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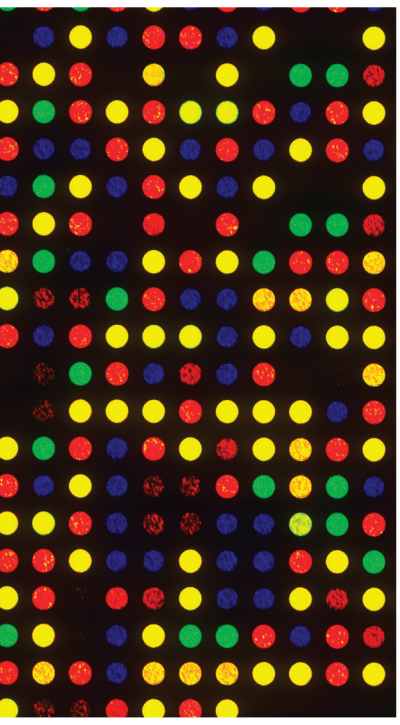
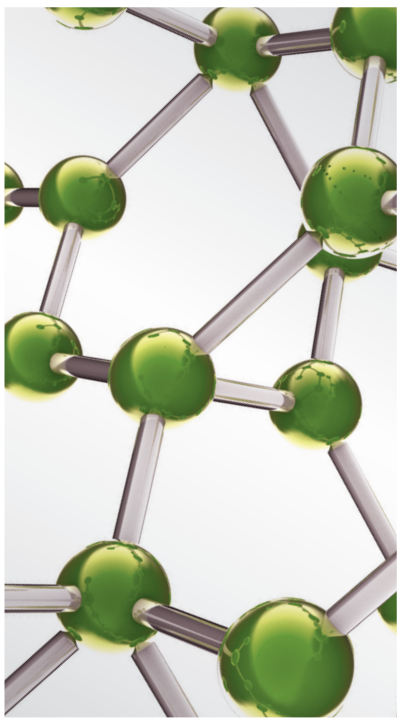
Guest Editors: Dazhuo Shi, Michael Y. H. Shen, Honglin Luo, and Yan Ma



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Editorial Board

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Editorial

Herbal Medicine after Interventional Therapy in Cardiovascular Diseases: Efficacy, Mechanisms, and Safety

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Despite recent medical advances, cardiovascular diseases (CVDs) remain the predominant cause of morbidity and mortality all over the world. Interventional therapy (IT) is the milestone of therapy of CVDs and has developed rapidly in recent years. Despite its proven benefit, recurrent cardiovascular events are still a big challenge in cardiology field. Plants have been used for medicinal purposes for as long as history has been recorded. Great varieties of plants are used for medicinal treatments and many new drugs have been discovered from herbal sources. Herbal medicine (HM) means use of natural plant substances (botanicals) to treat and prevent illness. Based on the integrative medicine of eastern and western, the application of HM has valuable significance in reducing the risk of cardiovascular event. During the past decades, some HM products went into Europe and United States for prevention and treatment of CVDs or prevention of in-stent restenosis after IT, however, as the complementary and alternative remedies. Widespread use has increased much demands that HM be regulated as drugs to insure the efficacy, mechanisms, and safety.

This special issue is a collection of seven articles describing the use of herbal medicines after interventional therapy in cardiovascular diseases. There are two clinical research articles describing the efficacy and mechanisms of HM. An article by S.-L. Wang et al. evaluated the 10-year effectiveness of HM plus conventional treatment versus conventional treatment alone with decision-analytic model for ACS after PCI. The authors found that treatment with HM, as an adjunctive therapy, in combination with conventional treatment for 6 months might improve the long-term clinical outcome in ACS patients after PCI. M. Xue et al.' study showed that Xuefuzhuyu oral liquid could effectively improve blood stasis syndrome and aspirin resistance by inhibiting ADP-induced platelet aggregation and patients with the rs5911 genetic variant exhibited better drug response. There are three experimental articles that make in-depth exploration about mechanisms of HM for cardiovascular diseases. Study by J. Su et al. showed luteolin could ameliorate hypertensive vascular remodeling mediated by the regulation of MAPK signaling pathway and the production of ROS. A study by W. Liu et al. demonstrated antihypertensive mechanism of gastrodin involved in regulation of the renin-angiotensin-aldosterone system (RAAS) and PPARγ. F. Wu et al. found that activation of local CSE-H2S-VEGF axis might participate in proangiogenesis effects of Danhong injection, suggesting a potential therapy for diabetic patients with critical limb ischemia. An interesting article by H. Berrougui et al. gives evidence from the beneficial role of extra virgin olive oil (EVOO) consumption towards oxidative stress and cardiovascular diseases. The only meta-analysis as a part of this special issue by A. Liu et al. demonstrated possible efficacy of active compounds of rhubarb root and rhizome that have potential neuroprotective effect for experimental ischemic stroke but should be interpreted with caution because of shortage of the methodology.

From the above-mentioned articles, this special issue provides recent evidence about efficacy, mechanisms, and safety of herbal medicine after interventional therapy in CVDs. We hope this special issue will offer a new scientific understanding of the effect of herbal medicine for CVDs.

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Research Article

Gastrodin Reduces Blood Pressure by Intervening with RAAS and PPARγ in SHRs

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Gastrodin is a bioactive compound extracted from traditional Chinese medicine, *Gastrodia elata* Bl. It has a definite effect on reducing blood pressure in hypertensive patients. However, the mechanisms of gastrodin in lowering blood pressure still remain unclear. In this study, 4 weeks of administration of gastrodin (100 mg/kg/d intraperitoneally injected) decreased the systolic blood pressure (SBP) in spontaneously hypertensive rats (SHRs) (190.2±8.9 versus 169.8±6.4, $P < 0.01$). Among SHRs receiving gastrodin treatment, angiotensin II (Ang II) and aldosterone (ALD) in serum were significantly decreased (2022.1 ± 53.0 versus 1528.7 ± 93.9, 213.33 ± 35.17 versus 179.65 ± 20.31, and $P < 0.01$, $P < 0.05$, resp.) and dramatically downregulated expression of angiotensin type 1 receptor (AT1R) (4.9 ± 0.9 versus 2.6 ± 0.9, $P < 0.05$) in myocardium in both mRNA and protein levels compared with their corresponding groups without gastrodin treatment. Additionally, gastrodin increased the mRNA expression (0.18 ± 0.07 versus 0.82 ± 0.10, $P < 0.01$) and protein synthesis (0.40 ± 0.10 versus 0.34 ± 0.10, $P < 0.01$) of peroxisome proliferator-activated receptor γ (PPARγ) in myocardium tissues. Overall, our data demonstrated that gastrodin was able to decrease the SBP in SHR. Furthermore, this study showed that gastrodin intervened with the renin-angiotensin-aldosterone system (RAAS) and PPARγ effectively, which indicates its antihypertensive mechanism.

1. Introduction

As one of the most common chronic diseases in the world, hypertension accelerates the progression of cardiovascular disease and severely threatens human health. It has been shown that the renin-angiotensin-aldosterone system (RAAS) plays a major role in the initiation and progression of hypertension [1]. RAAS is a cascade with effector molecules such as angiotensin II (Ang II) and aldosterone (ALD). Ang II has a strong biological activity in constricting blood vessels, enhancing aldosterone secretion, and ultimately elevating blood pressure level. Aldosterone enhances the reabsorption of sodium and water, thus increasing blood volume and blood pressure [2]. Therefore, inhibiting the activation of RAAS is one of the main strategies to lower blood pressure in patients with hypertension.

Peroxisome proliferator-activated receptor (PPAR) γ is a nuclear transcription factor regulated by ligands which are expressed in the cardiovascular system [3]. It has been reported that PPARγ can regulate the gene expression related with RAAS and may play a regulatory role in blood pressure modulation [4].

Gastrodin (PubChemCID:115067(2R,3S,4S,5R,6S)-2-(hydroxymethyl)-6-[4-(hydroxymethyl)phenoxy]oxane-3,4,5-triol, Figure 1) is one of the major bioactive components extracted from the Chinese herb *Gastrodia elata* Bl. (Figure 1). Gastrodin injection has been extensively used to treat cardiovascular and cerebrovascular diseases in China and has a certain efficacy to lower blood pressure in hypertensive patients [5]. However, the related mechanisms still remain cryptic. Therefore, we aimed to determine whether gastrodin could attenuate blood pressure by regulating RAAS and PPARγ. It is important to note that gastrodin is used clinically to manage cardiovascular disease and that the heart is the main target organ in the management of cardiovascular diseases. In this regard, we used echocardiography to evaluate cardiac function after gastrodin treatment.

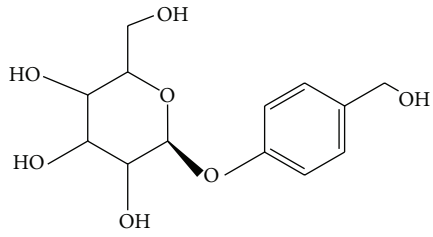

Figure 1: Structure of gastrodin.

Clinical and experimental evidence suggests that cardiac renin-angiotensin system (RAS) plays an important role in the regulation of SBP. Of note, PPARγ expression occurs in the heart and has been shown to possess key regulatory function on the cardiac RAS expression level. Our explicit objective was to study the biological action of gastrodin and its therapeutic significance on the regulation of PPARγ, ACE, and AT1R in the heart. We found that activation of cardiac PPARγ correlated with reduced expression level of myocardial ACE and AT1R expression levels. The subsequent reduction in blood pressure level suggests that gastrodin could reduce blood pressure by elevating the expression of PPARγ which negatively regulates cardiac ACE and AT1R.

2. Materials and Methods

2.1. Animals and Treatment. Ten-week-old male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs) were obtained commercially from Vital River Laboratories, Beijing, China. SHRs were randomly divided into model (SHR) and treatment groups (GAS) with 8 rats in each group. Eight WKY belonged to the control group (WKY). Rats were housed in a controlled environment (22 ± 2°C and 50% ± 5% humidity) receiving a circadian rhythm of 12 h/12 h light/dark. Rats were allowed food and water *ad libitum*. Bedding was refreshed daily for every cage. The first two weeks was the adaptation period, and rats were not given any intervention. Gastrodin therapy started from the third week during which rats were under therapeutic treatment. SHRs in the treatment group were injected intraperitoneally with gastrodin at the dose of 100 mg/(kg·d) for 4 weeks. The dose of gastrodin administration was arrived at by critical assessment of clinical dosage and analysis of previous studies [6]. Body weight and blood pressure of the rats were measured every week. All the procedures were approved by the Animal Ethics Review Committee of Tianjin University of Traditional Chinese Medicine.

2.2. Reagents. Gastrodin injections were purchased from Hainan Helpson Medicine & Biotechnique Co., Ltd. The concentration of gastrodin was 100 mg/mL, and it was stable for 2 years at room temperature. ELISA kit of aldosterone and angiotensin II were purchased from Beijing Sino-UK Institute of Biological Technology. Western blot reagents were purchased from Sigma, while antibodies were purchased from Santa Cruz. Real-time quantitative PCR reagents were purchased from Tianjin Hao Yang Biological Manufacture Co., Ltd., and the primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

2.3. Monitoring of Blood Pressure, Heart Rate, and Body Weight. Blood pressure (systolic blood pressure), heart rate, and body weight of rats were monitored once a week. Systolic blood pressure (SBP) and heart rate were monitored by the noninvasive tail-cuff method using animal sphygmomanometer (BP98AWU, Softron, Japan). All the blood pressure and heart rate measurements were performed on conscious animals. For each SHR and WKY rat, blood pressure and heart rate were measured with multiple readings, until 15 stable measurements in a row were obtained. Data were calculated as an average of blood pressure and heart rate values.

2.4. Cardiac Function Study by Echocardiography. Echocardiography was done by an observer blinded to the experiment, and measurements were taken before sacrifice. Echocardiography was performed by Visual Sonics Vevo 2100 imaging system. The LV was assessed in both parasternal long-axis and short-axis views at a frame rate of 50 Hz. End-systole or end-diastole was defined as the phase in which the smallest or largest area of LV, respectively, was obtained. Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level. These parameters were used to determine left ventricular ejection fraction.

2.5. Serum Collection and Harvest of the Tissue. After abdominal anesthesia in rats with chloral hydrate (5%, 6 mL/kg, i.p.), blood samples were collected via abdominal aorta puncture. Serum was then prepared by centrifugation of the collected blood (2000 rpm for 20 min). Serum samples were stored at −80°C and used to determine the levels of aldosterone (ALD) and angiotensin II (Ang II) with ELISA kits in a blinded manner following the manufacturer's instructions. Heart was removed from each rat.

2.6. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis. Total RNA was isolated from the myocardium tissues ($n = 8$ per group) using TRIZOL reagent (Tianjin Hao Yang Biological manufacture Co., Ltd., China). RNA was reverse-transcribed using SuperScript First Strand cDNA System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

The following primer sequences were used: β-actin (NM_031144), forward (TCA GGT CAT CAC TAT CGG CAA), reverse (AGC ACT GTG TTG GCA TAG AGG), ACE (NM_012544.1), forward (AAC AGG TTC GTG GAG GAG TAT), reverse (CAG GTG CCA TAT TTC AAG GTA). AT1R (NM_030985.4), forward (ATC TCG CCT TGG CTG ACT TAT), reverse (GAA GGA ACA CAC TGG CGT AGA), and PPARγ (NM_001145366.1), forward (AAG GGT GCC AGT TTC GAT CC), reverse (TAT TCA TCA GGG AGG CCA GCA). The sizes of the PCR products amplified with the primers were β-actin, 169 bp, ACE, 161 bp, AT1R, 150 bp, and PPARγ, 159 bp, respectively. In preliminary experiments,

we confirmed that the efficiency of these primer pairs is comparable (data not shown). qRT-PCR was done using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 μL on the 7900HT fast real-time PCR system (Applied Biosystems) as follows: 95°C for 15 min, 40 cycles of 95°C for 20 s, and 57°C for 20 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. β-actin was used as the reference gene. The relative levels of gene expression were represented as $\Delta Ct = Ct_{gene} - Ct_{reference}$, and the fold change of gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method [7].

2.7. Western Blotting Assay. Western blotting assay was performed to determine protein expression of AT1R and PPARγ ($n = 4$ per group). The myocardium tissue was homogenized in the lysis buffer using an ultrasound homogenizer at 50 Hz. The lysate was then centrifuged. The protein concentration of the supernatant was measured with the Bradford protein assay. Proteins were loaded into 8% SDS-polyacrylamide gels and transferred to PVDF membranes. After blocking in nonfat milk, the membranes were exposed to a rabbit polyclonal antibody against AT1R or PPARγ or β-actin overnight at 4°C. After incubation with HRP-linked secondary antibodies, the immune complexes were visualized with chemiluminescence (ECL Blotting Analysis System; Amersham, Arlington Heights, IL) and exposed to X-ray film, measured with Image J software, and normalized to β-actin.

2.8. Statistical Analysis. All results were expressed as mean ± S.E.M. Statistical comparisons between different groups were performed by one-way ANOVA with Dunnett's multiple comparison posttest. Differences with P value less than 0.05 were considered statistically significant.

3. Results

3.1. General Health of Rats in Each Group. Gastrodin did not significantly affect the body weight of the rats in this study; however, all rat weights increased during the experimental period. WKY's weights were more than the other two groups from the beginning to the end of the study. All the differences were statistically significant (Figure 2). Daily intraperitoneal injection of gastrodin did not affect food consumption (Figure 3). Moreover, gastrodin did not affect heart rate (Figure 4).

3.2. Systolic Blood Pressure. At the baseline, the systolic blood pressure (SBP) was no different than SHR and GAS. After two weeks of treatment, SBP of GAS began to reduce and was significantly decreased compared with SHR ($P < 0.05$). There was also a recorded steady reduction of SBP in GAS treated group, while elevated level of SBP in SHR was observed. Throughout the experiment, SBP of WKY was lower than in SHR and GAS ($P < 0.01$) (Figure 5). The data about the effects of gastrodin in the normotensive rats are not shown. First of all, there was no significant difference in the indices on the normotensive rats after daily administration of gastrodin injection.

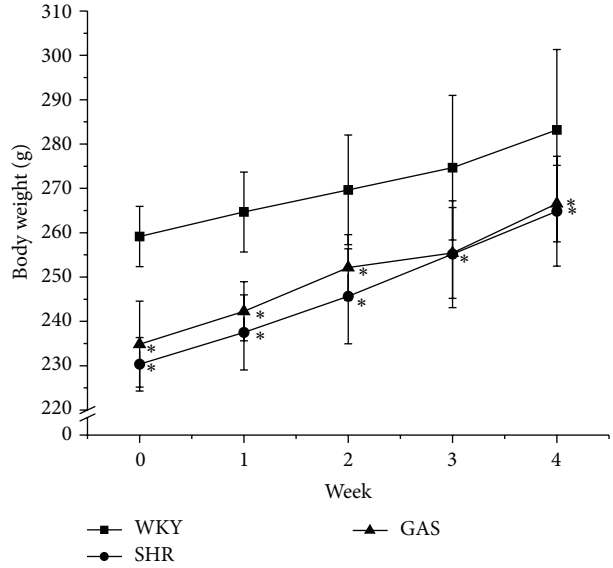

Figure 2: Effects of 4-week treatment with gastrodin on body weight of rats. Ten-week-old male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs) were obtained. SHRs were randomly divided into model (SHR) and treatment groups (GAS). WKY belonged to control group (WKY). The first two weeks was adaptation period. Starting from the third week, rats in GAS group were intraperitoneally injected with gastrodin at the dose of 100 mg/(kg·d) for 4 weeks. The rest of the rats were not given any intervention other than providing food and water. The body weights were recorded weekly during the experimental period. Values are shown as means ± SEM. $^*P < 0.05$ versus WKY.

