapeutic effects of *Alisma orientalis* [24]. In rats with experimental nonalcoholic fatty liver disease induced by high-fat diet, administration of *Alisma orientalis* (150, 300, and 600 mg/kg) markedly decreased the serum and liver lipids, decreased plasma glucose levels, improved insulin resistance, and reduced markers of liver injury, aminotransferase abnormalities, and hepatomegaly [25].

Some other herbs such as *Ganoderma lucidum* (Ling Zhi), *Stigma maydis* (corn silk or Yu Mi Xu in Chinese), and *Morus alba L.* (Sang Ye) have also been recommended for a long time for protection of CVD, partly through their potential benefits in reducing plasma lipids and glucose levels [26–28]. In obese diabetic mice, a water extract of *Ganoderma lucidum* lowered the plasma LDL-C levels without affecting the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in both hepatic and extrahepatic organs [29]. Double-blinded, placebo-controlled, and crossover intervention studies in healthy volunteers and in patients with diabetes showed that *Ganoderma lucidum* supplement (1.44 g daily) may improve plasma lipid profiles [30, 31]. *Stigma maydis* is rich in phenolic compounds, particularly flavonoids [28]. A recent animal study showed that the flavonoids from *Stigma maydis* extract significantly lowered plasma levels of LDL-C and triglycerides in rats fed high-cholesterol diet indicating that flavonoids from *Stigma maydis* extract may have potential antihyperlipidemic effects [32]. *Morus alba L.* has been used in traditional Chinese medicine for cardiovascular, liver, and spleen disorders. Several animal studies in rats have demonstrated that *Morus alba L.* reduced plasma triglycerides, free fatty acid, and/or LDL-C [33, 34]. DNA microarray analysis revealed that mulberry leaves upregulated expression of the genes involved in the peroxisome proliferator-activated receptor signaling pathway and downregulated the genes involved in lipogenesis [33]. In another study in hamsters fed with high fat/cholesterol diets, *Morus alba L.* extract (1% and 2%) significantly reduced plasma total cholesterol and triglyceride levels by 30–37% and 16–35%, respectively [35]. Low-density lipoprotein receptor gene expression and the uptake ability of LDL in HepG2 cells were upregulated, whereas the gene expressions of enzymes involved in triglyceride and cholesterol biosyntheses were decreased with *Morus alba L.* [35]. These studies suggested that *Stigma maydis* and *Morus alba L.* can be used as natural agents against hyperlipidaemia. However, to the best of our knowledge, there is no study reporting the effect of these natural products on plasma lipid profiles in human.

The present study for the first time examined the effect of a commercially available multiherb formula containing the herbs mentioned above (Table 1) on reducing plasma lipid and glucose levels in Chinese patients with dyslipidemia.

2. Materials and Methods

2.1. Participants

2.1.1. Inclusion Criteria. Subjects aged ≥18 years with dyslipidemia (familial or nonfamilial) were recruited from the patients who were regularly attending the Lipid Clinic in the Prince of Wales Hospital, Hong Kong. Dyslipidemia was defined as either having a documented history of dyslipidemia and receiving lipid-lowering therapy or having a documented elevated baseline fasting LDL-C cholesterol (≥4.1 mmol/L) or triglycerides ≥1.7 mmol/L based on local laboratory reference values. Patients were eligible if they had

Table 1: The multiherb formula tested in the study.

Herb extracts (Chinese pinyin names)	Weight (mg per capsule)	Proportions
Crataegus pinnatifida (Shan Zha)	129	30%
Alisma orientalis (Ze Xie)	86	20%
Stigma maydis (Yu Mi Xu)	86	20%
Ganoderma lucidum (Ling Zhi)	43	10%
Polygonum multiflorum (He Shou Wu)	43	10%
Marus alba (Sang Ye)	43	10%

a plasma level of LDL-C ≥2.6 mmol/L or ≥1.8 mmol/L for those with high cardiovascular risk (because of a history of coronary heart disease (CHD), other clinical evidence of atherosclerosis, diabetes mellitus, or calculated 10-year CHD risk score >20%) following advice on a lipid-lowering diet with lipid-lowering treatment or if they had elevated plasma triglyceride concentrations (≥1.7 mmol/L) following advice on a lipid-lowering diet with or without lipid-lowering treatment.

2.1.2. Exclusion Criteria. Exclusion criteria included a history of myocardial infarction, stroke, coronary artery bypass surgery or other revascularization procedures, unstable angina, or angioplasty within 3 months of screening; elevated liver enzymes (alanine aminotransferase [ALT] > 1.5 × ULN) or renal impairment (plasma creatinine > 200 μmol/L) or uncontrolled endocrine or metabolic disease known to influence serum lipids or lipoproteins; a history of alcohol or drug abuse; a history of hypersensitivity to any of the ingredients contained in the study herb formula; women who were pregnant or lactating; initiation of lipid-lowering therapy or antidiabetic treatment within 4 weeks prior to screening. Subjects were excluded if they took weight lowering agents with 6 months prior to screening or were currently engaging in vigorous exercise or aggressive diet regimens for weight control.

The study protocol and statement of informed consent were approved by the Joint Clinical Research Ethics Committee of The Chinese University of Hong Kong and New Territories East Cluster before the start of the study. The study was conducted in compliance with the Declaration of Helsinki and all participants gave written informed consent.

2.2. Study Medication. The herbal formula (blood fat droplets (control)) and matching placebo were manufactured and supplied by Vita Green Pharmaceutical (HK) Ltd (Hong Kong, China). This product is registered for use as a natural health product in Hong Kong. Pretreated *Crataegus pinnatifida*, *Alisma orientalis*, *Polygonum multiflorum*, *Ganoderma lucidum,* and *Stigma maydis* were extracted with water : ethanol (1 : 1) at 60°C and then extracted again with water alone. The extract was then concentrated. *Morus alba* was extracted with water at 80°C and then concentrated. The extracts of *Crataegus pinnatifida, Alisma orientalis, Stigma maydis, Polygonum multiflorum*, *Ganoderma lucidum*, and *Morus alba* were mixed (in a ratio of 3 : 2 : 2 : 1 : 1 : 1), vacuum dried, and ground into powder. The dosages of each herb in the final herb formula are shown in Table 1.

Ursolic acid was used as the quality marker of this herbal product and was quantified by HPLC. The mobile phase contained methanol-water-glacial acetic acid (88 : 12 : 0.2). UV detection was performed at 215 nm. The sample was defatted with petroleum ether and extracted using diethyl ether. The solvent was evaporated and the residue dissolved in methanol for injection. The specification is >0.01% ursolic acid in the final product. Heavy metal and toxic elements including arsenic, lead, and mercury were measured using in house methods. Microbial examination (total aerobic count, moulds and yeast count, and *Escherichia coli*) and pesticides residue analysis were also performed on the samples.

The placebo capsule contained starch and artificial food colouring. The herb formula and placebo were identical in packing, appearance, and colour.

2.3. Study Protocol. This study was a prospective, randomized, double-blinded, placebo-controlled, and parallel design study. After completion of a 2-week placebo run-in period, eligible patients were randomly assigned to receive the herbal formula or placebo which was consumed twice daily with or without food, four capsules in the morning and four capsules in the evening for a period of 12 weeks.

Randomization was performed using Random Allocation Software (Version 1.0, Isfahan University of Medical Sciences, Isfahan, Iran) that allows random lists to be generated with permuted block and designated seeds. Electronic and paper records of the randomization seed number and the randomization sequence were kept in the study center for operation. The allocation sequence was generated by Miao Hu (the first author), whereas participant enrollment and assignment were conducted by Brian Tomlinson and the study nurses. The patients, investigators, and the study staff were blinded to treatment assignment until the outcome assessment was completed.

Patients were assessed at baseline and at 6 and 12 weeks after the initiation of treatment with herb formula or placebo, with the last dose being consumed the evening before the visits. Anthropometric measurements, including body weight, waist circumference, hip circumference, and estimation of percentage body fat using an impedance device (TANITA Body Composition Analyzer BF-350, Tokyo, Japan), were performed by a research nurse. Blood pressure was measured by a semiautomatic sphygmomanometer (Critikon Dinamap; GE Medical Systems Information Technologies, Louisville, KY, USA). Fasting blood samples were taken for lipid profile, fasting glucose, and laboratory safety tests at the study visit. Adherence to study medication and tolerability were assessed at study visits. All subjects were asked to maintain their usual diet and other aspects of lifestyle during the study.

2.4. Sample Size Estimation. Some previous randomized, placebo-controlled studies with *Crataegus pinnatifida* or other herbs showed that a minimal number of 10–30 patients in each group were needed to demonstrate the significant effect of herb supplements on lipid and glucose levels depending on the effect of the herbs [30, 31, 36], but clinical data on the lipid-lowering effect of this herb product or with other similar herbal formulas in patients with dyslipidemia is still lacking. Assuming that this herb formula would show a mean of 20% greater reduction in LDL-C than placebo with the SD of the % reduction in LDL-C being 4% as some animal studies showed that some of these herbs might decrease plasma lipids by up to over 30% [35], 16 subjects in each group would be needed for 80% power with $\alpha = 0.05$. Considering a drop-out rate of 20%, we planned to recruit at least 40 patients with dyslipidemia in the present study.

2.5. Biochemistry Measurements. All biochemistry tests including lipid profiles, glucose, glycated haemoglobin (HbA1c), and laboratory safety parameters (e.g., creatinine, creatine kinase, total bilirubin, alkaline phosphatase (ALP), and ALT) were performed by standard methods in the Chemical Pathology laboratory at the Prince of Wales Hospital, which has international laboratory accreditation. Total cholesterol level was measured by the enzymatic method (Centrichem Chemistry System, Baker Instruments Co. Allentown). High-density lipoprotein cholesterol (HDL-C) level was determined by using the fractional precipitation of dextran sulphate with manganous ion. Triglyceride levels were measured by the glyceryl dehydrogenase reaction following the hydrolysis of the triglyceride (Centrichem Chemistry System, Baker Instruments Co., Allentown). LDL-C concentrations were calculated according to the Friedewald formula or directly measured if the triglyceride level was greater than 4.5 mmol/L [37].

2.6. Statistical Analysis. Per protocol analysis was performed in a blind manner in 40 patients who had completed all study visits. The primary end point of the study was percentage change in LDL-C from baseline at 12 weeks. The secondary end points included percentage changes in other lipid parameters, HbA1c, fasting plasma glucose, and laboratory safety tests at 6 and 12 weeks. Continuous variables were expressed as mean ± SD unless otherwise indicated. Skewed data were logarithmically transformed before analysis. The baseline characteristics and the primary and secondary outcomes between the two treatment groups were compared using Student's *t*-test for normally distributed parameters or Mann-Whitney test for continuous variables that could not be successfully transformed into normally distributed data and chi-square tests for categorical variables. Paired-sample *t*-test was performed to assess changes of parameters within the placebo or active treatment group. Differences were considered to be statistically significant, if the two-sided *P* value is <0.05. Statistical analysis was performed using the Statistical Package for the Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA).

Table 2: Baseline characteristics of the study participants.

	Placebo ($n = 20$)	Herb formula ($n = 20$)
Age, years	54.7 ± 9.1	57.5 ± 8.4
Males, *n* (%)	10 (50)	6 (30)
Body weight, kg	71.3 ± 17.2	65.0 ± 10.7
Body mass index, kg/m^2	27.2 ± 4.7	25.9 ± 2.8
Body fat, %	31.5 ± 7.3	31.9 ± 6.1
Waist, cm	94.3 ± 12.7	87.7 ± 7.7
SBP, mmHg	119.5 ± 12.4	118.9 ± 10.8
DBP, mmHg	77.1 ± 9.6	73.6 ± 9.6
Pulse, bpm	71.1 ± 10.1	67.4 ± 7.5
Diabetes, *n* (%)	8 (40)	11 (55)
Hypertension, *n* (%)	14 (70)	10 (50)
FH, *n* (%)	7 (35)	5 (25)
On lipid-lowering treatment	9 (45)	8 (40)
On antidiabetic treatment	5 (25)	5 (25)
Baseline TC, mmol/L	5.39 ± 0.89	5.90 ± 1.17
Baseline HDL-C, mmol/L	1.29 ± 0.39	1.28 ± 0.25
Baseline TG, mmol/L	2.35 ± 1.18	2.00 ± 0.71
Baseline LDL-C, mmol/L	3.04 ± 0.82	3.72 ± 1.17*
Baseline non-HDL-C, mmol/L	4.10 ± 0.68	4.63 ± 1.13
Fasting glucose, mmol/L	5.57 ± 1.23	5.84 ± 2.17
HbA1c, %	6.57 ± 0.98	6.59 ± 0.83

DBP: diastolic blood pressure; TC: total cholesterol; FH: familial hypercholesterolaemia; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; SBP: systolic blood pressure; TG: triglycerides.
*$P < 0.05$ versus placebo.

3. Results

3.1. Characteristics of Study Participants. A total of 43 patients were recruited for the study and 1 patient withdrew consent. A total of 42 patients were randomized and two patients dropped out due to adverse effects (one in each group) (Figure 2). Forty patients completed all study-related visits. Of those patients who completed the study, 16 (40%) were male, 19 (47.5%) had type 2 diabetes, and 12 (30%) had familial hypercholesterolemia (Table 2). The mean (±SD) age was 56 ± 8.8 years and the body mass index was 26.5 ± 3.9 kg/m^2. Seventeen patients were on a background of stable lipid-lowering treatment with 14 of them receiving statins and 3 patients receiving gemfibrozil. There were no statistically significant differences in the baseline characteristics between the two groups except that the baseline LDL-C levels were higher in the active treatment groups than those in the placebo group (3.72 ± 1.17 versus 3.04 ± 0.82 mmol/L, $P < 0.05$).

3.2. Effects on Lipids and Glucose Levels. There were no significant changes in body weight between baseline and after treatment for each of the groups during the study (Table 3). The baseline LDL-C levels were not associated with

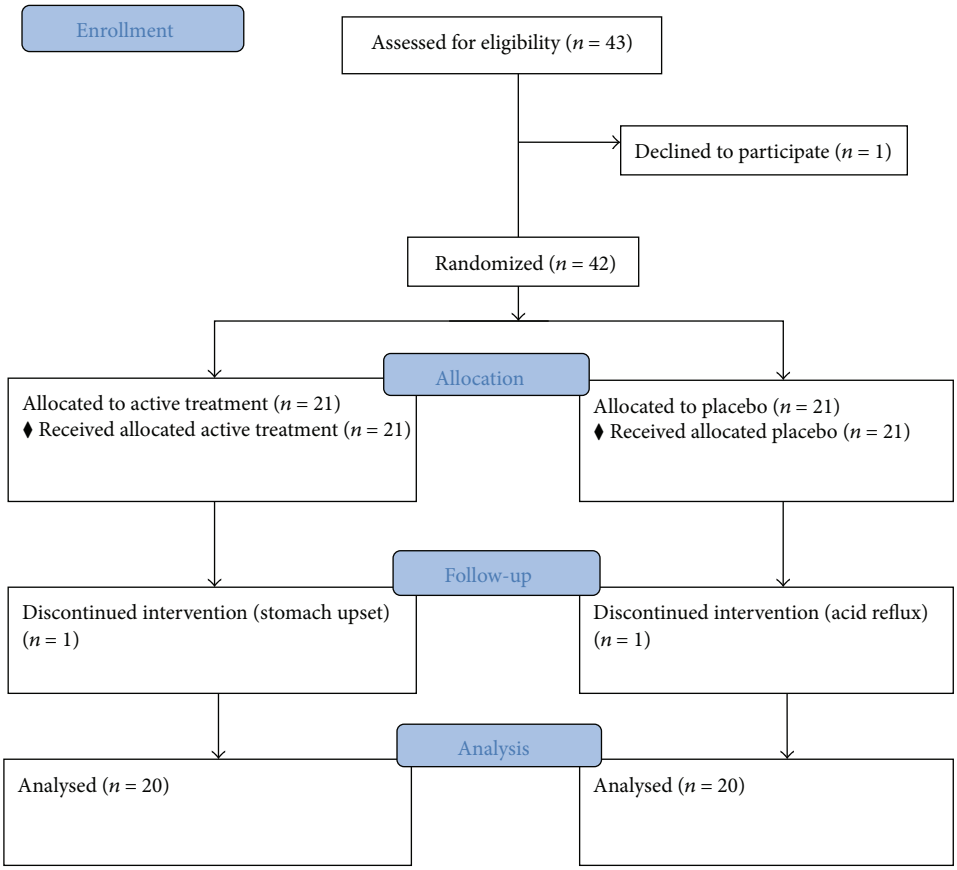

Figure 2: CONSORT flowchart of study recruitment and completion of the study.

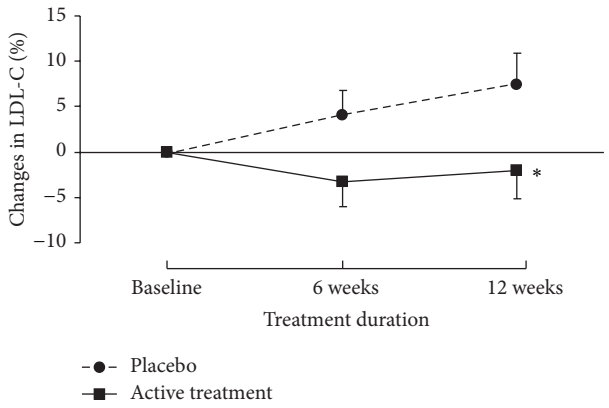

Figure 3: The percentage changes from baseline in LDL-C after 6 and 12 weeks of treatment. $^{*}P < 0.05$.

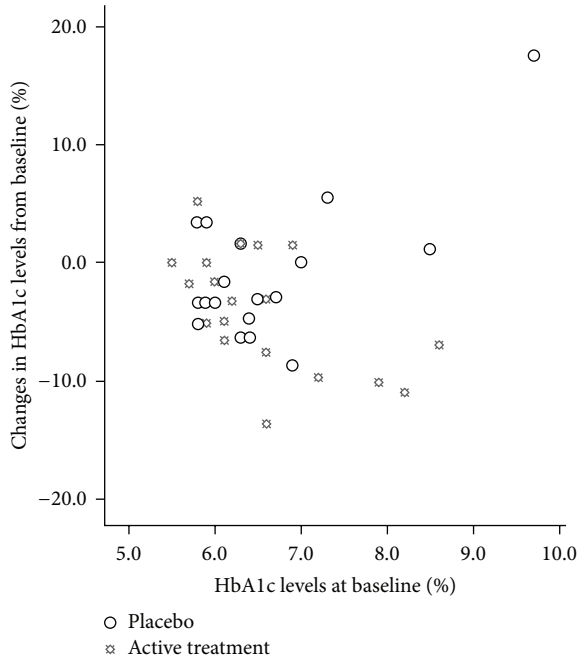

Figure 4: Association between the baseline levels of glycated haemoglobin and the percentage changes in glycated haemoglobin after 12 weeks of treatment.

the LDL-C response to the active treatment and placebo (data not shown). After 6 and 12 weeks of treatment, the LDL-C levels increased by 4% and 7.4% in the placebo group, while they decreased by −3% and −2% in the active treatment group, and the difference in changes in LDL-C at week 12 between placebo and active treatment groups was significant ($P = 0.062$ and $P < 0.05$ versus placebo, after 6 and 12 weeks,

resp.) (Figure 3). The mean level of triglycerides tended to be decreased in the placebo group and increased in the active treatment after 12 weeks of therapy, but this change was not statistically different compared to baseline or compared to the alternative regimen. The increase in the active treatment group was largely driven by an outlier who had an increase of triglycerides from 2.3 mmol/L to 8.5 mmol/L at the end of the study. His triglycerides after 6-week treatment with the herb product was 2.0 mmol/L suggesting that the increase in triglycerides at the end of study is likely related to changes in diet or other aspects of lifestyle which occurred during the longer follow-up period.

There was no significant difference in the changes in other lipid parameters and fasting plasma glucose between the two groups (Table 3). The HbA1c levels significantly decreased by −3.9% in the active treatment group after 12 weeks of treatment, but this was not significantly different from the change with placebo (−1.1%) ($P = 0.098$). There was an inverse correlation between baseline HbAlc levels and the changes in HbAlc in the active treatment group ($r = -0.565$, $P = 0.01$), so that patients with higher baseline levels tended to show a greater fall, but this was not seen in the placebo group with or without including one outlier ($P > 0.05$) (Figure 4). Background lipid-lowering treatment or antidiabetic treatment had no effect on the lipid and glucose response to the treatment (data not shown).

3.3. Adverse Effects. Both active treatment and placebo were well tolerated. Plasma biochemical parameters in the two groups are shown in Table 4. All the laboratory safety parameters were within the normal range during the study. The plasma urea level in the placebo group was increased by 10.2%, which was significantly different from the decrease of −3.6% in the active treatment group ($P < 0.05$). There was a significant increase in the plasma albumin levels in the active treatment group but no difference in the changes in albumin levels between the two groups.

A total of 28 adverse events were reported by 23 participants (12 in the placebo group and 11 in the active treatment group). Of the two patients who withdrew from the study, one patient randomized to the active treatment complained of stomach upset and one patient randomized to placebo developed acid reflux. Influenza and cough were the most common adverse events ($n = 8$) followed by shoulder or knee pain ($n = 5$) and headache ($n = 3$). None of the adverse events were considered clinically significant. There was no statistically significant difference in rates of any adverse events among the treatment groups.

4. Discussion

In this randomized clinical study of a multiherb product containing *Crataegus pinnatifida*, *Alisma orientalis*, *Stigma maydis,* and other herbs which have long been considered to have hypolipidaemic and/or hypoglycaemic effects, there was a significant improvement in plasma LDL-C levels in patients receiving the active treatment for 12 weeks compared to those assigned to placebo (difference 9.4%) and this trend was already observed at 6 weeks. However, the change in LDL-C in the active treatment group is very small and the difference in the LDL-C response in the two groups may be largely driven by the increase in the LDL-C levels in the placebo group and may be influenced by the difference in baseline LDL-C levels.

It was shown that hawthorn fruit drink 250 mL (containing 1.4 mg hawthorn flavones) twice daily significantly decreased the plasma LDL-C, apolipoprotein B, and triglycerides by 10.4%, 7.4%, and 9.3%, respectively, in 30 Chinese patients with dyslipidemia in an early uncontrolled study [22], although another placebo-controlled study showed a Chinese therapeutic food supplement with hawthorn fruit and Chinese kiwifruit-extract compound had no effect on plasma LDL-C or triglyceride levels but it increased the HDL-C by 5% in Caucasian patients with dyslipidemia [38]. Several factors may contribute to the limited lipid-lowering effect of the tested herbal product in the present study. Insufficient dosing is one of the possibilities. Furthermore, the relatively mild elevated baseline plasma levels of LDL-C and background of lipid-lowering drugs may also influence the lipid-lowering efficacy although there was no significant association between the baseline LDL-C level, the baseline lipid-lowering treatment, and the lipid responses to the herb product. Another potential confounder is that our study recruited a rather heterogeneous group of patients receiving different treatments for their comorbidities, and this may contribute to the variability of the lipid response to treatment and limit the power of the study to detect a significant effect of the supplement. However, herbal supplements are often used concomitantly with conventional drugs, especially in the elderly or those with chronic disease such as dyslipidemia and diabetes, and the goal of the study was to evaluate the real-world effect of the herbal product.

There was no statistically significant difference in the percentage change in HbA1c levels between the herb formula and placebo during the study, although there was a significant decrease in HbA1c levels in the active treatment group, particularly in patients with higher baseline levels, which is a typical finding with many antihyperglycaemic drugs. This result may suggest a potential beneficial effect of this supplement on the overall glycaemic control in patients with abnormal metabolic states of glucose regulation, for example, impaired glucose tolerance and diabetes. Several lines of evidence suggest the effectiveness of *Crataegus pinnatifida*, *Alisma orientalis*, and other herbs contained in this herb supplement on glucose and lipid metabolism [5, 6, 12, 24]. Flavonoids and triterpenes appear to be the main active components of these herbs to exert antihyperlipidaemia and antihyperglycaemia effect. However, this study was not designed to examine the effect of this herb formula in treating diabetes. Further research with larger sample size is needed to investigate the hypoglycaemic effect of this supplement in patients with diabetes.

This study has several limitations which need to be considered. Firstly, there was significant difference in the baseline LDL-C levels between the placebo group and the active treatment group. This may be related to the small sample size. In this study, patients were selected if they had

Table 3: Changes in body weight, lipids, and glucose at week 12.

	Placebo ($n = 20$)			Herb formula ($n = 20$)			P value (versus placebo)
	Baseline	Week 12	% change	Baseline	Week 12	% change	
Body weight, kg	71.3 ± 17.2	69.0 ± 20.9	−2.7 ± 15.0	65.0 ± 10.7	65.7 ± 10.0	0.8 ± 1.9	0.310
TC, mmol/L	5.39 ± 0.89	5.61 ± 1.01	4.5 ± 10.3	5.90 ± 1.17	5.96 ± 1.21	1.4 ± 10.5	0.362
HDL-C, mmol/L	1.29 ± 0.39	1.34 ± 0.46	0.7 ± 12.4	1.28 ± 0.25	1.27 ± 0.30	2.0 ± 12.4	0.276
TG, mmol/L	2.35 ± 1.18	2.21 ± 1.04	−6.7 (−18.3, 15.8)	2.00 ± 0.71	2.42 ± 1.61	5.8 (−14.8, 16.3)	0.383
LDL-C, mmol/L	3.04 ± 0.82	3.27 ± 0.95*	7.4 ± 15.5	3.72 ± 1.17	3.64 ± 1.17	−2.0 ± 13.6	0.049
Non-HDL-C, mmol/L	4.10 ± 0.68	4.28 ± 0.78	3.6 ± 9.2	4.63 ± 1.13	4.69 ± 1.16	0.4 ± 12.5	0.368
Fasting glucose, mmol/L	5.57 ± 1.23	5.80 ± 2.02	2.9 ± 12.1	5.84 ± 2.17	5.80 ± 1.19	0.3 ± 7.8	0.432
HbA1c, %	6.57 ± 0.98	6.54 ± 1.36	−1.1 ± 5.7	6.59 ± 0.83	6.31 ± 0.65**	−3.9 ± 4.9	0.098

TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides.
Data are presented as mean ± SD or median (interquartile range).
*$P < 0.05$; **$P < 0.01$ compared to baseline.

Table 4: Changes in biochemical parameters at week 12.

	Placebo ($n = 20$)			Herb formula ($n = 20$)			P value (versus placebo)
	Baseline	Week 12	% change	Baseline	Week 12	% change	
Creatinine, μmol/L	70.5 ± 13.1	68.1 ± 14.2	−3.3 ± 9.5	68.7 ± 12.5	66.7 ± 12.6	−2.5 ± 9.9	0.799
Creatine kinase, U/L	144.3 ± 72.8	135.7 ± 78.7	−1.0 (−26.8, 10.1)	144.3 ± 101.4	141.2 ± 73.6	1.2 (−7.5, 15.0)	0.563
Total protein, g/L	75.2 ± 3.3	76.1 ± 3.1	1.3 ± 3.7	74.4 ± 4.2	75.1 ± 3.3	1.1 ± 3.8	0.860
Urea, mmol/L	4.78 ± 1.18	5.13 ± 1.15	10.2 ± 24.6	5.35 ± 1.29	5.13 ± 1.36	−3.6 ± 15.6	0.041
Albumin, g/L	44.9 ± 2.4	45.3 ± 2.2	0.9 ± 4.5	43.5 ± 2.7	45.0 ± 2.6**	3.6 ± 5.4	0.092
Bilirubin, μmol/L	12.6 ± 5.5	13.1 ± 6.8	4.9 ± 25.2	12.1 ± 4.0	11.9 ± 5.2	1.4 ± 32.2	0.706
ALT, IU/L	31.4 ± 9.9	32.1 ± 14.3	3.2 ± 26.1	24.7 ± 9.8	26.6 ± 10.0	17.7 ± 60.5	0.332
ALP, U/L	73.8 ± 15.8	73.9 ± 14.2	0.8 ± 7.5	59.5 ± 16.8	60.0 ± 14.4	2.1 ± 11.5	0.672
Urate, mmol/L	0.38 ± 0.09	0.38 ± 0.08	3.1 ± 12.6	0.34 ± 0.06	0.35 ± 0.07	0.8 ± 13.2	0.567

ALT: alanine aminotransferase; ALP: alkaline phosphatase; data are presented as mean ± SD or median (interquartile range); **$P < 0.01$ compared to baseline.

either elevated LDL-C and/or elevated triglycerides, and thus they may have different types of dyslipidemia, contributing to the different lipid profiles of the two groups in this small study. The small sample size is another major limitation of the study. The studied herb formula only showed a marginal effect on plasma LDL-C levels compared to placebo and the study is underpowered to detect small differences between the two groups. In addition, it would be useful to measure various apolipoprotein levels, for example, apoAI, apoB, and apoE, although these apolipoprotein levels are usually closely associated with particular plasma lipid levels. It would be helpful to identify and quantify the active components in this herbal formula and to test whether these active components reduce the plasma lipids in a dose-dependent manner, but the study was designed to examine the real-world lipid-lowering effect of this commercially available herbal product in patients with dyslipidemia using a recommended dose, and thus the dose-response was not assessed.

In conclusion, this randomized, placebo-controlled study conducted in an ambulatory outpatient setting showed a marginal beneficial effect of the multiherb formula on reducing plasma LDL-C levels in subjects with dyslipidemia without any noticeable adverse effects. The finding was consistent with some of the previous experimental *in vitro* and animal studies with these herbs. Further well-designed clinical studies are warranted to support or refute the clinical use of herbal medicines in reducing cardiovascular risk.

Conflict of Interests

The authors have declared that there is no conflict of interests.

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Research Article

Ganoderma lucidum Polysaccharides Reduce Lipopolysaccharide-Induced Interleukin-1β Expression in Cultured Smooth Muscle Cells and in Thoracic Aortas in Mice

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The expression of inflammatory cytokines on vascular walls is a critical event in vascular diseases and inflammation. The aim of the present study was to examine the effects of an extract of *Ganoderma lucidum* (Reishi) polysaccharides (EORPs), which is effective against immunological disorders, on interleukin- (IL-) 1β expression by human aortic smooth muscle cells (HASMCs) and the underlying mechanism. The lipopolysaccharide- (LPS-) induced IL-1β expression was significantly reduced when HASMCs were pretreated with EORP by Western blot and immunofluorescent staining. Pretreatment with 10 μg/mL EORP decreased LPS-induced ERK, p38, JNK, and Akt phosphorylation. But the increase in IL-1β expression with LPS treatment was only inhibited by pretreatment with the ERK1/2 inhibitor, while the JNK and p38 inhibitors had no effect. In addition, EORP reduced the phosphorylation and nuclear translocation of nuclear factor- (NF-) κB p65 in LPS-treated HASMCs. Furthermore, *in vivo*, IL-1β expression was strongly expressed in thoracic aortas in LPS-treated mice. Oral administration of EORP decreased IL-1β expression. The level of IL-1β expression in LPS-treated or in LPS/EORP-treated group was very low and was similar to that of the saline-treated group in toll-like receptor 4-deficient ($TLR4^{-/-}$) mice. These findings suggest that EORP has the anti-inflammatory property and could prove useful in the prevention of vascular diseases and inflammatory responses.

1. Introduction

The inflammatory processes have a key role not only in the initiation and progression of atherosclerosis, but also in the stability of the established atherosclerotic plaques [1, 2]. Cytokines of the interleukin-1 (IL-1) family play a pivotal role in regulating the immunoinflammatory responses, and extensive studies have been performed to determine whether IL-1 modifies the inflammatory response [3, 4]. IL-1β upregulates a substantial increase in the expression of adhesion

molecules by vascular smooth muscle cells (VSMCs) *in vitro*, and these factors promote monocyte recruitment and infiltration into the arterial wall [5, 6] and induce VSMC migration and proliferation [3, 7, 8]. *In vivo* studies have shown increased levels of IL-1β mRNA in human atherosclerotic lesions [9] and of IL-1β mRNA and protein in VSMCs of atherosclerotic arteries from non-human primates [10]. Although the relationship between IL-1β and cardiovascular disease has been extensively studied, IL-1β expression and its related mechanism in LPS-induced human aortic smooth muscle cells (HASMCs) and in inflammatory vascular walls have not been studied in detail. Moreover, reducing IL-1β expression might be a beneficial approach for inhibiting the development of vascular inflammation and atherosclerosis [11].