3.3. Cardiac Function. After four weeks of treatment, there was no significant difference in cardiac function among the three groups (Table 1).

3.4. Effects of Gastrodin on RAAS. Since gastrodin showed effect on SBP in SHR, we explored the possible mechanisms. Previous studies have shed more light on the role RAAS plays in regulating blood pressure levels. In this regard, we sought to determine the effects of gastrodin on RAAS *in vivo*. After four weeks of treatment, the serum level of Ang II in GAS was lower than in SHR ($P < 0.01$). Surprisingly, the Ang II serum level in GAS was also lower than in WKY (Figure 6(a)). Gastrodin also significantly reduced ALD level in serum compared with SHR ($P < 0.05$). There was no significant difference between WKY and GAS treatment groups in the serum level of ALD (Figure 6(b)).

We further examined the mRNA expression of ACE and AT1R by qRT-PCR. After four weeks of treatment, compared with SHR, the mRNA expression of ACE in GAS declined, but the difference was not statistically significant (Figure 7(a)). Meanwhile, the mRNA expression of AT1R was lower in GAS than in SHR, and the difference was statistically significant ($P < 0.05$) (Figure 7(b)). There was no significant difference in mRNA expression levels of ACE and AT1R between WKY

Table 1: Cardiac function (means ± SEM).

	IVS; d (mm)	IVS; s (mm)	LVID; d (mm)	LVID; s (mm)	LVPW; d (mm)	LVPW; s (mm)	EF (%)
WKY	1.29 ± 0.07	2.21 ± 0.29	6.35 ± 0.43	3.77 ± 0.58	1.64 ± 0.15	2.48 ± 0.16	69.95 ± 8.40
SHR	1.70 ± 0.18	2.33 ± 0.37	6.24 ± 0.80	4.42 ± 1.23	2.02 ± 0.26	2.41 ± 0.47	55.16 ± 17.32
GAS	1.53 ± 0.24	2.09 ± 0.22	6.73 ± 0.42	4.88 ± 0.49	1.87 ± 0.25	2.35 ± 0.21	51.95 ± 7.87

After 4-week treatment, there was no difference in cardiac function among the three groups.

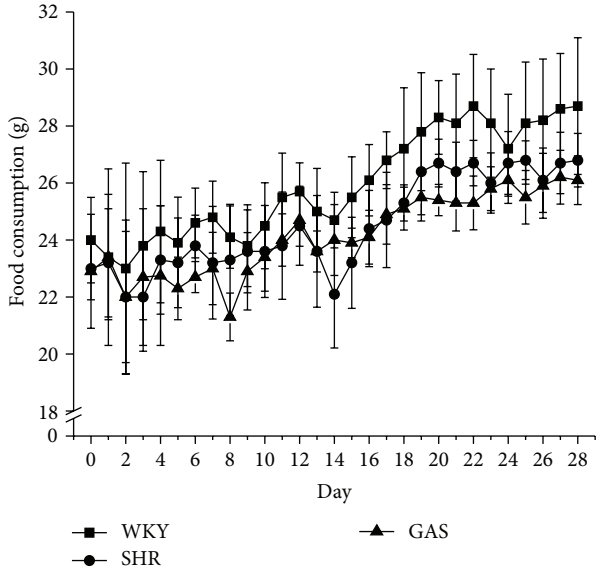

Figure 3: Effects of 4-week treatment with gastrodin on food consumption of rats. Ten-week-old male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs) were obtained. SHRs were randomly divided into model (SHR) and treatment groups (GAS). WKY belonged to control group (WKY). The first two weeks was adaptation period. Starting from the third week, rats in GAS group were intraperitoneally injected with gastrodin at the dose of 100 mg/(kg·d) for 4 weeks. The rest of the rats were not given any intervention. The food consumption was recorded daily during the experimental period. Values are shown as means ± SEM.

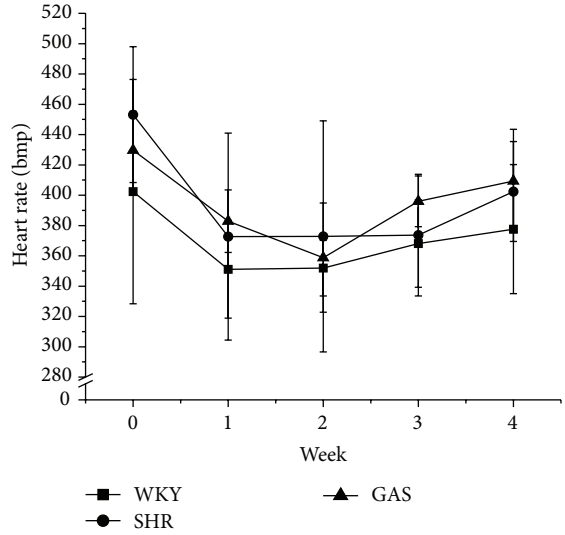

Figure 4: Effects of 4-week treatment with gastrodin on heart rate of rats. Ten-week-old male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs) were obtained. SHRs were randomly divided into model (SHR) and treatment groups (GAS). WKY belonged to control group (WKY). The first two weeks was adaptation period. Starting from the third week, rats in GAS group were intraperitoneally injected with gastrodin at the dose of 100 mg/(kg·d) for 4 weeks. The rest of the rats were not given any intervention. The heart rate was recorded weekly during the experimental period. Values are shown as means ± SEM.

and GAS groups (Figures 7(a) and 7(b)). Next, we examined the protein level of AT1R in myocardium by western blotting. The protein level of AT1R in GAS was significantly lower than in SHR ($P < 0.05$), while it was relatively higher compared with WKY (Figure 8).

3.5. Effects of Gastrodin on PPARγ. Considering that PPARγ had relevance with RAAS, we further examined the mRNA expression and protein level of PPARγ in myocardium.

After four weeks of treatment, the mRNA expression of PPARγ in GAS was higher than in SHR ($P < 0.01$). And it was lower than in WKY ($P < 0.05$) (Figure 9(a)).

Through western blotting, the protein level of PPARγ in GAS was significantly higher than in SHR ($P < 0.01$), while it was relatively lower compared with WKY (Figure 9(b)).

4. Discussion

Gastrodin is the main active ingredient obtained from the Chinese herb, *Tianma* (*Gastrodia elata* Bl.) [8–10]. Gastrodin is considered to have several beneficial properties. Gastrodin has been suggested to be effective as an anticonvulsant and analgesic and is a sedative effective against vertigo, general paralysis, epilepsy, and tetanus [11]. Clinical studies have shown that gastrodin has a good effect on treatment of vertigo and was able to improve hemodynamics, which quickly eliminated dizziness, vertigo, nausea, vomiting, tinnitus, and other symptoms caused by cervical spondylosis and atherosclerosis. Besides, gastrodin may improve microcirculation and cardiovascular compliance and promote fibrinolytic activity and anti-ischemic-reperfusion injury [12]. Additionally, gastrodin could also improve blood pressure, blood rheology, endothelin, and other indicators in patients with hypertension [5]. Some studies have shown that gastrodin inhibited cardiac hypertrophy and fibrosis through inhibiting ERK1/2 signaling pathway and activation of GATA-4 [6]. This study examined the effect of gastrodin on SBP and elucidated its possible mechanism via the activation of PPARγ.

Our investigation showed that treatment with gastrodin for 4 weeks was able to lower the SBP in SHR and did not

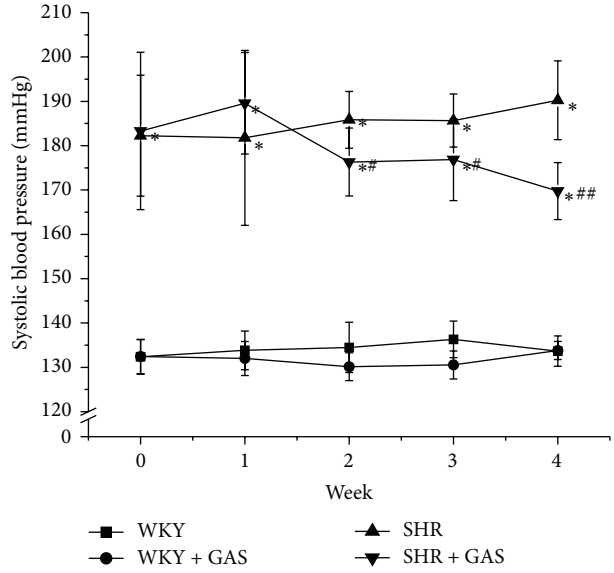

Figure 5: Effects of 4-week treatment with gastrodin on systolic blood pressure (SBP) of rats. Ten-week-old male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs) were obtained. SHRs were randomly divided into model (SHR) and treatment groups (SHR + GAS). WKY were randomly divided into control (WKY) and treatment groups (WKY + GAS). The first two weeks was adaptation period. Starting from the third week, rats in SHR + GAS and WKY + GAS groups were intraperitoneally injected with gastrodin at the dose of 100 mg/(kg·d) for 4 weeks. The rest of the rats were not given any intervention. The SBP of the animals was recorded weekly during the experimental period. Values are shown as means ± SEM. $^{*}P < 0.01$ versus WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus SHR.

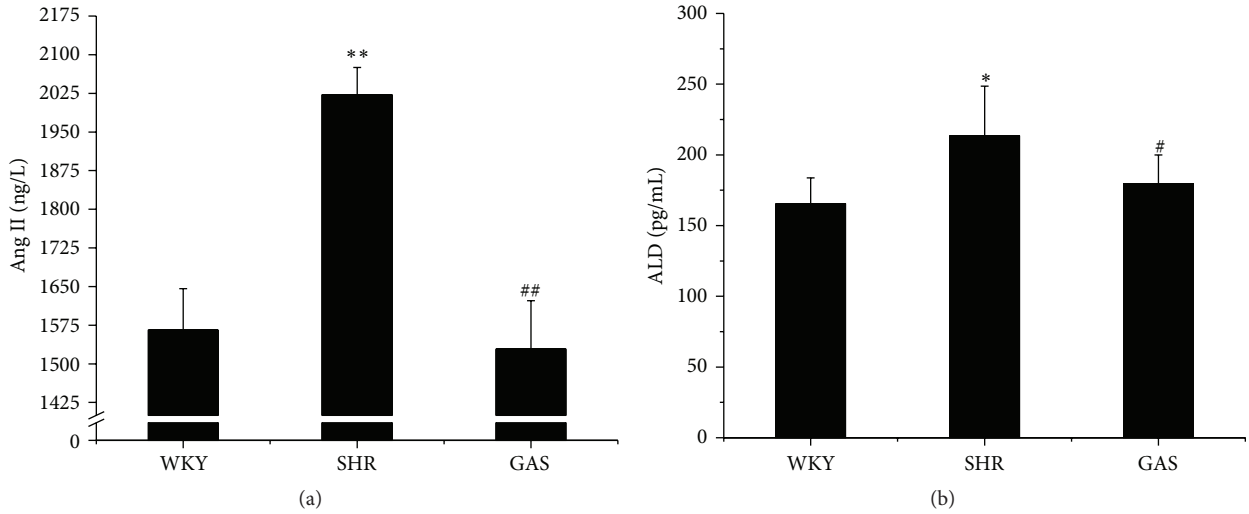

Figure 6: Effects of 4-week treatment with gastrodin on Ang II and ALD in serum of rats. (a) After 4-week treatment with gastrodin, levels of Ang II in serum were determined with ELISA kits. (b) After 4-week treatment with gastrodin, levels of ALD in serum were determined with ELISA kit. Values are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus SHR.

affect the general health of rats, including body weights, food consumption, and heart rate. The reduction of SBP by gastrodin was associated with the remarkable increase in the level of cardiac PPARγ and the subsequent reduction of the hypertensive effects of ACE and AT1R which can also be locally synthesized in the heart.

Renin-angiotensin-aldosterone system (RAAS) is an important factor in regulating blood pressure, within which there exists a proteolytic cascade. Circulating renin cleaves its substrate angiotensinogen to form the decapeptide angiotensin I (Ang I), which is converted by angiotensin-converting enzyme (ACE) to angiotensin II (Ang II). As the main mediator of RAAS, Ang II acts as a vasoconstrictor and thus elevates blood pressure. It also stimulates the release of aldosterone (ALD) which mediates sodium and water retention by directly acting at the distal tubule and eventually elevating the blood pressure level. The function of Ang II is mediated by the plasma membrane receptor AT1 (AT1R). AT1R stimulates vasoconstriction, vascular cell hypertrophy and hyperplasia, sodium retention, and reactive oxygen

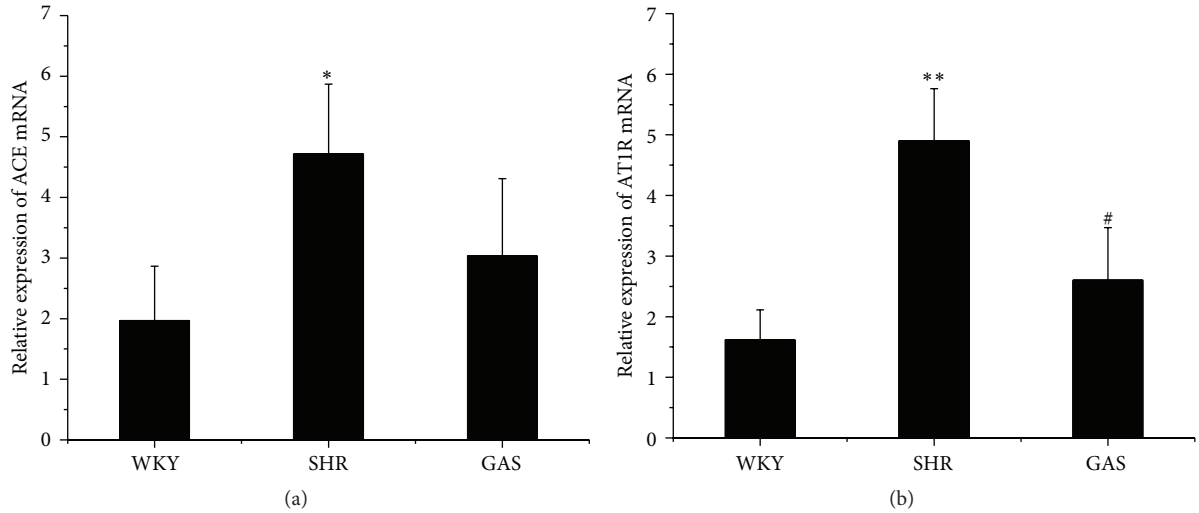

Figure 7: Effects of 4-week treatment with gastrodin on mRNA expression of ACE and AT1R. (a) After 4-week treatment with gastrodin, mRNA expressions of ACE in myocardium tissues were determined by qRT-PCR analysis. (b) After 4-week treatment with gastrodin, mRNA expressions of AT1R in myocardium tissues were determined by qRT-PCR analysis. Values are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus SHR.

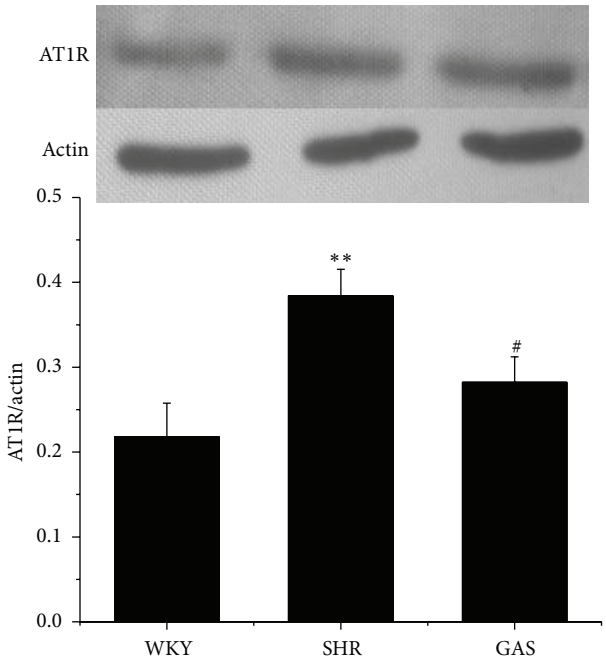

Figure 8: Effects of 4-week treatment with gastrodin on protein levels of AT1R in myocardium tissues of rats. After 4-week treatment with gastrodin, protein levels of AT1R in myocardium tissues were determined by western blotting assay. Values are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus SHR.

species generation. Furthermore, Ang II interacts with AT1R, which results in left ventricular remodeling, alterations in the morphology and mechanical properties of the vasculature, and the development of endothelial dysfunction [1, 13]. Thus, our finding that gastrodin improves blood pressure by targeting and inhibiting Ang II, ALD, ACE, and AT1R does not only have therapeutic significance in clinical management of hypertensive patients; but it may also enhance our understanding on the molecular mechanism underlying the antihypertensive effect of the traditional Chinese medicine.

As aforementioned, ACE, Ang II, AT1R, and ALD are the most important components in RAAS. In our study, we showed that gastrodin could reduce the concentration of Ang II and ALD in serum (Figures 6(a) and 6(b)), decrease the mRNA expression of AT1R (Figure 7(b)), and lower the protein level of AT1R (Figure 6). Surprisingly, gastrodin did not improve the cardiac function in spite of its effect on the expression of the abovementioned proteins (Table 1). It is possible that the intervention time was too short, which only brought changes in the level of protein molecules, and was not enough to cause changes in tissue function.

Peroxisome proliferator-activated receptor (PPAR) γ is a nuclear hormone receptor [14]. PPARγ is trans-activated by its agonists that have been reported to be able to lower blood pressure [15–18]. Some clinical studies have shown that the PPARγ agonist telmisartan could inhibit ACE and block AT1R [19, 20]. Besides, PPARγ agonist also blocked the action of Ang II [19, 21]. PPARγ ligands such as rosiglitazone could also reduce the blood pressure in hypertensive rats, increase urinary aldosterone excretion, reduce heart-to-body weight ratio, and diminish aldosterone-induced heart hypertrophy [22]. Therefore, PPARγ not only downregulates the expression of ACE and AT1R, but also blocks the action of Ang II and ultimately inhibits adrenal aldosterone synthesis/secretion.