Ganoderma lucidum (*G. lucidum*, Reishi), a popular home remedy, has long been known for its beneficial effects on human health and longevity and is used to treat chronic hepatopathy, hypertension, hyperglycemia, and neoplasia [12, 13]. *Ganoderma lucidum* intake caused an increase in plasma antioxidant capacity, and that 10 d supplementation was associated with a trend towards a decrease of plasma lipids in human subjects [14, 15]. Studies on the role of *G. lucidum* in regulating various body functions have revealed that *G. lucidum* polysaccharides are the bioactive constituents responsible for many of their health benefits, such as its antioxidant, anticancer, anti-inflammatory, and immunomodulatory activities [16–18]. The effects of *G. lucidum* on the immune system have been linked to the regulation of cytokine expression and differentiation of macrophages [19, 20] and the maturation of cultured murine bone marrow-derived dendritic cells [21]. *G. lucidum* have also been shown to suppress TNF-α expression from lipopolysaccharide- (LPS-) stimulated murine RAW 264.7 cells [22], reduce IL-1β secretion from activated microglia after LPS treatment [23], and prevent albumin-induced oxidative damage and chemokines synthesis in human proximal tubular epithelial cells [24]. Regulation of inflammatory cytokine expression requires a complex array of intracellular signaling pathways involving mitogen-activated protein kinases (MAPKs) and transcriptional factors [25, 26]. Although these multiple signaling molecules have received considerable attention, little is known about the effects of an extract of Reishi polysaccharides (EORPs) on IL-1β expression and the mechanisms of these effects, and a better understanding of this might provide important insights into the prevention of cardiovascular diseases and inflammation. We were therefore interested in understanding the mechanism of action of EORP on human aortic smooth muscle cells (HASMCs) stimulated by inflammatory cytokines and whether it affects the IL-1β expression, an important event in vascular diseases and inflammation. In addition, we studied the effects of EORP on IL-1β expression in thoracic aortas in LPS-treated mice. Our results showed that EORP attenuated IL-1β expression both *in vitro* and *in vivo* and that this effect was mediated, at least in part, through inhibition of extracellular signal-regulated kinase (ERK) phosphorylation, nuclear factor NF-κB activation, and TLR4 activation.

2. Materials and Methods

2.1. Preparation of EORP. A crude powder of *G. lucidum* prepared via alkaline extraction with 0.1 N NaOH, followed by neutralization and ethanol precipitation, was obtained from Pharmanex (Provo, UT). The biologically active compounds from Reishi are identified as the fucose-containing glycoprotein fraction and named EORPs. The molecular structure and the quality control of EORPs were described in detail in the previous reports [13, 19]. In brief, the carbohydrate composition analyses of crude water soluble Reishi extract indicated that glucose, mannose, and fucose exist as the major components. The crude extract contains 15.6% proteins. The saccharides contain either a polysaccharide backbone with β-1,3-linkages or a polymannose backbone with α-1,4-linkages, both with side chains of unknown structure. EORPs were prepared as GMP grade and carefully monitored to meet the Food and Drug Administration standard. The EORP is currently commercially available as the traditional Chinese medicine.

2.2. HASMC Cultures. HASMCs, purchased as cryopreserved tertiary cultures from Cascade Biologics (OR, USA), were grown in culture flasks in smooth muscle cell growth medium (M231, Cascade Biologics Inc.). The cells were used between passages 4 and 8. All cells were synchronized in serum-free medium for 24 h prior to experiments. For all data shown, the experiments were repeated at least 3 times in duplicate with different preparations of HASMCs. For Western blot analysis, the graphical analysis represents the results from three independent experiments and quantification by densitometry.

2.3. Effect of LPS and EORP on Cell Viability. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay. HASMCs were plated in 96-well plates (1×10^4 cells/well). After serum starvation, the cells were treated with different concentrations of LPS or EORP for 24 h, and then MTT solution was added to each well and incubated for another 2 h at 37°C in 5% CO_2. The supernatant was discarded and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The cellular viability was measured by ELISA plate reader at 540 nm.

2.4. Western Blot Analysis. Western blot analyses were performed as described previously [27]. In brief, samples of cell lysate (20 μg of protein) were subjected to 12% SDS-PAGE and transferred to PVDF membranes, which were then treated with 5% nonfat milk in 0.1 M phosphate buffer for 1 h at RT to block nonspecific binding of antibody. To measure IL-1β levels, the membranes were then incubated with rabbit anti-human IL-1β (1 : 1000 dilution, GeneTex, Irvine, CA, USA) and then with horseradish peroxidase- (HRP-) conjugated goat anti-rabbit IgG antibodies (1 : 6000 dilution, KPL, Gaithersburg, MA, USA) and bound antibody was detected using Chemiluminescence Reagent Plus. In other studies, the antibodies used were rabbit anti-human phospho-Jun N-treminal kinase (JNK), rabbit anti-human

phopho-ERK1/2, rabbit anti-human phospho-p38 (all 1 : 1000 dilution, Cell signaling, Danvers, MA, USA), rabbit anti-human phospho-p65, and rabbit anti-human p65 (all 1 : 1000 dilution, GeneTex) followed by HRP-conjugated goat anti-rabbit IgG antibodies. The intensity of each band was quantified using a densitometer. Antibodies against β-actin, α-tubulin, or GAPDH (all 1 : 1000 dilution, Gene Tex) were used as loading controls.

2.5. Immunocytochemical Staining. HASMCs were plated at a density of 10^4 cells/well in gelatin-coated coverslips. After starvation, the cells were treated with various concentrations and time of LPS and EORP. After treatment, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then incubated with IL-1β and NF-κB p65 antibodies at 4°C overnight. After incubation, the cells reacted with FITC-conjugated anti-rabbit IgG (both from Sigma) for 1 h at room temperature. DAPI stain was used as the nuclear counterstain and observation was conducted by fluorescence microscope.

2.6. IL-1β Enzyme-Linked Immunosorbent Assay (ELISA). The concentrations of IL-1β in cultured media of smooth muscle cell and in plasma from mice were determined using the human and mouse IL-1β ELISA kits (R&D Systems, Minneapolis, MN), respectively. The analysis was performed according to the instructions from the manufacturer.

2.7. Knockdown of Gene Expression Using Small Interfering RNA. Knockdown of ERK gene expression was performed by transfection with small interfering RNA (siRNA, Invitrogen, Carlsbad, CA, USA). HASMCs (10^6) were resuspended in 100 μL of Nucleofector solution (Amaxa Biosystems, Germany), and gene-specific ERK siRNA oligomers (1 μM) were electroporated according to the manufacturer's instruction manual. The ERK siRNAs (catalog number 10620319 124945 F11 and 10620318 124945 F12, Invitrogen) were AUA UUC UGU CAG GAA CCC UGU GUG A and UCA CAC AGG GUU CCU GAC AGA AUA U. Cells were transfected for 48 h. The siRNA results were evaluated by Western blotting.

2.8. RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay. Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The reverse-transcriptase reaction was carried out using M-MLV reverse transcriptase (Invitrogen). Complementary DNA was generated by addition of total RNA (1 μg) to a reaction mixture containing 0.5 μg/μL oligo-deoxythymidine, 20 mM dNTP, 0.1 M dithiothreitol, 250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$ and reaction at 37°C for 90 min. The oligonucleotide primers used were 5′-CAGACCATGATCACACAGGG-3′ (forward) and 5′-TGGAAAGATGGGCCTGTTAG-3′ (reverse) for ERK and 5′-GTAACCCGTTGAACCCCATT-3′ (forward) and 5′-CCATCCAATCGGTAGTAGCG-3′ (reverse) for 18 S subunit ribosomal RNA. The PCR conditions were an initial denaturation at 94°C for 5 min, followed by 29 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 5 min. The products of PCR were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

2.9. Mouse Model. All animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal care and Use Committee of the National Taiwan University (Permit number: 20110011). Eight-week-old male C57BL6J mice and toll-like receptor 4-deficient ($TLR4^{-/-}$) mice, weighing between 25 and 35 g, were used in the present study. To examine the effects of EORP administration on thoracic aortas in the LPS-treated mice, the C57BL6J mice were divided into two divisions. One division is defined as the acute inflammatory phase (2 days) and the other one is the chronic inflammatory phase (2 weeks). Each division was further divided into four groups. Groups 1 and 2 were given, respectively, either saline (control) or 1 mg/kg/day of LPS on day 1 or on days 1–13 by intraperitoneal (ip) injection, and group 3 was treated with LPS in the same way as group 2 but received oral EORP at 60 mg/kg/day on days 0-1 (acute inflammatory phase) or on days 0–13 (chronic inflammatory phase), and group 4 received only the oral EORP. Furthermore, the $TLR4^{-/-}$ mice were divided into 3 groups; groups 1–3 were treated the same way as groups 1–3 of the chronic inflammatory division. The selection of EORP dose was based on the previous reports [17, 27]. At the end of the 2 days or 2 weeks of the experiments, the animals were anesthetized by ip injection of 60 mg/kg sodium pentobarbital and sacrificed. Blood samples were collected, and soluble IL-1β in the plasma was measured by ELISA. One segment of the thoracic aortas was rinsed with ice-cold PBS, immersion-fixed with 4% buffered paraformaldehyde and paraffin-embedded, and then cross-sectioned for immunohistochemistry, while the remaining portion was immediately frozen in liquid nitrogen for protein extraction. The tissue sections (5-6 μm thick) were mounted on poly-L-lysine coated slides, deparaffinized, rehydrated, and washed with PBS. To study cellular expression and localization of IL-1β, serial sections were incubated with 1% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity and to permeabilize the cells; then nonspecific binding was blocked by incubation for 1 h at RT with PBS containing 5 mg/mL of bovine serum albumin. In the primary antibody step at 37°C for 1 h, the first serial section was incubated with goat anti-human CD31 antibody (EC marker, 1 : 100, Santa cruz, CA, USA), the second with goat anti-human IL-1β antibody (1 : 50), and the third with mouse anti-α-smooth muscle actin (SMC marker, 1 : 50, Dako Cytomation, CA, USA). The first and second slides were localized by an indirect immunoperoxidase technique (avidin-biotin-horseradish peroxidase complex) employing diaminobenzidine (Vector) as chromogen, while bound antibodies on the third section were then incubated for 1.5 h at RT with FITC-conjugated goat anti-mouse secondary antibody (1 : 400, Sigma Chemical Co., St. Louis, MO, USA) and observed by fluorescent microscopy. Each incubation was followed by three times of 5 min washes in PBS. Negative controls were performed by omitting the primary antibody.

2.10. Statistical Analysis. Values are expressed as the mean ± SEM. Student's t-test and one-way ANOVA were performed to compare the two groups and multiple groups, respectively. A P value <0.05 was considered statistically significant.

3. Result

3.1. EORP Reduces IL-1β Expression in LPS-Treated HASMCs. When the cytotoxicity of LPS or EORP for HASMCs was detected by the MTT assay after 24 h of incubation, cell viability was not affected by the presence of 1–15 μg/mL of LPS or 1–10 μg/mL of EORP (data no shown).

To determine the optimal conditions for LPS-induced IL-1β expression by HASMCs, we first performed dose-response studies in which HASMCs were cultured with various concentrations of LPS for various time intervals. As shown in Figure 1(a), IL-1β expression in HASMCs with 10 μg/mL LPS treatment reached a maximum after 24 h (2.3 ± 0.2). IL-1β expression was induced in a dose-dependent manner after treatment with 5, 10, or 15 μg/mL of LPS for 24 h (1.1 ± 0.2, 1.7 ± 0.1, 2.3 ± 0.1, resp., of control levels) (Figure 1(b)). The induction caused by the two highest concentrations was significant. As shown in Figure 1(c), when the HASMCs were pretreated with 10 μg/mL of EORP for 24 h before incubation with 10 μg/mL of LPS for 24 h, LPS-induced IL-1β expression was reduced to 0.9 ± 0.1 of control levels. The effect of EORP on IL-1β expression was also studied by immunofluorescence staining (Figure 1(d)). In untreated cells, IL-1β expression was weak. In contrast, cells treated for 24 h with LPS showed strong IL-1β expression and this effect was inhibited by pretreatment with EORP. Furthermore, unstimulated HASMCs produced low amounts of IL-1β in the cell culture supernatant (80 ± 23 pg/mL), and 24 h treatment with 10 μg/mL of LPS resulted in a marked increase in IL-1β levels (653 ± 90 pg/mL) by ELISA. This effect was significantly inhibited by 24 h preincubation with 10 μg/mL of EORP (398 ± 50 pg/mL). In all subsequent experiments, unless otherwise specified, 10 μg/mL LPS and 10 μg/mL EORP were used.

3.2. EORP Attenuated LPS-Induced IL-1β Expression is Partly Dependent on Inhibition of ERK1/2 Phosphorylation. The previous studies showed that LPS can activate MAPKs and Akt in the signaling pathway leading to inflammation [26, 28]. In the next set of experiments, the effects of LPS on the activation of the MAPK pathway (ERK1/2, JNK, p38) and Akt, a signaling cascade contributing to IL-1β expression, and the effects of EORP or MAPK inhibitors on LPS-stimulated IL-1β expression were studied. As shown in Figures 2(a)–2(d), phosphorylation of ERK1/2, p38, JNK, and Akt was significantly increased compared with that of control levels, respectively, at 30 min after addition of LPS. Pretreatment with 10 μg/mL EORP decreased LPS-induced ERK, p38, JNK, and Akt phosphorylation. As shown in Figure 2(e), the increase in IL-1β expression in response to LPS treatment was inhibited by 1 h pretreatment with 30 μM PD98059 (an ERK1/2 inhibitor), while SP600125 (a JNK inhibitor) or SB203580 (a p38 inhibitor) had no effect. The inhibitory effect of ERK1/2 was further confirmed by the transfection of ERK siRNA. The effectiveness of the siRNA treatment was validated by showing that ERK-specific siRNA caused a 50% reduction in ERK protein expression (Figure 2(f)). LPS-induced IL-1β expression was inhibited by transfection of HASMCs with ERK1/2-specific siRNA (1 μM) (Figure 2(g)). Additional experiments on ERK gene expression showed that it was detected in untreated cells; however, both LPS and EORP treatments did not affect its expression (Figure 2(h)). On the other hand, ERK-specific siRNA treatment decreased ERK gene expression. Both LPS and EORP treatments also did not alter its gene expression. These results indicate that EORP inhibits LPS-induced IL-1β expression by preventing LPS-induced phosphorylation of ERK1/2.

3.3. EORP Attenuates Phosphorylation and Nuclear Translocation of NF-κB p65 in LPS-Treated HASMCs. Transcriptional regulation involving NF-κB activation has been implicated in the stimulator-induced expression of inflammatory cytokines [25]. As shown in Figure 3(a), low basal levels of NF-κB p65 phosphorylation were detected in control cells, and phosphorylation was significantly increased by 90 min treatment with LPS. This increase was markedly inhibited by preincubation with 10 μg/mL EORP for 24 h. To determine whether NF-κB p65 translocation was involved in the pretranslational effects of EORP on IL-1β expression, we also studied NF-κB p65 protein levels in the nuclei of LPS-treated HASMCs by immunofluorescent staining. As shown in Figure 3(b), LPS-stimulated HASMCs showed marked NF-κB p65 staining in the nuclei, while EORP-pretreated cells showed weaker nuclear NF-κB expression, but stronger staining in the cytoplasm. Furthermore, the stimulatory effect of LPS on IL-1β levels was blocked by coincubation with parthenolide, a NF-κB inhibitor (Figure 3(c)). These results suggest that EORP inhibits LPS-induced IL-1β expression by preventing LPS-induced phosphorylation and translocation of NF-κB p65.

3.4. EORP Decreases IL-1β Protein Expression in Plasma and in Thoracic Aortas of LPS-Injected Mice. To determine the effect of EORP on IL-1β expression *in vivo*, mice were orally fed with EORP (60 mg/kg/day) prior to treatment with ip injection of LPS (1 mg/kg/day). Serial sections of thoracic aortas were performed to detect IL-1β expression and its association with endothelial cells and smooth muscle cells by using CD31 and α-actin antibodies as cell markers, respectively. As shown in Figure 4, in the saline- and EORP-treated groups, weak IL-1β staining was seen on the vascular wall, while in the LPS-treated group, strong IL-1β staining was seen on the vascular walls at the acute inflammatory phase. IL-1β expression was associated with endothelial cells and smooth muscle cells. Interestingly, IL-1β expression was mainly present in smooth muscle cells of the media rather than in endothelial cells of the intima. In contrast, administration of EORP resulted in weak IL-1β staining in the LPS-treated mice. The corresponding levels of IL-1β expression were similarly found in plasma and in thoracic aortas by ELISA (Figure 4(b)) and by Western blot (Figure 4(c)), respectively. Furthermore, in the LPS-treated

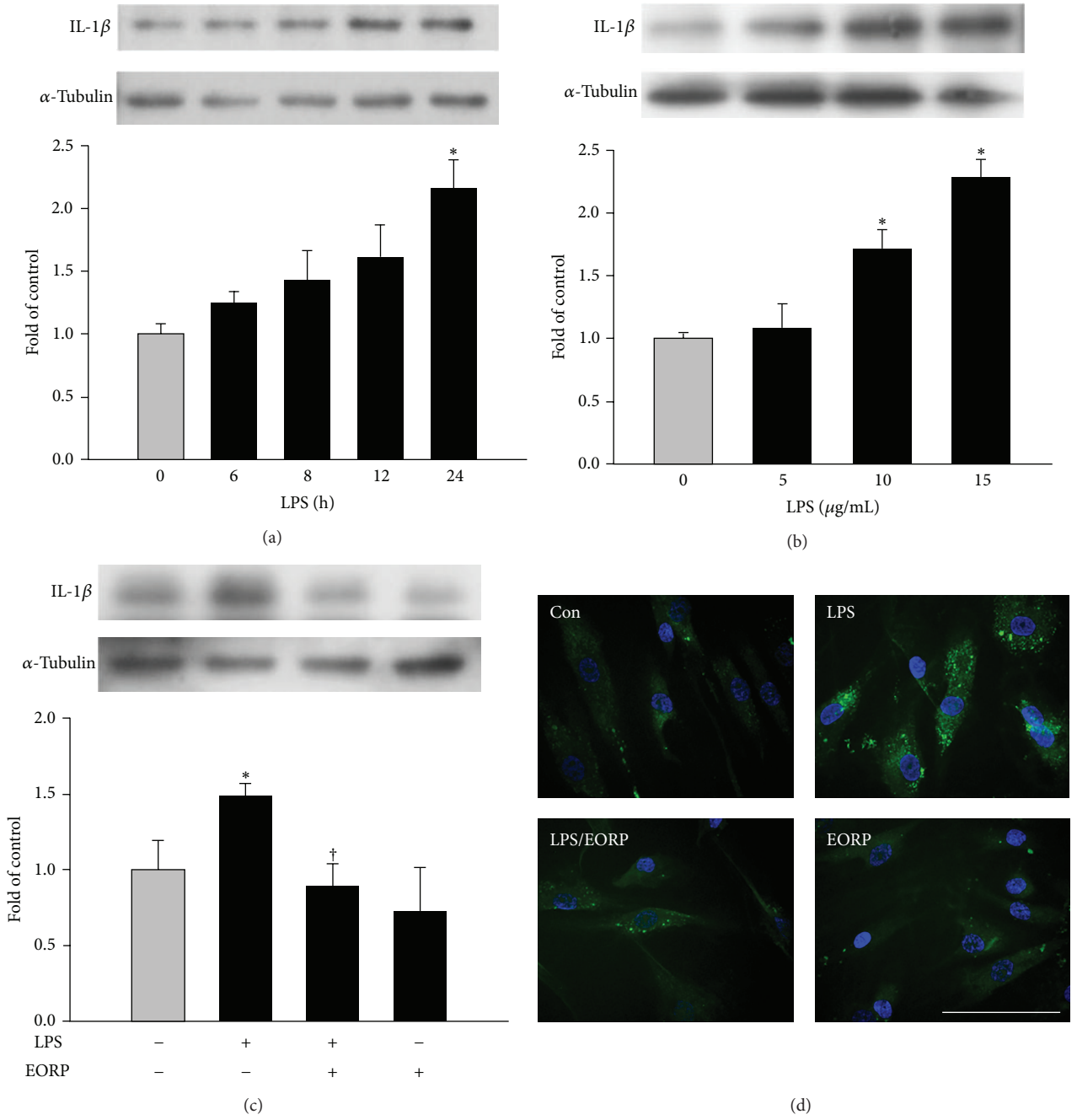

Figure 1: EORP reduces IL-1β protein expression in LPS-treated HASMCs. (a) and (b) HASMCs were treated with 10 μg/mL of LPS for the indicated time (a) or with the indicated concentration of LPS for 24 h (b). (c) HASMCs were incubated for 24 h with 10 μg/mL of EORP and then for 24 h with 10 μg/mL of LPS in the continued presence of the same concentration of EORP, and then IL-1β expression was measured in cell lysates by Western blotting. α-Tubulin was used as the loading control. (d) The cells were treated as in panel (c), and then the distribution of IL-1β was analyzed by immunofluorescent microscopy. IL-1β expression is indicated by green fluorescence (FITC) and nuclei by blue fluorescence (DAPI). Bar = 100 μm. In panels (a), (b), and (c), the data are expressed as a fold value compared to the control value and are the means ± SEMs ($n = 3$). $^{*}P < 0.05$ as compared to the untreated (control) cells. $^{\dagger}P < 0.05$ as compared to the LPS-treated cells.

group strong IL-1β staining was still seen on the vascular walls at the chronic inflammatory phase as shown in Figure 5. The administration of EORP resulted in weak IL-1β staining in the LPS-treated mice. ELISA (Figure 5(b)) showed higher levels of IL-1β expression in the LPS-treated group, and EORP treatment notably reduced IL-1β expression. The concentration of IL-1β in the chronic inflammatory phase was much higher than that in the acute inflammatory phase. Consistent with the *in situ* findings and ELISA, Western blot (Figure 5(c)) showed that IL-1β was significantly expressed

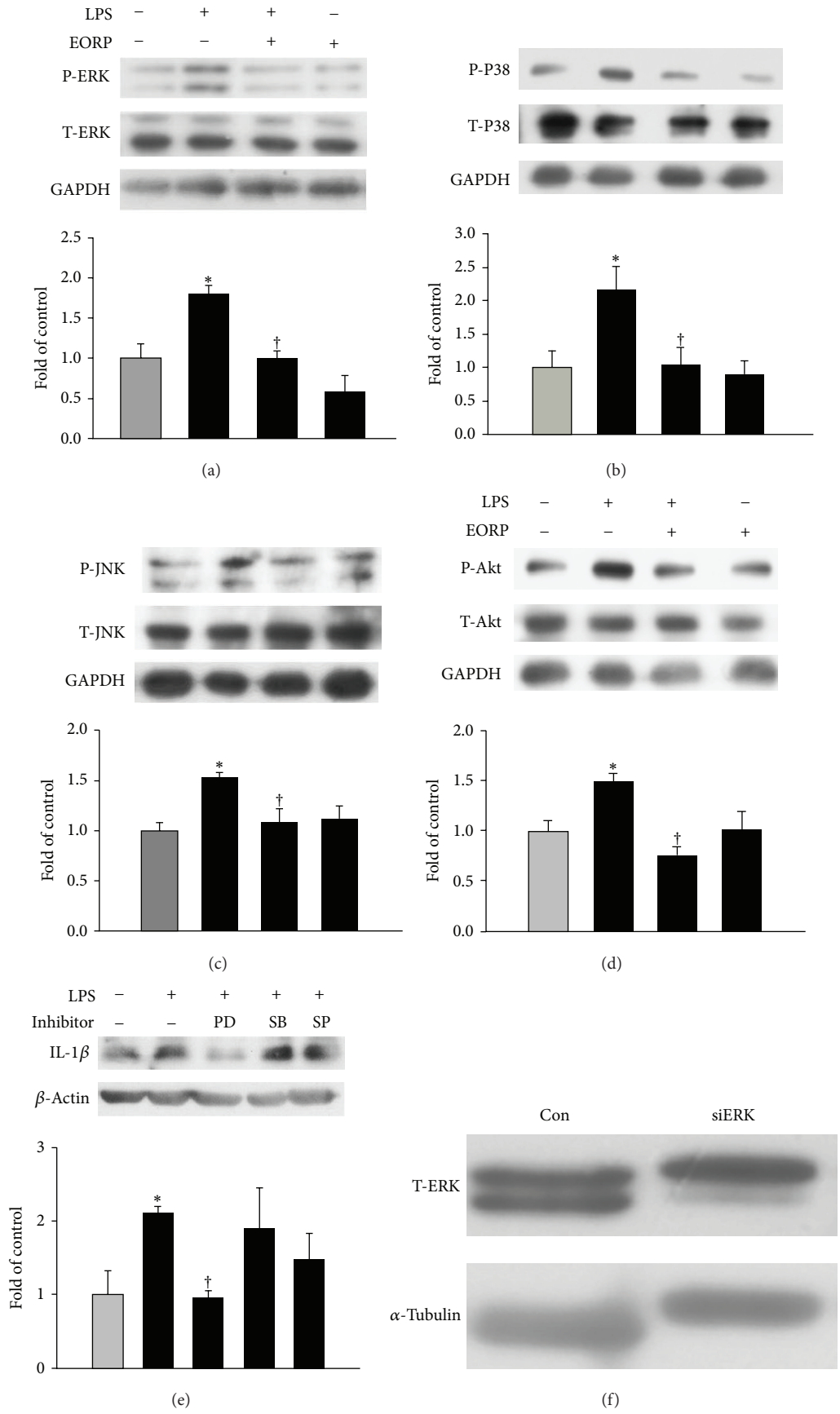

Figure 2: Continued.

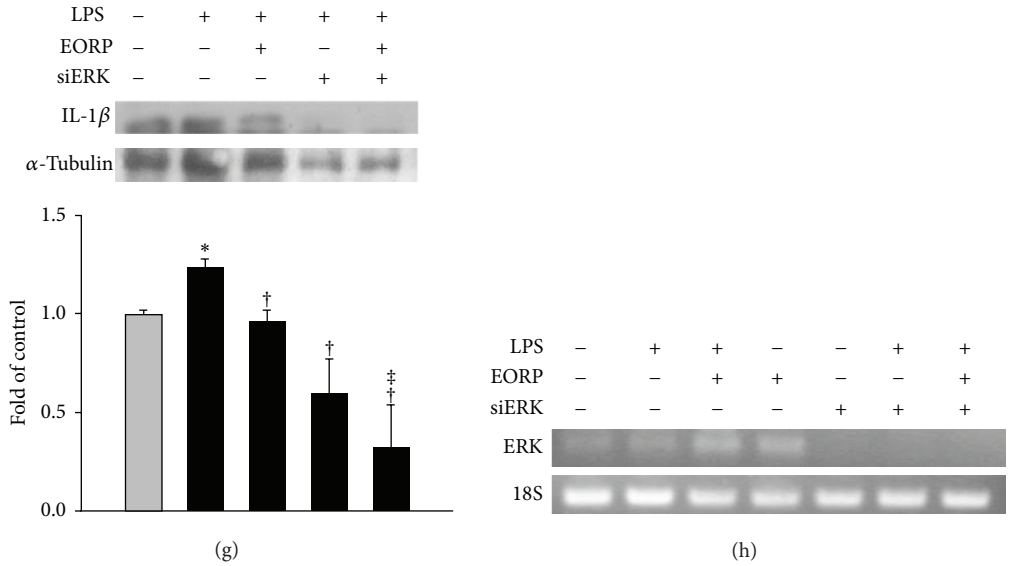

Figure 2: EORP-mediated reduction in LPS-induced IL-1β expression is partly dependent on inhibition of phosphorylation of ERK. (a)–(c) Western blot analysis showing the effect of EORP pretreatment on the phosphorylation of (a) ERK1/2, (b) p38, (c) JNK, or (d) Akt in LPS-treated HASMCs. HASMCs were incubated for 24 h with or without 10 μg/mL of EORP, and then the cells were incubated with 10 μg/mL of LPS for 30 min and aliquots of cell lysate containing equal amounts of protein subjected to immunoblotting with the indicated antibodies. (e) Effect of inhibitors of MAPK phosphorylation on IL-1β expression in control and LPS-treated HASMCs. HASMCs were incubated for 1 h with 30 μM PD98059 (an ERK1/2 inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor) and then for 24 h with or without 10 μg/mL of LPS in the continued presence of the inhibitor and then IL-1β expression was measured by Western blotting. (f) ERK-specific siRNA caused a 50% reduction in ERK protein expression by Western blotting. (g) The LPS-induced increase in IL-1β expression is inhibited by transfection of HASMCs with ERK1/2-specific siRNA (1 μM). HASMCs were transfected with either control siRNA or ERK1/2-specific siRNA (1 μM) for 48 h then were incubated with 10 μg/mL of EORP for 24 h and then with 10 μg/mL of LPS for 24 h in the continued presence of the same concentration of EORP, and IL-1β expression was measured in cell lysates by Western blotting. (h) ERK gene expression under different treatments was analyzed by the RT-PCR assay as described under Section 2. In panels (a)–(e) and (g), the data are expressed as a fold of the control value and are the means ± SEMs (n = 3). Total ERK and GAPDH, total p38 and GAPDH, total JNK and GAPDH, total Akt and GAPDH, β-actin, and α-tubulin were used as the loading control for panels (a), (b), (c), (d), (e), (f), and (g), respectively. $^*P < 0.05$ as compared to the untreated (control) cells. $^\dagger P < 0.05$ as compared to the LPS-treated cells.

in the LPS-treated group and EORP treatment reduced the expression.

3.5. EORP Regulating LPS-Induced IL-1β Expression Was Mediated through the TLR4 Receptor. The effects of LPS or EORP on the cytokine expression via TLR4-modulated protein kinase signaling pathways were studied by immunofluorescent microscopy. In untreated cells, TLR4 expression was weak (Figure 6(a)), whereas cells treated for 24 h with LPS showed strong TLR4 expression and this effect was reduced by pretreatment with EORP. To elucidate whether EORPs modulate LPS-induced IL-1β expression through the TLR4 receptor, TLR4$^{-/-}$ mice were used in the present study. As shown in Figures 6(b)–6(d), the level of IL-1β expression in LPS-treated or in LPS/EORP-treated group was very low and was similar to that of the saline-treated group examined by immunofluorescent staining, ELISA, and Western blot, respectively. The expression of TLR4 on TLR4$^{-/-}$ mice was confirmed by Western blot (Figure 6(e)). Based on these findings, the LPS-induced IL-1β expression was mainly mediated through the activation of TLR4 receptor.

4. Discussion

Herein, we demonstrated that EORP treatment effectively blocked IL-1β expression both *in vitro* in LPS-stimulated HASMCs and *in vivo* in thoracic aortas of LPS-treated mice. EORP decreased IL-1β expression in LPS-treated HASMCs, and the effect might be mediated through inhibition of ERK phosphorylation, NF-κB activation, and TLR4 receptor pathway.