Therefore our findings suggest that the activation of PPARγ by gastrodin inhibited the expression of ALD and Ang II that led to the reduction in SBP level.

We found that gastrodin was able to increase the mRNA expression of PPARγ, as well as increasing its protein synthesis (Figures 7(a) and 7(b)). Thus, we do not rule out the

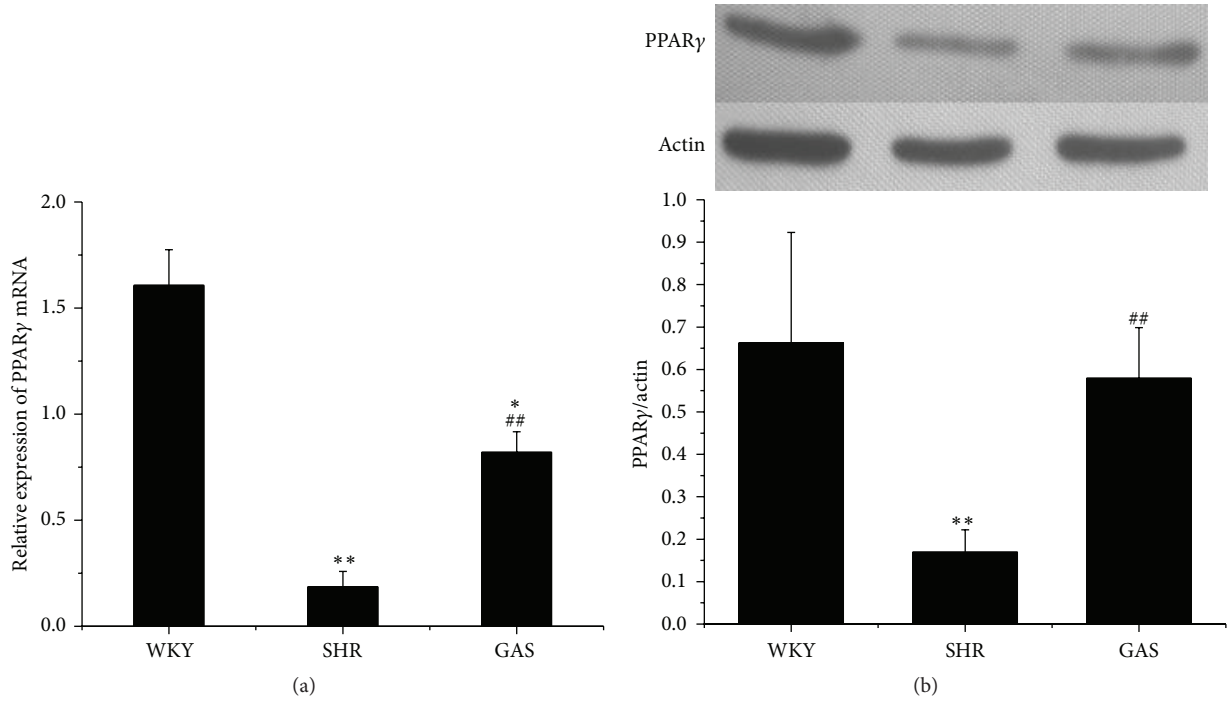

Figure 9: Effects of 4-week treatment with gastrodin on PPARγ in myocardium tissues of rats. (a) After 4-week treatment with gastrodin, mRNA expressions of PPARγ in myocardium tissues were determined by qRT-PCR analysis. (b) After 4-week treatment with gastrodin, protein levels of PPARγ in myocardium tissues were determined by western blotting assay. Values are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus SHR.

possibility that gastrodin could reduce blood pressure by activating PPARγ target, since activated PPARγ intervenes with the important components of RAAS, such as ACE, Ang II, AT1R, and ALD. What is more, clinical studies have demonstrated that gastrodin can improve the blood pressure level in patients with hypertension. This study was designed to explore the mechanism of lowering blood pressure. With regard to this, we did not include positive controls in this experiment. Although gastrodin may lower blood pressure by inhibiting RAAS, it can be inferred from our data that the inhibition was not strong enough to improve the overall cardiac function. This can be partially attributed to gastrodin's limited inhibition of RAAS which needs further investigation.

5. Conclusion

Despite our incomplete understanding of mechanisms involved in beneficial effects of gastrodin, the results of this study clearly demonstrated that gastrodin injected intraperitoneally at the dose of 100 mg/(kg·d) for 4 weeks decreased the SBP in SHR. Furthermore, this study showed that gastrodin intervened with RAAS effectively, including lowering the levels of Ang II and ALD in serum, reducing the mRNA expression of AT1R in myocardium, and decreasing the protein synthesis of AT1R. Meanwhile, gastrodin also increased the mRNA expression of PPARγ and its protein synthesis. According to previous studies, PPARγ could regulate RAAS and has antihypertensive effects. Therefore, the antihypertensive mechanism of gastrodin may be attributable to the direct intervention of RAAS, or indirect inhibition of RAAS via activation of PPARγ. This needs to be further explored.

Conflict of Interests

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

Acknowledgment

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Research Article

Active Compounds of Rhubarb Root and Rhizome in Animal Model Experiments of Focal Cerebral Ischemia

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Rhubarb root and rhizome (RRR) has been clinically used for stroke at least 2000 years and is still used in modern times in both China and elsewhere worldwide. The objective of present study was to evaluate the efficacy of active compounds of RRR (ACRRR) for experimental ischemic stroke. Studies of ACRRR in animal models of ischemic stroke were identified from 5 databases until April 2014. Study quality for each included article was evaluated according to the CAMARADES 10-item checklist. Outcome measures were neurological deficit score and infarct size. All the data were analyzed using RevMan 5.1 software. As a result, 20 studies were identified describing procedures involving 577 animals. The quality score of studies ranges from 2 to 6, and the median was 3.4. Six studies showed significant effects of ACRRR for improving infarct size compared with model group ($P < 0.01$). Six studies indicated significant effects of ACRRR for improving the neurological deficit scores according to Zea longa criterion or eight-point criterion ($P < 0.01$). In conclusion, these findings demonstrated a possible efficacy of ACRRR that have potential neuroprotective effect for experimental ischemic stroke. However, these apparently positive findings should be interpreted with caution because of the methodological flaws.

1. Introduction

Stroke is a major cause of disability and the second most common cause of death worldwide [1]. The burden of stroke will increase greatly during the next 20 years because of the aging population, especially in developing countries [2] such as in China where stroke has already become the leading cause of death [3]. Ischemic stroke is the most common type of stroke, accounting for almost 80% of all types of strokes. Unfortunately, intravenously recombinant tissue plasminogen activator (rtPA) is so far the only approved thrombolytic by Food and Drug Administration for treating ischemic stroke within 4.5 hours of stroke onset [4]. However, rtPA remains largely underutilized because of the short therapeutic window and the incidence of intracranial hemorrhages [5]. Owing to the limitations of the current available treatments, complementary and/or alternative medicine (CAM) is thus increasingly sought to treat stroke worldwide.

Traditional Chinese Medicine (TCM), as a form of CAM, has been used in stroke patients for thousands of years and is still being commonly used in modern times in both China and elsewhere worldwide [6]. In TCM treatment of stroke, the rhubarb root and rhizome (RRR) and RRR-based Chinese herbal prescriptions, known as Tongfu method in TCM theory, were one of the essential methods for acute stroke [7]. RRR, Dahuang in Chinese name and Radix et Rhizoma Rhei in Latin name, can purge accumulation, cool blood, drain damp-heat, and invigorate blood according to TCM theory. RRR has been clinically used for a long history of 2000 years [8], which was documented in the earliest complete Pharmacopoeia of China, *Shennongbencaojing* (*Shennong's Classic of Materia Medica*) at the Warring States Period to the Han Dynasty (221 BC-220 AD). The use of RRR in treatment of stroke can be traced back to the Eastern Han Dynasty (206 BC-220 AD). Doctor Zhang Zhongjing (AD152-219), one of the most eminent Chinese physicians, has first applied

RRR as one of the principal herbs in Fengyin Decoction to treat poststroke epilepsy patients induced evil-wind due to excessive heat [9]. In modern time, RRR is still being used to treat stroke and often present as a principle drug in Chinese herbal prescriptions for the treatment of stroke. In our group, we have conducted a systematic review assessing the effects of RRR-based prescriptions on patients suffering from acute ischemic stroke; the results indicated that this area is worthy of improvement and development for further research [7].

In the current Chinese Pharmacopoeia, RRR is listed as the dry root and rhizome of *Rheum officinale* Baill., *Rheum palmatum* L., and *Rheum tanguticum* Maxim. The extensive phytochemical research on RRR has isolated and identified about 200 chemical compounds [8], such as anthraquinones, dianthrones, stilbenes, anthocyanins, flavonoids, tannins, organic acids, and chromones [10]. Neuroprotection refers to the concept of applying a therapy that directly affects the brain tissue to salvage or delay the infarction of the still-viable ischemic penumbra, rather than reperfusing the tissue [4]. Pharmacological agents targeted the harmful molecular events that contribute to acute ischemic injury pathophysiology, including glutamate release, glutamate receptor activation, excitotoxicity, Ca^{2+} influx into cells, mitochondrial dysfunction, activation of many intracellular enzymes, free radical production, nitric oxide production, inflammation, necrosis, and apoptosis [11]. For example, the registered neuroprotective agents (Internet Stroke Center, 2011) included calcium channel blocker, calcium chelator, free radical scavenger/antioxidant, gamma aminobutyric acid (GABA) agonist, glutamate antagonist, growth factor, leukocyte adhesion inhibitor, nitric oxide inhibitor, opioid antagonist, phosphatidylcholine precursor, serotonin agonist, sodium channel blocker, potassium channel opener, and mechanism unknown or uncertain [12]. Although at least 26 phase 2 and 3 trials of neuroprotectants have completed since 2000, no definite pharmacological agents can limit the cellular effects of acute ischemia or reperfusion that demonstrate safety and efficacy after stroke in clinical studies [13]. Over the past decades, growing evidence indicates that the active compounds of RRR (ACRRR), including rhubarb aglycone (the five components including aloe-emodin, rhein, emodin, chrysophanol, and physcion), rhubarb glycosides (anthraquinone glycosides and double anthrone glycoside), chrysophanol, chrysophanol liposome, emodin, aloe-emodin, physcion, and rhein are responsible for the main pharmacological effects on the stroke and exert potentially neuroprotective function against cerebral ischemic injury [14–33]. The use of systematic review in the preclinical assessment of candidate neuroprotectants can more systematically assess the efficacy, identify an area for testing in further animal experiments, and provide robust information about the characteristics of individual drugs and the basis for a new classification of neuroprotective drugs [34]. In addition, systematic reviews of preclinical data can inform the planning and improve the likelihood of success of future clinical trials [35]. We thus conducted a preclinical systematic review to evaluate ACRRR for experimental ischemic stroke.

2. Methods

2.1. Database and Literature Search Strategies. We identified studies of ACRRR in animal models of ischemic stroke from PubMed, EMBASE, Chinese National Knowledge Infrastructure (CNKI), VIP information database, and Wanfang data Information Site. All of the searches were performed until April 2014. The search term used was ("ACRRR" OR "Rhubarb aglucone" OR "Rhubarb glycosides" OR "Chrysophanol" OR "Chrysophanol liposome" OR "Emodin" OR "Aloe-emodin" OR "Physcion" OR "Rhein") AND ["isch(a)emic stroke" OR "cerebral infarct" OR "middle carotid artery occlusion (MCAO)" OR "cerebral isch(a)emica/reperfution"]. All searches were limited to studies on animals. We also manually searched published abstracts of scientific meetings and asked senior authors of identified publications for references of other studies.

2.2. Inclusion Criteria. We included studies of the effect of ACRRR in animal models of focal cerebral ischemia, in which the outcome was measured as neurological function score (NFS) and (or) infarct size/infarct volume. To prevent bias, inclusion criteria were prespecified as follows: (1) experimental ischemic stroke was induced by temporary MCAO or permanent MCAO; (2) ACRRR referred to any chemical compounds of RRR; (3) infarct size/infarct volume and (or) NFS were compared with control animals receiving vehicle or no treatment. Prespecified exclusion criteria were treatment with single RRR or RRR-based prescriptions, nonfocal cerebral ischemia model, no control group, and duplicate publications.

2.3. Data Extraction. Two authors independently screened abstracts, and the resulting manuscripts were approved by corresponding author (Guo-qing Zheng). The following information was extracted from the complete manuscripts of the qualified studies: (1) publication year and the first author's name, model of ischemic stroke (transient or permanent); (2) the characteristics of animals used including animal number, species, sex, weight, age, and any comorbidity; (3) the information of treatment used in experimental group including the types of ACRRR, method of administration, and duration of treatment; (4) outcome measures and timing for outcomes assessments also included infarct size/infarct volume and (or) NFS were especially extracted separately. If outcomes were performed at different time points, only the final test was included. If the experimental group of animals received various doses of the drug therapy, only the data of highest dose of the drug was included. If the experimental group of animals received more than one kind of effective component of RRR intervention, the data of every intervention was included. If published data were incomplete, we contacted authors to obtain further information. For each comparison, we extracted data of mean value and standard deviation from each experimental and control group of every study.

2.4. Quality Assessment. We evaluated the methodological quality of the included studies using the collaborative

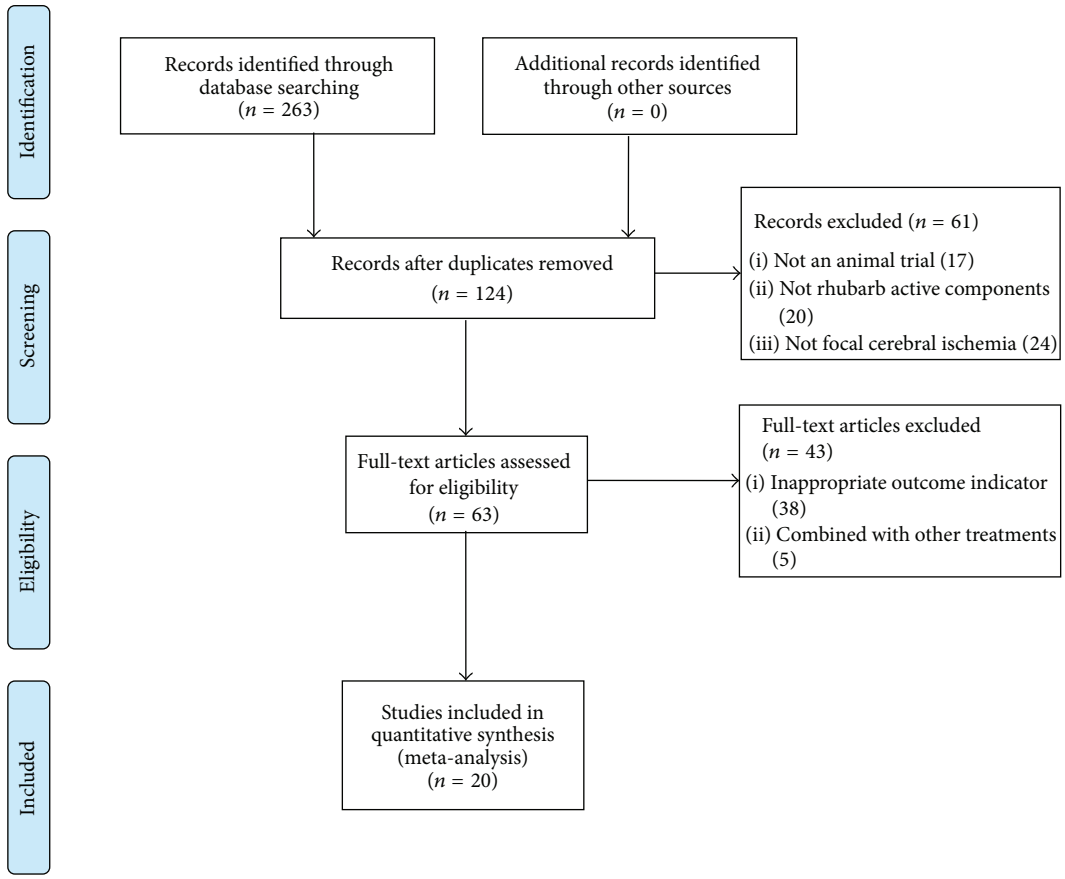

Figure 1: PRISMA 2009 flow diagram.

approach to meta-analysis and review of animal data in experimental stroke (CAMARADES) 10-item quality checklist [34]. One point was awarded for each of (1) publication in a peer-reviewed journal; (2) statement of temperature control; (3) random allocation to groups; (4) allocation concealment; (5) blinded assessment of outcome; (6) use of anesthetic without significant intrinsic neuroprotective activity; (7) appropriate animal model (aged, diabetic, or hypertensive); (8) sample size calculation; (9) compliance with animal welfare regulations; (10) statement of potential conflict of interests. Two authors independently assessed study quality and any disagreements were solved through discussion or consultation with corresponding author (Guoqing Zheng).

2.5. Statistical Analysis. All IS and NFS were considered as continuous data, and then an estimate of the combined effect sizes utilizing standard mean difference (SMD) with the random effects model was given. In the present meta-analysis, the results using the random effects model were presented because heterogeneity between multistudies has to be taken into account. I^2 statistic was used to assess heterogeneity. The significance of differences between n groups was assessed by partitioning heterogeneity and by using the χ^2 distribution with $n-1$ degrees of freedom (df), where n equals the number of groups. Publication bias was assessed using a funnel plot. Probability values 0.05 were considered significant. All analyses were performed with Revman version 5.1 provided by the Cochrane Collaboration.