Reishi extract was chosen for testing, as it has long been known as a healthy food and used as traditional Chinese medicines. Its beneficial effects are thought to be due to its anti-inflammatory, antitumor, antioxidant, and immunomodulatory actions [16–18]. A *Ganoderma* extract prevented albumin-induced oxidative damage of proximal tubular epithelial cells in an experimental setting, mimicking the proteinuric state [24], and reduced LPS-induced superoxide anion production by macrophages [29]. The triterpene extract from *G. lucidum* (GLT) suppressed the inflammatory response *in vitro* and *in vivo* by downregulating the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) and the production of TNF-α and IL-6

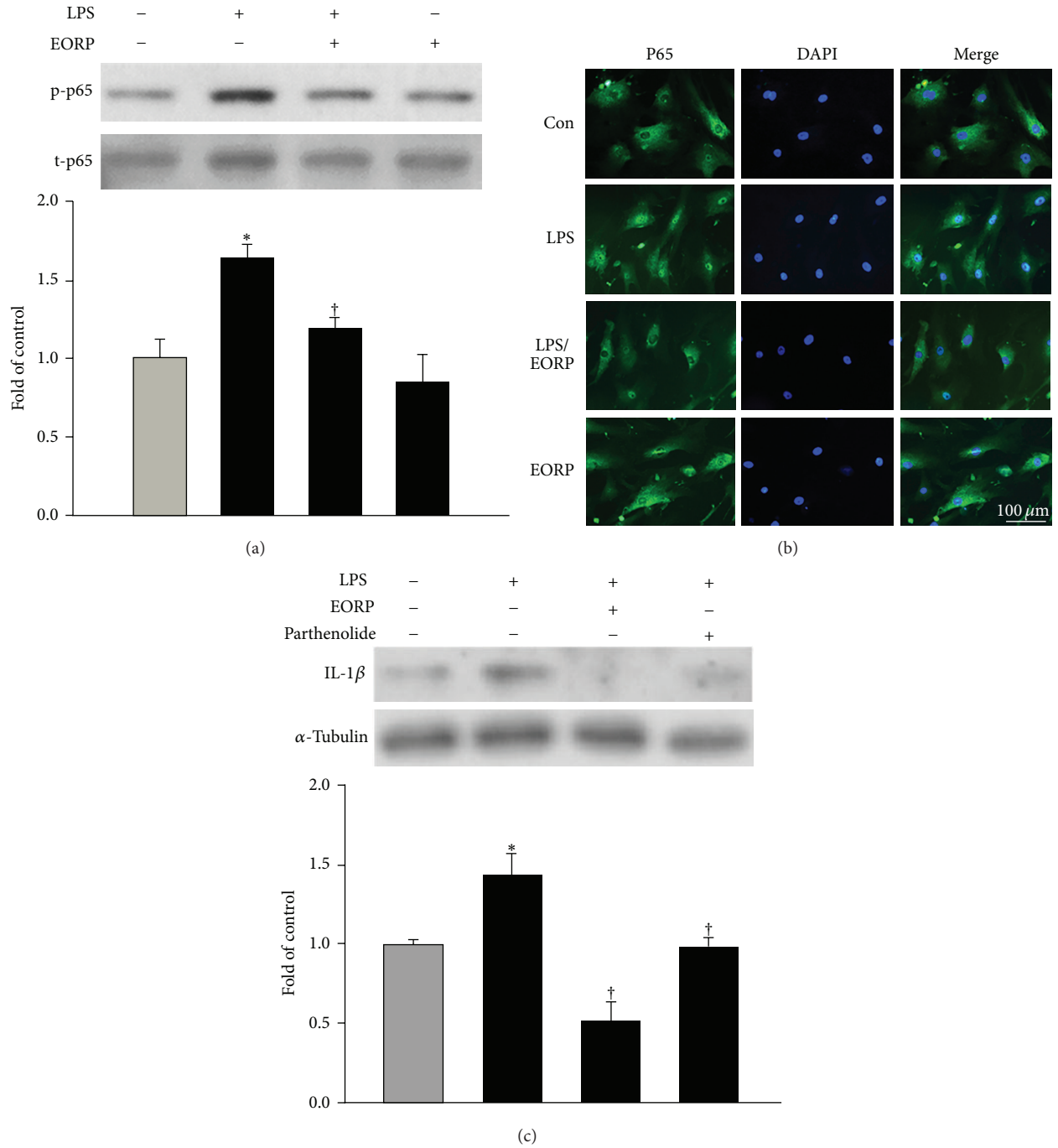

Figure 3: EORP-induced reduction in the upregulation of IL-1β expression in LPS-treated HASMCs is mediated by inhibition of both NF-κB p65 phosphorylation and nuclear translocation. (a, b) Western blotting and immunofluorescent staining for NF-κB p65. HASMCs were left untreated or incubated for 24 h with or without 10 μg/mL EORP and then with or without 10 μg/mL of LPS for 90 min in the continued presence of the EORP. (a) The phosphorylation of NF-κB p65 expression is examined by Western blotting. (b) NF-κB p65 expression is indicated by green fluorescence (FITC) and nuclei by blue fluorescence (DAPI). A representative result from three separate experiments is shown. Bar = 100 μm. (c) Cells were incubated for 1 h with 30 μM parthenolide (NF-κB inhibitor) and then coincubated for 24 h with 10 μg/mL of LPS, and then cell lysates were prepared and assayed for IL-1β on Western blots. The data are the mean ± SEM ($n = 3$). $^{*}P < 0.05$ as compared to the untreated (control) cells; $^{\dagger}P < 0.05$ as compared to the LPS-treated cells.

in LPS-induced endotoxemic mice [22]. *Ganoderma lucidum* extracts inhibited the production of microglia-derived proinflammatory and cytotoxic factors, including nitric oxide, TNF-α, and IL-1β in activated microglia [23]. *G. lucidum* polysaccharide-linked peptide reduced the production of proinflammatory cytokines (interleukin-6 and monocyte chemoattractant protein-1) by activated rheumatoid synovial fibroblasts [30]. Our previous report demonstrated

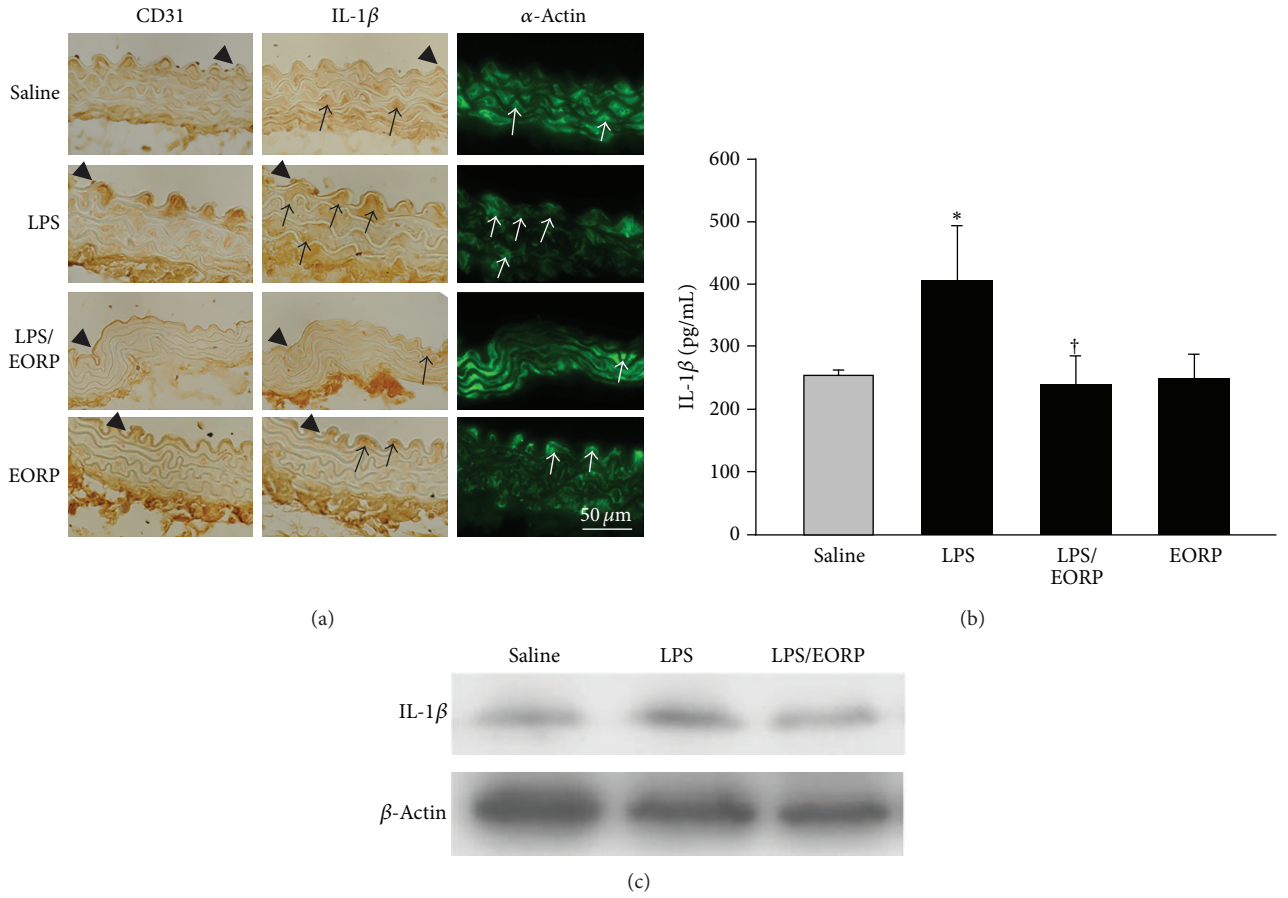

FIGURE 4: EORPs reduce IL-1β expression in LPS-treated mice at the acute inflammatory phase (2 days). (a) Immunohistochemical staining for CD31 (an endothelial cell marker, left panels), IL-1β (middle panels), and α-actin (a smooth muscle cell marker, right panels) antibodies in serial sections of thoracic aortas. The lumen is uppermost in all sections. The arrowhead and the arrow indicate IL-1β-positive overlapping with endothelial cells and smooth muscle cells specific staining, respectively. Bar = 50 μm. (b) IL-1β concentration in the plasma was detected by ELISA. The data are the mean ± SEM ($n = 6$). $^{*}P < 0.05$ as compared to the saline-treated mice (control); $^{\dagger}P < 0.05$ as compared to the LPS-treated mice. (c) Western blot analysis of IL-1β expression in thoracic aortas. β-actin was used as the loading control.

an EORP-associated protective mechanism against bacteria infection involving the clearance of LPS by macrophages [31]. We also demonstrated that EORP attenuates endotoxin-induced ICAM-1 expression in cultured smooth muscle cells and in the neointima in mice [27]. Moreover, EORP prevented PDGF-stimulated smooth muscle cell proliferation *in vitro* and neointimal hyperplasia in the endothelial-denuded artery *in vivo* [32]. The present study is the first to report that EORP strongly reduces the expression of IL-1β protein in LPS-treated HASMCs.

The activation of various intracellular pathways by inflammatory stimuli, such as LPS, is required for the production of these adhesion molecules and proinflammatory chemokines [28]. LPS-induced inflammatory responses, such as ICAM-1 expression in HASMCs, are regulated via TLR4 expression [33]. Most notably, the TLR4-mediated signaling pathway for LPS, leading to the activation of various intracellular kinases, including MAPKs and transcription factors, appears to be critical for the development of vascular inflammation and diseases [26]. Our study showed that LPS caused strong activation of three MAPK subtypes in HASMCs, as reported in a previous study [34]. However, the involvement of their activation in the protective mechanism of EORP remains unclear. In the present study, the increase in IL-1β expression induced by LPS was markedly suppressed in the presence of an ERK inhibitor (PD98059) but not a p38 inhibitor (SB203580) or a JNK inhibitor (SP600125). IL-1β expression was also inhibited by ERK-specific siRNA. EORP decreased LPS-induced ERK phosphorylation. Thus, one of the mechanisms by which EORP reduces LPS-induced IL-1β expression involves a reduction in ERK1/2 activation. Consistent with our results, a *G. lucidum* extract inhibited the oxidative stress-induced phosphorylation of ERK1/2 in breast cancer cells, resulting in suppression of IL-8 secretion and finally in inhibition of cell migration [35]. Another study showed that a *G. lucidum* extract inhibited prostate cancer-dependent angiogenesis by inhibition of phosphorylation of ERK1/2 and Akt kinases [36]. EORP has been shown to inhibit LPS-induced inflammatory cytokine in murine RAW264.7 cells by suppression of the phosphorylation of

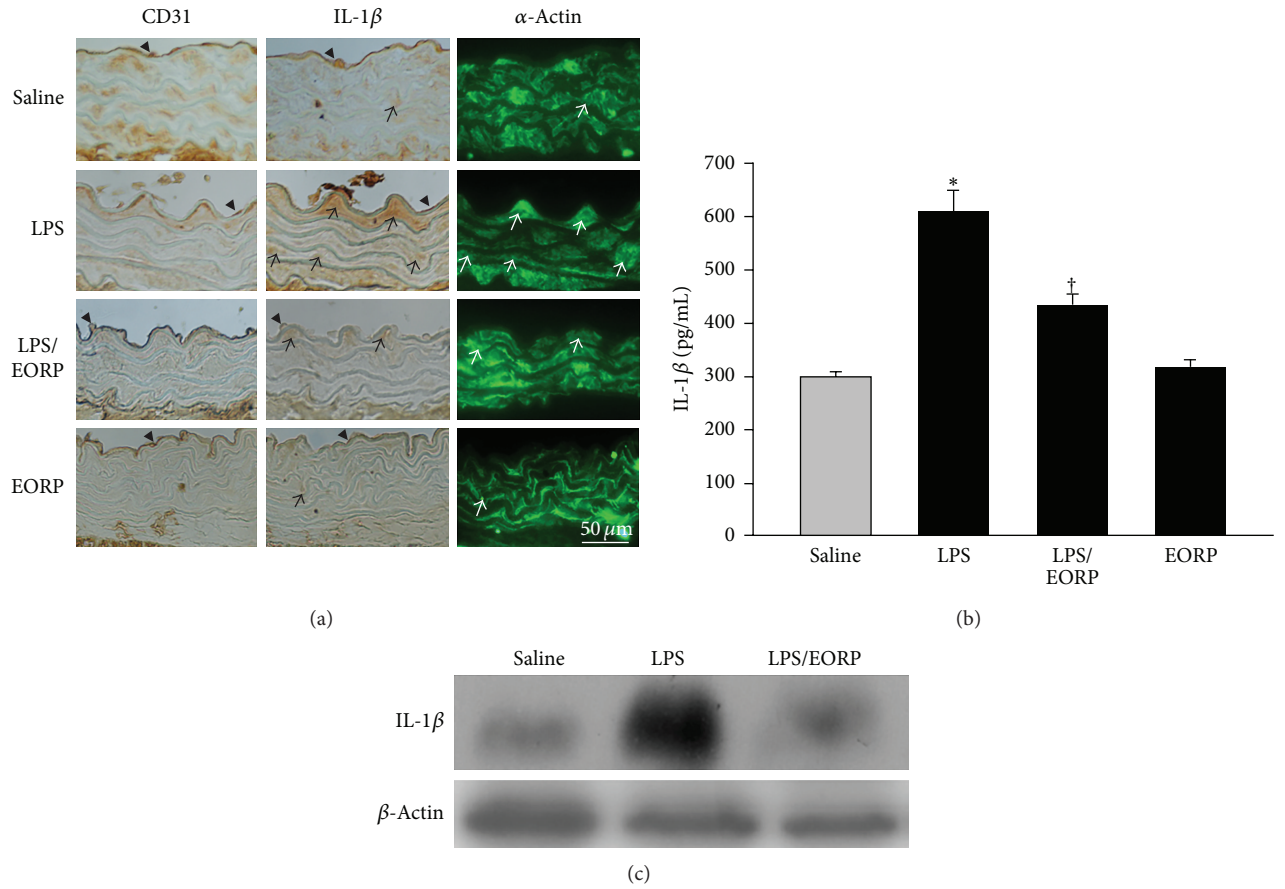

Figure 5: EORPs reduce IL-1β expression in LPS-treated mice at the chronic inflammatory phase (2 weeks). (a) Immunohistochemical staining for CD31 (left panels), IL-1β (middle panels), and α-actin (right panels) antibodies in serial sections on thoracic aortas. The lumen is uppermost in all sections. The arrowhead and the arrow indicate IL-1β-positive cells overlapping with endothelial cells and smooth muscle cells specific staining, respectively. Bar = 50 μm. (b) IL-1β concentration in the plasma was detected by ELISA. The data are the mean ± SEM ($n = 6$). $^{*}P < 0.05$ as compared to the saline-treated mice (control); $^{\dagger}P < 0.05$ as compared to the LPS-treated mice. (c) Western blot analysis of IL-1β protein level in thoracic aortas. The expression ratio (IL-1β/β-actin) was decreased in the EORP (LPS/EORP) group when compared to the LPS-treated group. β-actin was used as the loading control.

ERK1/2 and JNK [22]. Active lipids of *Ganoderma lucidum* spores are able to enhance apoptosis in THP-1 cells through inhibition of ERK1/2 and Akt and activation of JNK1/2 signaling pathways [32], whereas triterpenes from *Ganoderma lucidum* induce autophagy in colon cancer through the inhibition of P38 MAPK [37]. Our previous studies showed that EORP reduces LPS-induced the ICAM-1 expression by the decrease of ERK1/2 activation [27], and prevents the PDGF-stimulated HASMCs proliferation through inhibition of JNK activation [32]. The differences between the above results in terms of the pathways involved may be related to the different cell types used and the cytokines examined.

NF-κB is one of the most ubiquitous transcription factors and regulates the genes involved in cellular proliferation, inflammatory responses, and cell adhesion [25]. NF-κB transcriptional activity can be modulated by the phosphorylation of MAPKs. LPS-induced IL-1β, TNF-α, IL-6, COX-2, and iNOS in Raw264.7 macrophages via the NF-κB activation [38]. These findings raised the possibility that EORP reduces IL-1β expression through a reduction in NF-κB activity. Our study demonstrated that the EORP-induced decrease in IL-1β expression was mediated through inactivation of NF-κB binding activity. Pretreatment with an NF-κB inhibitor also suppressed the LPS-induced increase in IL-1β expression. This is consistent with a previous report that a *G. lucidum* extract inhibited the proliferation of human breast cancer cells by downregulation of NF-κB signaling [39]. *Ganoderma lucidum* polysaccharide peptide (GL-PP) reduced the production of proinflammatory cytokines in activated rheumatoid synovial fibroblast by inhibiting the NF-κB transcription pathway [30]. Another study also showed that a *Ganoderma lucidum* polysaccharides (Gl-PS) prevented pancreatic islets from alloxan-induced damage by inhibiting activation of NF-κB [40]. NF-κB is activated by signals possibly involving phosphorylation of the IκB subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active p50/p65 dimer to the nucleus [41]. We demonstrated that the EORP-induced decrease in IL-1β expression was mediated through inhibition of NF-κB p65 phosphorylation and translocation.

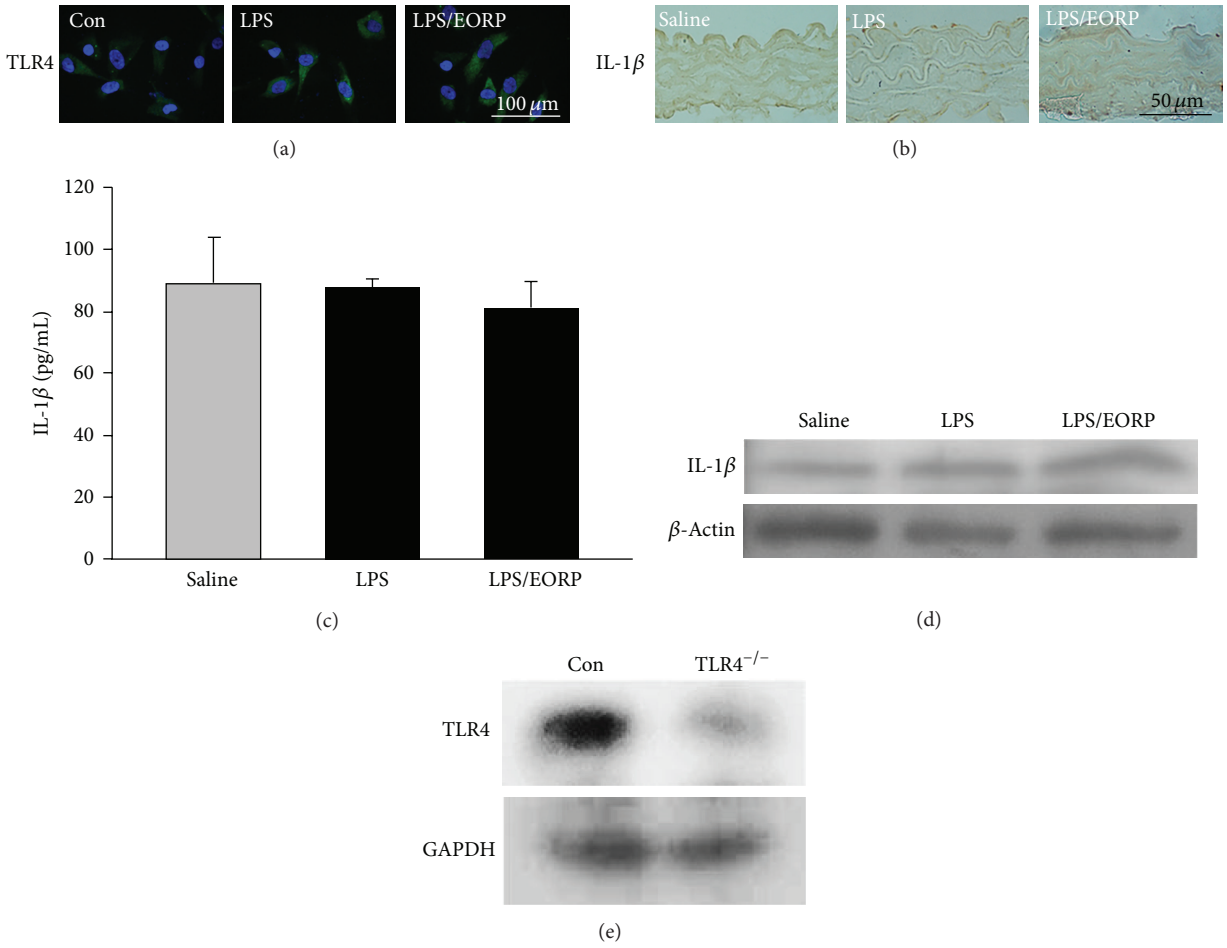

Figure 6: EORP regulating the LPS-induced IL-1β expression was mediated through the TLR4 receptor. (a) Immunofluorescent staining for TLR4. HASMCs were left untreated or incubated for 24 h with or without 10 μg/mL EORP and then incubated with the 10 μg/mL LPS for another 24 h. TLR4 expression was indicated by green fluorescence (FITC) and nuclei by blue fluorescence (DAPI). The untreated HASMCs were used as the control cells. Bar = 100 μm. (b) Immunohistochemical staining for IL-1β expression in thoracic aortas of TLR4$^{-/-}$ mice. The lumen is uppermost in all sections. Bar = 50 μm. (c) IL-1β concentration in plasma was detected by ELISA. The data are the mean ± SEM (n = 5). (d) Western blot analysis of IL-1β in thoracic aortas of TLR4$^{-/-}$ mice. The saline-treated TLR4$^{-/-}$ mice were used as the control group in (b), (c), and (d). β-actin was used as the loading control. (e) Western blot analysis of TLR4 expression in thoracic aortas of C57BL6J (con) and TLR4$^{-/-}$ mice. GAPDH was used as the loading control. F: CAGACCATGATCACACAGGG R: TGGAAAGATGGGCCTGTTAG.

LPS-induced systemic inflammatory responses increase neointimal formation after balloon injury and stent implantation, and inflammatory cytokines are produced by VSMCs in the neointima [42]. In the present study, EORP was shown to significantly reduce IL-1β expression in thoracic aortas in LPS-treated mice. On the basis of the probable involvement of IL-1β expression in migration and proliferation of SMCs, our findings suggest an additional mechanism by which EORP treatment may be important in preventing the progression of cardiovascular disorders and inflammation. It has been well known that the LPS induces the production of proinflammatory molecules through TLR4-activated signaling pathway, and IL-1 was the one of proinflammatory molecules [43]. Another study also showed that IL-1β expression is mediated by both TLR4 and Nod1 pathways in the cultured HAPI cells stimulated by LPS [44]. In addition, LPS-induced TLR4 protein expression and mRNA stabilization in HASMCs are mediated by NADPH oxidase-related ROS production and MAPK signaling pathways [45]. Consistent with the previous studies, LPS significantly induced TLR4 expression in HASMCs. Moreover, LPS did not affect IL-1β expression in thoracic aortas in TLR4$^{-/-}$ mice. There was no difference of IL-1β expression in all groups of TLR4$^{-/-}$ mice by using immunohistochemistry, cytokine ELISA, and Western blot. Based on these findings, we suggested that EORP suppressed the LPS-induced IL-1 expression through inhibition of TLR4 activation.

In conclusion, this study provides the first evidence that EORP reduces IL-1β expression in LPS-treated HAMSCs both *in vitro* and *in vivo*. The present data suggest that these effects might be mediated through inhibition of ERK phosphorylation and NF-κB activation. The results of the present

study suggest a possible therapeutic role for *G. lucidum* extract in cardiovascular disorders and in inflammatory diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Chan-Jung Liang, Chi-Yuan Li, Shu-Huei Wang, and Yuh-Lien Chen conceived and designed the experiments. Chan-Jung Liang, Chiang-Wen Lee, Hsin-Ching Sung, and Yung-Hsiang Chen performed the experiments. Hsin-Ching Sung, Yung-Hsiang Chen, Yao-Chang Chiang, Hsien-Yeh Hsu, and Ying-Chin Tseng contributed reagents/materials/analysis tools. Chi-Yuan Li, Shu-Huei Wang, and Yuh-Lien Chen wrote the paper.

Acknowledgments

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Review Article

Aspirin Resistance and Promoting Blood Circulation and Removing Blood Stasis: Current Situation and Prospectives

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Aspirin plays a crucial physiological and pathophysiological role in cardiovascular diseases and cerebrovascular diseases by irreversibly inhibiting thromboxane A2. However, some patients may be "resistant" to its effect. The resistance has close association with adverse cardiovascular outcomes and increased mortality, so that resolving the problem of aspirin resistance (AR) is widely concerned. By studying the correlation between AR and blood stasis syndrome (BSS), it is demonstrated that BSS may be one of the pathogenesis of AR in traditional Chinese medicine. Chinese herb and formulas definitely possess the advantage of whole body regulation by many ways and many targets. It is a new direction for treatment of AR to combine TCM and modern medicine to study the mechanism and prevention of AR.

1. Introduction

Despite recent medical advances, cardiovascular diseases (CVDs) remain the primary cause of morbidity and mortality throughout the world [1]. Activation of platelets plays a crucial physiological and pathophysiological role in thromboembolic events such as myocardial infarction, stroke, and acute limb ischemia [2]. Hence, drugs that inhibit platelet aggregation, particularly aspirin, are of essential significance for cardiovascular prevention. Currently, evidence of a number of large-scale clinical trials demonstrated that aspirin is a cornerstone in the primary and secondary prevention of CVDs [3, 4]. It is widely used in the medical management of acute coronary syndromes, in the prophylaxis of patients undergoing percutaneous angioplasty or vascular grafting and in long-term prevention of cardiovascular and cerebrovascular events [5]. Meta-analysis of 197 randomized controlled trials involving 135640 patients demonstrated that aspirin reduces the risk of a serious vascular event or cardiovascular death by 25% in high-risk patients [6]. Clinical and biochemical evidence revealed that a persistent thromboxane A2 (TXA2) production is the most likely cause of the residual platelet function [7]. Aspirin covalently and irreversibly inhibits COX-1 and COX-2 by acetylation of serine 530 and serine 516 in their respective active sites. Recent study showed that aspirin inhibits COX-1 approximately 170-fold more potently than COX-2 [8]. In short, aspirin can mainly affect the TXA2 pathway which is the single pathway of platelet activation by irreversibly inhibiting cyclooxygenase-1 (COX-1) by acetylation of serine 530, induce long lasting functional defects of the platelets, and display good antithrombotic activity [9, 10] (as shown in Figure 1).

However, some patients may be "resistant" to its effect. AR is widely manifested and associated with increased cardiovascular risk in aspirin-treated patients. This side effect can significantly influence the clinical effect. Measuring response to aspirin is often difficult and there is no accepted definition of AR [11]. On current, several definitions for resistance have been set. Some scholars defined "aspirin resistance" as the inability of aspirin to inhibit COX-1 dependent TXA2 production and consequently TXA2-dependent platelet functions [12]. Arguably, the most fundamental definition of AR is lower than the normal antiplatelet response to standard doses of aspirin [13]. That is to say, the term AR may mean different things to different individuals [7]. Studies in adults reported a 5%–51% prevalence of aspirin resistance, while the

Table 1: Causes of aspirin resistance and potential strategies to overcome resistance.

Cause of AR	Strategy to overcome AR
Noncompliance of patients	Patient education Minimisation of adverse effects
Insufficient dosing	Biofeedback use of platelet function assays and measurement of aspirin metabolites to guide increased dosing levels
Drug-drug interactions	Caution/avoid use of NSAIDs Caution/avoid use of proton pump inhibitors
Non-COX-1-mediated TXA2 production	Duel or increased dose of antiplatelet therapy Development of thromboxane receptor antagonists
Other pathways of platelet activation	Use of drugs to inhibit final common pathways of platelet aggregation
Pharmacogenetic factors	Exploitation of genetic polymorphisms-personalised antiplatelet therapy

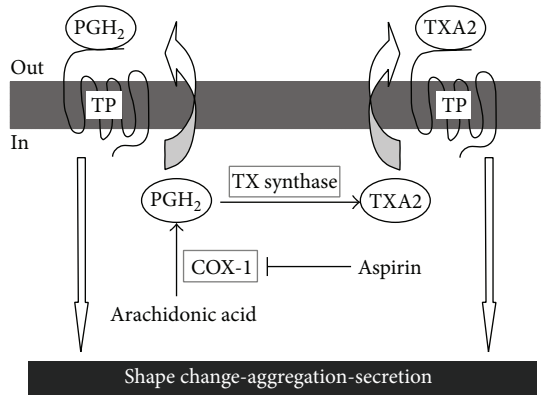

Figure 1: Aspirin inhibits COX-1 dependent TXA2 production.

ratio was 26% in children [14]. AR may be associated with an increased risk of dying from heart disease [15]. Nowadays, a prevalence investigation in Indian showed that incidence of AR in the cohort of patients with documented heart disease was 38.1% [16]. Several laboratory methods have been proposed to assess platelets' resistance (bleeding time, light transmission aggregation, impedance aggregation, platelet function analyser, rapid platelet function assay, TXB2, and flow cytometry), of which light transmittance aggregometry (LTA) is considered gold standard [17]. Besides there are also a variety of other techniques to assess aspirin responsiveness and there are advantages and drawbacks with each.

There are several proposed mechanisms of aspirin resistance, and true aspirin resistance is unclear. Aspirin noncompliance, insufficient dosing, platelet turnover, and interacting drugs, most notably nonsteroidal inflammatory drugs (NSAIDs), unfortunately are the most important contributors to the treatment failure. Aspirin resistance is a multifactorial phenomenon, so strategies of treatment should be directed to a number of COX-1 dependent and other independent factors, such as increasing patient compliance, increasing dose of levels, combining with other antiplatelet drugs, or applicating other kinds of drugs as alternatives. Also, adequate treatment of confounding clinical conditions such as smoking, hyperlipidemia, hyperglycemia, hypertension, heart failure, infection, and inflammation may further increase efficacy of antiplatelet treatment with aspirin (Table 1). However, there also exists similar phenomenon to AR, such as clopidogrel resistance (CR). On the other hand, the combination of the two types of antiplatelet drugs may cause increasing risk of serious bleeding. Considering the higher price of clopidogrel and other drugs, it is not suitable for patients for long-term use [18]. That is to say, it is not an ideal therapy to combine aspirin with other antiplatelet drugs or alternative medication. In such cases, we can seek solution from traditional Chinese medicine (TCM).

2. The Pathogenesis of AR in TCM Theory

With increasing application of complementary and alternative medicines all over the world, TCM became more popular and have drew more attention [19–23]. It has formed a particular way on diagnosis and treatment of disease [24–28]. Currently, blood stasis syndrome (BSS) and theory of promoting blood circulation and removing blood stasis (PBCRBS) is attached by scholars from various countries. In America, doctors are familiar to ABC drugs (activating blood circulation herbs), which were herbs with function of PBCRBS [29–32]. In Japan, doctors call BSS as Oketsu Syndrome [33–35]. In TCM, cardiovascular heart disease (CHD) belongs to category of "thoratic obstruction and cardialgia" or "chest stuffiness and pains." Ancient Chinese medicine had some understanding about thromboembolic disease. The "Five Pathogenic Factors" in the ancient book of "the Miraculous Pivot" recorded that "pathogenic factor (also called "xie qi")" lies in heart, so the disease is manifested as precordial pain." The pathogenesis of AR is related to obstruction of collaterals by blood stasis and obstruction of heart meridian. By theory of TCM, AR has a close association with cardiovascular and cerebrovascular thromboembolic diseases, especially the cardiovascular heart disease. BSS is the main syndrome of CHD and it is an important

TCM syndrome of CHD with cardiovascular risk [36]. BSS is defined as clinical syndrome caused by retardation or cessation of the blood flow and is regarded as the cause or product of many chronic diseases in TCM [37]. Recognition of ancient Chinese medicine to blood stasis is multifaceted and there were many narration about it. For example, there were 365 kinds of Chinese herbal medicine in "Classic of Materia Medica by God of Agriculture" (Shennong Ben Cao Jing), of which 41 kinds have function of PBCRBS. At least the following diseases of cardiovascular system may be related to manifestation of BSS, such as angina pectoris, acute myocardial infarction, rheumatic heart disease, heart failure, and various types of vasculitis [38, 39].

By theory of TCM, AR belongs to the category of "collaterals" disease. The pathological character of AR is blood stagnant. Accumulation of blood stasis can be transformed into turbid toxins. Finally, the blood stasis and the turbid toxin congest the "collaterals" and leaded to various diseases. In view of the characteristics of the disease, strategies of PBCRBS and removing toxic materials from meridians should be used to AR. AR will eventually lead to formation of BSS and cause blood stasis in channels and collateral branches by all kinds of reasons. Orient scholars studied the association of AR and BSS and concluded that AR is closely related to BSS [40–42]. BSS maybe pathogenesis of AR in TCM. Firstly, as we know, the clinical manifestation of platelet function disorder is one of indicators comprising diagnosis of BSS; also this indicator is a significant factor causing AR. Also, it is indicated that the incidence rate of AR was up to 64% in patients of cardiocerebral artery thrombotic disease with severe blood stasis, which is significantly more than non-BSS patients. Secondly, the pathogenesis of BSS is correlated with haemorheologic changes such as the deterioration of erythrocyte deformability, elevation of blood viscosity, and acceleration of erythrocyte aggregation, as well as abnormal status of microcirculatory dysfunction [43, 44], which is similar to mechanism of AR. Thirdly, Chinese herbs for treatment of BSS can also improve AR [45]. Study about mechanism of traditional Chinese herbs with function of PBSRBS showed that it can not only activate blood circulation (improving function of cardiovascular and cerebrovascular, changing the physical and chemical properties of blood, changing function of platelet and blood coagulation system, and improving microcirculation) but also remove stasis (antimyocardial ischemia, anticerebral ischemia, inhibiting platelet aggregation, anticoagulation, antiformation of thrombosis, etc.). In view of this, Chinese herbs for the therapy of BSS can show significant effect on AR aiming at the pathogenic mechanism.