3. Results

3.1. Study Inclusion. We identified 263 potentially relevant articles, and 139 were excluded because they were duplicates. Through screening titles and abstracts, 61 papers were excluded with at least one of following reasons: (1) not an animal research; (2) not ACRRR intervention; (3) not a research about stroke or ischemic stroke. By reading the full text of the remaining 63 articles, 38 were excluded because the outcome measure was neither NFS nor infarct size/infarct volume; 5 were excluded because of combination with other treatments. Ultimately, 20 eligible studies were identified [14–33]. The screening process is summarized in a flow diagram (Figure 1).

3.2. Study Characteristics. A total of 577 subjects were included in the 20 studies, of whom 282 were in the experimental group and 295 were in the control group. Two studies [15, 33] were published in English and eighteen studies [14, 16–32] were published in Chinese between 2004 and 2015. Seventeen studies [14, 16–31] used male/female Sprague-Dawley rat models; 1 study [15] used male Wistar rats; 1 study

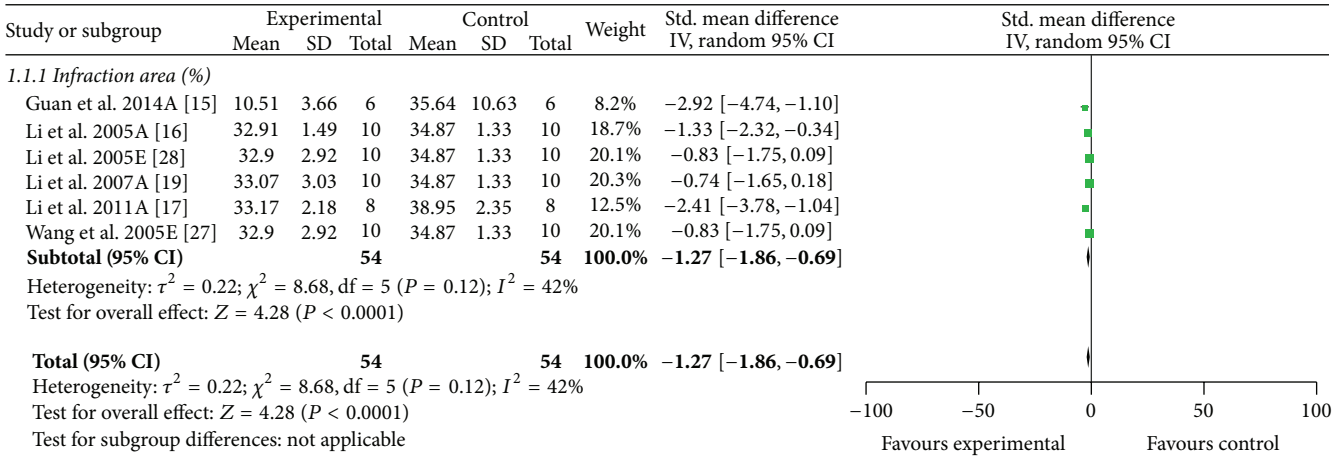

Figure 2: The forest plot: effects of active compounds of rhubarb root and rhizome for improving infarct size compared with middle carotid artery occlusion group.

[32] used male Kunming mice models; 1 study [33] used male CD1 mice model. Among 20 included studies, 12 studies [14–16, 18–24, 27, 28] used permanent MCAO models; 6 studies [25, 26, 29–31, 33] used temporary MCAO models; 1 study [17] used embolic MCAO models; the remaining 1 study [32] used Himori [36] method to induce mice models of cerebral ischemia/reperfusion. All 20 studies reported NFS, and 14 studies [14–17, 19, 20, 22, 25, 27, 28, 30, 31, 33] reported IS. However, there were three neurological grading systems which were used to measure NFS in 20 studies. Eight studies [14, 15, 25, 26, 29–31, 33] used Zea longa criterion [37]; eleven studies [16–24, 27, 28] adopt eight-point criterion [38]; the remaining one study [32] used Garcia criterion [39]. Four studies [25, 29, 32, 33] used anesthesia to execute the animals, whereas the rest of studies did not report the method of executing the animals. Eleven studies [14, 16, 18–20, 22–24, 26–28] used random digits table to generate experimental and control groups, whereas the rest of studies did not mention the random method, which only reported random allocation to groups. The characteristics of the 20 included studies were summarized in detail in Table 1.

3.3. Study Quality. All studies were publications in a peer-reviewed journal. Fourteen studies [15, 19, 20, 22, 23, 26–29, 32, 33] reported control of temperature, including control of the room and rats anal temperature. All studies described random allocation to groups, of which 11 studies used random number table method [14, 16, 18–20, 22–24, 26–28]. Masked assessment of outcome was used in 1 study [16]. Chloral hydrate was used as anesthetic in 8 studies [14–16, 25, 29, 30, 32, 33]; pentobarbital was used in 1 study [26], while there was no report of anesthetics in the remaining 11 studies. Six studies described a sample size calculation [16, 17, 20, 22, 26, 32]. One study [33] reported a compliance with animal welfare regulations or mentioned a statement of potential conflict of interests. None of studies described masked induction of ischemia and appropriate animal models (aged, diabetic, or hypertensive). The quality score of studies ranges from 2 to 6, and the median was 3.4. The methodological quality of each study was summarized in Table 2.

3.4. Effectiveness

3.4.1. Infarct Size/Infarct Volume. Fourteen studies [14–17, 19, 20, 22, 25, 27, 28, 30, 31, 33] used infarct size/infarct volume as primary outcome measures. Meta-analysis of seven studies [15–17, 19, 27, 28, 33] showed significant effects of ACRRR for improving infarct size compared with MCAO group (n = 120, SMD −1.60, 95% CI: −2.48~−0.72, P = 0.0004; heterogeneity χ^2 = 21.06, P = 0.002, I^2 = 72%). We used sensitivity analyses omitting one study at a time from the original analysis. One study [33] reported that the included animals were mice, while other six studies used rats. Thus, this study was considered as the potential sources of the heterogeneity. Meta-analysis of six studies [15–17, 19, 27, 28] indicated that the animal species may be the explanation for the heterogeneity. Six studies indicated that ACRRR significantly improved infarct size compared with MCAO group (n = 108, SMD −1.27, 95% CI: −1.86~−0.69, P < 0.0001; heterogeneity χ^2 = 8.68, df = 5, P = 0.12, I^2 = 42%, Figure 2). The remaining seven studies [14, 20, 22, 25, 29–31] failed to pool analysis due to data demonstrated in the form of infarct volume or the absence of primary data, but all of them reported the significant effects of ACRRR for reducing the infarct size/infarct volume compared with the control group (P < 0.05 or P < 0.01).

3.4.2. NFS. Based on the different neurological grading systems, eight studies [14, 15, 25, 26, 29–31, 33] used Zea longa criterion as measuring method of NFS. Meta-analysis of six studies [14, 15, 25, 26, 29, 30] indicated significant effects of ACRRR for improving the NFS according to Zea longa criterion (n = 142, SMD −0.85, 95% CI: −0.93~−0.78, P < 0.00001; heterogeneity χ^2 = 9.48, df = 5, P = 0.09, I^2 = 47%, Figure 3) compared with the control group. Two studies [31, 33] also showed the significant effects of ACRRR for reducing

Table 1: Study characteristics of included studies.

Study	Species (n)	Weight	Random method	Stroke model	Rhubarb effective component	Method of administration: Experimental group	Method of administration: Control group	The method to execute the animal	Outcome measure (experimental/control)	Intergroup differences
Li et al. 2005 [16]	Male, SD rats (10/10)	300 ± 50 g	Random digits table	Permanent MCAO	Rhubarb aglycone	4 d before occlusion; i.g; 103.68 mg/kg, daily	4 d before occlusion; i.g; same volume of normal saline, daily	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.05$ (2) $P < 0.05$
Li et al. 2011 [17]	Male and female, SD rats (8/8)	300 ± 50 g	Not mentioned	Embolic MCAO	Rhubarb aglycone	4 h before occlusion; i.g; 12.96 mg/kg, daily	4 h before occlusion; i.g; same volume of normal saline, daily	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.01$ (2) $P < 0.01$
Li et al. 2005 [18]	Male, SD rats (8/10)	300 ± 50 g	Random digits table	Permanent MCAO	Rhubarb aglycone	5 d before occlusion; i.g; 25.92 mg/kg, daily	MCAO without any intervention	Not mentioned	Neurobehavioral score	$P < 0.01$
Li et al. 2007 [19]	Male and female, SD rats (10/10)	300 ± 50 g	Random digits table	Permanent MCAO	Rhubarb aglycone	3 d before occlusion; i.p; 103.68 mg/kg, daily	3 d before occlusion; i.p; same volume of normal saline, daily	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.05$ (2) Not found
Li et al. 2005 [20]	Male and female, SD rats (6/6)	300 ± 50 g	Random digits table	Permanent MCAO	Rhubarb aglycone	3 d before occlusion; i.p; 103.68 mg/kg, daily	3 d before occlusion; i.p; same volume of normal saline, daily	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.01$ (2) $P < 0.01$
Liu et al. 2005 [21]	Male, SD rats (8/10; 8/10)	300 ± 50 g	Not mentioned	Permanent MCAO	Rhubarb aglycone Rhubarb glycosides	5 d before occlusion; i.g; 25.92 mg/kg, daily 5 d before occlusion; i.g; 174.96 mg/kg, daily	MCAO without any intervention	Not mentioned	Neurobehavioral score Neurobehavioral score	$P < 0.01$ $P < 0.01$
Li et al. 2004 [22]	Male, SD rats (8/10; 8/10)	300 ± 20 g	Random digits table	Permanent MCAO	Rhubarb aglycone Rhubarb glycosides	5 d before occlusion; i.g; 25.92 mg/kg, daily 5 d before occlusion; i.g; 174.96 mg/kg, daily	MCAO without any intervention	Not mentioned	Neurobehavioral score Neurobehavioral score	$P < 0.01$ $P < 0.01$
Li et al. 2004 [23]	Male, SD rats (8/10; 8/10)	300 ± 50 g	Random digits table	Permanent MCAO	Rhubarb glycosides Rhubarb aglycone	3 d before occlusion; i.p; 174.96 mg/kg, daily 3 d before occlusion; i.p; 25.92 mg/kg, daily	MCAO without any intervention	Not mentioned	Neurobehavioral score Neurobehavioral score	$P < 0.01$ $P < 0.01$

Table 1: Continued.

Study	Species (n)	Weight	Random method	Stroke model	Rhubarb effective component	Method of administration: Experimental group	Method of administration: Control group	The method to execute the animal	Outcome measure (experimental/control)	Intergroup differences
Liu et al. 2004 [24]	Male, SD rats (10/9; 9/9; 10/9; 8/9; 9/9; 9/9)	300 ± 50 g	Random digits table	Permanent MCAO	Rhubarb aglycone	3 d before occlusion; i.p; 25.92 mg/kg, daily	MCAO without any intervention	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.05$ (2) $P < 0.05$
					Emodin	3 d before occlusion; i.p; 1.404 mg/kg, daily			(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.01$ (2) $P < 0.05$
					Aloe-emodin	3 d before occlusion; i.p; 0.648 mg/kg, daily			(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.05$ (2) Not found
					Physcion	3 d before occlusion; i.p; 1.08 mg/kg, daily			(1) Neurobehavioral score (2) Infarction size	(1) Not found (2) Not found
					Rhein	3 d before occlusion; i.p; 3.46 mg/kg, daily			(1) Neurobehavioral score (2) Infarction size	(1) Not found (2) $P < 0.05$
					Chrysophanol	3 d before occlusion; i.p; 7.88 mg/kg, daily			(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.05$ (2) $P < 0.05$
Tan et al. 2010 [25]	Male, SD rats (5/5)	250–320 g	Not mentioned	Temporary MCAO	Emodin	30 min before occlusion; i.p; 25 mg/kg	MCAO without any intervention	Anesthetized	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.01$ (2) $P < 0.01$
Wu et al. 2009 [26]	Male, SD rats (6/6)	270–300 g	Random digits table	Temporary MCAO	Emodin	3 d before occlusion; i.p; 25 mg/kg, daily	3 d before occlusion; i.p; same volume of normal saline, daily	Not mentioned	Neurobehavioral score	$P < 0.05$
Wang et al. 2005 [27]	Male and female, SD rats (10/10; 10/10)	300 ± 50 g	Random digits table	Permanent MCAO	Emodin	3 d before occlusion; i.p; 5.616 mg/kg, daily	3 d before occlusion; i.p; same volume of normal saline, daily	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.01$ (2) $P < 0.05$
					Aloe-emodin	3 d before occlusion; i.p; 0.162 mg/kg, daily			(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.01$ (2) $P < 0.01$
Li et al. 2005 [28]	Male and female, SD rats (10/10)	300 ± 50 g	Random digits table	Permanent MCAO	Emodin	3 d before occlusion; i.p; 5.616 mg/kg, daily	3 d before occlusion; i.p; same volume of normal saline, daily	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.01$ (2) $P < 0.05$
Chen et al. 2006 [29]	Male, SD rats (12/12)	250–320 g	Not mentioned	Temporary MCAO	Physcion	3 d before occlusion; i.g; 40 mg/kg, daily	3 d before occlusion; i.p; same volume of normal saline, daily	Anesthetized	(1) Neurobehavioral score (2) Infarction volume	(1) $P < 0.01$ (2) $P < 0.01$

Table 1: Continued.

Study	Species (n)	Weight	Random method	Stroke model	Rhubarb effective component	Method of administration		The method to execute the animal	Outcome measure (experimental/control)	Intergroup differences
						Experimental group	Control group			
Mei et al. 2009 [30]	Male, SD rats (12/12)	250–320 g	Not mentioned	Temporary MCAO	Physcion	3 d before occlusion; i.g; 60 mg/kg, daily	MCAO without any intervention	Not mentioned	(1) Neurobehavioral score (2) Infarction volume	(1) $P < 0.01$ (2) $P < 0.01$
Chen et al. 2007 [31]	Male, SD rats (10/10)	250–320 g	Not mentioned	Temporary MCAO	Physcion	3 d before occlusion; i.g; 40 mg/kg, daily	MCAO without any intervention	Not mentioned	(1) Neurobehavioral score (2) Infarction volume	(1) $P < 0.01$ (2) $P < 0.01$
Song et al. 2011 [32]	Male, Kunming mice (15/15; 15/15)	28.0 ± 0.9 g	Not mentioned	Temporarily obstructing bilateral common carotid arteries (Himori method)	Chrysophanol	14 d after occlusion; i.p; 10.0 mg/kg, daily	14 d before occlusion; i.p; same volume of normal saline, daily	Anesthetized	Neurobehavioral score	Not found
					Chrysophanol liposome	14 d after occlusion; i.p; 10.0 mg/kg, daily			Neurobehavioral score	Not found
Zhang et al. 2014 [33]	Male, CD1 mice (6/6)	25~30 g	Not mentioned	Temporary MCAO	Chrysophanol	30 minutes before occlusion; i.p; 10.0 mg/kg, daily	30 minutes before occlusion; i.p; same volume of normal saline, daily	Anesthetized	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.05$ (2) $P < 0.05$
Chen et al. 2015 [14]	Male, SD rats (20/20)		Random digits table	Permanent MCAO	Physcion	3 d before occlusion; i.g; 40 mg/kg, daily	3 d before occlusion; i.p; same volume of normal saline, daily	Not mentioned	(1) Neurobehavioral score (2) Infarction volume	(1) $P < 0.01$ (2) $P < 0.01$
Guan et al. 2014 [15]	Male, Wistar rats (6/6)	280 ± 20 g	Not mentioned	Permanent MCAO	Rhubarb aglycone	4 d before occlusion; i.g; aloe-emodin 50 mg/kg, rhein 76 mg/kg, emodin 38 mg/kg, chrysophanol 105 mg/kg, physcion 68 mg/kg, daily	4 d before occlusion; i.g; same volume of 0.5% CMC-Na suspension	Not mentioned	(1) Neurobehavioral score (2) Infarction size	(1) $P < 0.05$ (2) $P < 0.01$

Note: rhubarb aglycone referred to the five components including aloe-emodin, rhein, emodin, chrysophanol, and physcion. Rhubarb glycosides referred to anthraquinone glycosides and double anthrone glycoside. IL-1β: interleukin-1β; MDH: malate dehydrogenase; MCAO: middle carotid artery occlusion; NALP3: NACHT domain-, leucine-rich repeat-, and pyrin domain-containing protein 3; NF-KB: nuclear factor-kappa B; SOD: superoxide dismutase; TGF-β: transforming growth factor beta; TNF-α: tumor necrosis factor-α; VCAM-1: vascular cell adhesion molecule.

Table 2: Quality characteristics of included studies.

Study	A	B	C	D	E	F	G	H	I	J	Score
Li et al. 2005 [16]	+	−	+	−	+	+	−	−	?	−	4
Li et al. 2011 [17]	+	−	+	−	−	?	−	+	?	−	3
Li et al. 2005 [18]	+	−	+	−	−	?	−	−	?	−	2
Li et al. 2007 [19]	+	+	+	−	−	?	−	−	?	−	3
Li et al. 2005 [20]	+	+	+	−	−	?	−	+	?	−	4
Liu et al. 2005 [21]	+	−	+	−	−	?	−	−	?	−	2
Li et al. 2004 [22]	+	+	+	−	−	?	−	+	?	−	4
Li et al. 2004 [23]	+	+	+	−	−	?	−	−	?	−	3
Liu et al. 2004 [24]	+	−	+	−	−	?	−	−	?	−	2
Tan et al. 2010 [25]	+	−	+	−	−	+	?	−	?	−	3
Wu et al. 2009 [26]	+	+	+	−	−	+	?	+	?	−	5
Wang et al. 2005 [27]	+	+	+	−	−	?	−	−	?	−	3
Li et al. 2005 [28]	+	+	+	−	−	?	−	−	?	−	3
Chen et al. 2006 [29]	+	+	+	−	−	+	?	−	?	−	4
Mei et al. 2009 [30]	+	−	+	−	−	+	?	−	?	−	3
Chen et al. 2007 [31]	+	−	+	−	−	?	?	−	?	−	2
Song et al. 2011 [32]	+	+	+	−	−	+	?	+	?	−	5
Zhang et al. 2014 [33]	+	+	+	−	−	+	?	−	+	+	6
Chen et al. 2015 [14]	+	−	+	−	−	+	−	−	?	−	3
Guan et al. 2014 [15]	+	+	+	−	−	+	−	−	?	−	4

Note: A: publication in a peer-reviewed journal, B: statement of temperature control, C: random allocation to groups, D: blinded induction of ischemia, E: blinded assessment of outcome, F: use of anaesthetic without significant intrinsic neuroprotective activity, G: appropriate animal model (aged, diabetic, or hypertensive), H: sample size calculation, I: compliance with animal welfare regulations, and J: statement of potential conflict of interests. +: yes, −: no, and ?: unclear.

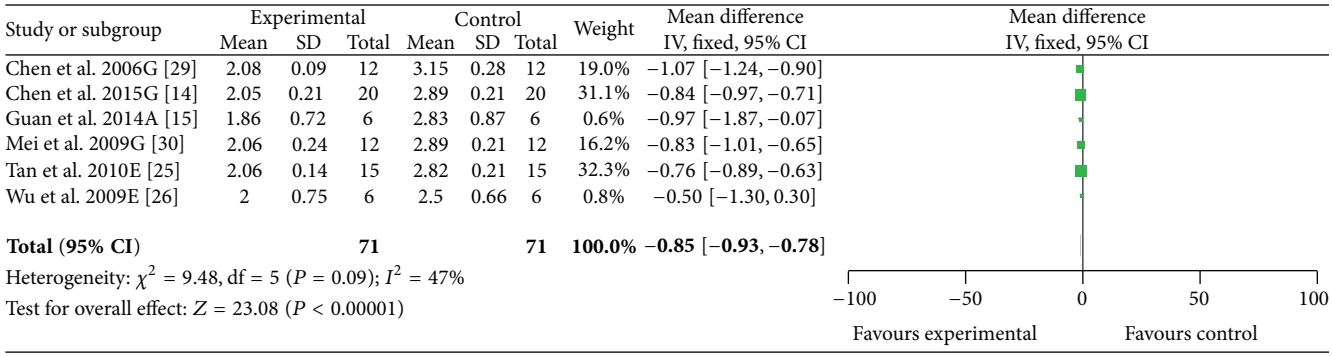

Figure 3: The forest plot: effects of active compounds of rhubarb root and rhizome for improving the neurological function score according to Zea longa criterion compared with middle carotid artery occlusion group.

NFS according to Zea longa criterion compared with the control group ($P < 0.01$) but failed to pool analysis due to the absence of primary data. One study [32] used Garcia criterion as measuring method of NFS. Meta-analysis of two comparisons of this study [32] showed significant effects of ACRRR for improving the NFS according to Garcia criterion compared with control group ($n = 60$, SMD 2.84, 95% CI: 1.83~3.85, $P < 0.00001$; heterogeneity $\chi^2 = 1.78$, $P = 0.18$, $I^2 = 44\%$, Figure 4). Eleven studies [16–24, 27, 28] used eight-point criterion as measuring method of NFS. Ten studies [16–19, 21–24, 27, 28] indicated that NFS was significantly improved in ACRRR group compared with control group according to eight-point criterion ($n = 350$, SMD −3.20, 95% CI: −4.03~−2.37, $P < 0.00001$; heterogeneity $\chi^2 = 117.41$, $P < 0.00001$, $I^2 = 85\%$, Figure 5). As the values of I^2 were greater than 50%, subgroup analyses were adopted according to stratification on gender of animals and the model construction. Effect size was greater in models of male rats than in male and female mixed models (Figure 6(a)) and was greater in the intragastric administration models

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. mean difference IV, random 95% CI
2.3.1 Garcia criterion								
Song et al. 2011D [32]	11.76	0.22	15	10.94	0.42	15	55.4%	2.38 [1.42, 3.34]
Song et al. 2011H [32]	12.22	0.3	15	10.94	0.42	15	44.6%	3.41 [2.24, 4.58]
Subtotal (95% CI)			**30**			**30**	**100.0%**	**2.84 [1.83, 3.85]**
Heterogeneity: $\tau^2 = 0.23$; $\chi^2 = 1.78$, df = 1 ($P = 0.18$); $I^2 = 44\%$								
Test for overall effect: $Z = 5.53$ ($P < 0.00001$)								
Total (95% CI)			**30**			**30**	**100.0%**	**2.84 [1.83, 3.85]**
Heterogeneity: $\tau^2 = 0.23$; $\chi^2 = 1.78$, df = 1 ($P = 0.18$); $I^2 = 44\%$								
Test for overall effect: $Z = 5.53$ ($P < 0.00001$)								
Test for subgroup differences: not applicable								

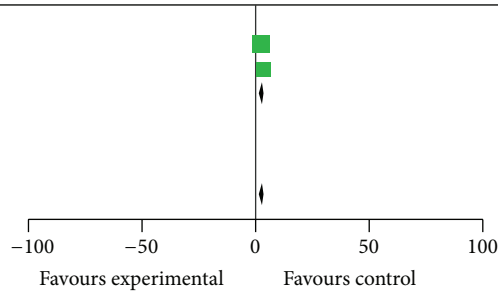

Figure 4: The forest plot: effects of active compounds of rhubarb root and rhizome for improving the neurological function score according to Garcia criterion compared with middle carotid artery occlusion group.

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. mean difference IV, random 95% CI
2.2.1 Eight-point criterion								
Li et al. 2004A [22]	1.5	0.53	8	5.86	0.61	10	3.8%	−7.20 [−10.02, −4.38]
Li et al. 2004B [22]	2.2	0.77	8	5.86	0.61	10	4.7%	−5.09 [−7.19, −2.99]
Li et al. 2004A [23]	1.5	0.53	8	5.86	0.61	10	3.8%	−7.20 [−10.02, −4.38]
Li et al. 2004B [23]	2.2	0.77	8	5.86	0.61	10	4.7%	−5.09 [−7.19, −2.99]
Li et al. 2005A [16]	5.08	0.79	10	5.75	0.57	10	6.2%	−0.93 [−1.87, 0.00]
Li et al. 2005A [18]	1.5	0.53	8	5.86	0.61	10	3.8%	−7.20 [−10.02, −4.38]
Li et al. 2005E [28]	4.5	0.52	10	5.75	0.57	10	6.0%	−2.19 [−3.35, −1.03]
Li et al. 2007A [19]	4.6	1.35	10	5.75	0.57	10	6.2%	−1.06 [−2.01, −0.11]
Li et al. 2011A [17]	5	0.93	8	6	0.53	8	6.0%	−1.25 [−2.35, −0.15]
Liu et al. 2004A [24]	4.2	0.2	10	5.78	0.44	9	5.1%	−4.50 [−6.35, −2.66]
Liu et al. 2004C [24]	4.78	2.05	9	5.78	0.44	9	6.2%	−0.64 [−1.60, 0.31]
Liu et al. 2004D [24]	3	1.51	8	5.78	0.44	9	5.7%	−2.44 [−3.78, −1.11]
Liu et al. 2004E [24]	2.22	1.56	9	5.78	0.44	9	5.6%	−2.96 [−4.39, −1.53]
Liu et al. 2004F [24]	4	1.41	10	5.78	0.44	9	6.1%	−1.59 [−2.65, −0.53]
Liu et al. 2004G [24]	5.13	1.25	8	5.78	0.44	9	6.2%	−0.68 [−1.66, 0.31]
Liu et al. 2005A [21]	1.5	0.53	8	5.86	0.61	10	3.8%	−7.20 [−10.02, −4.38]
Liu et al. 2005B [21]	2.2	0.77	8	5.86	0.61	10	4.7%	−5.09 [−7.19, −2.99]
Wang et al. 2005E [27]	4.5	0.52	10	5.75	0.57	10	6.0%	−2.19 [−3.35, −1.03]
Wang et al. 2005F [27]	3.1	0.73	10	5.75	0.57	10	5.4%	−3.88 [−5.48, −2.27]
Subtotal (95% CI)			**168**			**182**	**100.0%**	**−3.20 [−4.03, −2.37]**
Heterogeneity: $\tau^2 = 2.67$; $\chi^2 = 117.41$, df = 18 ($P < 0.00001$); $I^2 = 85\%$								
Test for overall effect: $Z = 7.54$ ($P < 0.00001$)								
Total (95% CI)			**168**			**182**	**100.0%**	**−3.20 [−4.03, −2.37]**
Heterogeneity: $\tau^2 = 2.67$; $\chi^2 = 117.41$, df = 18 ($P < 0.00001$); $I^2 = 85\%$								
Test for overall effect: $Z = 7.54$ ($P < 0.00001$)								
Test for subgroup differences: not applicable								

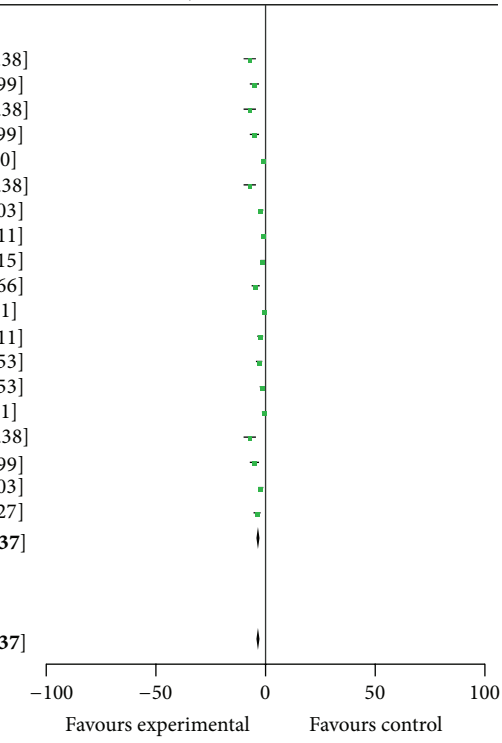

Figure 5: The forest plot: effects of active compounds of rhubarb root and rhizome for improving the neurological function score according to eight-point criterion compared with middle carotid artery occlusion group.

than in intraperitoneal injection models (Figure 6(b)). One study [20] also reported the significant effects of ACRRR for reducing NFS according to eight-point criterion compared with control group ($P < 0.01$), but it did not provide primary data and failed for pool analysis.

3.5. Assessment of Publication Bias. The funnel plot revealed a roughly symmetrical distribution of studies around the line of identity, indicating no obvious publication bias existed in this review (Figure 7).

4. Discussion

4.1. Summary of Evidences. This is the first preclinical systematic review evaluating the ACRRR for animal model of ischemic stroke with NFS and infarct size as the outcome measures. Twenty studies, involving a total of 577 experimental subjects, were identified. The quality of studies included in systematic review was generally low. The present study demonstrated that the ACRRR substantially reduced infarct size and improved NFS in animal models experiments of focal cerebral ischemia.

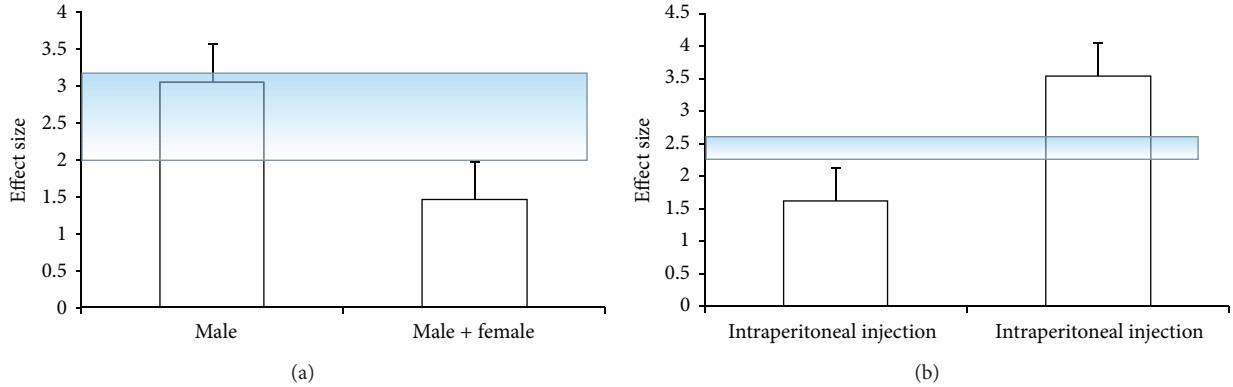

Figure 6: Subgroup analysis: (a) point estimates of effect size and 95% CIs by animal species; (b) point estimates of effect size and 95% CIs by route of drug delivery.

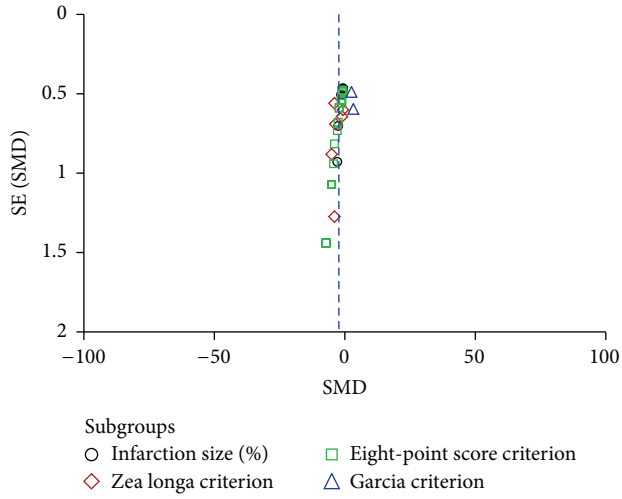

Figure 7: The funnel plot of assessing publication bias.

4.2. Methodological Considerations. This systematic review is subject to possible methodological weaknesses. First, our analysis can only include available data, and negative studies are often not published and obtained. Thus, the analysis may overestimate effect size. Second, we cannot rule out the possibility of missing relevant studies because our search strategy used only English and Chinese databases, which may lead to certain degree of selective bias. Third, the analysis rested with inherent limitations in the primary studies. Methodological quality of animal experiments is a significant concern because studies that report items such as blinding of outcomes and randomization are less prone to bias than are more rigorous studies [40]. Only 1 study [16] mentioned masked assessment of outcome, which may result in performance bias and detection bias. An adequate sample size is crucial to the design of randomized controlled trials [41]. Only six studies described a sample size calculation [16, 17, 20, 22, 26, 32]. Ischemic stroke generally occurs in elderly patients with associated medical problems such as hypertension and hyperglycemia. However, none of the studies investigated stroke in models with comorbidities such as diabetes, hypertension, or aged animals. Some anesthetic agents, including ketamine, have significant intrinsic neuroprotective activity [34], and experiments using these anesthetics may overestimate effect size, but no report of anesthetics in the 12 out of 20 studies.

4.3. Possible Neuroprotective Mechanism. The possible mechanisms, especially neuroprotective mechanism against cerebral ischemic injury, are summarized as follows: (i) rhubarb aglycone can reduce thrombosis, blood coagulation, and the aggregation and adhesion of platelet, decrease expression of fibrinogen, downregulate levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and vascular cell adhesion molecule (VCAM-1), and upregulate transforming growth factor beta (TGF-β) in brain tissues [16, 20]; (ii) rhubarb glycosides can reduce the level of TNF-α and IL-1β, extracellular Ca^{2+} influx, and malate dehydrogenase (MDH) contents and increase superoxide dismutase (SOD) activity in brain tissue of MCAO rats [21, 22]; (iii) chrysophanol can reduce TNF-α level in mouse brain [24] and inhibit the NACHT domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 (NALP3) inflammasome activation and it ameliorates cerebral ischemia/reperfusion in mice [33]; (iv) chrysophanol liposome has beneficial effects on neurobehavioral score and hippocampal pathological damage via increasing B-cell lymphoma-2 (Bcl-2) expression and reducing caspase-3 and Bax level in ischemic mice [32]; (v) emodin can reduce inflammatory cascade and increase TGF-β level [28] and inhibit the activation of caspase-3 in the cerebral ischemic model of SD rats [25]; (vi) aloe-emodin can provide neuroprotection against cerebral ischemic injury of SD rats by reduction of TNF-α level [24, 27]; (vii) physcion can enhance ischemic tolerance induced by brain ischemic preconditioning through decreasing IL-1β, TNF-α, ICAM-1, and caspase-3 expression in MCAO rats [42]; (viii) rhein has neuroprotective effects through reduction of level of nitric oxide (NO) and TNF-α in ischemic brain tissue of mice [43]. Thus, ACRRR have been demonstrated to be beneficial effects on multiaspects of the pathophysiology of stroke.

4.4. Implication for Further Practices and Studies. Although the relationship between study quality and the estimate of size of effect is not yet conclusive [44], some previous studies suggested that the quality of the research design is an important factor affecting the observed size of effect [34, 45]. On the practice level, we recommended the principles of randomization to treatment group, performance of surgery blinded to treatment allocation, blinded assessment of outcome, minimization of use of anesthetics with intrinsic neuroprotective activity, increased use of hypertensive, diabetic, and aged animals, and full reporting of potential conflicts of interests. In particular, ACRRR should be tested in aged, hyperpietic, and diabetic animals in future stroke studies because a metaepidemiologic approach by Crossley et al. [46] indicated that studies using healthy animals may overestimate the effectiveness of an intervention. On the study side, the relationship between study quality and the estimate of size of effect is an important area for future research.