3. Principles of AR Treatment in Traditional Chinese Medicine

Dysfunction of "zang" and "fu" can lead to blood stasis of heart meridian. So that therapeutic of PBCRBS is the first choice of CHD. But, in concrete applications, it is imperative to differentiate TCM syndrome according to the etiology and pathogenesis, the involved zang-fu organs, and clinical manifestation of BSS when using PBCRBS as the main therapy. Also, it is necessary to modulate the liver, the spleen, the lung, and the kidney at the same time and use the corresponding treatment to improve effect of diagnosis and treatment, such as promoting qi circulation, dispelling cold, resolving phlegm, clearing away heat, supplementing qi, supplementing blood, warming yang, nourishing yin, and supplementing kidney. Chinese herbs of PBCRBS have effect on antibacterial, antiviral, inhibiting inflammation, treatmenting infectious diseases, regulating immune system, improving body resistance, and so on [46]. Besides, recent research demonstrated that CHD is closely related to inflammation, so that maybe principal of dissolving blood stasis and detoxification for treatment of BSS is more effective. Current researches demonstrated that Chinese herb and formulas definitely possess the advantage of whole body regulation by many ways and many targets, so that we can resolve AR issues with higher efficacy to prevent the occurrence of cardiovascular disease.

Nowadays, alternative and synergistic treatment of TCM has become an ideal therapeutics. Researches demonstrated that some Chinese herbal compounds (such as composite salvia dropping pill, Nao xin tong capsule, Tong xin luo capsule, Xuefu zhuyu decoction, Huo xue capsule, and Zhilong huoxue capsule) and several traditional medicine monomer or Chinese herbal extract (Di'ao xinxuekang capsule, Xinnao shutong capsule, ginkgo biloba extract, etc.) can effectively inhibit platelet activity. These drugs can be used combining with aspirin to achieve the purpose of enhancing pharmacy efficiency. Also, it can be used as alternative medicine of aspirin for secondary prevention of cardiovascular and cerebrovascular diseases [47]. It is demonstrated that most of the decoctions have effect on promoting blood circulation to remove stasis and this fact can also verify the previous mentioned theory.

4. TCM of PBCRBS for the Treatment of AR

Currently, traditional medicine monomer and Chinese formulas used alone or combined with antiplatelet pertensive drugs have been widely used as an alternative and effective method for the treatment of AR with coronary heart diseases in clinical treatment in China. Until now, many clinical studies of formulas used for therapy of AR verified 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 these Chinese formulas for AR to justify their clinical use and their recommendation. This paper aims to assess the current clinical evidence of the frequently used Chinese decoctions or traditional medicine monomer for aspirin resistance in patients with coronary heart diseases.

We selected all the clinical trials about Chinese decoctions or traditional medicine monomer for treatment of AR using the search terms of "aspirin resistance," "coronary heart disease," "herb," "Chinese drug," "compound prescription," "traditional Chinese medicine," "decoction," "Chinese

Table 2: Clinical trials of Chinese herbal interventions in treating AR with a concomitant population.

Reference (year)	Study design	Participants T/C	Intervention (herb included)	Control	Outcome measure	Treatment duration	Main finding (P value)
Chai et al. (2008) [48]	RCT	10/10	Composite salvia dropping pill (10 pills, tid); aspirin (100 mg, qd)	Aspirin (100 mg, qd)	Platelet aggregation rate induced by AA and ADP	2 w	Composite salvia dropping pill can enhance patients' sensitivity to aspirin. The combining use can synergisticly exert antiplatelet effect.
Peng et al. (2011) [49]	RCT	12/13	Huo xue capsule (4 pills, tid); aspirin (100 mg, qd)	Aspirin (100 mg, qd)	Platelet aggregation rate induced by AA and ADP	12 w	The combination of huo xue capsule and aspirin can decrease the platelet aggregation rate significantly.
Yang et al. (2011) [50]	RCT	130/130	Nao xin tong capsule (2 pills, tid); aspirin (100 mg, qd)	Aspirin (100 mg, qd)	Platelet aggregation rate induced by AA and ADP, the incidence of AR and ASR of each group.	24 w	The combining use of nao xin tong capsule and aspirin can significantly decrease the platelet aggregation rate. Incidence of AR and ASR is lower than single aspirin group.
Yin et al. (2010) [51]	RCT	30/29	Tong xin luo capsule (3 pills, tid); aspirin (100 mg, qd)	Aspirin (100 mg, qd)	COL and platelet aggregation rate induced by AA and ADP	30 d	Tong xin luo capsule has some effects on decreasing the platelet aggregation rate induced by ADP and COL.
Song (2008) [52]	RCT	24/23	Tong xinluo capsule (4 pills, tid)	Clopidogrel (75 mg, qd)	CRP, TXB2, and platelet aggregation rate induced by AA and ADP	30 d	Tongxinluo capsule has antiplatelet aggregation and anti-inflammatory effect in ACS patients with AR and can improve AR in ACS patients in a certain degree.
Liu (2011) [53]	RCT	30/30	Xinnao shutong capsule (9 pills, qd); aspirin (100 mg, qd)	Aspirin (100 mg, qd)	Uric acid, Blood lipid, Hs-CRP, TXB2, and platelet aggregation rate induced by AA and ADP AA, ADP, and so on.	1 y	The combining use of aspirin and xinnao shutong capsule can decrease the adverse cadiovascular events of patients with AR.
Wu (2012) [54]	RCT	30/30	Xuefu zhuyu decoction (1 dose, qd); aspirin (100 mg, qd)	Aspirin (100 mg, qd)	The expression of AA and ADP in serum.	4 w	The combining use of aspirin and Xuefu zhuyu decoction can significantly decrease the platelet aggregation rate induced by ADP and AA.
Luo et al. (2012) [55]	RCT	30/30	Zhilong huoxue capsule (1.6 g, tid)	Aspirin (300 mg, qd)	The mean platelet aggregation rate, TXB2, and 6-K-PGF1α.	4 w	Capsule zhilong huoxue of aspirin resistance has better efficacy and safety. The mechanism may be related to its effect of decreasing TXB2, increasing 6-K-PGF1α, and decreasing TXB2/6-K-PGF1α.
Xiu (2012) [56]	RCT	30/30	Di'ao xinxuekang capsule (1.6 g, tid)	Aspirin (300 mg, qd)	Platelet aggregation rate induced by AA and ADP	4 w	Capsule di'ao xinxuekang of aspirin resistance has better efficacy and safety. The mechanism may be related to its effect of decreasing TXB2, increasing 6-K-PGF1α, and decreasing TXB2/6-K-PGF1α.

Table 2: Continued.

Reference (year)	Study design	Participants T/C	Intervention (herb included)	Control	Outcome measure	Treatment duration	Main finding (P value)
Yang et al. (2011) [57]	RCT	50/50	Ginkgo biloba extract (19.2 mg, tid); aspirin (100 mg, qd)	Aspirin (100 mg, qd)	Platelet aggregation rate induced by AA, ADP, and FAP	2 w	Combination of ginkgo biloba extract and low dosage of aspirin can decrease AR and decrease incidence of adverse cadiovascular events.

Notes: T/C: treatment group and control group; CT: clinical trial; RCT: randomized clinical trial; NR: not reported; ADP: adenosine diphosphate; COL: collagen.

formula," "controlled clinical trial," "random," and "blind" individually or combined in five databases, such as Chinese National Knowledge Infrastructure (CNKI), Chinese Biomedical Literature Database (CBM), PubMed, Embase, and the Cochrane Central Register of Controlled Trials in the Cochrane Library (January 2013). After primary search of five databases, ten RCTs were reviewed [48–57]. All the trials were conducted in China and published in Chinese. The characteristics of the fourteen randomized trials were summarized in Table 2. The majority of the included trials were assessed to be of general poor methodological quality according to the predefined quality assessment criteria. The randomized allocation of participants was mentioned in all trials. However, only 2 trials stated the methods for sequence generation by random number table [51, 56].

751 patients with AR were included. Intervention included all the prescriptions of Chinese decoctions or traditional medicine monomer, or combined with antiplatelet-aggregation drugs. The Chinese decoction includes composite salvia dropping pill, Huo xue capsule, Nao xin tong capsule, Tong xinluo capsule, Xuefu zhuyu decoction, and Zhilong huoxue capsule. The traditional medicine extract or monomer can only be reported by five researches [53, 56, 57], such as Di'ao xinxuekang capsule, Ginkgo biloba extract, and Xinnao shutong capsule. The different ingredient of frequently used Chinese formulas is shown in Table 3. The controls included antiplatelet-aggregation drugs alone. The standard therapy of control group included aspirin (100 mg, qd) [48–51, 53, 54, 57], routine treatment plus clopidogrel (75 mg, qd) [52], and aspirin (300 mg, qd) [55, 56]. Only three trials investigated the prescriptions of Chinese medicine used alone versus antiplatelet-aggregation drugs [52, 55, 56]. The other eleven clinical trials investigated the Chinese medicine combined with antiplatelet-aggregation drugs as therapy intervention versus antiplatelet-aggregation drugs [49–51, 53, 54, 57].

The treatment duration ranged from 14 days to 1 year. All of the 10 trials used platelet aggregation as the outcome measure. In addition, the outcome measurement also includes the unfrequently used standards, such as uric acid, blood lipid, CRP, hs-CRP, TXB2, COL, 6-K-PGF1α, platelet counts, and coagulation time. The main finding showed that the combining use of Chinese medicine and antiplatelet-aggregation drugs can decrease the platelet aggregation rate in a different degree more effectively. Of the ten trials, only one research does not show specific data [50].

The meta-analysis showed significant beneficial effect of Chinese formula plus antiplatelet-aggregation drugs compared with antiplatelet-aggregation drugs in platelet aggregation rate induced by AA (MD: −9.36 [−10.48, −8.25]; $P < 0.00001$) and ADP (MD: −17.61 [−19.56, −15.65]; $P < 0.00001$). Also, there was beneficial effect of Chinese formula used alone compared with antiplatelet-aggregation drugs in platelet aggregation rate induced by AA (MD: −2.53 [−4.28, −0.78]; $P = 0.005$). However, the meta-analysis showed no significant effect of Chinese formula used alone compared with antiplatelet-aggregation drugs in platelet aggregation rate induced by ADP (MD: 0.70 [−11.07, 12.48]; $P = 0.91$) (as shown in Tables 4 and 5).

Six out of ten trials mentioned the adverse effect [50, 51, 53, 55–57]. Two trials reported six specific symptoms in Chinese formulas including distending feeling in head, dizziness, headache, facial flush, abdominal discomfort, and nausea. And adverse events were found in both of two trials [55, 56]. Three trials reported adverse effect in aspirin group. Two of it showed symptoms including distending feeling in head, dizziness, headache, facial flush, abdominal discomfort, and nausea [55, 56]. Another one trial reported symptoms of faulty in the mouth, gastrointestinal discomfort, gum haemorrhage, retinal hemorrhage, and positive of fecal occult blood test [53].

Our previous meta-analysis demonstrated that Chinese decoctions or traditional medicine monomer for treatment of AR showed significant beneficial effect compared with Western medicine. Adverse effect of Chinese formulas was reported by Luo et al. and Xiu [55, 56]. The adverse effect was not severe, and it spontaneously recovered without special treatment. Five trials demonstrated that there was no significant difference between the two treatments. Six trials did not mention adverse effect. Therefore, due to the limited and inadequate evidence provided by the eligible trials, conclusions about the safety of Chinese formulas combined with antiplatelet-aggregation drugs cannot be made from this paper. Large-scale clinical trials with long-term follow-up were warranted to assess the safety of new integrative medicine therapy properly.

5. Conclusion and Prospective

BSS is defined by retardation or cessation of the blood flow and is regarded as the cause or product of many chronic

Table 3: The ingredient of frequently used Chinese Formulas.

Formulas	Components	TCM Efficacy
Composite salvia dropping pill	Salvia miltiorrhiza [Dan shen, 丹参], Panax notoginseng [San qi, 三七], and Borneolum Syntheticum [Bing pian, 冰片].	Promoting blood circulation to remove stasis and regulating the circulation of qi and alleviating pain.
Huo xue capsule	Radix astragali [Huang qi, 黄芪], Semen persicae [Tao ren, 桃仁], Safflower [Hong hua, 红花], Bidentate achyranthes [Niu xi, 牛膝], 酸枣仁, Rhizoma chuanxiong [Chuan xiong, 川芎], Citrus aurtantium [Zhi qiao, 枳壳], Radix rehmanniae [Di huang, 地黄], Platycodon grandiflorum [Jie geng, 桔梗], Angelica sinensis [Dang gui, 当归], and Glycyrrhiza [Gan cao, 甘草].	Suplementing qi, nourishing blood, promoting blood circulation for removing obstruction, and regulating qi-flowing for tranquilization.
Nao xin tong capsule	Radix astragali [Huang qi, 黄芪], Radix Paeoniae Rubra [Chi shao, 赤芍], Salvia miltiorrhiza [Dan shen, 丹参], Angelica sinensis [Dang gui, 当归], Rhizoma chuanxiong [Chuan xiong, 川芎], Semen persicae [Tao ren, 桃仁], Olibanum [Ru xiang, 乳香], Myrrha [Mo yao, 没药], Bidentate achyranthes [Niu xi, 牛膝], Ramulus cinnamomi [Gui zhi, 桂枝], Ramulus mori [Sang zhi, 桑枝], Earthworm [Di long, 地龙], Caulis Spatholobi [Ji xue teng, 鸡血藤], Scorpio [Quan xie, 全蝎], and Hirudo [Shui zhi, 水蛭].	Benefiting qi for activating blood circulation and promoting blood circulation for removing obstruction in collaterals.
Tong xin luo capsule	Radix Ginseng [Ren shen, 人参], Hirudo [Shui zhi, 水蛭], Scorpio [Quan xie, 全蝎], Eupolyphaga Seu Steleophaga [Tu bie chong, 土鳖虫], Scolopendra [Wu gong, 蜈蚣], Periostracum Cicadae [Chuan tui, 蝉蜕], Radix Paeoniae Rubra [Chi shao, 赤芍], Lignum Santali Albi [Tan xiang, 檀香], Lignum Dalbergiae Odoriferae [Jiang xiang, 降香], Olibanum [Ru xiang, 乳香], Semen Ziziphi Spinosae [Suan zao ren, 酸枣仁], and Borneolum Syntheticum [Bing pian, 冰片].	Benefiting qi for activating blood circulation and promoting blood circulation for removing obstruction in collaterals and relieving pain.
Zhilong huoxue tongyu capsule	Radix astragali [Huang qi, 黄芪], Radix Ginseng [Ren shen, 人参], Hirudo [Shui zhi, 水蛭], Ramulus cinnamomi [Gui zhi, 桂枝], Caulis Spatholobi [Ji xue teng, 鸡血藤], Earthworm [Di long, 地龙], and others.	Benefiting qi for activating blood circulation and removing obstruction in collaterals.
Xuefu zhuyu decoction	Semen persicae [Tao ren, 桃仁], Angelica sinensis [Dang gui, 当归], Citrus aurtantium [Zhi qiao, 枳壳], Rhizoma chuanxiong [Chuan xiong, 川芎], Bupleurum [Chai hu, 柴胡], Safflower [Hong hua, 红花], Bidentate achyranthes [Niu xi, 牛膝], Radix Paeoniae Rubra [Chi shao, 赤芍], Radix rehmanniae [Di huang, 地黄], Platycodon grandiflorum [Jie geng, 桔梗], and Glycyrrhiza [Gan cao, 甘草].	Promoting blood circulation to remove stasis and regulating the circulation of qi and alleviating pain.
Di'ao xinxuekang capsule	Extract from root of haicaet Burkill [Huang shan yao, 黄山药] and Dioscorea niponica Makino [Chuan long shu yu, 穿龙薯蓣]	Promoting blood circulation for removing obstruction and stimulating qi circulation to relieve pain.
Xinnao shutong capsule	Extract from aboveground part of Sandbur [Ji li, 蒺藜]	Resolving stagnation for alleviation of pain and activating blood circulation to dredge channel blockage.
Ginkgo leaf extract	Extract from leaf of Ginkgo [Yin xing, 银杏]	Benefiting qi for activating blood circulation and removing obstruction in collaterals

Table 4: Analysis of platelet aggregation rate induced by AA.

Trials		MD (95% CI)	*P* value
Chinese formula versus anti-platelet-aggregation drugs			
Tong xin luo capsule versus clopidogrel	1	0.86 [−3.38, 5.10]	0.69
Zhilong huoxue capsule versus aspirin	1	−3.23 [−7.34, 0.88]	0.02
Di'ao xinxuekang capsule versus aspirin	1	−3.23 [−7.34, 0.88]	0.02
Meta-analysis	3	−2.53 [−4.28, −0.78]	0.005
Chinese formula plus anti-platelet-aggregation drugs versus anti-platelet-aggregation drugs			
Composite salvia dropping pill plus aspirin versus aspirin	1	−11.09 [−14.84, −7.34]	<0.00001
Huo xue capsule plus aspirin versus aspirin	1	−14.22 [−16.59, −11.85]	<0.00001
Xinnao shutong capsule plus aspirin versus aspirin	1	−12.58 [−14.56, −10.60]	<0.00001
Xuefu zhuyu decoction plus aspirin versus aspirin	1	−7.20 [−11.73, −2.67]	0.002
Ginkgo biloba extract plus aspirin versus aspirin	1	−2.53 [−4.53, −0.53]	0.01
Meta-analysis	5	−9.36 [−10.48, −8.25]	<0.00001

Table 5: Analysis of platelet aggregation rate induced by ADP.

Trials		MD (95% CI)	*P* value
Chinese formula versus anti-platelet-aggregation drugs			
Tong xin luo capsule versus clopidogrel	1	13.32 [7.68, 18.96]	<0.00001
Zhilong huoxue capsule versus aspirin	1	−5.50 [−10.49, −0.51]	0.03
Di'ao xinxuekang capsule versus aspirin	1	−5.50 [−10.49, −0.51]	0.03
Meta-analysis	3	0.70 [−11.07, 12.48]	0.91
Chinese formula plus anti-platelet-aggregation drugs versus anti-platelet-aggregation drugs			
Composite salvia dropping pill plus aspirin versus aspirin	1	−13.92 [−19.58, −8.26]	<0.00001
Huo xue capsule plus aspirin versus aspirin	1	−40.97 [−49.64, −32.30]	<0.00001
Tong xin luo capsule plus aspirin versus aspirin	1	−2.51 [−4.46, −0.56]	0.01
Xinnao shutong capsule plus aspirin versus aspirin	1	−23.26 [−26.11, −20.41]	<0.00001
Xuefu zhuyu decoction plus aspirin versus aspirin	1	−16.82 [−22.12, −11.52]	<0.00001
Ginkgo biloba extract plus aspirin versus aspirin	1	−2.87 [−7.00, 1.26]	0.17
Meta-analysis	5	−17.61 [−19.56, −15.65]	<0.00001

diseases. It is a diagnosis that indicates a very strong sense of TCM [58–61]. Usually, chronic diseases and slow progressing diseases mostly involve blood stasis. Also, severe warm diseases and trauma mostly involve acute blood stasis. Ancient Chinese medical texts describe commonly phenomenon of a disorder in the blood circulation as "Yu Xue" in Chinese, "Eohyul" in Korean, and "Oketsu" in Japanese [62, 63]. It has close correlation with various diseases. In cardiovascular system, there was close association between BSS and coronary artery disease, angina pectoris, myocardial infarction, rheumatic heart disease, heart failure, and vasculitis. With increasing popularity of complementary and alternative medicine among AR patients, TCM is becoming more and more frequently used both in China and Western countries [64–66]. Clinical use of PBCRBS herbs in associated diseases also becomes more plausible. Their therapeutic effect can be clearly shown. Thus, the "PBCRBS phenomenon" becomes more widely discussed and becomes a hot topic of integrative medicine.

Statistical analysis on sixteen Chinese traditional medicine materia medica classics showed that there are 150 commonly used PBCRBS herbs. In addition, Chinese herbal medicine can be widely used for AR. We statis frequency of each Chinese herbs used for aspirin resistance in current frequently Chinese formulas; the frequent appearance of herbs is hirudo. Hirudo, also called leech, the herb is some bitter and salty in flavor and enters liver and bladder meridian. Modern pharmacological studies demonstrated that the herb contains peptides, heparin, and antithrombin [67]. Besides, at least four experiments confirmed that leech and its effective ingredients can reduce or inhibit activation of platelet [68–71]. Furthermore, the semen persicae, radix paeoniae rubra, and radix astragali were used frequently, of which, semen persicae is a commonly used traditional

Chinese herbal medicine with function of PBCRBS. It showed therapeutic effect on treatment of coronary heart disease, myocardial infarction, and traumatic injury. Related study showed that semen persicae can effectively inhibit platelet aggregation by multiple pathways [72]. Radix paeoniae rubra is another commonly used traditional Chinese herbal medicine with function of PBCRBS. It is some bitter and slightly cold in flavor and enters liver meridian and has function of resolving blood stasis, relieving pain, cooling the blood and detumescence. The paeonilorin demonstrated the effective pharmacological components (such as monoterpene and glycosides), gallic acid and its derivatives. Total paeony glycoside (TPG) is one of the main effective components of radix paeoniae rubra. It has been demonstrated that TPG has anticoagulant and antithrombotic effects [73]. The animal experiment showed that TPG can improve the microcirculation of mice, reduce serum and plasma viscosity in rats, and inhibit platelet aggregation induced by adenosine diphosphate (ADP) [74]. Radix astragali was mostly used for qi deficiency disease. According to theory of traditional Chinese medicine, invigorating qi can promote blood circulation. So radix astragali can be used for treatment of BSS combined with PBS herbs. Bu yang huan wu decoction is a typical example using this treatmen rule [75]. At present, at least five experiments showed that astragalus could reduce platelet adhesion and aggregation, reduce plasma fibrinogen, and show antithrombus formation effect [76–79]. In addition, Panax notoginseng, salvia miltiorrhiza, and bidentate achyranthes were also used frequently.

Under what circumstance can we use PBCRBS? For a long time, PBCRBS herbs were maily used for traumatic injury, "zhengjia" agglomeration, and gynecological diseases. They were seldom used in treatment of coronary heart disease. Professor Chen keji sticks to the point that we should associate the main pathological link of the coronary disease (such as formation of thrombosis, platelet activation, vascular stenosis, and spasm) with BSS, so that etiology and pathogenesis of the disease can be clearly clarified by theory of TCM. Traditionally, the diagnosis of BSS depended on subjective diagnostic methods such as inspection and palpation of the patient [80]. In 1988, academia from Japan, Korea, Singapore and so forth attended the "International Conference on Blood Stasis Syndrome" and recognized a standard for diagnosing blood stasis syndrome. In China, Chen improved the above standards and grading system in more detail and formed a commonly used Chinese criteria diagnosis of BSS [81].

There are still some problems existing which seriously limited the research and progress on the treatment and should be solved as soon as possible. Researchers of clinical trials in TCM should also pay more attention to experimental design and methodological quality and improve the reporting quality according to the consolidated standards of reporting trials (CONSORT) [82]. Also, it is imperative to establish a rapid, accurate, and practical method for monitoring of AR. Monitoring platelet activity can be just as easy as blood pressure, blood glucose, and cholesterol. Currently, Chinese scholars study the mechanism of BSS mainly on direction of microcirculation, hemorheology, platelet function, deformability of red blood cell, prostaglandin, thromboxane metabolism, and fibrinolytic system change. There were many researches duplicating previous work. So that, it is necessary to carry out further targeted research and design observation index according to different kinds of diseases. In addition, Chinese scholars used too many types of decoctions for treatment of BSS. Maybe it is better to concentrate on study of the generally accepted formulas or decoctions. In addition, the abuse in using Chinese herbs for treatment BSS should be avoided. Caution should a1ways be taken to differentiate clinical application.

Abbreviations

PBCRBS: Promoting blood circulation and removing blood stasis
AR: Aspirin resistance
BSS: Blood stasis syndrome
TCM: Traditional Chinese medicine
CVDs: Cardiovascular disease
TXA2: Thromboxane A2
COX-1: Cyclo-oxygenase-1
LTA: Light transmittance aggregometry
CR: Clopidogrel resistance
CHD: Coronary heart diseases
TPG: Total paeony glycoside
ADP: Adenosine diphosphate.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jie Wang and Xingjiang Xiong contributed equally to this paper.

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Review Article

Traditional Chinese Medicine Syndromes for Essential Hypertension: A Literature Analysis of 13,272 Patients

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Background. To simplify traditional Chinese medicine syndrome differentiation and allow researchers to master syndrome differentiation for hypertension, this paper retrospectively studied the literature and analyzed syndrome elements corresponding to hypertension syndromes. *Methods*. Six databases including PubMed, EMBASE, Chinese Bio-Medical Literature Database, Chinese National Knowledge Infrastructure, Chinese Scientific Journal Database, and Wan-fang Data were searched from 1/January/2003 to 30/October/2013. We included all clinical literature testing hypertension syndromes and retrospectively studied the hypertension literature published from 2003 to 2013. Descriptive statistics calculated frequencies and percentages. *Results*. 13,272 patients with essential hypertension were included. Clinical features of hypertension could be attributed to 11 kinds of syndrome factors. Among them, seven syndrome factors were excess, while four syndrome factors were deficient. Syndrome targets were mainly in the liver and related to the kidney and spleen. There were 33 combination syndromes. Frequency of single-factor syndromes was 31.77% and frequency of two-factor syndromes was 62.26%. *Conclusions*. Excess syndrome factors of hypertension patients include yang hyperactivity, blood stasis, phlegm turbidity, internal dampness, and internal fire. Deficient syndrome factors of hypertension patients are yin deficiency and yang deficiency. Yin deficiency with yang hyperactivity, phlegm-dampness retention, and deficiency of both yin and yang were the three most common syndromes in clinical combination.

1. Introduction

Hypertension is an important public health issue worldwide because of its high prevalence and concomitant increase in disease risk [1–3]. It has been estimated that 29% of the world's adult population, or approximately 1.56 billion people, will have hypertension by 2025 [4, 5]. Complementary and alternative medicine (CAM) is becoming increasingly popular [6–13] and numerous interventions are regularly recommended to lower elevated blood pressure (BP) [14–17]. Traditional Chinese medicine (TCM), including herbal medicine and acupuncture, is an important component of CAM therapies [18–21]. Hypertension could be improved by insights from TCM and considerable progress has been made in lowering BP by TCM [22–26].

Syndrome differentiation is a diagnostic and treatment method used in TCM [27, 28]. It plays an important role in the therapeutic process and affects the therapeutic result of certain diseases [29–31]. The syndrome is not only the basic unit of TCM theory and syndrome differentiation, but also the bridge to associating disease and formula [32–35]. TCM syndrome, which is different from a disease or symptoms, is the abstraction of a major pathogenesis. Syndromes are identified from a comprehensive analysis of all symptoms and signs (including tongue appearance and pulse feeling) from the four main diagnostic TCM methods: observation, listening, questioning, and pulse analyses [36–40]. However, syndromes are the product of speculation in TCM. Therefore, they depend on medical experience, academic origins, and other factors. Therefore, the concept of syndromes is vague and broad, which makes clinical application difficult. Syndrome elements, which are the minimum units of syndromes, contribute to simplifying syndrome differentiation and understanding TCM syndromes. Each element has specific symptoms.

To simplify TCM syndrome differentiation and enable researchers not familiar with Chinese medicine to master the laws of hypertension syndrome differentiation, this paper

retrospectively studied the literature for 13,272 patients with hypertension, published from 2003 to 2013. This study is beneficial to deepening of the understanding of hypertension and providing a basis and reference for clinical treatment using TCM syndrome differentiation.

2. Materials and Methods

2.1. Database and Search Strategies. Six databases including PubMed, EMBASE, Chinese Bio-Medical Literature Database (CBM), Chinese National Knowledge Infrastructure (CNKI), Chinese Scientific Journal Database (VIP), and Wan-fang Data were searched from 1/January/2003 to 30/October/2013. Databases in Chinese were searched to retrieve the maximum possible number of trials of syndrome differentiation for essential hypertension (EH) because syndrome differentiation is mainly used in China. Ongoing registered clinical trials were searched at the International Clinical Trial Registry by the U.S. National Institutes of Health (http://clinicaltrials.gov/). The following search terms were used individually or combined: "hypertension," "blood pressure," "essential hypertension," "syndrome differentiation," "vertigo," "headache," "parting," and "traditional Chinese medicine therapy." The bibliographies of included studies were searched for additional references.

2.2. Inclusion and Exclusion Criteria. Systolic blood pressure (SBP) ≥140 mmHg (1 mmHg = 0.133 kPa) and diastolic blood pressure (DBP) ≥90 mmHg from the literature were based on 1999 WHO-ISH Guidelines for the Management of Hypertension (1999 WHO-ISH GMH), 1998 WHO-ISH Guidelines for the Management of Hypertension (1998 WHO-ISH GMH), 2000 WHO-ISH Guidelines for the Management of Hypertension (2000 WHO-ISH GMH), Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005), China Guidelines on Prevention and Management of High Blood Pressure-2006 (CGPMHBP-2006), and Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7). Syndrome differentiation of TCM diagnosis used the Standard of TCM Diagnosis and Curative Effect of Disease-Syndrome, published by the State Administration of Traditional Chinese Medicine in 1994. Standards of dialectical classification used Clinical Research Guiding Principles of New Medicine of Chinese Herbs revised by the State Food and Drug Administration in 2002. Exclusion criteria were secondary hypertension, gestational hypertension, repeated literature, reviews, and literature with no clear classification.

2.3. Classification Criteria of Syndrome Elements. According to the classification criteria of syndrome elements proposed by Wang, statistical analysis was conducted for syndromes included in the cases. The classification criteria of syndrome elements were (1) six-excess external contraction: wind, cold, dampness, dryness, and fire; (2) five endogenous qi: internal wind, internal cold, internal dampness, internal dryness, and internal fire; (3) factors related to gas: qi deficiency, qi stagnation, qi block, qi counterflow, qi fall, and qi collapse; (4) factors related to blood: blood deficiency, blood stasis, blood collapse, blood dryness, and bleeding; (5) factors related to yin and yang: yin deficiency, yang deficiency, yin exuberance, and yang hyperactivity; (6) others: poison, excessive fluid, and phlegm turbidity.

2.4. Statistical Methods. Two authors conducted the literature search, study selection, and data extraction independently. Disagreements were resolved by discussion and consensus was met through a third party. SPSS 11.5 statistical software was used for data analyses (Chicago, IL, USA). Descriptive statistics procedures calculated frequency and percentage.

3. Results

3.1. Description of Included Literature. After a primary search of the databases, 503 articles were screened. After reading the titles and abstracts, 398 articles were excluded the reasons included; retrospective study that did not match the included criteria of this review ($n = 42$) and duplicated titles ($n = 356$). The full texts of 83 articles [41–123] were retrieved, and 22 articles were excluded for the following reasons: participants not meeting the inclusion criteria ($n = 11$), duplicated data ($n = 5$), patients having other diseases ($n = 5$), and no data for extraction ($n = 1$). In the end, 83 articles [41–123] were included, and all trials were conducted in China (Figure 1). The characteristics of included trials are listed in Table 1.

Overall, 13,272 patients with essential hypertension were included, with an average of 160 per trial, ranging from 23 to 703. Among them, 7075 were men, accounting for 53.3%, while 6197 were women, accounting for 46.7%. There was a wide range in patient age (18–92 years). Sources of cases included 24 provinces and the number of papers in each region is shown in Table 2.

3.2. Extraction of Syndrome Elements of EH. According to the definition of syndrome elements and classification criteria, syndrome elements were obtained and classified from the literature as follows: blood stasis (qi stagnation and blood stasis, qi deficiency with blood stasis, kidney deficiency and blood stasis, stasis blocking channels, phlegm and blood stasis resistance winding); qi stagnation (liver qi stagnation, qi stagnation and blood stasis); phlegm (phlegm turbidity resistance, phlegm-dampness retention); internal fire (intense liver fire, internal harassment of phlegm-heat); internal dampness (spleen deficiency with dampness encumbrance, phlegm-damp retention); internal wind (internal stirring of liver wind, wind-yang interference); qi deficiency (dual deficiency of qi and yin, dual deficiency of qi and blood, and qi deficiency with blood stasis); yang hyperactivity (ascendant hyperactivity of liver yang, yin deficiency with yang hyperactivity); yin deficiency (yin deficiency with yang hyperactivity, liver-kidney yin deficiency, dual deficiency of qi and yin, and deficiency of both yin and yang); yang deficiency (kidney yang deficiency, deficiency of both yin and yang); blood deficiency (dual deficiency of qi and blood).