It is worth noting that the neuroprotective activity of ACRRR for acute ischemic stroke may identify an area that other chemical compounds of RRR possess this activity. Second, which type of ACRRR possesses better neuroprotective function needs to be further clarified. Third, future studies of neuroprotective agents need to be tested in combination with different types of ACRRR to reduce the cellular effects of acute ischemia and to restore perfusion. Fourth, most of the studies in this field are explanatory on the therapeutic potential of ACRRR with little explanation of mechanism of action, especially on the causal relationship of the molecular or biological changes induced by ACRRR on therapeutic action. Thus, whether the neuroprotective effects of different types of ACRRR in acute ischemic stroke may have same or different molecular and biological mechanisms is worthy of further exploration. Fifth, further experimental studies with delayed ACRRR administration are required in order to assess when the optimum time window closes and to determine the time of administration under which maximum efficacy can be achieved.

5. Conclusion

The ACRRR can improve NFS and infarct size and exert potential neuroprotective effect for experimental ischemic stroke. However, these apparently positive findings should be interpreted with caution because of the methodological flaws. Future research should examine the presence of possible experimental bias and clinical trials of ACRRR are needed.

Conflict of Interests

The authors have declared that no competing interests exist.

Authors' Contribution

Ai-ju Liu, Liang Song, and Yan Li contributed equally to this work.

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Research Article

The Effect of Xuefuzhuyu Oral Liquid on Aspirin Resistance and Its Association with rs5911, rs5787, and rs3842788 Gene Polymorphisms

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Aspirin should be continued indefinitely in patients after interventional therapy, but 10% to 40% of patients experience recurrent vascular events despite adequate aspirin therapy, a condition known as aspirin resistance (AR). Xuefuzhuyu oral liquid, derived from the classic recipe Xuefuzhuyu decoction, has been well documented to inhibit platelet aggregation and to improve hemorheology. The aims of this study were to investigate the effects of Xuefuzhuyu oral liquid on AR in patients with chronic stable angina after percutaneous coronary intervention (PCI) and the possible genetic markers related to the drug response. 43 patients diagnosed as having aspirin resistance or semi-resistance were randomly divided into control and treatment groups after screening 207 stable CHD patients. Platelet aggregation rate was determined using turbidimetry. Three single nucleotide polymorphisms in COX-1 (rs5787, rs3842788) and GP IIb (rs5911) were genotyped in whole blood samples using ABI PRISM 7900 HT Fast Real-Time instrument and ABI PRISM 3730 DNA Sequencer. The results showed that Xuefuzhuyu oral liquid could effectively improve blood stasis syndrome and AR by inhibiting ADP-induced platelet aggregation and that patients with the rs5911 genetic variant exhibited better drug response upon treatment with Xuefuzhuyu oral liquid, which suggests Xuefuzhuyu oral liquid as a new possible drug for the prevention of AR.

1. Introduction

Platelet activation and aggregation have a pivotal role in the thrombotic complications that occur in patients undergoing percutaneous coronary intervention (PCI) [1]. Aspirin and clopidogrel (dual) antiplatelet therapy is recommended by the guidelines for the prevention of ischemic complications after PCI [2, 3]. However, bleeding events limit their clinical application. It is also recommended in the guidelines that if the risk of morbidity from bleeding outweighs the antiplatelet benefit of a recommended duration of P2Y12 inhibitor therapy after stent implantation, earlier discontinuation (<12 months) of P2Y12 inhibitor therapy is reasonable [4]. After PCI, aspirin administration should be continued indefinitely at a low dose. However, some patients still experience cardiovascular events despite its regular intake, a phenomenon which is known as aspirin resistance (AR) [5].

Gene polymorphisms can affect individual drug response. Detecting genetic variation may help to predict a patient's response to drugs and could be used as a tool to optimize therapy strategy, tailor dosage regimens, and improve clinical outcomes [6]. A number of studies have examined the association of AR with single nucleotide polymorphisms (SNPs) in the genes for COX-1 and for several receptors on the surface of platelets [7–9]. Maree reported COX-1 haplotypes (A-842G, C22T (R8W), G128A (Q41Q), C644A (G213G), and C714A (L237M)) were significantly associated with aspirin response determined by AA-induced platelet aggregation ($P = 0.004$;

4 d.f.) in patients ($n = 144$) with stable coronary heart disease (CHD) from Ireland [7]. Platelet glycoprotein (GP) IIb/IIIa receptors play an inevitable role in platelet aggregation [10]. Pamukcu reported that GP IIIa (PlA) polymorphism is related to aspirin resistance in Turkish patients with intracoronary stent restenosis, while our previous study has shown that there are only PlA1, A1 alleles of GP IIIa in 212 CHD patients and 39 healthy volunteers in the Chinese Han population [11, 12]. Therefore, it is important to find specific genetic markers for different ethnic groups.

Traditional Chinese medicines exhibiting good antiplatelet effects are the most commonly used drugs for patients after interventional therapy for activating blood circulation to remove blood stasis [13]. Xuefuzhuyu oral liquid, derived from the classic recipe Xuefuzhuyu decoction, can effectively inhibit platelet activation and reduce platelet aggregation and showed good effects in the clinical treatment of CHD [12]. But it still remains unknown whether Xuefuzhuyu oral liquid could relieve AR in patients after interventional therapy and if there are some specific gene polymorphisms related to the drug response. Therefore a control randomized study was designed to investigate the effects of Xuefuzhuyu oral liquid on AR in patients with chronic stable angina after PCI and the possible associated genetic markers for the drug response.

2. Materials and Methods

2.1. Patients. Patients were recruited from Xiyuan hospital, China Academy of Chinese Medical Sciences, from March 2012 to November 2014. The protocol was approved by the institutional Ethics Committee of China Academy of Chinese Medical Sciences, and all patients gave written informed consent. Trial is registered with Chinese Clinical Trial Register number ChiCTR-TRC-12002416.

2.2. Diagnostic Criteria. Chronic stable angina patients with coronary angiography showed stenosis ≥50% in at least one coronary artery or previous myocardial infarction [14]. Classification of CHD syndrome referred to the "Criterion of Syndrome Differentiation for CHD" by Cardiovascular Specialty Committee, China Association of Integrative Medicine [15]. Stasis syndrome differentiation and scores were made according to the "diagnostic criteria of blood stasis syndrome (BSS)" [16].

2.3. Inclusion and Exclusion Criteria. Inclusion criteria included (1) stable angina patients after postrevascularization or myocardial infarction, (2) 35 years ≤ age ≤ 75 years, (3) taking aspirin for more than 7 days, and (4) aspirin resistance. Aspirin resistance is defined when patients showed both (i) platelet aggregation rate ≥70% induced by diphosphate adenosine (ADP, 10 μM) and (ii) platelet aggregation rate ≥20% induced by arachidonic acid (AA, 0.5 mg/mL). Aspirin semi-resistance is defined when either (i) or (ii) was observed. Exclusion criteria included (1) family or personal history of bleeding disorders, (2) platelet count $<100 \times 10^9$/L, or $>450 \times 10^9$/L, (3) hemoglobin <90 g/L, (4) taking other antiplatelet, anticoagulant drugs or nonsteroidal anti-inflammatory drugs, (5) taking other herbs besides Xuefuzhuyu oral liquid which are activating blood circulation to remove blood stasis within the latest two weeks, (6) history of trauma or surgery in the latest two weeks, (7) severe primary diseases like renal insufficiency, liver dysfunction, hematopoietic system diseases, mental disorder, or malignant tumor, and (8) female in pregnancy or lactation period.

2.4. Clinical Design and Treatment Procedure. The enrolled aspirin resistance or semi-resistance patients were randomly divided into control and treatment groups using a randomized block design. Conventional western medicine treatment and aspirin (100 mg) once daily were used in the control group, while Xuefuzhuyu oral liquid (10 mL, three times per day) was added in the treatment group for four consecutive weeks. The enrolled patients should not take antiplatelet, anticoagulant drugs, nonsteroidal anti-inflammatory drugs, and any other herbs activating blood circulation to remove blood stasis besides Xuefuzhuyu oral liquid during the research period. Biochemical indicators of liver and kidney function, platelet aggregation rate, and BSS scores were detected before and after treatment.

2.5. Xuefuzhuyu Oral Liquid Preparation. Xuefuzhuyu oral liquid (national medicine permit number Z10950063, batch number 1211010) was kindly provided by Jilin Aodong Yanbian Pharmaceutical Co., Ltd. (Jilin, China). It contains water extracts of semen *persicae*, safflower, *Angelica*, rhizoma *ligustici wallichii*, rehmanniae, rot of peony, *Achyranthes*, *Bupleurum*, fructus aurantii immaturus, *Platycodon grandiflorum*, and liquorice. The main active components used for quality control in Xuefuzhuyu oral liquid are paeoniflorin (≥1.4 mg/mL) and ferulic acid (≥0.15 mg/mL), which meet the requirement of China State Food and Drug Administration (the state drug standards number YBZ11722004) [17].

2.6. Platelet Aggregation Studies. Platelet aggregation rate was determined among different patients groups using turbidimetry (Platelet Aggregation Instrument, LBYNJ2, Beijing Lipusheng Co., China). The inducer of platelet aggregation was ADP (10 μM, Chrono-log Co., Havertown, USA) and AA (0.5 mg/mL, Chrono-log Co., Havertown, USA).

2.7. DNA Preparation and Genotyping. Genomic DNA was isolated from whole blood using a Wizard Genomic DNA Purification Kit (Promega Co., USA) in accordance with the manufacturer's instructions as previously described [18]. Patients were genotyped for three single nucleotide polymorphisms in COX-1 (rs5787, rs3842788) and GP IIb (rs5911) (Table 1). Genotyping was performed using Taqman probe technique (rs5787 and rs5911) and gene sequencing technology (rs3842788) on an ABI PRISM 7900 HT Fast Real-Time instrument (Applied Biosystems, Foster City, CA) and an ABI PRISM 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA), respectively, as has previously been described [12, 18].

2.8. Statistical Analysis. Continuous variables were expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was carried out for the comparison of

Table 1: COX-1 and GP IIb single nucleotide polymorphisms.

	Region	Contig position	mRNA position	dbSNP rs cluster id number	RefSNP allele	Protein residue	Codon position	Amino acid position
COX-1	Exon_4	32462028	458	rs5787	G	Arg [R]	2	108
					A	Gln [Q]	2	108
	Exon_3	32461411	258	rs3842788	A	Gln [Q]	3	41
					G	Gln [Q]	3	41
GP IIb	Exon_26	1106870	2653	rs5911	A	Ile [I]	2	874
					C	Ser [S]	2	874

Notes: dbSNP: single nucleotide polymorphism database; RefSNP: reference single nucleotide polymorphism.

Table 2: Baseline characteristics of study participants.

	Control group ($n = 21$)	Treatment group ($n = 22$)
Age, y	62.8 ± 6.3	67.0 ± 8.1
Male sex, *n* (%)	6 (28.6)	10 (45.5)
Body mass index, kg/m^2	24.7 ± 2.9	26.9 ± 6.1
Statins, *n* (%)	15 (71.4)	15 (68.2)
Myocardial infarction history, *n* (%)	3 (14.3)	5 (22.7)
Hypertension, *n* (%)	14 (66.7)	18 (81.8)
Dyslipidemia, *n* (%)	16 (76.2)	16 (72.2)
Diabetes, *n* (%)	10 (47.6)	11 (50)
rs5787 GG, *n* (%)	21 (100)	22 (100)
rs5911 AA/(AC + CC)	5/16	6/16
rs3842788 GG, *n* (%)	21 (100)	22 (100)

means. Categorical data were described by frequency tables, percentage, or constituent ratio and analyzed by Chi-square test. All statistical analysis was performed with SPSS version 13.0, and P value of less than 0.05 was considered statistically significant.

3. Results

3.1. General Clinical Characteristics. 43 patients diagnosed as having aspirin resistance or semi-resistance were randomly divided into control and treatment groups after screening 207 stable CHD patients, and there were no significant adverse reactions occurring before and after treatment. There was no statistical difference between the two groups in age, sex, and body mass index ($P > 0.05$). The risk factors (myocardial infarction, hypertension, dyslipidemia, and diabetes history) and statins medication history were comparable between the two groups ($P > 0.05$) (Table 2). All the patients enrolled carried only the G/G allele in both rs5787 and rs3842788 gene polymorphisms. The C haplotype of rs5911 was carried by 76.2% and 72.7% of patients in the two groups, respectively, without significant difference ($P > 0.05$).

3.2. Improvement of Aspirin Resistance before and after Treatment. 90.5% of patients retained aspirin resistance or aspirin semi-resistance in the control group after conventional western medicine treatment, while only 18.2% retained resistance in the treatment group after combination therapy with Xuefuzhuyu oral liquid ($P < 0.01$) (Table 3).

Table 3: Improvement of aspirin resistance before and after treatment.

	Aspirin resistance/aspirin semi-resistance	
	Before treatment *n* (%)	After treatment *n* (%)
Control group	21 (100)	19 (90.5)
Treatment group	22 (100)	4 (18.2)$^{**\wedge\wedge}$

Notes: $^{**}P < 0.01$, compared with the control group; $^{\wedge\wedge}P < 0.01$, compared with before treatment.

3.3. Comparison of BSS Patients and BSS Scores between Groups. There were 12 patients (57.1%) and 18 patients (81.8%) with BSS, respectively, in the control and treatment groups with no statistical difference ($P > 0.05$) (Table 4). The BSS scores in the treatment group were reduced significantly after combination therapy with Xuefuzhuyu oral liquid compared to the control group, which indicated that Xuefuzhuyu oral liquid could reduce the degree of blood stasis in patients.

3.4. Correlation between Gene Polymorphism and the Effects of Xuefuzhuyu Oral Liquid on AR. ADP-induced platelet aggregation was significantly lower ($P < 0.05$, $P < 0.01$) after treatment in combination with Xuefuzhuyu oral liquid, no matter what kind of genotypes (A/A or A/C + C/C) the patients had, while AA-induced platelet aggregation provoked no significant change (Table 5), which indicated that Xuefuzhuyu oral liquid improves aspirin resistance by inhibiting ADP-induced platelet aggregation.

3.5. Comparison of BSS Scores in Patients with Different Genotypes before and after Treatment. After treatment in combination with Xuefuzhuyu oral liquid, BSS scores decreased significantly in patients with A/C or C/C genotype (Table 6), which illustrated that patients carrying the C allele were more responsive to the improvement of blood stasis symptoms and more sensitive to the treatment of Xuefuzhuyu oral liquid.

Table 4: Comparison of BSS patients and BSS scores between groups.

	BSS patients n (%)	Non-BSS patients n (%)	BSS scores	
			Before treatment	After treatment
Control group	12 (57.1)	9 (42.9)	20.25 ± 5.59	19.92 ± 4.81
Treatment group	18 (81.8)	4 (18.2)	21.11 ± 3.38	13.5 ± 3.36*^

Notes: $^{*}P < 0.05$, compared with the control group; $^{\wedge}P < 0.05$, compared with before treatment.

Table 5: Effects of Xuefuzhuyu oral liquid on patients' platelet aggregation with different genotyping of rs5911.

rs5911 genotyping	Group	n	ADP-induced platelet aggregation rate	AA-induced platelet aggregation rate
A/A	Before treatment	6	77.06 ± 6.48	12.21 ± 7.17
	After treatment	6	63.65 ± 4.27**	11.48 ± 4.73
A/C + C/C	Before treatment	16	72.09 ± 14.20	16.38 ± 7.18
	After treatment	16	60.88 ± 13.37*	14.03 ± 3.32

Notes: $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with before treatment.

Table 6: Comparison of BSS scores in patients with different genotypes before and after treatment.

rs5911 genotyping	Group	n	BSS scores
A/A	Before treatment	8	19.88 ± 3.23
	After treatment	8	15.75 ± 3.65
A/C + C/C	Before treatment	22	21.09 ± 4.69
	After treatment	22	16.18 ± 5.56**

Notes: $^{**}P < 0.01$, compared with before treatment.

4. Discussion

An aspirin maintenance dose should be continued indefinitely in patients after interventional therapy, and it was reported that aspirin could reduce serious vascular events by 25% in patients with high risk conditions [19]. However, its effectiveness is limited because 10% to 40% of patients with arterial thrombosis who are treated with aspirin have recurrent vascular events during long-term follow-up [20]. Eikelboom reported that AR patients, defined as failure of suppression of thromboxane generation, had a 2-times-higher risk of myocardial infarction and a 3.5-times-higher risk of cardiovascular death than those with low expression of thromboxane [21]. It has been suggested that higher doses of aspirin or dual antiplatelet therapy may be required in AR patients to achieve the optimal antithrombotic effect [22]. However, bleeding and upper gastrointestinal damage have been serious complications of this therapeutic strategy with high morbidity and mortality [23, 24]. Therefore, it is urgent to find novel effective and safe antiplatelet agents, which provides a great opportunity for traditional Chinese medicine with multitarget effects.

Xuefuzhuyu oral liquid, a Chinese herbal patent medicine (containing water extracts of semen *persicae*, safflower, *Angelica*, rhizoma *ligustici wallichii*, rehmanniae, rot of peony, *Achyranthes*, *Bupleurum*, fructus aurantii immaturus, *Platycodon grandiflorum*, and liquorice) approved by the China State Food and Drug Administration (national medicine permit number Z10950063), has been used in the treatment of ischemic cardiovascular diseases in mainland China for more than 20 years [17]. Xuefuzhuyu oral liquid, derived from the classic recipe Xuefuzhuyu decoction, has been well documented to inhibit platelet aggregation and to improve hemorheology [13]. In the present study, 43 enrolled patients with chronic stable angina after PCI exhibiting aspirin resistance or semi-resistance were randomly divided into control and treatment groups using a randomized block design. Only 18.2% of patients retained AR or ASR in the treatment group after combination therapy with Xuefuzhuyu oral liquid, while 90.5% retained resistance in the control group, which illustrated that Xuefuzhuyu oral liquid could effectively improve AR in patients with chronic stable angina after PCI. The BSS scores in the treatment group were reduced significantly after combination therapy with Xuefuzhuyu oral liquid compared to the control group, which indicated that Xuefuzhuyu oral liquid could reduce the degree of blood stasis in patients. Aspirin exerts its major antithrombotic effect by irreversibly acetylating platelet cyclooxygenase-1 (COX-1). One of the possible explanations for AR is that platelets can be activated by pathways that are not blocked by aspirin [21]. ADP-induced platelet aggregation was significantly lower after treatment in combination with Xuefuzhuyu oral liquid, while AA-induced platelet aggregation provoked no significant change, which demonstrated that Xuefuzhuyu oral liquid improves AR by inhibiting ADP-induced platelet aggregation. But the possible mechanism of Xuefuzhuyu oral liquid on improving AR remained unknown. Because the main active components in Xuefuzhuyu oral liquid are considered to be paeoniflorin and ferulic acid, which are reported to have good antiplatelet effects [17], the further study on the mechanisms may be focused on the ADP-pathway of these ingredients.

Many researches are currently focusing on identifying variants of genes that affect drug response. Because aspirin exhibited antiplatelet aggregation effects by irreversible inhibition of COX-1, polymorphisms of the COX-1 gene are in

the focus of many researches, but the roles of COX-1 SNPs in the mechanism of AR have not been fully elucidated. rs3842788 has been shown to be significantly associated with aspirin response determined by AA-induced platelet aggregation and serum TXB2 generation in Irish patients with cardiovascular disease, while Xu et al. reported that the mutation of rs3842788 (4.44% mutant) was not related to AR in patients accepting aspirin treatment in China [7, 8]. An in vitro study proved rs5787 variants exert the largest functional effects on decreasing the antiplatelet effectiveness of aspirin among four SNPs, with evidence for impaired interactions with COX substrate and inhibitors [25]. In our present study, no variants of rs5787 and rs3842788 were detected among 207 stable CHD patients enrolled, and because the mutations of rs5787 and rs3842788 were very uncommon, they were not suitable as representative gene polymorphisms for AR in the Chinese population. The GP IIb/IIIa receptor is critical in the process of thrombus formation since it serves as the final common pathway for platelet aggregation [26]. Several polymorphisms of the GP IIb/IIIa receptor have been identified in the general population. As compared to rs5911 C/C homozygotes, individuals with the rs5911 A/C genotype showed significantly increased inhibition of platelet aggregation in healthy Chinese male volunteers [27]. In the present study, BSS scores decreased significantly in patients with A/C or C/C genotype after treatment with Xuefuzhuyu oral liquid, which showed that patients carrying the C allele were more responsive to the improvement of blood stasis symptoms and more sensitive to the treatment of Xuefuzhuyu oral liquid.

Therefore, Xuefuzhuyu oral liquid therapy in addition to aspirin administration for the treatment of chronic stable angina patients after PCI leads to greater protection from AR, and the patients with the rs5911 variants of GP IIb exhibited better drug response upon treatment with Xuefuzhuyu oral liquid. More rigorous randomized controlled trials are necessary to provide clinicians with evidence regarding the use of Xuefuzhuyu oral liquid in the treatment of AR.

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 Medicines Might Improve the Long-Term Clinical Outcomes in Patients with Acute Coronary Syndrome after Percutaneous Coronary Intervention: Results of a Decision-Analytic Markov Model

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Aims. The priority of Chinese herbal medicines (CHMs) plus conventional treatment over conventional treatment alone for acute coronary syndrome (ACS) after percutaneous coronary intervention (PCI) was documented in the 5C trial (chictr.org number: ChiCTR-TRC-07000021). The study was designed to evaluate the 10-year effectiveness of CHMs plus conventional treatment versus conventional treatment alone with decision-analytic model for ACS after PCI. *Methods and Results*. We constructed a decision-analytic Markov model to compare additional CHMs for 6 months plus conventional treatment versus conventional treatment alone for ACS patients after PCI. Sources of data came from 5C trial and published reports. Outcomes were expressed in terms of quality-adjusted life years (QALYs). Sensitivity analyses were performed to test the robustness of the model. The model predicted that over the 10-year horizon the survival probability was 77.49% in patients with CHMs plus conventional treatment versus 77.29% in patients with conventional treatment alone. In combination with conventional treatment, 6-month CHMs might be associated with a gained 0.20% survival probability and 0.111 accumulated QALYs, respectively. *Conclusions*. The model suggested that treatment with CHMs, as an adjunctive therapy, in combination with conventional treatment for 6 months might improve the long-term clinical outcome in ACS patients after PCI.

1. Introduction

Percutaneous coronary intervention (PCI) as well as pharmacological treatments has significantly reduced but did not eliminate the risk of major adverse cardiovascular events (MACE) in ACS patients. It is reported that approximately 10% ~ 18% of ACS survivors after PCI ultimately suffer a second myocardial infarction (MI), stroke, or cardiovascular death despite the availability of timely and appropriate treatments. With the raising concern of recurrent cardiovascular events in ACS patients undergoing primary PCI, it is necessary to substantiate the effectiveness and outcomes of adjunctive therapies, such as Chinese herbal medicines (CHMs) (e.g., xin mai tong capsule, Shexiang Baoxin Pill, and tongxinluo capsule) and acupuncture, when added to conventional medication.

CHMs have been widely used in clinical practice for thousands of years. Previously, we published the 1-year clinical outcomes of the 5C trial [1]. This multicenter, open-label, randomized controlled trial (chictr.org number: ChiCTR-TRC-07000021) showed that CHMs, Xinyue Capsule, and Fufang *Chuanxiong* Capsule, in combination with conventional treatment, further prevent 1-year occurrence of cardiovascular events in ACS patients after primary PCI without increasing risk of major bleeding, as compared with conventional treatment alone. Owing to the only 1-year follow-up period in this trial and other limited CHMs trial resources, the priority of CHMs in combination with

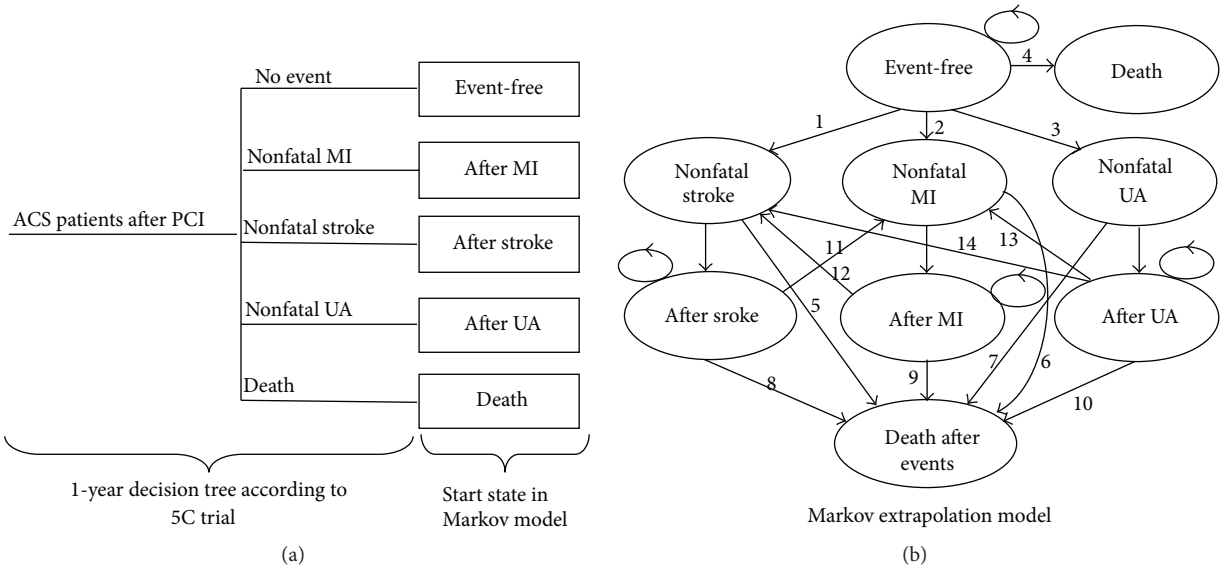


Figure 1: Two-component decision-analytic model structure. Part (a) is a decision tree representing the 5 clinical outcomes of the 5C trial during the 1-year period: event-free, nonfatal myocardial infarction (MI), nonfatal stroke, nonfatal unstable angina (UA), or all-cause death. Part (b) is long-term Markov model. (1) Risk of nonfatal stroke for event-free patients. (2) Risk of nonfatal MI for event-free patients. (3) Risk of nonfatal UA for event-free patients. (4) Mortality risk for event-free patients. (5) Mortality risk at the first year after a nonfatal stroke. (6) Mortality risk at the first year after a nonfatal MI. (7) Mortality risk at the first year after a nonfatal UA. (8) Mortality risk at second and subsequent years after a nonfatal stroke. (9) Mortality risk at second and subsequent years after a nonfatal MI. (10) Mortality risk at second and subsequent years after a nonfatal UA. (11) Risk of nonfatal MI for patients with stroke. (12) Risk of nonfatal stroke for patients with MI. (13) Risk of nonfatal MI for patients with UA. (14) Risk of nonfatal stroke for patients with UA.

conventional treatment over conventional treatment alone on long-term outcome of ACS patients has not been established.

Therefore, we constructed a decision-analysis Markov model to assess the effectiveness of CHMs plus conventional treatment versus conventional treatment alone in ACS patients after primary PCI. During 10 years, whether ACS patients after PCI may benefit from reducing the risk of MACE and increasing the quality of life (QOL) when treated by additional CHMs for 6 months, as an adjunctive therapy, in combination with conventional treatment, remains unclear.

2. Methods

2.1. Study Design. To capture the short- and long-term clinical outcomes in ACS patients after PCI receiving additional CHMs for 6 months plus conventional treatment versus conventional treatment alone, a decision-analytic Markov model was developed, by following generally accepted principles of design [2]. Referring to the model developed by previous studies [3–5], the model in the study comprises two components: the first part contains a decision tree which was in line with the period of the 5C trial (one year); the other part is that the subsequent events were modelled as a Markov structure with the potential for a recurrent event (subsequent years). The health outcomes modelled in the study were quality-adjusted life years (QALYs), which take into account both the quantity and QOL generated by the interventions. The model was based on the 5C trial's population, which included a broad spectrum of ACS patients, that is, ST-segment elevation MI, non-ST-elevation MI, and unstable angina (UA), who underwent successful PCI. The patients were randomized to receive additional CHMs for 6 months plus conventional treatment or conventional treatment alone. The aim of the modelling exercise was to adhere closely to the 5C trial and the model structure is based on the key clinical outcomes of 5C trial.

2.2. Model Structure. The 1-year decision tree was modelled based on the clinical outcomes in 5C trial. During the first year, the patients who received additional CHMs for 6 months plus conventional treatment or conventional treatment alone could suffer a nonfatal MI, a nonfatal stroke, a nonfatal UA, or death from all causes. Those patients who experienced no events were considered as event-free.

To simulate the long-term clinical outcomes in post-PCI ACS patients in 5C trial, the 1-year decision tree was extended to a long-term Markov model. A Markov model consists of a number of mutually exclusive and collectively exhaustive health states, usually named as Markov states, representing the disease progression process from entry to death or end of the time horizon of the analysis [6]. Disease progression or occurrence is modelled as transitions between states over time. In any given interval of time, referred to as a cycle or stage, a cohort member is in one and only one of the states. Patients who remain alive with an event spend 1 cycle in the first state of the corresponding event and then move on to the corresponding state for following cycles. The cycle length used in the model is 1 year.

In the study, the Markov model (Figure 1) had 8 health states, which were event-free, nonfatal AMI, post-MI,

Table 1: Model parameters (1-year decision-analysis model).

Variables	Probability	
ACS after PCI	CHMs plus conventional treatment	Conventional treatment alone
Nonfatal AMI	0.005 (0, 0.0119)	0.0175 (0.0047, 0.0303)
Nonfatal stroke	0.0074 (0, 0.0158)	0.0150 (0.0031, 0.0269)
Nonfatal UA	0.0149 (0.0031, 0.0267)	0.0399 (0.0207, 0.0591)
Death	0.0050 (0, 0.0119)	0.0075 (0, 0.0159)

ACS: acute coronary syndrome; PCI: percutaneous coronary intervention; AMI: acute myocardial infarction; CHMs: Chinese herbal medicines; UA: unstable angina.

nonfatal stroke, poststroke, nonfatal UA, post-UA, and death (all-cause). Patients entered the Markov model based on the events in the 1-year decision tree. Patients who experienced no events during the first year in the decision tree entered the Markov model in the "event-free" state. These patients could suffer a fatal MI, stroke, or UA in every subsequent year and could also transit to a nonfatal MI, nonfatal stroke, or nonfatal UA state. Patients who suffered an MI, stroke, or UA in the 1-year decision tree entered the new MI, stoke, and new UA states in the Markov model, respectively. After 1 year in the new MI, new stroke, and new UA states, patients would transit to the corresponding postevent state. In each cycle, patients could experience a new MI, new stroke, and all-cause death or remain in a postevent state. The model assumed that patients could not enter the new UA state from the poststroke state and post-MI state due to the limitation of the relative data. Patients with a fatal event in the 1-year decision tree entered the Markov model as "dead," the same as patients who died from the "no event" state. Patients who die in a nonfatal event state or postevent state pass to the dead postevents state.

The model was run up to a time horizon of 10 years. Half-cycle correction was performed in this study with assigning one-half of the state reward for simulated individuals starting in each state.

2.3. Transition Probabilities. Transition probabilities, which characterize how a cohort member may pass in successive cycles, vary over time and depend on patient characteristics but not on previous events as the model has no memory [7]. In 1-year decision tree, the probabilities of the patients experiencing nonfatal MI, stroke, or all-cause death were calculated from the data in 5C trial (Table 1).

In Markov model, as the intervention period of additional CHMs was only 6 months and no long-term data were available after the first year of treatment, we conservatively assumed that the transition probabilities were identical for patients receiving CHMs plus conventional treatment or conventional treatment alone for year 2 and onwards. And the only difference between the two treatment strategies was caused by the different distribution of patients in the different Markov states after the first year. To obtain the transition probabilities for the Markov model, data beyond the duration of the 5C trial were required. By following the methods used to derive the transition probabilities which had been previously published and validated in some trial-based economic analyses [8], the transition probabilities for nonfatal MI, nonfatal UA, nonfatal stroke, and death were also extrapolated on the basis of the available data drawn from the published reports. We conducted a literature search of PubMed, OVID, and the Cochrane Library websites for reaching results from cardiovascular trials from January 1980 up to December 2012. For reflecting the clinical outcomes in post-ACS patients, these probabilities were obtained from registry-based studies, analyses of randomized controlled trials, and systematic reviews which were preferred when available. The selection of the studies finally included in the model was performed in a nonsystematic way and conditioned on the adequacy of the data to the decision problem [9]. When necessary, reported and calculated rates from published reports were converted to probabilities for use in the model with the assumption of a constant hazard over time [10]. Details of the data sources and the transition probabilities are summarized in Table 2.

2.4. Utility Values. The outcomes of each treatment strategy were quantified in terms of QALYs over a 10-year horizon, as noted previously. To calculate QALYs, the utility weights were multiplied by the duration in each health state. An annual utility was assigned for each health state in the study.

Utility values describe the health-related QOL correlated with different health states on a scale of zero to one, where zero and one represent death and best imaginable health, respectively. The baseline utility values for patients of event-free during 1-year follow-up in CHM plus conventional treatment arm and conventional treatment alone arm were taken from 5C trial, in which health-related QOL was assessed at 1 year after PCI using EuroQol (EQ-5D). EQ-5D scores were derived using Japanese population tariff values [11]. Due to lack of the local utility values for patients with nonfatal AMI, nonfatal stroke, or nonfatal UA, the values proposed by published studies in the literatures were applied in the present analysis [12]. We calculated the disutility values by taking the difference in health-related QOL values between a patient with and without an event based on the method reported by Bagust et al. [13] and Chaplin et al. [14]. Patients experiencing an event (AMI, stroke, or UA) were assigned disutility weights to take into account the one-off decrease in their health status due to the event. For patients experiencing a MI, UA, or stroke, we attributed a disutility of 0.127, 0.117, and 0.139, respectively, at the time of the occurrence of an event until end of follow-up, which was obtained from a previous published study [15]. The baseline utility values used for each health states in the model as well as the ranges used within the sensitivity analyses are presented in Table 3.

Table 2: Transition probabilities among health states in the long-term Markov model.

Variables	Baseline probability	Range	Source
Event-free followed by			
Nonfatal AMI	0.018	0.010–0.020	[26–35]
Nonfatal stroke	0.007	0.001–0.009	[36–38]
Nonfatal UA	0.03	0.02–0.05	[39–41]
Death	0.027	0.014–0.033	[26–35]
Post-MI followed by			
Death, 1st year	0.039	0.008–0.076	[33, 42–56]
Death, after 1st year	0.021	0.003–0.027	[33, 42, 45–53, 56]
Nonfatal AMI, 1st year	0.024	0.002–0.060	[33, 42–56]
Nonfatal AMI, after 1st year	0.018	0.001–0.008	[33, 42, 44–53, 56]
Nonfatal stroke, 1st year	0.010	0.0024–0.024	[33, 43, 44, 57–61]
Nonfatal stroke, after 1st year	0.007	0.0008–0.022	[58, 59]
Post-UA followed by			
Death, 1st year	0.034	0.012–0.050	[30, 32, 33]
Death, after 1st year	0.020	0.016–0.028	[30, 32, 33]
Nonfatal AMI, 1st year	0.036	0.01–0.05	[30, 33, 59, 62]
Nonfatal AMI, after 1st year	0.011	0.010–0.063	[30, 33, 59, 62]
Nonfatal stroke, 1st year	0.018	0.014–0.023	[33, 62]
Nonfatal stroke, after 1st year	0.008	0.006–0.01	[33, 62]
Post stroke followed by			
Death, 1st year	0.115	0.066–0.189	[58, 62–69]
Death, after 1st year	0.035	0.016–0.061	[58, 62, 64–67, 69]
Nonfatal AMI, 1st year	0.003	0.002–0.006	[58, 59]
Nonfatal AMI, after 1st year	0.004	0.002–0.006	[58, 59]
Nonfatal stroke, 1st year	0.128	0.064–0.189	[68, 70, 71]
Nonfatal stroke, after 1st year	0.040	0.030–0.080	[71, 72]
Rate of age-related MACE (OR/10 years)	0.5	0.33–0.87	[28, 30, 31, 39, 69]

AMI: acute myocardial infarction; UA: unstable angina; MACE: major adverse cardiovascular events.