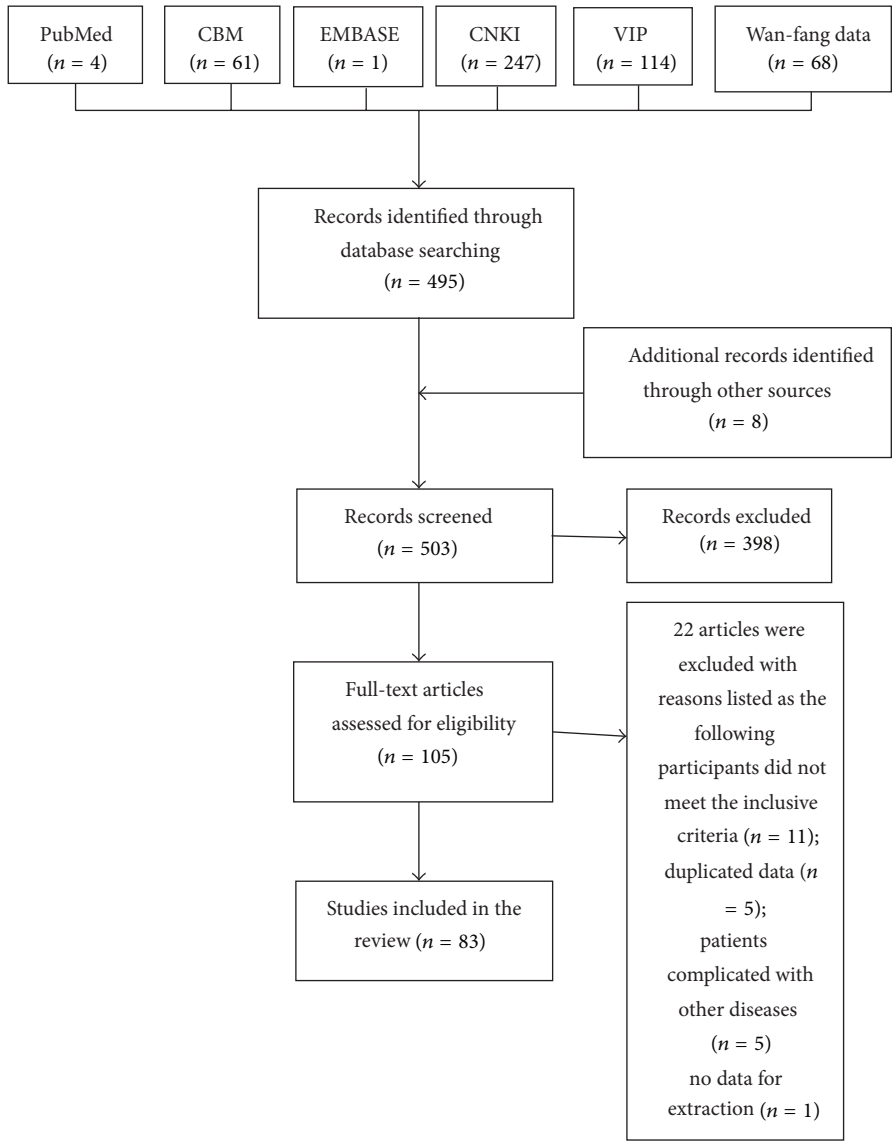

Figure 1: Screening process of articles.

As a result, 13,272 cases of hypertension syndrome were classified as 11 syndrome element types, which cover all cases.

3.3. Analysis of Syndrome Elements of EH. Syndrome elements of 13,272 patients with hypertension were divided into excessive syndrome elements and deficient syndrome elements (Table 3, Figure 2). The proportions of excessive syndrome elements are yang hyperactivity (19.08%), phlegm turbidity (13.68%), internal fire (13.21%), internal dampness (11.04%), blood stasis (4.86%), internal wind (1.21%), and qi stagnation (0.78%). The proportion of deficient syndrome elements are yin deficiency (26.27%), yang deficiency (7.89%), qi deficiency (1.80%), and blood deficiency (0.18%). Excessive syndrome elements greater than 10% included yang hyperactivity, phlegm turbidity, internal fire, and internal dampness. Deficient syndrome elements greater than 10% included yin deficiency. Yang hyperactivity and yin deficiency were the most common syndrome elements of hypertension.

3.4. Targets of Syndrome Elements of EH. The targets of syndrome elements are the disease locations of individual syndrome elements. Disease location of syndrome elements was confirmed according to the five zang-organs and six fu-organs, chi heng fu, and meridians.

As a result, 9091 cases (68.50%) had clear targets of syndrome elements related to liver, kidney, and spleen (Table 4). There were 7789 cases of liver syndromes (85.68%). Among them, there were 2793 cases of internal fire of liver (35.86%), 4033 cases of ascendant hyperactivity of liver yang (51.78%), 543 cases of liver yin deficiency (6.97%), 164 cases of liver qi stagnation (2.11%), and 256 cases of internal stirring of liver wind (3.29%). There were 903 cases of kidney syndromes

Table 1: Characteristics of included studies.

Study ID	Sample (M/F)	Age (years)	Diagnosis standard	TCM syndrome differentiation (number of patients)	Region of China
Xu and Chen 2012 [13]	122 (58/64)	60–79	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Phlegm and blood stasis resistance winding (39), yin deficiency with yang hyperactivity (44), and idney deficiency (39)	Beijing
Ferreira and Lopes 2011 [14]	448 (243/205)	M: 62.1 ± 10.9 F: 59.3 ± 8.7	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (284), yin deficiency with yang hyperactivity (43), phlegm-damp retention (74), and deficiency of both yin and yang (47)	Jiangsu
Wang et al. 2013 [15]	99 (50/49)	73 ± 6.1	1999 WHO-ISH GMH	Qi stagnation and blood stasis (99)	Guangdong
Lee et al. 2004 [16]	87 (48/39)	M: 62.7 ± 8.3 F: 58.9 ± 7.5	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Ascendant hyperactivity of liver yang (32), phlegm-damp retention (27), and qi deficiency with blood stasis (28)	Guangdong
Wang et al. 2013 [17]	140 (83/57)	56.5 ± 9.8	Chinese Guidelines for the Management of Hypertension-2010 (CGMH-2010)	Intense liver fire (28), yin deficiency with yang hyperactivity (39), phlegm-damp retention (45), and deficiency of both yin and yang (28)	Heibei
Wang and Xiong 2012 [18]	76 (38/38)	Not reported	Chinese Guidelines for the Management of Hypertension-2010 (CGMH-2010)	Kidney deficiency and blood stasis (76)	Fujian
Xiong et al. 2013 [19]	395 (228/167)	53 ± 17	Hypertension diagnostic criteria (unclear)	Intense liver fire (54), yin deficiency with yang hyperactivity (177), phlegm-damp retention (62), and deficiency of both yin and yang (102)	Liaoning
Wang et al. 2013 [20]	120 (60/60)	29–62	Hypertension diagnostic criteria (unclear)	Ascendant hyperactivity of liver yang (30), yin deficiency with yang hyperactivity (30), phlegm-damp retention (30), and deficiency of both yin and yang (30)	Hainan
Wang et al. 2013 [21]	184 (83/101)	18–80	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (21), yin deficiency with yang hyperactivity (75), phlegm-damp retention (81), and deficiency of both yin and yang (7)	Jiangsu
Chen 1993 [22]	60 (30/30)	T: 48 ± 8.1 C: 47 ± 6.7	1999 WHO-ISH GMH	Dual deficiency of qi and yin (60)	Guangdong
Wang et al. 2012 [23]	53 (16/37)	40–80	1999 WHO-ISH GMH	Insufficiency of spleen with overabundance of dampness (19), dual deficiency of qi and blood (20), and liver-kidney yin deficiency (14)	Neimenggu
Wang et al. 2013 [24]	112 (83/29)	53.5 ± 11.04	1999 WHO-ISH GMH	Intense liver fire (19), yin deficiency with yang hyperactivity (23), phlegm-damp retention (16), and deficiency of both yin and yang (22)	Jiangxi
Wang and Xiong 2012 [25]	61 (M/F not reported)	T: 57.1 ± 6.16 C: 55.67 ± 6.28	Chinese Guidelines for the Management of Hypertension-2004 (CGMH-2004)	Blood stasis (61)	Guangdong
Chen et al. 2011 [26]	259 (108/151)	65.58 ± 12.17	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (35), yin deficiency with yang hyperactivity (89), phlegm-damp retention (88), and deficiency of both yin and yang (47)	Beijing
Xu and Chen 2011 [27]	81 (53/28)	52.79 ± 12.83	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (42), yin deficiency with yang hyperactivity (18), phlegm-damp retention (14), and deficiency of both yin and yang (7)	Zhejiang

Table 1: Continued.

Study ID	Sample (M/F)	Age (years)	Diagnosis standard	TCM syndrome differentiation (number of patients)	Region of China
Chen et al. 2012 [28]	183 (85/98)	66.81 ± 8.81	Chinese Guidelines for the Management of Hypertension-2004 (CGMH-2004)	Intense liver fire (28), yin deficiency with yang hyperactivity (53), phlegm-damp retention (57), and deficiency of both yin and yang (45)	Jiangsu
Liu et al. 2011 [29]	89 (45/44)	M: 59.5 ± 10.9 F: 59.3 ± 11.0	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (59), yin deficiency with yang hyperactivity (5), phlegm-damp retention (13), and deficiency of both yin and yang (12)	Jiangsu
Dobos and Tao 2011 [30]	342 (213/129)	M: 59.43 ± 16.76 F: 59.43 ± 11.82	1999 WHO-ISH GMH	Intense liver fire (51), yin deficiency with yang hyperactivity (139), phlegm-damp retention (85), and deficiency of both yin and yang (67)	Guangdong
Xiong et al. 2011 [31]	562 (297/265)	M: 62.1 ± 10.8 F: 58.5 ± 9.1	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (352), yin deficiency with yang hyperactivity (58), phlegm-damp retention (97), and deficiency of both yin and yang (55)	Jiangsu
Wang and Xiong 2012 [32]	398 (199/199)	59.20 ± 9.54	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (88), yin deficiency with yang hyperactivity (196), phlegm-damp retention (89), and deficiency of both yin and yang (25)	Jiangsu
Wang et al. 2013 [33]	178 (81/97)	18–80	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (49), yin deficiency with yang hyperactivity (43), phlegm-damp retention (57), and deficiency of both yin and yang (29)	Shanghai
Tian 2011 [34]	200 (109/91)	30–75	1999 WHO-ISH GMH	Intense liver fire (37), yin deficiency with yang hyperactivity (55), phlegm-damp retention (82), and deficiency of both yin and yang (26)	Tianjin
Wang and Xiong 2012 [35]	120 64/56	T: 62.77 ± 9.18 C: 59.63 ± 8.77	1999 WHO-ISH GMH	Intense liver fire (37), yin deficiency with yang hyperactivity (55), phlegm-damp retention (82), and deficiency of both yin and yang (26)	Hunan
Wang et al. 2012 [36]	494 (264/230)	M: 61.6 ± 10.6 F: 58.3 ± 8.5	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Ascendant hyperactivity of liver yang (313), yin deficiency with yang hyperactivity (52), deficiency of both yin and yang (83), and liver-kidney yin deficiency (46)	Jiangsu
Xu and Chen 2008 [37]	150 (M/F not reported)	Not reported	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (29), randomized stagnation of phlegm (53), dual deficiency of qi and yin (30), and stasis blocking channels (38)	Xinjiang
Cheung 2011 [38]	109 (68/41)	65.6 ± 10.6	1999 WHO-ISH GMH	Intense liver fire (19), yin deficiency with yang hyperactivity (18), phlegm-damp retention (34), and deficiency of both yin and yang (38)	Fujian
Xiong et al. 2013 [39]	102 (58/44)	37–85	1999 WHO-ISH GMH	Intense liver fire (18), yin deficiency with yang hyperactivity (31), phlegm-damp retention (23), and deficiency of both yin and yang (30)	Guizhou
Lu et al. 2004 [40]	40 (23/17)	Not reported	1999 WHO-ISH GMH	Blood stasis (40)	Guangdong
Zhao et al. 2012 [41]	60 (41/19)	T: 62.07 ± 8.88 C: 57.3 ± 9.09	1999 WHO-ISH GMH and Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Blood stasis (60)	Guangdong

Table 1: Continued.

Study ID	Sample (M/F)	Age (years)	Diagnosis standard	TCM syndrome differentiation (number of patients)	Region of China
Liu et al. 2009 [42]	60 (36/24)	T: 53.87 ± 5.92 C: 52.97 ± 5.40	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Phlegm-damp retention (60)	Shanghai
Wang et al. 2012 [43]	82 (49/33)	60–75	1999 WHO-ISH GMH	Liver-kidney yin deficiency (82)	Heilongjiang
Luo et al. 2011 [44]	100 (42/58)	36–81	Hypertension diagnostic criteria (unclear)	Ascendant hyperactivity of liver yang (12), yin deficiency with yang hyperactivity (60), phlegm-damp retention (18), and kidney deficiency (10)	Guangxi
Wang et al. 2012 [45]	80 (48/32)	68.05 ± 5.41	1999 WHO-ISH GMH	Liver-kidney yin deficiency (80)	Guangxi
Wang et al. 2011 [46]	251 (148/103)	55 ± 19	1999 WHO-ISH GMH	Intense liver fire (71), yin deficiency with yang hyperactivity (62), phlegm-damp retention (60), and deficiency of both yin and yang (58)	Liaoning
Bai et al. 2005 [47]	122 (71/51)	T: 44.7 ± 11.6 C: 46.2 ± 9.5	1999 WHO-ISH GMH	Ascendant hyperactivity of liver yang (35), liver-kidney yin deficiency (18), phlegm-damp retention (32), dual deficiency of qi and yin (25), and stasis blocking channels (12)	Hebei
Yang et al. 2005 [48]	80 (41/39)	M: 51.28 ± 6.96 F: 52.71 ± 6.57	1999 WHO-ISH GMH	Ascendant hyperactivity of liver yang (80)	Henan
Xia et al. 2010 [49]	40 (M/F not reported)	T: 55.23 ± 6.01 C: 55.13 ± 6.34	1999 WHO-ISH GMH	Ascendant hyperactivity of liver yang (40)	Gansu
Liu et al. 2003 [50]	60 (43/17)	45–73	Chinese Guidelines for the Management of Hypertension-2009 (CGMH-2009)	Yang hyperactivity (29), phlegm turbidity resistance (31)	Zhejiang
Yin and Liu 2005 [51]	36 (M/F not reported)	40.50 ± 11.51	1999 WHO-ISH GMH	Phlegm-damp retention (36)	Jiangsu
Wu et al. 2010 [52]	90 (41/39)	32–78	1999 WHO-ISH GMH	Ascendant hyperactivity of liver yang and blood stasis (90)	Hebei
Deng 2008 [53]	60 (45/15)	T: 61 ± 4.12 C: 61 ± 4.02	1999 WHO-ISH GMH	Qi deficiency with blood stasis (60)	Hebei
Wu and Xu 2010 [54]	60 (32/28)	Not reported	1999 WHO-ISH GMH	Dual deficiency of qi and yin (60)	Guizhou
Wang et al. 2011 [55]	276 170/106	M: 53.4 ± 21.1 F: 55.6 ± 17.3	1999 WHO-ISH GMH	Wind-yang interference (22), stasis blocking channels (73), yin deficiency with yang hyperactivity (134), and phlegm turbidity resistance (47)	Guangxi
Wu et al. 2010 [56]	156 (79/77)	T: 48 ± 6.9 C: 49 ± 8.2	Hypertension diagnostic criteria (unclear)	Ascendant hyperactivity of liver yang (52), yin deficiency with yang hyperactivity (53), and deficiency of both yin and yang (51)	Zhejiang
Fan and Liu 2010 [57]	395 (203/192)	30–80	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Qi deficiency with blood stasis (65), intense liver fire (91), yin deficiency with yang hyperactivity (63), phlegm-damp retention (57), deficiency of both yin and yang (39), and dual deficiency of qi and blood (18)	Beijing

Table 1: Continued.

Study ID	Sample (M/F)	Age (years)	Diagnosis standard	TCM syndrome differentiation (number of patients)	Region of China
Zhu et al. 2009 [58]	54 (30/24)	61.74 ± 14.89	1999 WHO-ISH GMH	Kidney yang deficiency (24), kidney yin deficiency (30)	Yunnan
Liu et al. 2009 [59]	140 (68/72)	34–79	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (16), yin deficiency with yang hyperactivity (52), phlegm-damp retention (41), and deficiency of both yin and yang (31)	Guangxi
He et al. 2013 [60]	230 (65/165)	43–74	1999 WHO-ISH GMH	Intense liver fire (28), ascendant hyperactivity of liver yang (148), and liver-kidney yin deficiency (54)	Guangdong
Tang et al. 2012 [61]	100 (37/63)	55.1 ± 6.2	1999 WHO-ISH GMH	Intense liver fire (19), yin deficiency with yang hyperactivity (29), deficiency of both yin and yang (20), and liver-kidney yin deficiency (32)	Shanghai
Gong et al. 2010 [62]	120 (60/60)	T: 55.38 ± 8.01 C: 56.80 ± 8.58	1999 WHO-ISH GMH	Intense liver fire (30), yin deficiency with yang hyperactivity (30), phlegm-damp retention (30), and deficiency of both yin and yang (30)	Shandong
Zhang et al. 2005 [63]	60 (32/28)	62.22 ± 6.12	Chinese Guidelines for the Management of Hypertension-2004 (CGMH-2004)	Intense liver fire (8), yin deficiency with yang hyperactivity (28), phlegm-damp retention (14), and deficiency of both yin and yang (10)	Guangxi
Liu et al. 2009 [64]	200 (105/95)	M: 61.88 ± 11.91 F: 63.07 ± 12.45	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (51), yin deficiency with yang hyperactivity (49), phlegm-damp retention (50), and deficiency of both yin and yang (50)	Guangxi
Wang 2012 [65]	200 (103/94)	46.4 ± 15.46	1999 WHO-ISH GMH	Intense liver fire (96), yin deficiency with yang hyperactivity (46), phlegm-damp retention (18), and deficiency of both yin and yang (37)	Shanxi
Yao and Huang 2007 [66]	47 (22/25)	66.00 ± 12.35	Chinese Guidelines for the Management of Hypertension-2004 (CGMH-2004)	Intense liver fire (12), yin deficiency with yang hyperactivity (11), phlegm-damp retention (12), and deficiency of both yin and yang (12)	Tianjin
Guo et al. 2002 [67]	120 (62/58)	T: 63.64 ± 9.22 C: 60.30 ± 3.36	Chinese Guidelines for the Management of Hypertension-2004 (CGMH-2004)	Intense liver fire (30), yin deficiency with yang hyperactivity (30), phlegm-damp retention (30), and deficiency of both yin and yang (30)	Anhui
Zhang et al. 2011 [68]	320 (135/185)	66.40 ± 12.56	2007 WHO-ISH GMH	Intense liver fire (36), yin deficiency with yang hyperactivity (101), phlegm-damp retention (125), and deficiency of both yin and yang (58)	Jiangsu
Liao et al. 2010 [69]	23 (14/9)	T: 65 ± 5 C: 65 ± 8	1999 WHO-ISH GMH	Blood stasis (23)	Fujian
Xiong 2010 [70]	70 (37/33)	53.06 ± 8.62	Hypertension diagnostic criteria (unclear)	Intense liver fire (13), yin deficiency with yang hyperactivity (21), phlegm-damp retention (25), and deficiency of both yin and yang (11)	Heilongjiang
Jiang et al. 2012 [71]	86 (50/36)	36–81	Hypertension diagnostic criteria (unclear)	Yin deficiency with yang hyperactivity (86)	Guangdong
Huang and Wei 2012 [72]	260 (119/141)	65.56 ± 8.42	Chinese Guidelines for the Management of Hypertension-2010 (CGMH-2010)	Intense liver fire (56), yin deficiency with yang hyperactivity (77), phlegm-damp retention (73), and deficiency of both yin and yang (54)	Beijing

Table 1: Continued.

Study ID	Sample (M/F)	Age (years)	Diagnosis standard	TCM syndrome differentiation (number of patients)	Region of China
Lu 2004 [73]	138 (97/41)	61.84 ± 5.25	1999 WHO-ISH GMH	Intense liver fire (16), yin deficiency with yang hyperactivity (43), phlegm-damp retention (45), and deficiency of both yin and yang (34)	Fujian
Sun and Wang 2005 [74]	703 (382/321)	50–79	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Yin deficiency with yang hyperactivity (215), phlegm-damp retention (83), deficiency of both yin and yang (91), ascendant hyperactivity of liver yang (135), liver-kidney yin deficiency (92), yang deficiency (11), qi deficiency (14), dual deficiency of qi and yin (14), blood stasis (11), qi deficiency with blood stasis (3), internal harassment of phlegm-heat (22), internal harassment of phlegm-heat and blood stasis (3), liver-kidney yin deficiency and blood stasis (2), internal harassment of phlegm-heat and qi deficiency (1), deficiency of both yin and yang and internal harassment of phlegm-heat (1), liver-kidney yin deficiency and phlegm-damp retention (1), yin deficiency with yang hyperactivity and blood stasis (1), ascendant hyperactivity of liver yang and internal harassment of phlegm-heat (1), ascendant hyperactivity of liver yang and blood stasis (1), and deficiency of both yin and yang and phlegm-damp retention (1)	Guangdong
Xiang et al. 2012 [75]	125 (75/50)	55–72	Hypertension diagnostic criteria (unclear)	Kidney deficiency and blood stasis (15), internal stirring of liver wind (68), qi deficiency with blood stasis (21), and intermingled phlegm and blood stasis (21)	Guangdong
Zhu 2009 [76]	97 (41/56)	37–79	Hypertension diagnostic criteria (unclear)	Ascendant hyperactivity of liver yang (13), yin deficiency with yang hyperactivity (59), liver-kidney yin deficiency (16), and deficiency of both yin and yang (21)	Shandong
Xu and Wang 2009 [77]	80 (49/31)	40–83	1999 WHO-ISH GMH	Intense liver fire (18), yin deficiency with yang hyperactivity (17), phlegm-damp retention (35), and deficiency of both yin and yang (10)	Xinjiang
Lin and Kang 2012 [78]	69 (37/32)	T: 53.48 ± 10.02 C: 59.20 ± 5.610	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Liver-kidney yin deficiency (69)	Zhejiang
Feng et al. 2013 [79]	60 (60 M)	63.0 ± 7.5	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Ascendant hyperactivity of liver yang (60)	Fujian
Yu and Xing 2010 [80]	168 (108/60)	T: 58 ± 12 C: 54 ± 12	1999 WHO-ISH GMH	Intense liver fire (54), yin deficiency with yang hyperactivity (45), phlegm-damp retention (36), and deficiency of both yin and yang (33)	Beijing
Qiu et al. 2011 [81]	170 (122/48)	54 ± 11.6	Chinese Guidelines for the Management of Hypertension-2004 (CGMH-2004)	Intense liver fire (43), yin deficiency with yang hyperactivity (40), phlegm-damp retention (38), and deficiency of both yin and yang (49)	Beijing

Table 1: Continued.

Study ID	Sample (M/F)	Age (years)	Diagnosis standard	TCM syndrome differentiation (number of patients)	Region of China
Wu and Xu 2012 [82]	149 (74/75)	61.22 ± 9.36	Hypertension diagnostic criteria (unclear)	Intense liver fire (48), yin deficiency with yang hyperactivity (32), phlegm-damp retention (49), and deficiency of both yin and yang (20)	Hubei
Fang et al. 2007 [83]	220 (128/92)	34–73	1999 WHO-ISH GMH	Intense liver fire (98), yin deficiency with yang hyperactivity (79), phlegm-damp retention (19), and deficiency of both yin and yang (24)	Gansu
Fang et al. 2003 [84]	229 (113/116)	>35	Hypertension diagnostic criteria (unclear)	Liver-kidney yin deficiency (60), yin deficiency with yang hyperactivity (73), phlegm-damp retention (85), and deficiency of both yin and yang (11)	Hangzhou
Peng and Shi 2010 [85]	122 (57/65)	64.62 ± 8.86	1999 WHO-ISH GMH	Qi deficiency with blood stasis (26), intense liver fire (23), yin deficiency with yang hyperactivity (26), phlegm-damp retention (25), and deficiency of both yin and yang (22)	Anhui
Yang et al. 2004 [86]	151 (110/41)	Not reported	Hypertension diagnostic criteria (unclear)	Ascendant hyperactivity of liver yang (151)	Shandong
Shi et al. 2013 [87]	60 (29/31)	52.6 ± 12.3	Clinical research guiding principles of new medicine of Chinese traditional medicine	Phlegm-damp retention (60)	Zhejiang
Han 2004 [88]	377 (182/195)	20–60	Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005)	Intense liver fire (108), yin deficiency with yang hyperactivity (70), phlegm-damp retention (154), and deficiency of both yin and yang (45)	Anhui
Shen et al. 2008 [89]	79 (40/39)	T: 51.70 ± 4.53 C: 51.67 ± 4.36	Clinical research guiding principles of new medicine of Chinese traditional medicine	Phlegm and blood stasis resistance winding and ascendant hyperactivity of liver yang (79)	Guangzhou
Shen et al. 2005 [90]	290 (120/170)	66.2 ± 1.37	Chinese Guidelines for the Management of Hypertension-2004 (CGMH-2004)	Intense liver fire (34), yin deficiency with yang hyperactivity (99), phlegm-damp retention (114), and deficiency of both yin and yang (43)	Jiangsu
Liu et al. 2009 [91]	240 (120/120)	18–65	Hypertension diagnostic criteria (unclear)	Intense liver fire (240)	Anhui
Lu et al. 2011 [92]	80 (56/24)	T: 66.07 ± 7.15 C: 67.10 ± 7.32	1999 WHO-ISH GMH	Blood stasis (80)	Guangxi
Guo et al. 2006 [93]	60 (30/30)	Not reported	Chinese Guidelines for the Management of Hypertension-2010 (CGMH-2010)	Yin deficiency with yang hyperactivity (60)	Fujian
Zhang et al. 2012 [94]	140 (83/57)	56±10	Chinese Guidelines for the Management of Hypertension-2010 (CGMH-2010)	Ascendant hyperactivity of liver yang (28), yin deficiency with yang hyperactivity (39), phlegm-damp retention (45), and deficiency of both yin and yang (28)	Hebei
Dong et al. 2010 [95]	166 (106/60)	63–82	Hypertension diagnostic criteria (unclear)	Kidney yin deficiency and wind-phlegm (166)	Sichuan

(9.93%). Among them, there were 879 cases of kidney yin deficiency (97.34%) and 24 cases of kidney yang deficiency (2.66%). There were 399 cases of spleen syndromes (4.39%), all of which were spleen qi deficiency.

3.5. Combining Forms of Syndrome Elements of EH. We found that 13,272 cases of hypertension contained 33 syndrome types. According to the definition of syndrome elements, all syndromes were divided into four types: single factor,

Table 2: Number of papers and cases in region.

Region (China)	Provinces	Papers (pieces)	Cases	Male	Female
North China	Hebei	5	552	323	229
	Beijing	6	1374	718	656
	Inner Mongolia	1	53	16	37
	Tianjin	2	247	131	116
Northeast	Liaoning	2	646	376	270
	Heilongjiang	2	152	86	66
Northwest	Xinjiang	2	230	124	106
	Shanxi	1	197	103	94
	Gansu	2	260	148	112
Central China	Henan	2	200	101	99
	Hubei	1	149	74	75
	Hunan	1	120	64	56
East China	Shandong	3	368	211	157
	Jiangsu	10	3004	1489	1515
	Anhui	4	859	421	438
	Zhejiang	6	655	354	301
	Fujian	6	466	307	159
	Jiangxi	1	112	83	29
	Shanghai	3	338	154	184
South China	Guangdong	12	1972	1047	925
	Guangxi	7	936	519	417
Southwest China	Yunnan	1	54	30	24
	Guizhou	2	162	90	72
	Sichuan	1	166	106	60
Total		83	13272	7075	6197

Table 3: Syndrome elements of 13,272 patients with essential hypertension.

Syndrome factors	Frequency	Percentage (%)
Yin deficiency	5554	26.27
Yang hyperactivity	4033	19.08
Phlegm turbidity	2892	13.68
Internal fire	2793	13.21
Internal dampness	2333	11.04
Yang deficiency	1668	7.89
Blood stasis	1027	4.86
Qi deficiency	380	1.80
Internal wind	256	1.21
Qi stagnation	164	0.78
Blood deficiency	38	0.18

Table 4: Targets of syndrome elements.

Target	Percentage (%)
Liver	7789 (85.68)
Kidney	903 (9.93)
Spleen	399 (4.39)
Total	100

two-factor, three-factor, and four-factor syndromes. The statistics of the combined forms of syndrome and their frequency (proportion more than 1%) are shown in Table 5. Internal fire is the most common in the single factor group, while yin deficiency with yang hyperactivity is the most common in the two-factor group. From highest to lowest frequency in the two-factor group are phlegm-damp retention, deficiency of both yin and yang, Liver-kidney yin deficiency, dual deficiency of qi and yin, qi stagnation and blood stasis, and qi deficiency with blood stasis. The syndrome, yin deficiency and wind-phlegm, is the most common in the three-factor category. There were no four-factor combinations that reached a frequency of greater than 1%.

4. Discussion and Perspectives

4.1. Pathogenesis of Hypertension. Syndrome elements are the expression of pathogenesis of a disease [36]. According to the statistical results of syndrome elements, pathogenesis of EH can be summarized as simultaneous insufficiency and excess. Deficiency syndrome included yin deficiency, yang deficiency, qi deficiency, and blood deficiency. Excess

Table 5: Combined syndrome forms.

Combination Class	Combination Forms	Frequency	Percentage (%)
Single-factor	Internal fire	2765	20.98
	Yang hyperactivity	875	6.64
	Blood stasis	398	3.02
	Phlegm turbidity	149	1.13
Two-factor	Yin deficiency with yang hyperactivity	3059	23.21
	Phlegm-damp retention	2508	19.03
	Deficiency of both yin and yang	1605	12.18
	Liver-kidney yin deficiency	543	4.12
	Dual deficiency of qi and yin	189	1.43
	Qi stagnation and blood stasis	164	1.24
	Qi deficiency with blood stasis	138	1.05
Three-factor	Yin deficiency and wind-phlegm	166	1.26
Total		12559	95.29

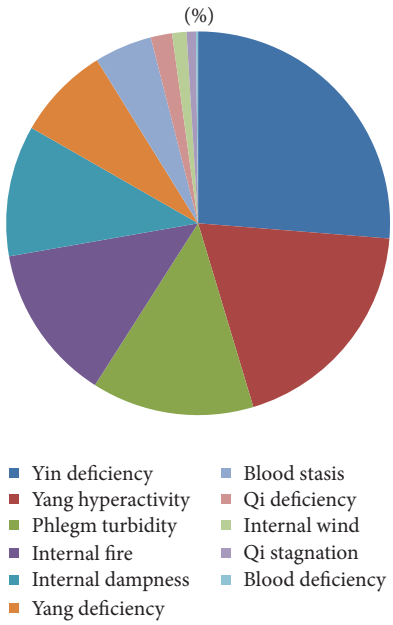

Figure 2: Percentage of syndrome factors.

syndrome included blood stasis, phlegm turbidity, qi stagnation, yang hyperactivity, internal fire, internal dampness, and internal wind. Among them, yin deficiency was the most common, followed by yang hyperactivity. Other elements, included in descending order, were phlegm turbidity, internal fire, internal dampness, yang deficiency, blood stasis, qi deficiency, and internal wind. The main disease location is the liver, which is closely related to the kidney and spleen.

4.2. Characteristics of Combined Syndrome Elements of EH. The combined forms of syndrome elements of hypertension have certain characteristics according to the literature, summarized as follows. (1) The combined forms of syndrome elements of hypertension have three forms, single-factor, two-factor, and three-factor forms. (2) Excess syndromes are more common than deficiency syndromes for single-factor syndromes, with internal fire, yang hyperactivity, blood stasis, and phlegm turbidity as the main syndrome factors. (3) Deficiency syndrome and excess syndrome was the most common two-factor syndrome, followed by excess syndrome and excess syndrome and deficiency syndrome and deficiency syndrome, respectively. (4) Syndrome of yin deficiency and wind-phlegm was the most common three-factor syndrome.

4.3. Implications for Instructing Clinical Application. The discovery of distributing characteristics of syndrome elements is conducive to instructing clinical application. Several Chinese herbs and classical formulas can lower BP and improve symptoms according to syndrome differentiation (Table 6). First, when aiming to cure internal fire syndrome, use *Huanglian Jie Du Tang* (detoxicant decoction of *Coptis*) to clear heat and toxins of the liver [35]. Chinese herbs such as *Xiakucao* (*Prunella vulgaris* L.) [123], *Huanglian* (*Coptis chinensis*) [124], *Huangqin* (*Scutellaria baicalensis* Georgi), *Huang-bai* (*Phellodendron* bark), and *Zhizi* (*Gardenia*) [125] can lower BP. Second, when aiming to cure yin deficiency with yang hyperactivity, use *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*). Chinese herbs such as *Tianma* (*Gastrodia*) [126] and *Gouteng* (*Uncaria*) [127] could suppress liver yang hyperactivity. *Niuxi* (*Achyranthes* root) [128] *and Duzhong* (*Eucommia ulmoides*) [129–131] had antihypertensive effects by nourishing the kidney. Third, when aiming to cure phlegm-damp retention, use *Wuling* powder [132], *Zexie Tang* (decoction of *Alisma*) [133], and *Wendantang jiawei* decoction (modified decoction for clearing away gallbladder heat). In addition, when aiming at wind-phlegm syndrome, use *Banxia Baizhu Tianma Tang* (decoction of *Pinellia ternata, Atractylodes macrocephala,* and *Gastrodia elata*) to calm the liver, strengthen the spleen, remove dampness, and reduce phlegm [35]. Chinese herbs such as *Zexie* (*Alisma*), *Fuling* (*Poria cocos*) [134], *Zhuling*

Table 6: Chinese herbs and classical formulas that lower BP and improve symptoms according to syndrome differentiation.

Syndrome	Formula	Components	TCM efficacy	Label	Chinese herbs
Internal fire syndrome	*Huanglian Jie Du Tang (detoxicant decoction of Coptis)*	*Rhizoma Coptidis, Radix Scutellariae, Radix et Rhizoma Rhei, and Cortex Phellodendri Chinensis*	Clear heat and toxins from liver	Classical prescription of *Arcane Essentials from the Imperial Library* dispensed by Wang Tao in Tang dynasty	*Xiakucao (Prunella vulgaris* L.*), Huanglian (Coptis chinensis), Huangqin (Scutellaria baicalensis* Georgi*), Huang-bai (Phellodendron* bark*), and Zhizi (Gardenia)*
Yin deficiency with yang hyperactivity	*Tianma Gouteng Yin (decoction of Gastrodia and Uncaria)*	*Rhizoma Gastrodiae, Ramulus Uncariae cum Uncis, Concha Haliotidis, Cortex Eucommiae, Radix Achyranthis Bidentatae, Herba Taxilli, Fructus Gardeniae, Radix Scutellariae, Herba Leonuri, Sclerotium Poriae Pararadicis, and Caulis Polygoni Multiflori*	Suppressing liver yang hyperactivity, clearing heat, activating blood, and nourishing the kidney	Classical prescription of *New Meanings of Treatment in Miscellaneous Diseases with Traditional Chinese Medicine*	*Tianma (Gastrodia), Gouteng (Uncaria), Niuxi (Achyranthes* root*), and Duzhong (Eucommia ulmoides)*
Phlegm-dampness retention	*Wuling powder*	*Alisma, Polyporus, Poria cocos, Ramulus Cinnamomi, Rhizoma Atractylodis Macrocephalae*	Dissolving phlegm, draining water-dampness, and warming Yang	Classical prescription of *Treatise on Febrile and Miscellaneous Diseases* by Zhang Zhongjing in the Eastern Han Dynasty	*Zexie (Alisma), Zhuling (Polyporus), Fuling (Poria cocos), Banxia* (*The tuber of pinellia*), *baizhu (Rhizoma Atractylodis Macrocephalae), Zelan (Herba Lycopi), and Shichangpu (Rhizoma Acori Tatarinowii)*
	Zexie Tang (decoction of Alisma)	*Alisma, Rhizoma Atractylodis Macrocephalae*	Dissolving phlegm and draining water-dampness	Classical prescription of *Treatise on Febrile and Miscellaneous Diseases* by Zhang Zhongjing in the Eastern Han Dynasty	
	Wendan Tang jiawei decoction (modified decoction for clearing away gallbladder heat)	*Caulis Bambusae in Taenia, Fructus Aurantii Immaturus, Rhizoma Pinelliae, Pericarpium Citri Reticulatae* (aged tangerine peel), *Poria, Radix et Rhizoma Glycyrrhizae, Radix Codonopsis, Radix Curcumae, and so forth.*	Dissolving phlegm and boosting qi	Modified classical prescription of *Prescriptions Assigned to the Three Categories of Pathogenic Factors of Diseases*	
Wind-phlegm	*Banxia Baizhu Tianma Tang (decoction of Pinellia ternata, Atractylodes macrocephala, and Gastrodia elata)*	*Rhizoma Pinelliae Praeparatum, Rhizoma Gastrodiae, Pericarpium Citri Reticulatae, Poria,* and *Radix et Rhizoma Glycyrrhizae*	Calmed the liver, strengthened the spleen, removed dampness, and reduced phlegm	Classical prescription of *Medical Revelations* dispensed by Cheng Zhongling in Qing dynasty	*Fuling (Poria cocos), Banxia (Pinellia ternata), Baizhu (Rhizoma Pinelliae Praeparatum), Tianma (Rhizoma Gastrodiae), and Chenpi (Pericarpium Citri Reticulatae)*

Table 6: Continued.

Syndrome	Formula	Components	TCM efficacy	Label	Chinese herbs
Blood stasis	*Xuefu Zhuyu Tang*	*Radix Angelicae Sinensis, Radix Rehmanniae, Semen Pruni Persicae, red flower, Fructus Aurantii, Chinese thorowax root, red peony root, Radix et Rhizoma Glycyrrhizae, Platycodon grandiflorum, Ligusticum chuanxiong* Hort, *and Radix Achyranthis Bidentatae*	Removing blood stasis and promoting Qi	Classical prescription of *Yi Lin Gai Cuo* (correction of the errors of medical works) by Wang Qingren in the Qing Dynasty	*Danggui (Radix Angelicae Sinensis), Chishao* (red peony root*), Danshen (Salvia miltiorrhiza), Yimucao (Leonurus japonicus), Chuanxiong (Ligusticum chuanxiong* Hort*), and Shengdi (Radix Rehmanniae)*
Liver-kidney yin deficiency	*Liu Wei Dihuang Wan (pill of Rehmannia)*	*Rehmannia glutinosa, Fructus corni, Rhizoma Dioscoreae, Alisma, Poria cocos, and Cortex Moutan Radicis*	Replenish liver and kidney yin	*Xiaoer Yaozheng Zhijue* (*Pediatric medicine card straight*) by *Qianyi* in the Song Dynasty	*Shanyurou (Fructus corni), Duzhong (Eucommia), Shudi (Rehmannia glutinosa), Gouqizi (Lycium barbarum* L.*), and Huangjing (Rhizoma Polygonati)*
Yang deficiency	*Shen qi Wan* (*kidney qi pill*)	*Rehmannia glutinosa, Fructus corni, Rhizoma Dioscoreae, Alisma, Poria cocos, Cortex Moutan Radicis, Cortex Cinnamomi, and Radix Aconiti Carmichaeli*	Recuperate kidney yang	Classical prescription of *Treatise on Febrile and Miscellaneous Diseases* by Zhang Zhongjing in the Eastern Han Dynasty	*Fuzi (Radix Aconiti Carmichaeli), Bajitian (Morinda officinalis), Yinyanghuo (Epimedium), Buguzhi (Psoralea* fruits*), and Rousongrong (Cistanche)*
Qi deficiency	*Buzhong yiqi Tang*	*Codonopsis pilosula, Astragalus membranaceus, Rhizoma Atractylodis Macrocephalae, Tangerine Peel, Rattletop, Radix Bupleuri, Angelica sinensis, and Liquorice*	Replenish qi to invigorate the spleen	Classical prescription of *Treatise on Spleen and Stomach* by Li Dongyuan in the Jin Dynasty	*Dangshen (Codonopsis pilosula), Huangqi (Astragalus membranaceus), and Baizhu (Rhizoma Atractylodis Macrocephalae)*
Blood deficiency	*Danggui siwu Tang*	*Angelica sinensis, Radix Paeoniae Rubra, Ligusticum chuanxiong* Hort, *and Rehmannia glutinosa* Libosch	Enrich and nourish blood	Classical prescription of *Treatise on Febrile and Miscellaneous Diseases* by Zhang Zhongjing in the Eastern Han Dynasty	*Danggui (Angelica sinensis), Chuanxiong (Ligusticum chuanxiong* Hort*), Shudihuang (Rehmannia glutinosa* Libosch*), and Baishao (Radix Paeoniae Rubra)*

(*Polyporus*) [135], and *Banxia* (*The tuber of pinellia*) [136] could effectively reduce BP as well. Fourthly, to remove blood stasis, use *Xuefu Zhuyu Tang*, a famous classical prescription recorded in *Yi Lin Gai Cuo* (*Correction of the Errors of Medical Works*) by Wang Qingren in the Qing Dynasty. It is effective in removing blood stasis and promoting Qi. Herbs such as *Chishao* (red peony root) [137], *Danshen* (*Salvia miltiorrhiza*) [138], *Yimucao* (*Leonurus japonicus*), and *Chuanxiong* (*Ligusticum chuanxiong* Hort) [139] could also lower BP. When aiming to remove qi stagnation and blood stasis, use herbs to promote qi circulation by taking herbs to remove blood stasis. Herbs that promote qi circulation include *Chaihu* (Chinese thorowax root) [137], *Cangzhu* (*Rhizoma Atractylodis*), and *Zhiqiao* (*Fructus Aurantii*). Finally, deficiency syndromes including liver-kidney yin deficiency, yang deficiency, qi deficiency, and blood deficiency are common in hypertension. When curing liver-kidney yin deficiency, use *Liu Wei Dihuang Wan* (pill of *Rehmannia*) [23]. *Liu Wei Dihuang Wan* was recorded in *Xiaoer Yaozheng Zhijue* (*Pediatric Medicine Card Straight*) by *Qianyi* in the Song Dynasty, and it can replenish liver and kidney yin. When treating yang deficiency, use *Shen qi Wan* (kidney qi pill) to recuperate kidney yang. When aiming to treat qi deficiency, use *Huangqi* (*Astragalus membranaceus*) [140–142] and *Baizhu* (*Rhizoma Atractylodis Macrocephalae*). When aiming to treat blood deficiency, use *Danggui* (*Angelica sinensis*) [143], *Shengdihuang* (*Radix Rehmanniae*), *Chuanxiong* (*Ligusticum chuanxiong* Hort) [144], and *Baishao* (*Radix Paeoniae Rubra*).

In summary, the syndrome elements of hypertension are limited and are combined into syndromes. Single and the combined syndrome elements of hypertension are the basis of syndrome differentiation for EH and the key to the standardization of this syndrome. In this paper, we retrospectively confirmed the validity and reliability of the theory of syndrome elements and the combined forms of syndrome elements of hypertension. This study can provide new ideas and methods for the treatment of hypertension by syndrome differentiation, and has laid a foundation for researching syndrome standardization of hypertension.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jie Wang and Xingjiang Xiong contributed equally in this paper.

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Review Article

Effect of *Crataegus* Usage in Cardiovascular Disease Prevention: An Evidence-Based Approach

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Hawthorn (*Crataegus oxyacantha*) is a widely used Chinese herb for treatment of gastrointestinal ailments and heart problems and consumed as food. In North America, the role of treatment for heart problems dates back to 1800. Currently, evidence is accumulating from various in vivo and in vitro studies that hawthorn extracts exert a wide range of cardiovascular pharmacological properties, including antioxidant activity, positive inotropic effect, anti-inflammatory effect, anticardiac remodeling effect, antiplatelet aggregation effect, vasodilating effect, endothelial protective effect, reduction of smooth muscle cell migration and proliferation, protective effect against ischemia/reperfusion injury, antiarrhythmic effect, lipid-lowering effect and decrease of arterial blood pressure effect. On the other hand, reviews of placebo-controlled trials have reported both subjective and objective improvement in patients with mild forms of heart failure (NYHA I–III), hypertension, and hyperlipidemia. This paper discussed the underlying pharmacology mechanisms in potential cardioprotective effects and elucidated the clinical applications of *Crataegus* and its various extracts.

1. Introduction

Hawthorn (*Crataegus oxyacantha*), also known as haw, maybush, or whitehorn, is part of a genus of spiny shrubs and trees native to temperate regions in the Northern Hemisphere in Europe, Asia, and North America [1]. It belongs to the Rosaceae family and consists of bright green leaves, white flowers, and bright red berries (as shown in Figure 1). Hawthorn has been used in folk medicine for the treatment of diarrhea, gall bladder disease, insomnia, and as an antispasmodic agent in the treatment of asthma [2]. In Chinese, hawthorn was also used for a variety of conditions including digestive problems, hyperlipidemia, poor circulation, and dyspnea [3, 4]. For example, the dried fruits are traditionally used as a digestive aid and are often made into jam, jelly, candies, or wine [5]. Also, preparations of hawthorn are available in various forms ranging from infusions and tinctures to standardized extracts and may be available variously as authorized prescription drugs, over-the-counter (OTC) medications, authorized herbal medicinal products, dietary supplements, or unregulated herbal remedies. The use of hawthorn for the treatment of cardiovascular heart disease dates back to the late 1800s [6, 7]. Current claims suggested that hawthorn could be used as an alternative therapy for various cardiovascular diseases, such as angina, hypertension, hyperlipidemia, arrhythmia, and New York Heart Association (NYHA) functional class II congestive heart failure [8, 9]. Nowadays, it is gaining attention for its potential cardiovascular enhancing and protective properties [10] and numerous laboratory tests and clinical trials have demonstrated hawthorn's efficacy in the treatment or prevention of cardiovascular diseases and the most substantial evidence for clinical benefits of hawthorn is its use in chronic congestive heart failure (CHF) [11]. A meta-analysis of randomized, placebo-controlled trials of hawthorn extract in combination with standard CHF therapy suggested several beneficial cardiovascular effects of hawthorn as compared to placebo [12]. Similarly, a 2008 Cochrane review, wherein all primary literature pertaining to the health effects of hawthorn on humans was assessed, found a significant benefit in symptom control and physiologic outcomes from hawthorn extract as an adjunctive treatment for chronic heart failure [13]. Besides, the antioxidant, positive inotropic, anti-inflammatory, and anticardiac remodeling effects and

(a)

(b)

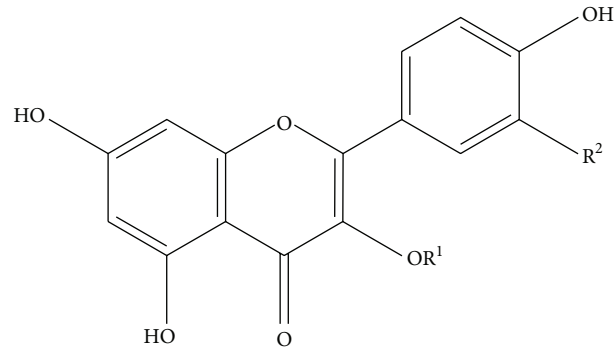
(c)

Figure 1: Different parts of *Crataegus monogyna* used as traditional food and folk medicine in China. (a) Flowers. (b) Ripened fruits. (c) Dried fruit for pharmaceutical use.

other cardiovascular protective effect of the hawthorn active ingredients were demonstrated in various in vivo and in vitro experiments. *Crataegus* has a number of pharmacological properties, but the specific mechanism is not clear.

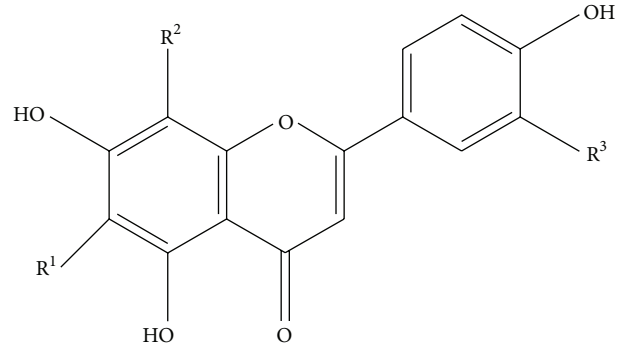
2. Chemical Constituents

Crataegus oxyacantha is popularly known for its cardioprotective action. *Crataegus monogyna* and *Crataegus laevigata* are the major hawthorn species in middle Europe, *Crataegus pentagyna*, *Crataegus nigra*, and *Crataegus azarolus* in southern and southeastern Europe, and *Crataegus pinnatifida* and *Crataegus scabrifolia* in China [14, 15]. Available products include tinctures, tablets, teas, and aqueous extracts [16, 17]. Extracts may be prepared using hydroalcoholic



R^1 = H or sugar
R^2 = H, OH or O-sugar

(a)



R^1 = H or sugar
R^2 = H or sugar
R^3 = H or OH

(b)

Figure 2: Example of flavonols (a) and flavones (b) in *Crataegus* leaves and flowers.

(methanol or ethanol) or water-based extraction and are derived from various plant parts including, most commonly, berries or leaves and flowers [18]. The source material contains a range of pharmacologically active substances, of which the most widespread compounds reported are flavonoids, triterpenic acids, and phenol carboxylic acids [19]. Flavonoids (as shown in Figure 2) such as vitexin, hyperoside, rutin, or vitexin-2″-O-α-L-rhamnoside, and catechin/epicatechin derived oligomeric procyanidins (OPC) (as shown in Figure 3) are the most important constituent. Triterpenic acids (ursolic, oleanolic, and crataegolic acids) and phenol carboxylic acids (chlorogenic and caffeic acids and various amines) are thoroughly also investigated in in vitro experiments, in animal studies, and in human clinical trials [20–23]. Currently, the most studied hawthorn extracts are WS 1442 (45% ethanol extract) and LI 132 (70% methanol extract) [24]. WS 1442 is a standardized dry extract adjusted to a content of 18.75% OPC with a starting plant material/extract ratio of 4 to 7 : 1, while LI 132 is adapted to a content of 2.2% flavonoids [25, 26].

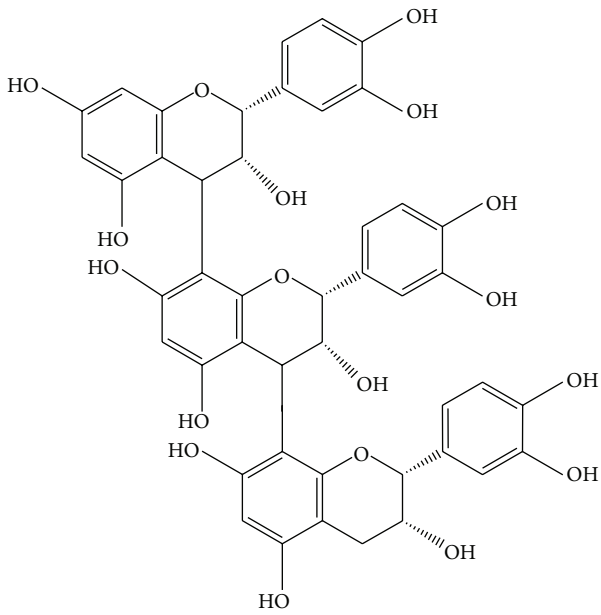


Figure 3: Example of an oligomeric procyanidin (OPC) consisting of three epicatechin monomers.

3. Cardiovascular Effect

3.1. Antioxidant Activity. Oxidative stress is a major concern in the pathogenesis of myocardial ischaemia. Therapeutic intervention showing antioxidant or free radical scavenging activity should exert beneficial effects against oxidative stress associated with various cardiovascular diseases (CVDs) [27]. Possible mechanisms of tincture of *Crataegus* (TCR) include preventing the increase in lipid peroxidation and activity of marker enzymes, preventing the isoproterenol-induced decrease in antioxidant enzymes in the heart, and increasing the rate of ADP-stimulated oxygen uptake and respiratory coupling ratio in isoproterenol-induced rats [28]. As we know, CVDs are associated with the structural and functional disturbances in heart mitochondria. As mitochondria produce 95% of energy necessary for heart function, therapeutic agents that could influence mitochondrial dysfunction are of special importance. Alcoholic extract of *Crataegus oxyacantha* (AEC) pretreatment maintained mitochondrial antioxidant status and prevented mitochondrial lipid peroxidative damage and decrease in Krebs cycle enzymes induced by isoproterenol in rat heart [29]. Another research showed that *Crataegus* fruit extracts decreased the mitochondrial membrane potential by 1.2–4.4 mV measured with a tetraphenylphosphonium-selective electrode and H_2O_2 production measured fluorometrically. Also it slightly reduced the maximal ADP-stimulated and uncoupled respiration, which might be due to inhibition of the mitochondrial respiratory chain between flavoprotein and cytochrome [30].

3.2. Positive Inotropic Effect. One research elucidated the potential inotropic mode of action of *Crataegus* special extract WS 1442. It is demonstrated that WS 1442 as well as its lipophilic ethyl acetate-soluble fraction A increased force of contraction in left ventricular papillary muscle strips through a cAMP-independent mechanism. As suggested by the concentration-dependent displacement of specifically bound ^{3}H-ouabain from its receptor, the sarcolemmal Na^+/K^+-ATPase, WS 1442 seems to increase the force of contraction by inhibition of the sodium pump. Also, they can enhance the peak intracellular Ca^{2+} concentration as well in human myocardium from patients with congestive heart failure [31]. Similarly, hawthorn most probably acts on the Na^+/K^+-ATPase and increases the efficiency of calcium transport in cardiomyocytes [32].

3.3. Anti-Inflammatory Effect. Chronic and uncontrolled inflammation plays an important role in CVDs. Inflammation has been increasingly recognized as an important pathogenic component of chronic heart failure [33, 34]. Many transcriptional factors, inflammatory cytokines, enzymes, and other mediators have been shown to be related to these effects [35]. The observed anti-inflammatory effects of the water fraction of hawthorn fruit might be attributed to the downregulation of COX-2, TNF-α, IL-1β, and IL-6 expression in LPS-stimulated RAW 264.7 cells [36]. AEC most likely achieves its myocardial protection by reducing nitritive stress and oxidative stress and decreasing apoptosis. This conclusion is supported by reduced iNOS expression, nitrite levels, downregulated COX-2, decreased lipid peroxidation, decreased release of cytochrome c, and protection of DNA fragmentation [37]. Besides, hawthorn extract inhibited N-formyl-Met-Leu-Phe (FMLP-) induced superoxide anion generation, elastase release, and chemotactic migration and reduced leukotriene B4 production and lipopolysaccharide-induced generation of TNF-α and IL-8. Also the extract inhibited intracellular calcium signal and the extracellular calcium entry into calcium-depleted neutrophils [38]. Moreover, the anti-inflammatory mechanism also illustrated that the activity of triterpene fraction isolated from *Crataegus* was closely related to inhibition of peritoneal leukocyte infiltration and weak inhibition of phospholipase A2 (PLA2) in vitro [39].

3.4. Anticardiac Remodeling Effect. Cardiac remodeling comprises changes in heart structure such as alterations in cardiac wall thickness, chamber size, cell dimension, cell number, and extracellular matrix volume. These structural changes can influence heart function [40]. Hawthorn markedly reduced LV chamber volumes (VOL) after aortic constriction (AC) and augmented relative wall thickness and attenuated the AC-induced decrease in velocity of circumferential shortening (Vcfc) showing antileft ventricular remodeling and antimyocardial dysfunction in early pressure overload-induced cardiac hypertrophy [41].

3.5. Antiplatelet Aggregation Effect. Activated platelets play a crucial role in the pathological development of several arterial disorders, including strokes and acute coronary syndromes, which are initiated by plaque disruption and subsequent platelet-thrombus formation [42–44]. *Crataegus* extract had effective antiplatelet activity at low doses of 100, 200, and

500 mg/kg as indicated by the increase in bleeding time, decrease in platelet aggregation as assessed by PFA-100, and reduction in serum levels of thromboxane B2 [45].

3.6. Vasodilating Effect. Vascular protection might be associated with the direct action on endothelial cells. The endothelium regulates the contractility of the underlying vascular smooth muscle cells by releasing a number of factors, the most important of which are the nitric oxide (NO) and endothelium derived hyperpolarizing factor (EDHF). These two factors play a major role in the controlling of vascular homeostasis [46–49]. Endothelial NO-release is related to an activation of the endothelial nitric oxide synthase (eNOS) and can be stimulated by various agonists. It is concluded in vitro and vivo research that WS 1442 induced an endothelium-dependent, NO-mediated vasorelaxation via eNOS phosphorylation at serine 1177 [50]. Besides, WS 1442 induced endothelium-dependent No-mediated relaxations of coronary artery rings through the redox-sensitive Src/PI3-kinase/Akt-dependent phosphorylation of eNOS [51]. Moreover, it preserves endothelium-dependent relaxation and vascular contraction in STZ-induced diabetes, possibly by reducing iNOS expression in the aorta, by decreasing plasma levels of TNF-α and IL-6, and by preventing lipid peroxidation [52]. There is evidence that NO may increase activation of both the ATP-dependent K^+-channel and the Ca^{2+}-dependent K^+-channel in vascular smooth muscle cells [53]. Similar experiment showed that procyanidins in *Crataegus* extract may be responsible for the endothelium-dependent NO-mediated relaxation, possibly via activation of tetraethylammonium sensitive K^+ channels in isolated rat aorta [54]. Quite recently it has been demonstrated that red blood cells (RBCs) express a functional NO-synthase (rbcNOS) and rbcNOS activation has been associated with increased RBC deformability. WS 1442 activates rbcNOS and causes NO-formation in RBCs [55]. There is another opinion that hawthorn does have a vasodilating action both in the coronary circulation and the peripheral vasculature that may be mediated by inhibition of angiotensin-converting enzyme (ACE) [56].

3.7. Endothelial Protective Effect. Endothelial hyperpermeability, that is, a compromised endothelial barrier function, and the subsequent formation of edema are hallmarks of many severe disorders, such as atherosclerosis, asthma, sepsis, or heart failure [57–60]. One research showed that the herbal drug WS 1442 effectively protects against endothelial barrier dysfunction by its action on key determinants of endothelial permeability (adherens junctions, actin cytoskeleton, and contractile apparatus) by inhibiting the barrier-destabilizing calcium/PKC/Rho A signaling and activating the barrier-stabilizing cAMP/Epac1/Rap1 pathway [61]. Another research showed that WS 1442 prevented the deleterious hyperpermeability-associated rise of $[Ca^{2+}]_i$ by interferring with sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and the inositol 1,4,5-trisphosphate (IP3) pathway without inducing store-operated calcium entry (SOCE) [62]. Past and ongoing studies also suggest that chronic intake of *Crataegus* prevented aging-related endothelial dysfunction by reducing the prostanoid-mediated contractile responses, most likely by improving the increased oxidative stress and the over expression of COX-1 and COX-2 [63].

3.8. Reduction of Smooth Muscle Cell Migration and Proliferation. There have been few studies on the migration and proliferation effects of herbal medications such as hawthorn. Hawthorn appears to exhibit some cardioprotective effects due to reduction of smooth muscle cell migration and proliferation properties. Currently, up to 50% of patients undergo conventional balloon angioplasty recurrent stenosis [64]. After vessel injury, biologically active components are released that trigger a dedifferentiation of vascular smooth muscle cell (VSMCs). They start to migrate and proliferate resulting in neointimal hyperplasia. Mediators involved in these processes are platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and to a lesser extent epidermal growth factor (EGF). WS 1442 decreased VSMC migration by 38% and proliferation by 44%. It inhibited VSMC DNA synthesis induced by PDGF, blocked recombinant human PDGF receptor (PDGFR)-β kinase activity and decreased PDGFR-β activation and extracellular signal-regulated kinase (ERK) activation in VSMCs [65].

3.9. Protective Effect against Ischemia/Reperfusion Injury. Ischemia and reperfusion (I/R) exerts multiple injuries in microcirculation, frequently accompanied by endothelial cell injury, enhanced adhesion of leukocytes, macromolecular efflux, production of oxygen free radicals, and mast cell degranulation [66]. Thus, much effort has been made to attenuate the microcirculatory disturbance by ablating one of the insults in the pathogenetic process. Preliminary research demonstrated the cardioprotective effects of hawthorn in vivo models of ischemia/reperfusion. There are at least three experiments showing the effect. Hawthorn extract WS 1442 significantly reduced the deterioration of contractile function and infarct size in rat myocardium exposed to prolonged ischemia and reperfusion [67]. Besides, it showed evident effect against reperfusion arrhythmias by reducing the average prevalence of malignant arrhythmias (VF + Flutter) and the average prevalence of ventricular tachycardia (VT) [68]. Moreover, it prevented the isoproterenol-induced decrease in antioxidant enzyme activity [69].

3.10. Antiarrhythmic Effect. Hawthorn extract may produce some antiarrhythmic effects in the rat heart, but the mechanism underlying the effect remains elusive. One result shows that *Crataegus* extract prolongs action potential duration and delays recovery of V_{max} [70]. On the other hand, concerns have been raised regarding blocking repolarising potassium currents in ventricular myocytes. This effect is similar to the action of class III antiarrhythmic drugs and might be the basis of the antiarrthemic effects described for *Crataegus* extract [71]. Another mechanism showed that extract from *Crataegus* resulted in a significant decrease in the total number of ventricular ectopic beats, mainly by reduction of

beats occurring as ventricular tachycardia. Also it reduced the total number of ventricular ectopic beats but this reduction was due to the decrease of single extrasystoles [72].

3.11. Lipid-Lowering Effect. As we know, oxidation of the low-density lipoprotein (LDL) cholesterol plays an important role in atherosclerosis [73]. This accumulation causes a cascade of inflammatory processes, resulting in an unstable atherosclerotic plaque that ultimately bursts, causing myocardial infarction [74]. Many herbs can reduce low-density lipoprotein oxidation. One research investigated the effects of seven Chinese herbs and concluded that Shan Zha (Hawthorn Fruit) is effective in lowering blood lipid levels [75]. Similar study showed that Hawthorn fruit compound lowered blood lipids in atherogenic diet fed, ApoE gene deficient atherosclerotic mice. The results showed that Hawthorn fruit compound significantly reduced the ratio between low-density lipoprotein cholesterol (LDL-C) and serum cholesterol (TC): (LDL-C)/TC, especially the triglyceride (TG) levels [76]. Besides, TCR can significantly increase the binding of ^{125}I-LDL to the liver plasma membranes in vitro. This may be related to enhancement of the LDL-receptor activity. TCR was also shown to increase bile acid excretion and to depress hepatic cholesterol synthesis in atherogenic diet fed rats by upregulating hepatic LDL-receptors resulting in greater influx of plasma cholesterol into the liver [77]. Treatment using hawthorn fruit can decrease serum cholesterol that involves the inhibition of cholesterol absorption mediated by downregulation of intestinal acyl CoA: cholesterol acyltransferase (ACAT) activity in Caco-2 cells. In animals research, hawthorn significantly lowered plasma non-HDL (VLDL + LDL) cholesterol concentrations and decreased hepatic cholesterol ester content [78]. The flavonoids fraction showed inhibitory effects on TG and glucose absorption and accelerating effects on gastrointestinal transit in vivo and suppressed the accumulation of TG and free fatty acid. It also suppressed the gene expressions of C/EBPα, PPARγ, SREBP 1c, aP2, and adiponectin in vitro [79]. As we know, LPL plays an important role in lipoprotein metabolism and is expressed in various tissues, especially adipose and muscle tissue, where it plays different roles. Hawthorn flavonoids increase LPL expression through a PPARγ-dependent mechanism directed towards identification of the components [80]. TCR prevented the elevation of lipids in the serum and heart and caused a significant decrease in lipid accumulation in the liver and aorta reverting the hyperlipidemic condition of these rats. The extract significantly restored the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione, thereby restoring the antioxidant status of the organism to almost normal levels [81]. One research used the larval Zebrafish as model to test plant-based dietary intervention of hypercholesterolemia and it was demonstrated that hawthorn leaves and flowers have the potential to affect cardiac output as well as intravascular cholesterol levels [82].

3.12. Decrease of Arterial Blood Pressure Effect. It was observed that *Crataegus*, especially the hyperoside fraction, prevented L-NAME-induced hypertension in rats and had beneficial effects on the cardiovascular system [83]. *Crataegus* administered at escalating doses produced a dose-time-dependent decrease in heart rate (HR) and mean arterial pressure (MAP). Higher doses produced the most significant reduction in both HR and MAP and induced sinus node suppression and progressive atrio-ventricular blockade. The underlying mechanism appeared to be related to the direct stimulation of the muscarinic receptor M2 and possible blockade of beta-receptors, while the hypotension was caused by enhanced nitric oxide release [84]. (All above cardiovascular effects were shown in Tables 1 and 2).

4. *Crataegus* for Clinical Cardiovascular Disease Prevention

CVD are considered a serious health public problem due to the high morbidity and mortality rates. It caused 17.1 million deaths yearly worldwide according to the World Health Organization (WHO) [85]. Role of *Crataegus* in CVD prevention has been a topic of concerns for many years. However, these claimed benefits were not supported by evidence-based clinical studies. In recent years, *Crataegus* has been a focus of attention because of its potential role in the prevention of various aspects of cardiovascular disease. Evidence from numerous studies suggests that *Crataegus* works through various mechanisms to achieve this favorable effect. Majority of the studies have shown positive impact for various CVD; however, one contradictory study showed that CSE does not reduce heart failure progression, even to increase the early risk of HF progression [86]. This paper critically examines the current scientific literature concerning claims of cardiovascular benefits from *Crataegus* and its extract since 1990. We searched all human studies of clinical trials in English assessing the effect of *Crataegus* on cardiovascular disease prevention among patients (congestive heart failure, hyperlipidemia, hypertension, or c Arrhythmias were included) in five major electronic databases, including CNKI, CBMdisc, VIP, PubMed, and the Cochrane Library, to retrieve any potential randomized controlled trials (RCTs). A number of keywords were used for data searching including *Crataegus* and cardiovascular disease clinical trial, *Crataegus* hypertension and hyperlipidemia, *Crataegus* arrhythmias platelet aggregation, and clinical trial. Finally, there were 15 trials included in the review, of which 8 trials were therapies of heart failure, 4 trials were therapies of hypotension, and 3 trials of hyperlipidemia (as shown in Table 3).

4.1. CHF. Contemporary therapies of heart failure such as ACE inhibitors, beta-blockers, spironolactone, implantable cardioverter defibrillators, and biventricular pacemakers have produced remarkable reductions in morbidity and mortality. However, quality of life (QOL) for patients with heart failure remains impaired and improved treatment regimens are still needed. *Crataegus* extract is an adjunct to conventional treatment in patients with HF (New York Heart Association classes I–III) due to its positive inotropic, antiarrhythmic, and vasodilating properties. The hawthorn extract

Table 1: Compounds derived from *Crataegus* in vivo cardiovascular effects.

Target	Compounds	Animal/organs	Effect	References
Antioxidant effect	TCR	Rat heart	Lipid peroxidation; Activity of marker enzymes; Antioxidant enzymes; Oxygen uptake; Respiratory coupling ratio	Jayalakshmi and Devaraj, 2004 [28]
Positive inotropic effect	WS 1442	Human myocardial tissue	cAMP-independent mechanism; Sarcolemmal Na^+/K^+-ATPase; Sodium pump; Intracellular Ca^{2+} concentration;	Schwinger et al., 2000 [31]
Anti-inflammatory effect	AEC	Rat heart	Nitritive stress; Oxidative stress; iNOS expression; COX-2; Lipid peroxidation; Cytochrome c;	Vijayan et al., 2012 [37]
Anti-cardiac remodeling effect	WS 1442	Rat	LV chamber volumes (VOL); Relative wall thickness; Vcfc;	Hwang et al., 2008 [41]
Antiplatelet aggregation	*Crataegus aronia* syn. Azarolus (L)	Rat blood	Bleeding time; Platelet aggregation; Serum levels of TXB2;	Abdullah et al., 2012 [45]
Vasodilating effect	WS 1442	Isolated rings of rat aorta	Endothelium-dependent, NO-mediated vasorelaxation; eNOS phosphorylation;	Brixuis et al., 2006 [50]
	Crataegus microphylla CM extract	Rat thoracic aorta	Endothelium-dependent relaxation; Vascular contraction; iNOS expression; Plasma levels of TNF-α, IL-6;	Gökçe et al., 2013 [52]
	CE	Rat aorta	Lipid peroxidation; Endothelium-dependent nitric oxide(NO)-mediated relaxation; Tetraethylammonium-sensitive K^+ channels;	Kim et al., 2000 [54]
Endothelial protection	WS 1442	Rat main mesenteric arteries	Prostanoid-mediated contractile responses; Oxidative stress; COX-1 and COX-2;	Idris-Khodja et al., 2012 [63]
Protect I/R injury	WS 1442	Rat myocardium	Contractile function; Infarct size;	Veveris et al., 2004 [67]
	Crataegus oxyacantha	Rat heart	Average prevalence of malignant arrhythmias (VF + Flutter); Average prevalence of VT;	Makdessi et al., 1999 [68]
	TCR	Rat heart	Antioxidant enzyme activity;	Jayalakshim and Devaraj, 2004 [69]
Antiarrhythmic effect	*Crataegus meyeri* extracts	Rat	Ventricular ectopic beats; Single extrasystoles;	Garjani et al., 2000 [72]

Table 1: Continued.

Target	Compounds	Animal/organs	Effect	References
Lipid-lowering effect	HFC	Mice	Blood lipids; (LDL-C)/TC; TG levels;	Xu et al., 2009 [76]
	HFC	Hamsters	Serum cholesterol; VLDL + LDL cholesterol; Hepatic cholesterol ester content;	Zhang et al., 2002 [78]
	The leaf of *Crataegus pinnatifida*	Mice	TG and glucose absorption; Gastrointestinal transit; TG and free fatty acid;	Wang et al., 2011 [79]
	Hawthorn flavonoids	Mice	LPL expression; PPARγ-dependent mechanism;	Fan et al., 2006 [80]
	TCR	Rat	Lipids in the serum and heart; Lipid in liver and aorta; Activity of antioxidant enzymes;	Akila and Devaraj, 2008 [81]
	Whole plant of *Crataegus*	Zebrafish	Intravascular cholesterol levels;	Robert et al., 2012 [82]
Decrease of arterial blood pressure	Crataegus *tanacetifolia* leaf extract	Rats coronary artery	L-NAME-induced hypertension; Beneficial effects on cardiovascular system; HR and MAP;	Koçyõldõz et al., 2006 [83]
	Aqueous extract of Crataegus	Rat	Sinus node suppression; Atrioventricular blockade; Muscarinic receptor M2; Blockade of beta-receptors;	Shatoor, 2013 [84]

Notes: TCR: tincture of *Crataegus*; AEC: alcoholic extract of *Crataegus oxycantha*; Vcfc: velocity of circumferential shortening; TXB2: thromboxane B2; CE: crataegus fruit extracts; CM: *Crataegus microphylla*; VT: ventricular tachycardia; TC: serum cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglyceride; HFC: hawthorn fruit compound; HR: heart rate; MAP: mean arterial pressure.

may provide additional benefit in symptoms control (fatigue, listlessness, dyspnoea under strain, pretibial oedema, and rapid exhaustion) and frequency of nocturnal urinations and exercise tolerance (distance walked and number of stairs ascended without fatigue) [88]. A number of randomized, controlled trials were carried out to study the effect of different preparation of *Crataegus* on congestive heart failure. The majority of hawthorn clinical trials have been performed with WS 1442, a dry extract from hawthorn leaves with flowers (4–6.6 : 1), extraction solvent ethanol 45% (w/w).

In a 3-year open cohort study, 372 patients (261 females and 111 males) of stage NYHA II taking 900 mg/day WS 1442 in addition to their standard medication were followed for three years by their treating office-based physicians. Outcome parameters demonstrated that maximal workload (MWL), left ventricular ejection fraction (LVEF) and pressure-heart rate product increase (PHRPI) at 50 W ergometric exercise improved more in active treatment than in placebo patients. In addition improvement of typical symptoms like reduced exercise tolerance, exertional dyspnea, weakness, fatigue, and palpitations improved more with active treatment and in patients with more severe symptoms [89]. Similarly, a multicentre nonrandomized cohort study in patients aged 50–75 years in received Cralonin ($n = 110$) or ACE inhibitorydiuretics ($n = 102$) for 8 weeks. The trial using *Crataegus* preparation Cralonin among NYHA class II. Patients demonstrated that the Cralonin is noninferior to usual ACE inhibitorydiuretics treatment for mild cardiac insufficiency on all parameters except BP reduction [90].

Another placebo-controlled, randomized, parallel group, multicentre trial recruiting 143 patients and treated with 3 times 30 drops of the extract ($n = 69$) or placebo ($n = 74$) for 8 weeks showed the efficacy and safety of a standardised extract of fresh berries of *Crataegus oxyacantha* L. and *monogyna* Jacq. (*Crataegisan*) in patients with cardiac failure NYHA class II. The result is confirmed that changes in blood pressure-heart rate product (BHP) at 50 watts and at comparable maximum load were in favour of *Crataegus* extract but the results are not statistically significant. What is more, an improvement in their heart failure condition may be achieved under long term therapy [91]. Effective therapy in patients was also seen in other clinical trials. In a randomized, placebo-controlled, double-blind clinical study for 12 weeks with either WS 1442 ($n = 20$) or placebo ($n = 20$), the difference between the groups was borderline statistically significant in the exercise tolerance and the

Table 2: Compounds derived from *Crataegus* in vitro cardiovascular effects.

Target	Compounds	Cell/tissues	Effect	References
Antioxidant effect	AEC	Mitochondria from rat heart	Mitochondrial lipid peroxidative damage; Kreb's cycle enzymes; Mitochondrial membrane potential;	Jayalakshim et al., 2006 [29]
	CE	Mitochondria from rat heart	H_2O_2 production; Maximal respiration; Mitochondrial respiratory chain;	Bernatoniene et al., 2009 [30]
Positive inotropic effect	Two alcohol extracts	Neonatal rat cardiomyocytes	Na^+/K^+-ATPase; Calcium transport;	Rodriguez et al., 2008 [32]
Anti-inflammatory effect	Water fraction from hawthorn fruit	LPS-stimulated RAW 264.7 cells	COX-2, TNF-α, IL-1β, and IL-6 expression;	Li and Wang, 2011 [36]
	Hawthorn extract	Human blood neutrophils	Superoxide anion generation; Elastase release; Chemotactic migration; Leukotriene B4 production; TNF-α and IL-8; Intracellular calcium signal;	Dalli et al., 2008 [38]
	Triterpene fraction isolated from *Crataegus*	Peritoneal exudates	Peritoneal leucocyte infiltration Phospholipase A2;	Ahumada et al., 1997 [39]
Vasodilating effect	WS 1442	HCAEC	eNOS phosphorylation;	Brixuis et al., 2006 [50]
	WS 1442	Porcine coronary artery endothelial cells	Src/PI3-kinase/Akt-dependent phosphorylation of eNOS;	Anselm et al., 2009 [51]
	Hawthorn extract	VSMCs	ATP-dependent K+-channel; Ca^{2+}-dependent K^+-channel;	Waldron and Cole, 1999 [53]
	WS 144	Human venous blood cell	rbcNOS and NO-formation;	Rieckeheer et al., 2011 [55]
Endothelial protection	WS 1442	HUVECs	Endothelial permeability; Calcium/PKC/Rho A signaling pathway; cAMP/Epac1/Rap1 pathway;	Bubik et al., 2012 [61]
	WS 1442	HUVECs	Hyperpermeability-associated rise of $[Ca^{2+}]_i$; SERCA and IP pathway;	Elisabeth et al., 2012 [62]
Reduction of smooth muscle cell migration and proliferation	WS 1442	Rat aortic VSMCs	VSMC migration and proliferation; VSMC DNA synthesis; PDGFR-β kinase activity; ERK activation;	Fürst et al., 2010 [65]
Antiarrhythmic effect	LI 132	Guinea pig ventricular myocytes	Block repolarizing potassium currents;	Müller et al., 1999 [71]
Lipid-lowering effect	TCR	Rat liver plasma membranes	Binding of ^{125}I-LDL to the liver plasma; LDL-receptor activity; Increase bile acid excretion; Depress hepatic cholesterol synthesis;	Rajendran et al., 1996 [77]

Table 2: Continued.

Target	Compounds	Cell/tissues	Effect	References
Lipid-lowering effect	HFC	Caco-2 cells	ACAT activity;	Zhang et al., 2011 [78]
	The leaf of *Crataegus pinnatifida*	3T3-L1 cells	Gene expressions of C/EBPα, PPARγ, SREBP 1c, aP2 and adiponectin	Wang et al., 2011 [79];

Notes: AEC: alcoholic extract of *Crataegus oxycantha*; CE: *Crataegus* fruit extracts; ACAT: acyl CoA (Coenzyme A): cholesterol acyltransferase; HCAEC: human coronary artery endothelial cells; HUVECs: human umbilical vein endothelial cells; SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; IP3: inositol 1,4,5-trisphosphate; ERK: extracellular signal-regulated kinase; HFC: hawthorn fruit compound; NYHA: New York Heart Association.

double product (heart rate × systolic blood pressure × 10^{-2}). It is demonstrated that WS 1442 was safe and well tolerated and was clinically effective in patients with congestive heart failure corresponding to NYHA class II [92].

One RCT studied effect of the extract LI 132 on chronic heart failure defined as NYHA functional class II. Patients were treated either with *Crataegus* extract ($n = 50$) or with a placebo preparation ($n = 50$) for a period of 8 weeks, with a wash-out phase of one week. Outcomes of MWL, LVEF, PHRPI and typical symptoms were statistically significant with the LI 132 preparation compared to the patients treated with the placebo preparation. Apart from that, a significant reduction of the systolic blood pressure, of the heart rate and of the pressure/rate product was observed for the patients treated with the verum preparation. Besides, there were no severe side effects observed [93].

However, controversy result showed that hawthorn provides no symptomatic or functional benefit when given with standard medical therapy to patients with heart failure. The research performed a randomized, double-blind, placebo-controlled trial in 120 ambulatory patients with NYHA class II-III chronic heart failure. All patients were randomized to either hawthorn 450 mg twice daily or placebo for 6 months. But there were no significant differences between groups in the change in 6 min walk distance or on measures of QOL, functional capacity, neurohormones, oxidative stress, or inflammation [94].

Two clinical trials used WS 1442 to investigate the efficacy and safety of an add-on treatment in patients with congestive heart failure. In one of this randomized, double-blind, placebo-controlled multicenter study, 2681 patients (WS 1442: 1338; placebo: 1343) were included. Results showed that WS 1442 reduced sudden cardiac death by 39.7%, so that it was safe to be used in patients receiving optimal medication for heart failure [95]. Another research included 209 patients randomized to treatment with 1800 mg of WS 1442, 900 mg of WS 1442, or with placebo for 16 weeks. The data from the study confirm that there is a dose-dependent effect of WS 1442 on the exercise capacity of patients with heart failure and on typical heart failure-related clinical signs and symptoms. The maximal tolerated workload during bicycle exercise showed that increase and typical heart failure symptoms as rated by the patients were reduced to a greater extent by WS 1442 than by placebo, so that the drug was shown to be well tolerated and safe [87]. To ascertain the effectiveness of *Crataegus* in CHF therapy, meta-analyses are required to prove its efficacy.

4.2. Hypertension. Hypertension is an increasingly important medical and public health issue, which could lead to severe complications [96]. It is an important risk factor for CVDs. Currently, it affects 1 billion people worldwide, and this number is expected to rise to 1.6 billion by 2025 [97, 98]. Although there have also been significant advances in therapeutic concepts and measures; however, hypertension in most individuals remains untreated or uncontrolled. With the popularity and prevalence of Chinese medicine (CM), there has been a growing interest in Chinese herbal medicine (CHM) for patients with hypertension both in China and the West [99–104]. Several small clinical trials with hawthorn have demonstrated modest blood pressure reduction. Randomised controlled trial was designed to investigate the effects of hawthorn for hypertension in type 2 diabetes patients who were randomized to daily 1200 mg hawthorn extract ($n = 39$) or placebo ($n = 40$) for 16 weeks, taking prescribed drugs. Results demonstrated that there was a significant group difference in mean diastolic blood pressure reductions: the hawthorn group showed greater reductions than the placebo group. What is more, this is the first randomized controlled trial to demonstrate a hypotensive effect of hawthorn in patients with diabetes taking medication [105].

One pilot study was aimed at investigating the hypotensive potential of hawthorn extract and magnesium dietary supplements individually and in combination, compared with a placebo. Volunteers were then randomly assigned to four groups: 600 mg hawthorn extract, 500 mg hawthorn extract, a combination of the previous two groups and placebo. Results showed that there was a decline in both systolic and diastolic blood pressure in all treatment groups or placebo, but hawthorn extract group showed a promising reduction in the resting diastolic blood pressure at week 10 in the 19 subjects, compared with the other groups. Furthermore, a trend towards a reduction in anxiety was also observed in those taking hawthorn compared with the other groups [106].

Similarly, in order to test the efficacy of a camphor-*Crataegus* berry combination (CCC) on orthostatic hypotension, two similar, controlled, randomized studies were carried out in a balanced crossover design in 24 patients each with orthostatic dysregulation. Results showed that CCC drops

Table 3: Randomized, controlled, double-blind trials of Hawthorn extract for cardiovascular diseases.

Study	Design[a]	Target	Duration	Dose	Case/control	Primary outcome measures
Eggeling et al., 2011 [89][b]	OPC	Early chronic heart failure	156 w	900 mg, qd	372/—	Improve outcomes of MWL, LVEF, PHRPI, BP, HR, DP, and typical symptoms.
Schröder et al., 2003 [90][e]	Double-blind, Nonrandomized controlled trial	Mild cardiac insufficiency (NYHA II)	8 w	100 mL, tid	110/102	Change HR, BP, DP, symptoms, frequency of nocturnal urinations, and exercise tolerance.
Degenring et al., 2003 [91][c]	RCT, pg	Congestive heart failure (NYHA II)	8 w	2.25 mL, qd	69/74	Change BHP and maximum load.
Zapfe Jun, 2001 [92][b]	RCT, pg	Congestive heart failure (NYHA II)	12 w	240 mg, qd	20/20	Increase exercise tolerance and reduce the DP.
Schmidt et al., 1994 [93][d]	RCT, pg	Congestive heart failure (NYHA II)	8 w	600 mg, qd	50/50	Reduce the SBP, HR, and DP.
Zick et al., 2009 [94][b]	Randomized controlled trial	Chronic heart failure (NYHA II-III)	24 w	450 mg, bid	60/60	No symptomatic or functional benefit when given with standard medical therapy.
Holubarsch et al., 2008 [95][b]	RCT, pg	Chronic heart failure (NYHA II/III)	48 w	900 mg, qd	1338/1343	Reduce the incidence of sudden cardiac death.
Tauchert, 2002 [87][b]	RCT, pg	Congestive heart failure (NYHA III)	16 w	900/1800 mg, qd	70/69	The treatment is safe and well tolerated.
Belz et al., 2002 [105][e]	RCT, pg	Hypertension	16 w	1200 mg, qd	39/40	Lower mean DBP.
Walker et al., 2006 [106][e]	RCT, pg	Hypertension	10 w	500 mg, qd	19/17	Lower both SBP and DBP, especially DBP.
Walker et al., 2002 [107][e]	RCT, co	Hypertension	5 min.	80 drops, qd	24/24	Lower DBP.
Asher et al., 2012 [108][e]	RCT, co	Hypertension	3 d	1000/1500/2000 mg, bid	15/6	No evidence of a dose-response effect of hawthorn extract on FMD.
Dalli et al., 2011 [109][e]	RCT, pg	Hyperlipidemia	24 w	400 mg, tid	24/21	Decrease NE and lower LDL-C.
Liang and ye, 2004 [110][e]	Randomized controlled trial	Hyperlipidemia	5 w	60 mg, tid	60/52	Decrease TC, TG, LDL-C.
Shen et al., 2000 [111][e]	Clinical controlled trial	Hyperlipidemia	4 w	5 pills, tid	120/20	Decrease TC, TG, and LP(a) and increase HDC.

[a]RCT: randomized, double blind, placebo-controlled trial; co: crossover; pg: parallel group; OPC: open prospective cohort study; [b]*Crataegus* extract WS 1442; [c]Crataegisan; [d]*Crataegus* extract LI 132; [e]Other extracts or preparations of *Crataegus*; MWL: maximal workload; LVEF: left ventricular ejection fraction; PHRPI: pressure-heart rate product increase; BHP: blood pressure-heart rate product; HR: heart rate; BP: blood pressure; DP: double product (evaluated on a bicycle ergometric test and defined as heart rate × systolic blood pressure × 10^{-2} where HR is heart rate in bpm and BP blood pressure in mmHg); NYHA: New York Heart Association; SBP: systolic blood pressure; DBP: diastolic blood pressure; NE: neutrophil elastase; LDL-C: LDL cholesterol; FMD: flow mediated dilation.

decreased the orthostatic fall in blood pressure, especially affecting diastolic blood pressure after 1 minute of orthostasis in all dosages as compared to placebo. A statistically significant effect of the highest dose of 80 drops on diastolic blood pressure could be demonstrated after 1-, 3-, and 5-minute orthostasis [107].

Clinical investigations exploring the effects of *Crataegus* and its various preparations in hypertension have demonstrated somewhat contradictory results. One randomized, controlled cross-over designed trial was to investigate the relationship between hawthorn extract dose and brachial artery flow mediated dilation (FMD), an indirect measure of

nitric oxide release. Randomly sequenced doses of hawthorn extract (1000 mg, 1500 mg, and 2500 mg) and placebo were assigned to each participant. However, results showed that there was no evidence of a dose-response effect for our main outcome (FMD percent) or any of our secondary outcomes, such as absolute change in brachial artery diameter and blood pressure [108].

4.3. Hyperlipidemia. Although the lipid-lowering property of the hawthorn extract has been shown in a number of animal studies by means of reducing in total cholesterol, low density lipoprotein, and ApoB synthesis, there are still few well-designed clinical trials. One study included 49 diabetic subjects with chronic CHD who were randomly assigned to either a micronized flower and leaf preparation of *C. laevigata* group or a matching placebo. The main results were that *C. laevigata* decreased NE and showed a trend to lower LDL-C compared to placebo as add-on treatment for diabetic subjects with chronic CHD [109]. Two Chinese clinical trials used Shan Zha Jingjiangzhi pill as therapy drugs compared with Duoxokang pill and placebo, separately. Results showed that, compared with Duoxikang pill, Shanzha Jingjiangzhi pill can lower TG and TC [110]. While compared with placebo, more benefits about decreasing TC, TG, and LP(a) and increasing HDL-C were attained from Shanzha Jingjiangzhi pill [111].

5. Dosage and Side Effects

Then what is the adverse effect of *Crataegus*? How to use the hawthorn properly? Although modern drugs are effective in preventing cardiovascular disorders, their use is often limited because of their side effects [112]. Most of the adverse events were mild to moderate and majority of studies indicate that oral hawthorn is well tolerated. One systematic review included 29 clinical studies of 7311 patients. Overall, 166 adverse events were reported. Eight severe adverse events have been reported with the LI 132 extract such as dizziness/vertigo, gastrointestinal complaints, headache, migraine, and palpitation. Hawthorn is a slow-acting herb and should be used for at least 4 to 8 weeks for full benefit. The dosage depends on the type of preparation and source material [113]. Most effective dosage was unknown currently. Recommended dosages range from 160 to 1800 mg per day in two or three divided days. There were no reports of drug interactions.

6. Conclusions and Perspectives

In the last 20 years, over 60% of new drugs for the treatment of cancer and 75% of new drugs used to treat infectious diseases were of natural health products [114]. In North America, however, natural health products (NHPs) are considered as food and dietary supplements and are therefore sold in health food stores [115]. In North America, and Canada in particular, NHPs are considered mainly used in the treatment of heart-related problems [116]. *Crataegus* and its various extracts were such NHPs. Currently, *Crataegus* products are currently marketed as an alternative treatment for hypertension, angina, arrhythmia, and the early stages of congestive heart failure by regulating whole body on multilevel and multitargets. What is the mechanism of *Crataegus* for cardiovascular diseases? The previous described animal studies have suggested that hawthorn extracts exert a wide range of cardiovascular pharmacological properties, including antioxidant activity, positive inotropic effect, anti-inflammatory effect, anticardiac remodeling effect, antiplatelet aggregation effect, vasodilating effect, endothelial protective effect, reduction of smooth muscle cell migration and proliferation, protective effect against ischemia/reperfusion injury, antiarrhythmic effect, lipid-lowering effect, and decrease of arterial blood pressure effect. Moreover, numerous clinical studies have demonstrated that hawthorn preparations are very effective in early stages of congestive heart failure. A few researches were reported on therapy of hypertension and hyperlipidemia.

In China, *Crataegus* was first mentioned in "New Materia Medica." The herb was used widely in traditional Chinese medicine, particularly in department of internal medicine, such as the food stagnation, nausea or vomiting, abdominal pain or diarrhea, hernia pain, hematocele in bosom, and postpartum lochia. Seldom narration was seen in ancient literature on *Crataegus* for treatment of cardiovascular diseases but many literature showed that *Crataegus* had the effect of "activating blood and dissolving stasis." Since *Crataegus* has effect of eliminating food mass and removing blood stasis, it can be used for treatment of stomach disease and cardiovascular disease. In traditional Chinese medicine, there is a theory of "Treating heartache by regulating the spleen and stomach." It comes from "gastric collaterals goes into heart" in the ancient literature of "The Miraculous Pivot." The theory regarded that chest-bi had close association with dysfunction of spleen and stomach in physiological and pathological aspects, so it is important for treating heartache by regulating the spleen and stomach. In future, carrying out the research of traditional Chinese medical theory combined with modern pharmacological achievement is beneficial to the treatment of heart disease.

Nowadays, with the population of NHPs, finding the high efficiency and fewer adverse effects of cardiovascular-protective drugs from Chinese herb and formulas attracts great attention of researchers, and the study of target or mechanism of Chinese herb and formulas for hypertension is to be the hot topic of research and development of antihypertensive drugs. But there are still some problems we need to arise. On current, anminal research of *Crataegus* on vasodilating effect and lipid-lowering effect were performed more frequently than those other effect of studies. Nevertheless there are only a few studies that have been published about the anticardiac remodeling effect and effect of reducting smooth muscle cell migration and proliferation. So, further systematic in vivo and in vitro researches are warranted to explore and verify the potential effect to provide precise guidance for clinical use and new drug discovery. Besides, with the studies published, the strength of the evidence, however, was often limited by lack of controls or placebos, nonrandomization, non-blinded design, or small numbers of patients. 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. In addition, there are so many active ingredients in *Crataegus*, so that large quantity of active ingredients should be identified, extracted, and purified from the herb. What is more, some active ingredients are chemically unstable, which have limited the large-scale synthesis. All these pressing issues should be resolved in future researches.

Abbreviations

AC: Aortic constriction
ACAT, acyl CoA: Cholesterol acyltransferase
ACE: Angiotensin-converting enzyme
AEC: Alcoholic extract of *Crataegus oxyacantha*
BHP: Blood pressure-heart rate product
BP: Blood pressure
CVD: Cardiovascular diseases
CCC: Camphor-*Crataegus* berry combination
CE: *Crataegus* fruit extracts
CHF: Congestive heart failure
CHM: Chinese herbal medicine
CM: *Crataegus* microphylla
DBP: Diastolic blood pressure
DP: Double product
EDHF: Endothelium derived hyperpolarizing factor
EGF: Epidermal growth factor
ERK: Extracellular signal-regulated kinase
FGF: Fibroblast growth factor
FMD: Flow mediated dilation
FMLP: Formyl-Met-Leu-Phe
HCAEC: Human coronary artery endothelial cells
HFC: Hawthorn fruit compound
HR: Heart rate
HUVECs: Human umbilical vein endothelial cells
I/R: Ischemia and reperfusion
IP3: Inositol 1,4,5-trisphosphate
LDL: Low-density lipoprotein
LDL-C: Low-density lipoprotein cholesterol
LVEF: Left ventricular ejection fraction
MAP: Mean arterial pressure
MWL: Maximal workload
NE: Neutrophil elastase
NO: Nitric oxide
NYHA: New York Heart Association
OA: Oleanolic acid
OPC: Oligomeric procyanidins
PHRPI: Pressure-heart rate product increase
PLA2: Phospholipase A2
QOL: Quality of life
RBCs: Red blood cells
SBP: Systolic blood pressure
SERCA: Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SOCE: Store-operated calcium entry
TC: Serum cholesterol
TCR: Tincture of *Crataegus*
TG: Triglyceride
TXB2: Thromboxane B2
UA: Ursolic acid
Vcfc: Velocity of circumferential shortening
VSMCs: Vascular smooth muscle cell
VT: Ventricular tachycardia.

Conflict of Interests

All authors declare that there is no conflict of interests.

Authors' Contribution

Jie Wang and Xingjiang Xiong contributed equally to this paper.

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Research Article

Protective Effects of Shen-Yuan-Dan, a Traditional Chinese Medicine, against Myocardial Ischemia/Reperfusion Injury *In Vivo* and *In Vitro*

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Objectives. The study was to investigate the effects and mechanisms of Shen-Yuan-Dan (SYD) pharmacological postconditioning on myocardial ischemia/reperfusion (I/R) injury. *Methods.* In the *in vivo* experiment, myocardial injury markers and histopathology staining were examined. In the *in vitro* experiment, cell viability and cell apoptosis were, respectively, detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and Hoechst 33342 fluorochrome staining. The protein expressions of Bcl-2 and Bax were determined by immunocytochemistry assay. *Results.* Both low and high doses of SYD protected myocardium against I/R injury in rat model by reducing lactic dehydrogenase (LDH) and creatine kinase-MB (CK-MB) activity and malondialdehyde (MDA) content, increasing superoxide dismutase (SOD) activity and attenuating histopathology injury. Meanwhile, in the *in vitro* experiment, SYD promoted cell viability and inhibited the cardiomyocyte apoptosis. The level of Bcl-2 protein was restored to the normal level by SYD pharmacological postconditioning. In contrast, the Bax protein level was markedly reduced by SYD pharmacological postconditioning. These effects of SYD were inhibited by LY294002. *Conclusions.* The results of this study suggested that SYD pharmacological postconditioning has protective effects against myocardial I/R injury in both *in vivo* and *in vitro* models, which are related to activating the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway.

1. Introduction

Ischemic heart disease (IHD) is associated with high morbidity and mortality, and its prevalence is continuously increasing in China and worldwide [1]. Myocardial ischemia/reperfusion (I/R) injury is a pathophysiological phenomenon commonly seen during thrombolysis, percutaneous transluminal coronary angioplasty (PTCA), and coronary artery bypass grafting (CABG). It is defined as restoration of blood flow to a previously ischemic region followed by complex pathological events leading to tissue injury greater than the original ischemic insult [2–4]. The outcomes of the myocardium I/R injury include reperfusion arrhythmias, myocardial stunning, myocardial hibernation, and final myocardial dysfunction which inevitably result from myocardium apoptosis subsequent to I/R injury [2]. Therefore, antiapoptotic agents that prevent I/R injury may be a novel therapeutic opportunity for IHD patients.

In recent years, ischemic preconditioning (IPC) and ischemic postconditioning (IPoC) became important approaches in endogenous cardioprotection, which yield a potential to significantly reduce the I/R-induced myocardial cell damage [5–7]. Pharmacological postconditioning (PPoC) is an extension of ischemic postconditioning, in which a drug is applied to ischemic myocardium or hypoxic cardiomyocytes during the early reperfusion or reoxygenation phases, and significantly attenuates cardiomyocyte injury and apoptosis [8].

The phosphatidylinositol 3-kinase/Akt (PI3K/Akt) is a powerful survival signaling pathway in many cell types [9, 10].

Activation of the PI3K/Akt pathway may be useful to promote myocytes survival in the damaged heart [11, 12], while administration of inhibitor of PI3K-Akt pathway like wortmannin and LY294002 was reported to abolish cardioprotection caused by IPoC [13]. This highlights the potentially beneficial role of PI3K in IPoC-mediated cardioprotection [13, 14]. Inhibition of PI3K accelerates apoptosis, while activation of Akt blocks apoptosis [15].

Traditional Chinese medicine has been used in the treatment of IHD for nearly three thousands of years. Shen-Yuan-Dan (SYD), a widely used traditional Chinese medicine prescription, consists of eight crude Chinese medicinal agents named *Salvia miltiorrhiza* Bge, *Astragalus membranaceus* Bge, root of Pilose Asiabell, Radix Scrophulariae, *Hirudo nipponica* (Whitman), *Lumbricus, Eupolyphaga sinensis* (Walker), and Rhizoma Corydalis, and has been confirmed to be effective in the treatment of IHD [16, 17]. Our previous studies demonstrated that oral supplementation for four weeks with SYD decoction at 60 g per day does not only relieve symptoms of angina but also promotes recovery of cardiac dysfunction [16, 17]. This involved reduction of myocardium infarct size [18], promotion of endothelial function [19], and inhibition of oxidative injury [20]. However, the effects and mechanisms of SYD postconditioning on I/R cell apoptosis have not been clarified yet. Therefore, the purpose of our study was to examine the *in vivo* and *in vitro* effects of SYD postconditioning on protecting against myocardial I/R injury. Myocardial injury markers and histopathology staining were examined in a rat model. To further examine the involvement of the PI3K/Akt pathway in the cardioprotection by SYD postconditioning, Bcl-2 and Bax protein levels were studied by immunocytochemistry.

2. Materials and Methods

2.1. Reagents. We utilized the following reagents and assay kits: lactic dehydrogenase (LDH) detection kit (Yatai Co., Ningbo, China), creatine kinase-MB (CK-MB) detection kit (Leadmanbio Co., Beijing, China), malondialdehyde (MDA) and superoxide dismutase (SOD) detection kit (Jiancheng Co., Nanjing, China), Dulbecco's Modified Eagle Medium/Nutrient Mixture F12Ham (DMEM/F12) (Sigma Aldrich Co., St. Louis, MO, USA), fetal bovine serum (FBS) (Hyclone Co., Rockford, IL, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco Co., Solon, OH, USA), hoechst 33342 (Dojindo Laboratories Co., Tokyo, Japan), rabbit anti-rat Bcl-2 IgG, rabbit anti-rat Bax IgG (Santa Cruz Co., Santa Cruz, CA, USA), Dylight 488 labeled goat anti-rabbit IgG (Goldenbridge Co., Beijing, China), and LY294002 (Biyuntian Co., Beijing, China).

2.2. Preparation of SYD Aqueous Extracts and Pharmacological Serum. SYD consists of eight crude medicinal agents including *Salvia miltiorrhiza* Bge (15 g), *Astragalus membranaceus* Bge (12 g), root of Pilose Asiabell (10 g), Radix Scrophulariae (5 g), *Hirudo nipponica* (Whitman) (3 g), *Lumbricus* (5 g), *Eupolyphaga sinensis* (Walker) (5 g), and Rhizoma Corydalis (5 g). All medicinal herbs were purchased from Beijing Xinglin Pharmaceutical Co. (Beijing, China) and were authenticated by Kechen Mao, a professional herbalist from Beijing TCM Hospital, Capital Medical University. The mixtures were soaked in distilled water for 30 min, boiled in 10 volumes of water (v/w) for 1 hour, and extracted three times. The filtered and mixed solution from three decoctions was concentrated under vacuum by using a rotary evaporator to a final concentration of 1 g/mL (w/v) followed by centrifugation at 3000 rpm for 30 min, which was then stored at −20°C for the following experiment.

To obtain SYD pharmacological serum, fifty Wistar rats (weight: 220–250 g) which were purchased from the Institute of Laboratory of Animal Sciences, China Academy of Medical Science (Beijing, China), were divided into two groups, and SYD (6 mL/kg) or saline (6 mL/Kg) as control was administered by oral gavage, twice daily, for five days. One hour after the final administration, rats from each group were anesthetized, blood specimens were drawn from the abdominal aortic artery and centrifuged at 3000 rpm for 10 min. Serum from each rat was collected and centrifuged at 1000 rpm for another 10 min. Individual serum samples from the same treatment group were combined in a 4 mL tube, inactivated at 56°C for 30 min, and kept at −20°C until processing. Before cell experiments, both SYD and control sera were diluted to 5% or 10% (v/v) with DMEM/F12 culture medium. As there were many gradients in SYD formula, we only analyzed a main constituent in SYD pharmacological serum called Danshensu salvianic acid Afrom *Salvia miltiorrhiza* Bge by a HPLC fingerprint. Based on the fingerprint, as shown in Figure 1, we established an optimal and easily controlled procedure for preparing SYD pharmacological serum.

2.3. I/R Injury in Rats and Neonatal Rat Cardiomyocyte. The rat model of I/R was established according to our previously published protocol [19]. Briefly, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). Then, coronary artery ligation was achieved with a gab occluder fixed onto the left anterior descending (LAD) coronary artery. A 5-0 silk suture was passed underneath the LAD (2-3 mm inferior to the left auricle) and tied. The ischemia was confirmed by myocardial blanching and ECG evidence of injury. Myocardial I/R model was induced by 30 min of ischemia followed by 3 hours of reperfusion. Rats surviving for 5 min after the reperfusion were randomized into five groups ($n = 8$ per group): sham-operated group (sham), I/R group (I/R), ischemic postconditioning (IPoC) group, SYD low-dose group (L-SYD, 3 g/kg), and SYD high-dose group (H-SYD, 6 g/kg). All drugs were administered via duodenal injection at the onset of reperfusion. Sham-operated and I/R groups were given equal volume of saline.

Primary cultures of neonatal rat cardiomyocytes from 1- to 3-days-old Wistar rats were prepared and cultured as described previously [21]. After 72 hours of cell culture, cardiomyocytes (cultured in DMEM/F12 containing 10% FBS at 37°C in CO_2 incubation) were subjected to various treatments and subsequent experimental protocols. In order to simulate the extracellular environment of myocardial

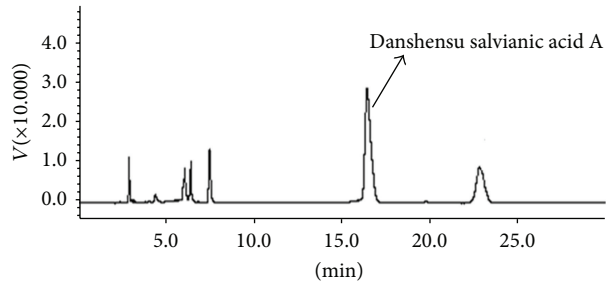

Figure 1: Chromatographic profile of SYD pharmacological serum. A Shimadzu LC9A series HPLC system (SHIMADZU, Japan) with a SPD-6AV detector and a Kromasil 100-5C18 column (4.6 mm × 250 mm, 5 μm particle size) was used for HPLC analysis. The UV spectra were recorded in the range of 230–400 nm, and chromatographic peaks were measured at a wavelength of 280 nm. Mobile phase consisted of acetonitrile and 1% acetic acid (v/v). The flow rate was 1.0 mL/min and the column temperature was set at 25°C.

I/R injury, a simulated hypoxia/reoxygenation model was performed as described previously with some modifications [4]. Briefly, cells were randomly divided into 7 groups. Cells in the normal control group were kept in normoxic culture for 6 hours (normal). In the hypoxia/reoxygenation group (H/R), the medium was replaced with glucose-free Earle's balanced salt solution (bubbled with 95% N_2 + 5% CO_2 for 15 min to remove soluble oxygen) prior to hypoxia, and the cells were immediately transferred into a hypoxic incubator in a humidified atmosphere equilibrated with 95% N_2 + 5% CO_2 for 4 hours (hypoxia). Thereafter, Earle's solution was replaced with DMEM/F12 containing 10% FBS to simulate reperfusion, followed by normoxic culture (for reoxygenation) for 4 hours. In the next four groups, Earle's solution was replaced with DMEM/F12 + 10% FBS containing, respectively, 5% or 10% of SYD pharmacological serum (5% SYD, 10% SYD), or 5% or 10% control serum (5% control, 10% control) prior to reoxygenation with other procedures identical to the H/R group. In the LY294002 + SYD postconditioning group, Earle's solution was replaced with DMEM/F12 + 10% FBS medium containing 30 ng/mL of LY294002 and 5% SYD pharmacological serum (5% SYD + LY294002) prior to reoxygenation, while other procedures were unchanged.

Animal use conformed with the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, Revised in 1996) and was approved by the Animal care and Use Committee, Beijing TCM Hospital, Capital Medical University (Beijing, China).

2.4. Measurement of LDH, CK-MB, SOD, and MDA. At the end of reperfusion, blood samples were drawn from the abdominal aortic artery, and serum samples were obtained by centrifugation of the specimens at 3000 rpm for 10 min at room temperature. Activities of LDH, CK-MB, SOD, and MDA were measured at 25°C using commercial kits according to the manufacturer's instructions on a spectrophotometer (Bio-Tek ELX800, Beijing, USA) at wavelengths of 340 nm (LDH and CK-MB), 532 nm (MDA), and 550 nm (SOD).

2.5. Histological Examination. At the end of reperfusion, rats were sacrificed. Left ventricles were sectioned, fixed for 24 hours in 10% formalin at room temperature, dehydrated by graded ethanol, and embedded in paraffin. Tissue sections (thickness of 5 μm) were deparaffinised with xylene, stained with haematoxylin-eosin (H&E), and viewed under light microscopy (Leica DM2000, Wetzlar, Germany). All histological evaluations were performed in a blinded manner.

2.6. MTT Assay. Myocardial cells were cultured in a 96-well plate at a density of 2×10^5 cells/well. Following experiments, cells were treated with 20 μL MTT (5 mg/mL) and incubated for 4 hours in darkness at 37°C. Afterwards, medium and MTT were removed from the wells. The remaining MTT-formazan crystals were dissolved in 150 μL DMSO (lysis for 10 min). Optical densities (OD) were analyzed spectrophotometrically at a wavelength of 540 nm, using 150 μL of DMSO as blank. All experiments were performed three times.

2.7. Hoechst 33342 Assay. Exponentially growing cells were plated in 12-well plates at a density of 2×10^5 cells/well and cultured for 72 hours. Following simulated I/R procedures, cells were fixed for 8 min with the precooled (−20°C) formaldehyde and acetone solution (1 : 1, *v/v*) and washed with PBS 3 × 3 min, followed by staining with Hoechst 33342 solution (50 mmol/L) at 37°C in darkness for 5 min. Apoptotic cells were observed and images were taken using fluorescence microscope (Olympus BX51, Tokyo, Japan), with excitation wavelength of 350 nm and emission wavelength of 460 nm.

2.8. Immunocytochemistry Assay. Exponentially growing cells were plated in 12-well plates at a density of 2×10^5 cells/well and cultured for 72 hours. Following simulated I/R procedures, cells in each group were fixed and washed with PBS as above. Afterwards, primary antibodies (rabbit anti-rat Bcl-2 or Bax IgG) were added and incubated with cells at 37°C for 60 min. Then, cells were washed three times with cold PBS, and secondary antibody (Dylight 488 labeled goat anti-rabbit IgG) was added and incubated with cells at 37°C for 20 min. Cells were observed and images were taken using fluorescence microscope. The integral optical density (IOD) was measured in fluorescence positive stained cell by Image-Pro Plus v5.0 (Media cybernetics, USA).

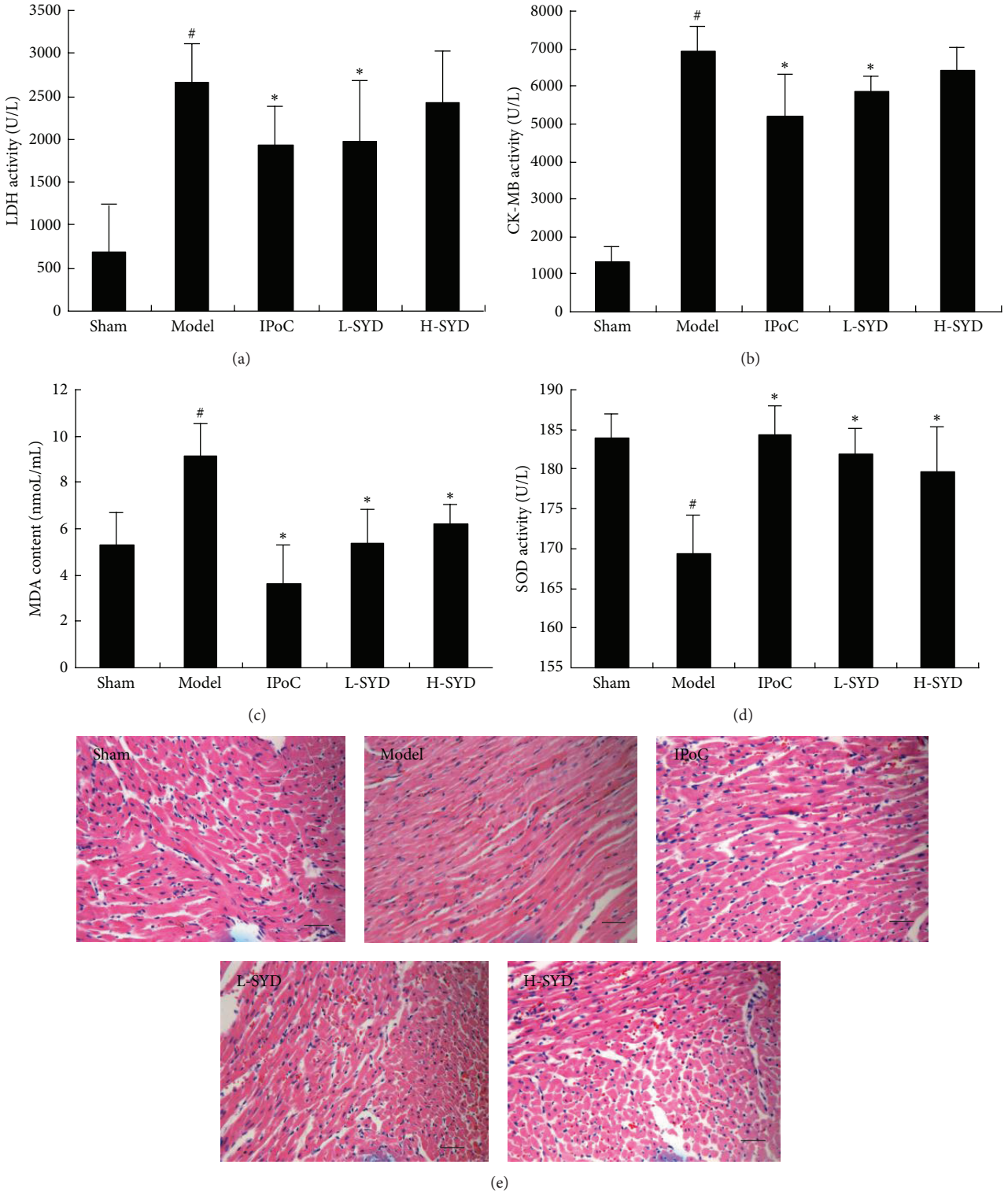

FIGURE 2: Effects of SYD postconditioning on ischemia-reperfusion (I/R) injury in the sham, I/R, IPoC, low-dose (L)-SYD (3 g/kg/day), and high-dose (H)-SYD (6 g/kg/day) groups. (a) serum LDH activity; (b) serum CK-MB activity; (c) Serum MDA content; and (d) serum SOD activity. Data are expressed as mean ± SD ($n = 8$). $^{\#}P < 0.05$ versus sham; $^{*}P < 0.05$ versus I/R. (e) Representative images of H&E-stained sections. In the I/R group, myocardial fiber loss and disruption are evident, and this is reversed by SYD treatment. SYD (both L- and H-dose) was administered via duodenal injection. Scale bar represents 50 μm.

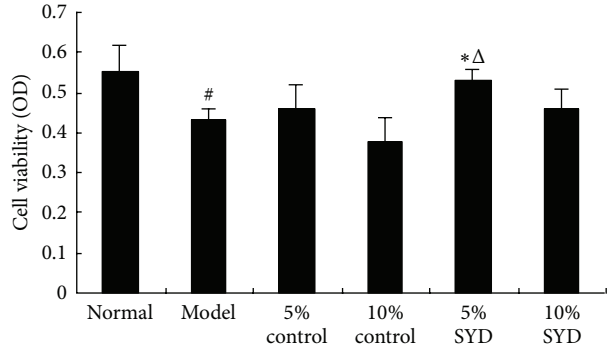

Figure 3: Effects of different doses of SYD pharmacological serum on cell viability in H/R cardiomyocytes. The cells were exposed to 6 hours of normoxic culture (normal), 2 hours of hypoxia followed by 4 hours of reoxygenation (H/R), and postconditioning with two concentrations of SYD or control serum (5% or 10% each). Data are expressed as mean ± SEM ($n = 3$). $^{\#}P < 0.05$ versus normal, $^{*}P < 0.05$ versus 5% control serum, and $^{\Delta}P < 0.05$ versus 10% SYD.

2.9. Statistical Analysis. All data are expressed as mean ± SEM. Differences between groups were analyzed by one-way ANOVA, with the Student-Newman-Keuls (SNK) assay used for post hoc analysis. The two-sided $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS software (version 11.0, SPSS Inc., Chicago, USA).

3. Results

3.1. Effects of SYD Pharmacological Postconditioning on I/R Injury in Rats. As a consequence of I/R injury, serum activities of LDH and CK-MB were significantly increased in I/R group as compared with sham group ($P < 0.05$; Figures 2(a) and 2(b)). Both IPoC and low dose of SYD (3 g/kg) significantly inhibited elevation of LDH and CK-MB activity ($P < 0.05$ versus I/R group; Figures 2(a) and 2(b)), while no significant difference was found between H-SYD group and I/R group with regard to these markers (Figures 2(a) and 2(b)). After I/R, MDA activity increased, activity of SOD decreased, in the I/R group compared with sham group ($P < 0.05$; Figures 2(c) and 2(d)), while both IPoC and SYD treatments (L- and H-dose) significantly inhibited elevation of MDA activity and promoted SOD activity compared with the I/R group ($P < 0.05$; Figures 2(c) and 2(d)).

Three hours after reperfusion, no lesions were observed in the sham group. In the I/R group, apparent perivascular edema and structural disarray were present, and neutrophil influx was documented. After treatment with SYD (both L- and H-dose), histological features became typical of normal cardiac structure or mild architectural damage (Figure 2(e)).

3.2. Effects of SYD Pharmacological Serum on Cell Viability in H/R Cardiomyocytes. Effects of SYD pharmacological postconditioning on cell viability in H/R myocardial cells were assessed by MTT assay. As shown in Figure 3, viability was decreased in H/R group compared with normal group ($P < 0.05$), and 5% SYD postconditioning significantly increased cell viability compared with 5% control or 10% SYD groups ($P < 0.05$, in both comparisons). The results indicated that SYD preserves cell viability effect, however, only at an optimal dose.

3.3. Effects of SYD Pharmacological Serum and LY294002 Postconditioning on Apoptosis in H/R Cardiomyocytes. Effects of SYD postconditioning on the H/R induced cell apoptosis were assayed by Hoechst 33342 staining. As shown in Figure 4, few apoptotic cells were present in the normal group. As expected, there were many apoptotic (i.e., Hoechst 33342-positive) cells in the H/R group and in the group that received 5% SYD + LY294002 postconditioning. By contrast, fewer apoptotic cells were observed when SYD alone was used for postconditioning ($P < 0.01$; Figure 4(a)). Further, 5% SYD exhibited a better effect compared with postconditioning with 10% SYD group ($P < 0.01$; Figure 4(b)).

3.4. Effects of SYD Pharmacological Postconditioning on Expressions of Bcl-2 and Bax in H/R Cardiomyocytes. The Bcl-2 and Bax positive cells were observed in cytoplasm of cells cultured under normoxic conditions (Figures 5(a) and 6(a)). In the SYD postconditioning group, expression of Bcl-2 was significantly increased compared with H/R group ($P < 0.01$; Figure 5(a)), while expression of Bax was significantly decreased ($P < 0.01$; Figure 6(a)). By contrast, expression of Bcl-2 was decreased when pre-conditioning with 5% SYD complemented with LY294002, whereas expression of Bax was increased ($P < 0.01$; Figures 5 and 6). The pattern of the changes in Bcl-2 and Bax expressions was compatible with the patterns observed in the cell viability and apoptosis experiments.

4. Discussion

Myocardial I/R injury can be defined as damage to the heart when blood supply is restored after a prolonged period of ischemia resulting in oxidative damage, inflammation, cell apoptosis, and cardiac dysfunction [3, 22]. IPoC is defined as brief episodes of coronary occlusion and reperfusion at the onset of reperfusion after sustained ischemic insult and has been confirmed to have beneficial effects in protecting against

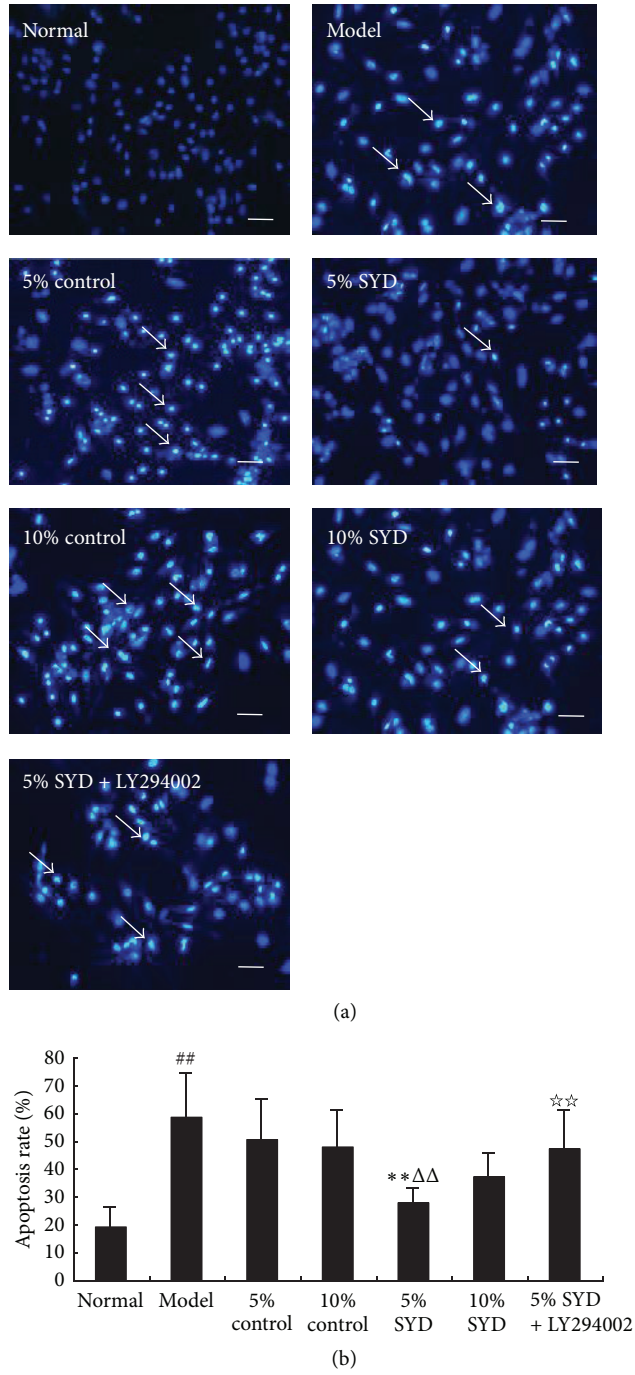

Figure 4: Effects of SYD and LY294002 on cell apoptosis. (a) Representative images of Hoechst 33342 staining. (b) Quantitative analysis of apoptosis rate. The cells were exposed to 6 hours of normoxic culture (normal), 2 hours of hypoxia followed by 4 hours of reoxygenation (H/R), postconditioning with two concentrations of SYD or control serum (5% or 10% each). In some experiments, 5% SYD was complemented with LY294002 (5% SYD + LY294002). Data are expressed as mean ± SEM ($n = 3$), $^{\#\#}P < 0.01$ versus normal, $^{**}P < 0.01$ versus 5% control serum. $^{\Delta\Delta}P < 0.01$ versus 10% SYD, and $^{\star\star}P < 0.01$ versus 5% SYD. Scale bar represents 50 μm.

I/R injury in dogs, cats, rats, and rabbits [6, 23–25]. Further, PPoC is a condition when a drug is applied to ischemic myocardium or hypoxic cardiomyocytes during the early reperfusion or reoxygenation phases; PPoC has similar protective effects in attenuating cardiomyocyte injury and apoptosis. And a diverse array of pharmacological agents such as bradykinin-B2 receptor activator [26], PKC-adenosine A2b receptor activator [27], phytoestrogen genistein [28], and several natural drug components administered at the time of reperfusion was reported to be cardioprotective [29–31]. Our previous studies confirmed that SYD, an adjunctive traditional Chinese medicine prescription in the treatment

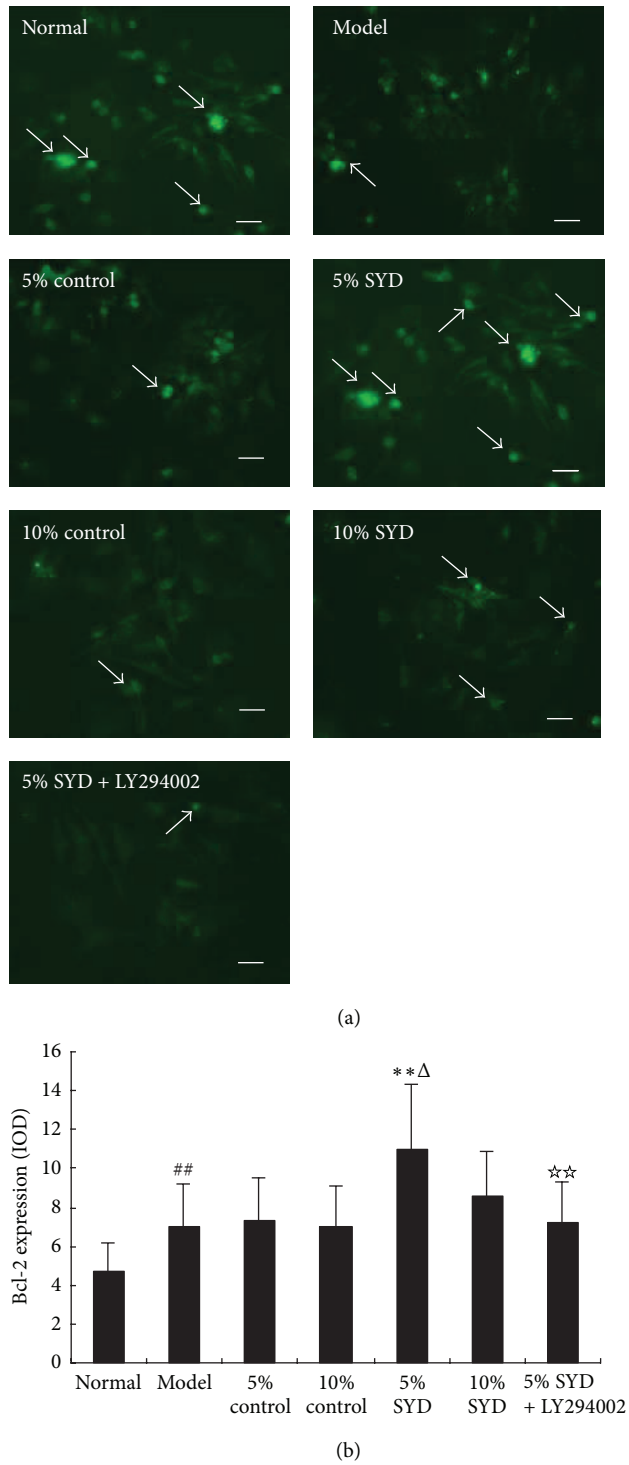

Figure 5: Effects of SYD and LY294002 on Bcl-2 expression. (a) Representative images of Bcl-2 staining. (b) Quantitative analysis of Bcl-2 expression (IOD). The cells were exposed to 6 hours of normoxic culture (normal), 2 hours of hypoxia followed by 4 hours of reoxygenation (H/R), and postconditioning with two concentrations of SYD or control serum (5% or 10% each). In some experiments, 5% SYD was complemented with LY294002 (5% SYD + LY294002). Data are expressed as mean ± SEM ($n = 3$). $^{\#\#}P < 0.01$ versus normal, $^{**}P < 0.01$ versus 5% control serum, and $^{\Delta\Delta}P < 0.01$ versus 10% SYD, $^{\star\star}P < 0.01$ versus 5% SYD. Scale bar represents 50 μm.

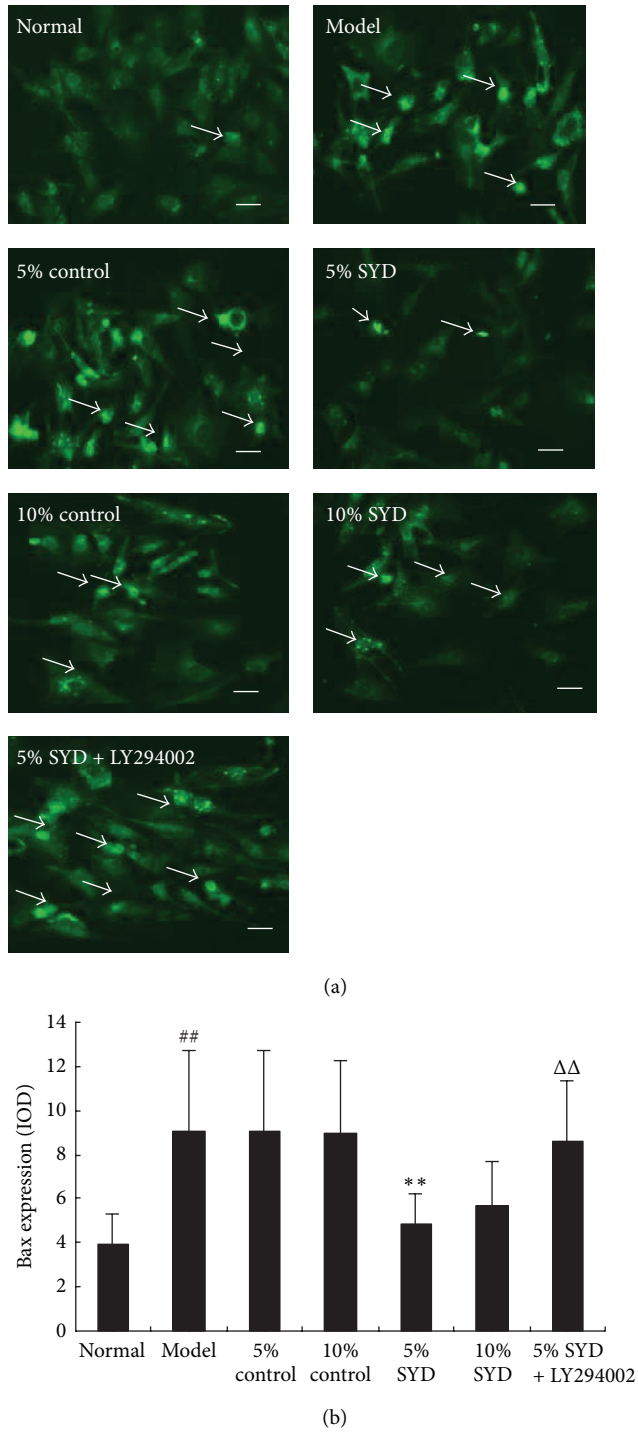

Figure 6: Effects of SYD and LY294002 on Bax expression. (a) Representative images of Bax staining. (b) Quantitative analysis of Bax expression (IOD). The cells were exposed to 6 hours of normoxic culture (normal), 2 hours of hypoxia followed by 4 hours of reoxygenation (H/R), and postconditioning with two concentrations of SYD or control serum (5% or 10% each). In some experiments, 5% SYD was complemented with LY294002 (5% SYD + LY294002). Data are expressed as mean ± SEM ($n = 3$). $^{\#\#}P < 0.01$ versus normal, $^{**}P < 0.01$ versus 5% control serum, $^{\Delta\Delta}P < 0.01$ versus 10% SYD, and $^{\star\star}P < 0.01$ versus 5% SYD. Scale bar represents 50 μm.

of ischemic heart disease, has beneficial effects in reducing myocardium infarct size [18], promoting endothelial function [19], and inhibiting oxidative damage [20]. The mechanisms behind these beneficial effects include activation of the PKC signaling [18].

Traditional Chinese medicine (TCM) has been widely used in treating many kinds of cardiovascular and metabolic diseases, such as hypertension, hyperlipidemia, and diabetes [32–34]. Shen-Yuan-Dan (SYD), a widely used traditional Chinese medicine prescription, has been confirmed to be

effective in management of IHD, but the effects of SYD postconditioning on myocardial I/R injury and cell apoptosis and the involved mechanism still remain unclear. In the present study, we investigated the effects and mechanisms of SYD postconditioning in protecting from myocardium I/R injury and apoptosis, both *in vivo* and *in vitro*. Our findings demonstrate that both low (3 g/kg) and high dose (6 g/kg) of SYD protected myocardium against I/R injury in rat model, as demonstrated by reduced serum LDH and CK-MB activity and MDA content, increased SOD activity and attenuated histopathology injury. In the *in vitro* studies, SYD pharmacological serum promoted cell viability and inhibited the cardiomyocyte apoptosis. Expression of Bcl-2 in cells treated with SYD pharmacological serum was significantly increased, while Bax expression was markedly reduced. These effects of SYD were inhibited by LY294002, an inhibitor of PI3K/Akt. The above results suggest that SYD is capable of protecting myocardium from I/R injury and inhibit the H/R-induced cell apoptosis and that this protection involves activating PI3K/Akt signaling pathway.

LDH and CK-MB are two specific myocardium injury biomarkers, which are often elevated in myocardial infarction and other ischemic injuries [35]. It is reported that myocardial I/R injury can also significantly increase the activity of LDL and CK-MB, and evidence from previous studies confirmed that IPC and IPoC can significantly reduce the activity of LDH and CK-MB and attenuate histopathology injury in myocardium, indicating great potential of IPC and IPoC in the treatment of I/R injury [36]. As described above, both low and high doses of SYD significantly inhibited the elevation of LDH and CK-MB in our study.

It is widely accepted that oxidative stress, which is associated with increased formation of reactive oxygen species (ROS), plays an important role in the pathogenesis of I/R injury [37]. Various lines of clinical and experimental evidence suggested that myocardium injury after I/R can be attributed to oxygen-free radicals mediated lipid peroxidation, a process that can be measured through its by-products, specifically MDA, and the activity of endogenous antioxidant enzymes such as SOD can be decreased after I/R injury [37]. In our study, the MDA content was significantly increased and SOD activity was significantly decreased in I/R group and both low and high dose of SYD can significantly decrease the serum MDA content and increase SOD activity at the end of the experiment. The results indicated that SYD postconditioning is protective in attenuating the reperfusion mediated lipid peroxidation damage.

Apoptosis has been shown to play an important role in the pathogenesis of myocardial I/R injury [38]. The balance between the up- and downregulations of the members of proapoptotic (Bax and Bad) and antiapoptotic (Bcl-xL and Bcl-2) family proteins determines the fate of the cells either to undergo apoptosis or to survive [38]. It is reported that the process of cell apoptosis after myocardial I/R showed a remarkable decrease of Bcl-2 gene expression and increased expression of Bax, and evidence from various *in vivo* and *in vitro* studies have confirmed the beneficial effects of ischemic postconditioning and pharmacological postconditioning in protecting against I/R injury mediated apoptosis [39]. In this study, we first investigated the effects of different concentrations of SYD pharmacological serum in protecting against simulated hypoxia/reoxygenation injury *in vitro* by MTT assay. The results showed that simulated hypoxia/reoxygenation (I/R *in vitro*) can significantly decrease the cell viability in cultured cardiomyocyte and postconditioning with different concentrations (5% and 10%) of SYD pharmacological serum greatly decreased the loss of cell viability (Figure 3). These results indicate that SYD pharmacological postconditioning significantly protected cardiomyocyte from I/R-induced cytotoxicity.

PI3K/Akt plays a key role in the reperfusion injury salvage kinase (RISK) pathway. Its activation leads to cardiac protection during myocardial I/R injury [8]. Our experiments confirmed that protective effects of SYD pharmacological serum were associated with PI3-kinase/Akt signaling pathway, as its inhibition reversed beneficial effects of SYD.

Finally, our study also demonstrated some interesting results. Comparing with postconditioning with 10% SYD, 5% SYD better improved the rate of myocardial cells' survival and more efficiently reduced the rate of apoptosis.

In conclusion, the results of our study demonstrate that SYD has a beneficial effect in protecting ischemic myocardium from the I/R injury and inhibiting cell apoptosis in H/R cardiomyocytes. The mechanism by which SYD exhibits its cardioprotective effects is associated with activation of the PI3K/Akt pathway. Better effects were observed in the low-dose SYD indicating that an optimal treatment dose may be needed for maximal cardioprotection.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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