Table 3: Estimated utilities and disutilities.

Events	Base-case value	Range	Source
Event-free			
CHMs plus conventional treatment	0.818	0.418 to 0.848	5C trial
Conventional treatment alone	0.809	0.252 to 0.848	5C trial
Disutilities (QALYs)			
Nonfatal AMI	0.127	0.108 to 0.147	[15]
Nonfatal Stroke	0.139	0.118 to 0.160	[15]
Nonfatal UA	0.117	0.100 to 0.135	[15]
Death		0	

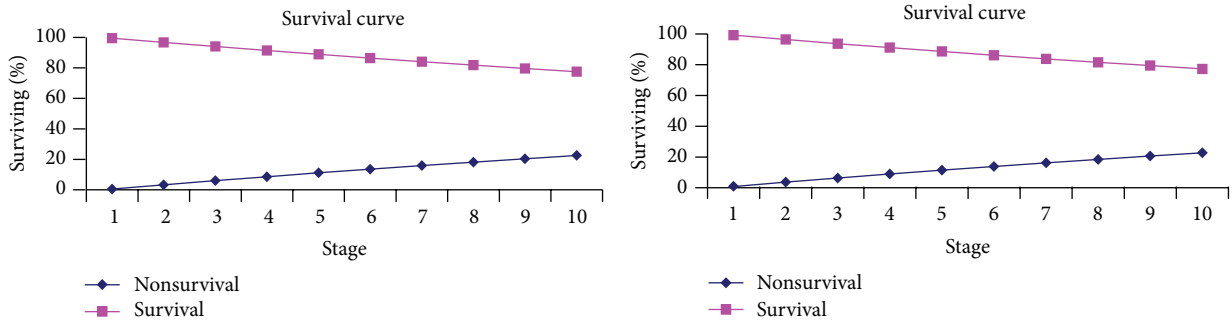
CHMs: Chinese herbal medicines; AMI: acute myocardial infarction; UA: unstable angina.

2.5. Analytic Method and Univariate Sensitivity Analysis. For the 2 strategies, CHMs plus conventional treatment versus conventional treatment alone, we calculated QALYs and considered the strategy associated with a higher value to be preferred. Since our model was based on a number of assumptions and weighted average of published literature-derived probabilities, we performed univariate sensitivity analyses, in which we allowed any one of the variables of the model to vary at a time according to its estimates range, to determine whether and how plausible parameters in these assumptions and risks would alter our findings [10].

The Markov model was designed and all analyses were performed with TreeAge Pro Suite 2011 software package.

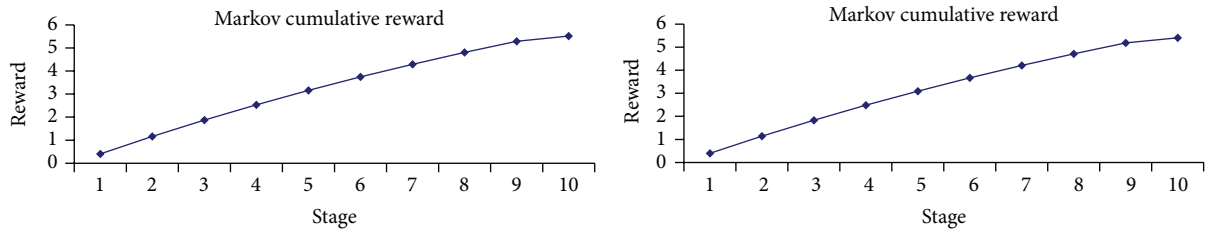
3. Results

3.1. Base-Case Analysis. The Markov model predicted that the discounted survival was higher in the CHMs plus



(a) CHMs plus conventional treatment (b) Conventional treatment alone

Figure 2: Survival curve.



(a) CHMs plus conventional treatment (b) Conventional treatment alone

Figure 3: Cumulative QALYs over 10-year horizon.

conventional treatment arm when compared with the conventional treatment alone arm by 0.20% survival probability. The survival probability over the 10-year horizon was 77.49% in the CHMs plus conventional treatment arm and 77.29% in the conventional treatment alone arm, respectively (Figure 2).

In a cohort of 1000 patients over 10 years, the CHMs plus conventional treatment, compared with conventional treatment alone, would gain 22 patients remaining event-free, prevent 7, 9, and 5 patients further suffering from nonfatal MI, nonfatal UA, and nonfatal stroke, respectively, and avoid 20 patients dying from all causes.

The model predicted that participants after PCI who received CHMs plus conventional treatment would live an average of 0.405 discounted QALYs in 1-year and 5.519 discounted accumulated QALYs over 10-year horizon. And those who received conventional treatment alone would live an average of 0.396 QALYs in 1-year and 5.408 QALYs over 10-year horizon (Figure 3). Comparing with conventional treatment alone, CHMs plus conventional treatment would save 0.009 QALYs in 1 year and 0.111 QALYs for the time horizon of 10 years.

3.2. Sensitivity Analyses. The priority of CHMs plus conventional treatment over conventional treatment alone for all the parameters was considered in the univariate sensitivity analysis. The analysis showed that changes of every input parameter had no impact on the interpretation of the results. Thus, CHMs plus conventional treatment remained a dominant therapy over a broad range of the input parameters. The 5 most sensitive input parameters were annual mortality risk for event-free patients, annual risk of nonfatal UA for event-free patients, annual risk of nonfatal stroke for event-free patients, annual risk of nonfatal MI for event-free patients, and disutility of UA. The annual mortality risk for event-free patients (varied from 0.014 to 0.033) had the largest influence on the QALYs (5.505 to 5.532 for patients in the CHMs plus conventional treatment arm versus 5.393 to 5.423 for patients in the conventional treatment alone arm).

4. Discussion

In the present study, a two-component decision-analytic model approach was used to predict the short- and long-term effectiveness of CHMs plus conventional treatment and conventional treatment alone in the treatment of ACS after PCI. The results showed that CHMs plus conventional treatment would reduce the risk of death in ACS participants after PCI over 10-year period compared to conventional treatment alone, as well as the risk of nonfatal MI, nonfatal stroke, and nonfatal UA. Comparing with conventional treatment alone, CHMs plus conventional treatment would save 0.111 QALYs for the time horizon of 10 years. Given a cohort of 1000 patients over 10 years, the CHMs plus conventional treatment, compared with conventional treatment alone, would gain 22 patients benefit from no events and prevent 20 patients from all-cause death.

As far as we are concerned, this is the first study that has accounted for QOL and generated QALYs in estimating the long-term effectiveness of CHMs plus conventional treatment in ACS patients after PCI compared with conventional treatment alone. The study showed that the estimated gain

with CHMs plus conventional treatment, compared with conventional treatment alone, was accrued due to an increase in survival probability and QOL as well. And the reduction in mortality and the increase in remaining event-free were the majority contributors to the survival probability. The favorable effectiveness of CHMs plus conventional treatment in our analysis was supported by earlier clinical studies, showing that CHMs plus conventional treatment were expected to reduce the risk of MI and improve myocardial reperfusion after PCI in patients with AMI during 3 months [16, 17], as well as playing roles in decreasing recurrent angina in patients with coronary heart disease [18, 19]. It should be noted that our findings extended the previous studies and dynamically analyzed the favorable effectiveness of CHMs plus conventional treatment over conventional treatment alone in the 10-year period, indicating that CHMs plus conventional treatment might be an adjunctive therapy in further improving the long-term clinical prognosis in patients with ACS after PCI. Since there was no study regarding long-term QALYs among ACS patients after PCI up to now in China, which used CHMs plus conventional treatment, more evidences were needed in the future to support the estimation of our study.

In the present study, the input parameters were derived from 5C trial and medical literatures. Over the first year, transition probabilities and baseline utility for patients in CHMs plus conventional treatment and conventional treatment alone arms were mainly taken from 5C trial. For some parameters, however, no data in 5C trial were available, so we used international data instead. In the absence of long-term data after 1 year (i.e., beyond the duration of 5C trial), the transition probabilities were obtained based on the collected data from registry-based studies, analyses of randomized controlled trials, and systematic reviews. Additionally, there were no previous published evidences of disutility values for patients experiencing MI, UA, or stroke after PCI in China; we used data from a published study [15] to perform the analysis as well.

In 5C trial, the intervention period of CHMs plus conventional treatment was only 6 months and there was no intervention difference in the subsequent 6 months between CHMs plus conventional treatment and conventional treatment alone. Our model made the conservative assumption that there was no incremental clinical benefit from CHMs plus conventional treatment versus conventional treatment alone beyond the first year of treatment; that is, the benefits of CHMs only worked in 6 months and beyond 1 year the transition probabilities were identical for both treatment arms. Advantages of using the decision-analytic model approach in long-term effectiveness evaluation are to be able to extend analyses beyond trial durations, to integrate data from a variety of sources, and to be able to explore the impact of the therapy in various treatment settings [20, 21]. The assumptions in the model, however, may not necessarily hold true. And we do not expect these assumptions to have a major influence on our results of the present study. Since further uncertainty arises through methodological and modeling structure uncertainty, which can be addressed with univariate sensitivity analysis [22], we performed univariate sensitivity analyses on various parameters and assumptions to assess the rigour of the assumptions on the effectiveness estimation. The analysis showed that our results are robust to very wide variations in model inputs.

Our study has several limitations. First, the study was performed based on a decision-analytic Markov model which was a simplification of reality, where probability data of outcomes occurred in another population and different scenarios from that of 5C trial were inevitably employed, although we used a resource estimate adapted to our reality [23]. The data sources about probabilities beyond 1 year were partly driven by the clinical event rates observed in the patients with NSTE-ACS or STEMI patients, while the population in 5C trial were combined NSTE-ACS patients and STEMI patients together. Even if GUSTO-IIb trial showed that the mortality rate at 30 days was greater among patients with ST-segment elevation than among those without ST elevation, this difference narrowed at 6 months and disappeared at 1 year [24], and the study reported by Singh et al. [25] also demonstrated that patients with STEMI and NSTEMI experienced similar outcomes; it was still difficult to exactly match the patients recruited in 5C trial. Somewhat, transition probabilities in our study were measured with some degree of error. Secondly, the study was supposed to predict the whole spectrum of consequences of therapy with a 10-year horizon, which requires making a series of difficult-to-demonstrate assumptions [9]. On the basis of the data currently available, our study might conservatively estimate the effectiveness of CHMs plus conventional treatment.

5. Conclusion

On the basis of the decision-analytic Markov model, the analysis suggested that treatment with CHMs, as an adjunctive therapy, in combination with conventional treatment for 6 months might improve the long-term clinical outcome in ACS patients after PCI. However, the larger long-term clinical trials are needed to prove the long-term effectiveness of CHMs plus conventional treatment in the treatment of ACS after PCI in the future.

Disclosure

The sponsor of the study had no role in the study design, data collection, data analysis, data interpretation, or paper preparation.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

All authors contributed to the data collection, paper writing, and final approval of this study.

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Research Article

Extra Virgin Olive Oil Polyphenols Promote Cholesterol Efflux and Improve HDL Functionality

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Results of the present work give evidence from the beneficial role of extra virgin olive of oil (EVOO) consumption towards oxidative stress and cardiovascular diseases. Polyphenols contained in EVOO are responsible for inhibiting lipoproteins oxidative damages and promoting reverse cholesterol transport process via ABCA1 pathway.

1. Introduction

Coronary heart disease (CHD) is the main cause of mortality in the Western world. The oxidation of low-density lipoproteins (LDL) is an early event in the development of atherosclerosis, the underlying cause of CHD [1]. Oxidized LDL are not recognized by the LDL-receptor Apo (B/E) but are taken up by macrophages in a nonregulated manner through the scavenger-receptor pathway, which leads to the formation of foam cells, the hallmark of arteriosclerotic lesions [1].

Macrophage-specific reverse cholesterol transport (RCT) is thought to be one of the most important HDL-mediated cardioprotective mechanisms. RCT is the process by which cholesterol in peripheral cells is effluxed onto circulating HDL and is transported back to the liver for excretion in bile and feces [2, 3]. The promotion of RCT is considered a major antiatherogenic function of HDL [4, 5]. The efflux of cholesterol from cells to HDL is the first and rate-limiting step of RCT [6]. Two major macrophages cholesterol efflux pathways have been described: SR-BI receptor-mediated cholesterol efflux and ABCA1/ABCG1-mediated cholesterol efflux. ABCA1 promotes the efflux of phospholipids and cholesterol to lipid-poor apo-AI via a process that involves the direct binding of apo-AI to the ABCA1 transporter, whereas ABCG1 and SR-BI are key mediators of macrophage cholesterol efflux to mature HDL [7]. Evidence from a recent study indicates that the inflammatory process induces changes in HDL composition and metabolism that impair RCT [8]. Interestingly, we recently showed that RCT is also impaired with aging, especially by changes to the ABCA1-mediated cholesterol efflux pathway [9, 10].

Polyphenol-rich vegetable oils and monounsaturated fatty acids provide protection against an array of human diseases such as cancer, atherosclerosis, and CVD, including those involving the central nervous system. Olive oil, which is known for its healthful properties, which are often attributed to its high monounsaturated fatty acid content, including oleic acid (18:1 n-9), is a prominent member of the family of polyphenol- and monounsaturated fatty acid-rich oils. However, olive oil, unlike other vegetable oils, contains high amounts of several micronutrient constituents, including polyphenolic compounds (100–1000 mg/Kg) such as hydroxytyrosol, tyrosol, and oleuropein [11]. *In vitro* and *in vivo* human and animal studies have shown that EVOO reduces blood pressure [12], improves the lipid profile by increasing HDL-cholesterol and reducing LDL-cholesterol and triglyceride levels [13–15], reduces oxidative stress, and inhibits human lipoprotein oxidation, making LDL, for instance, less atherogenic [16, 17]. Olive oil dietary supplementation decreases the levels of high inflammatory and endothelial

dysfunction markers in the serum. Experimental and clinical studies have shown that olive oil downregulates vascular cell adhesion molecule-1 (VCAM-1), human soluble intercellular adhesion molecule-1 (sICAM-1), and E-selectin expression in the endothelium [18] and decreases plasma levels of sICAM-1, soluble E-selectin, interleukin-6 (IL-6), and high-sensitive C-reactive protein (CRP) in high-risk patients [19].

The beneficial effects of polyphenols appear to be mediated via a plethora of biochemical pathways and signaling mechanisms that act either independently or synergistically. In the present study, we investigated the atheroprotective effect of the phenolic compounds in EVOO on cholesterol efflux and on oxidative stress damage in healthy subjects.

2. Methods

2.1. Subjects. Twenty-four healthy volunteers (30.92 ± 2.55 years) with normal serum lipid profiles and blood pressure were recruited. They were all nonsmokers and were not taking any medication, including lipid-lowering treatments or oral antioxidants. None of the female subjects was undergoing estrogen replacement therapy for menopause. None of the participants showed clinical signs of inflammation, obesity, or diabetes. The physical and biochemical parameters of the participants are presented in Table 1. The Ethics Committee of the Sherbrooke Geriatric University Institute approved the study, and all subjects provided written informed consent before being enrolled.

Table 1: Clinical and biochemical parameters of participants.

	Mean ± esm
$n = 24$ (w/m)	14/10
Age (mean ± SD years)	30.92 ± 2.55
Body mass index (kg/m^2)	23.7 ± 1.65
System blood pressure (mmHg)	127 ± 4.65
Dias. blood pressure (mmHg)	78.23 ± 2.09
Total cholesterol (mmol/L)	5.06 ± 0.2
Triglycerides (mmol/L)	1.32 ± 0.15
HDL-c (mmol/L)	1.42 ± 0.09
LDL-c (mmol/L)	3.05 ± 0.15
Apo A1 (g/L)	1.56 ± 0.05
Apo B (g/L)	0.90 ± 0.04
Apo B/Apo A1	0.8 ± 0.04
TC/HDL-c	3.81 ± 0.23
LDL-c/HDL-c	2.5 ± 0.2
TG/HDL-c	1.08 ± 0.17
Glucose (mmol/L)	4.43 ± 0.10
Insulin (pmol/L)	38.32 ± 5.26
CRP (mg/L)	3.16 ± 0.13

TC (total cholesterol); HDL-C (HDL-cholesterol); LDL-C (LDL-cholesterol); CRP (C-reactive protein).

2.2. Phytochemistry. The phenolic compounds were extracted from EVOO using the method of Pirisi et al. [20]. Briefly, EVOO was mixed with *n*-hexane and methanol/water and was stirred in a vortex apparatus overnight at 4°C. The mixture was then centrifuged, and the hydroalcoholic solution was washed with *n*-hexane and then lyophilized overnight.

2.3. Lipoprotein Isolation. Fasting human plasma was collected in heparin tubes, and the HDL was immediately isolated using the method of Sattler et al. [21]. The isolated lipoproteins were dialyzed overnight at 4°C against 10^{-2} M sodium phosphate buffer (pH 7.0). The protein concentrations were measured using commercial assay kits (Bio-Rad, Canada) using the manufacture's protocol.

2.4. Lipoprotein Enrichment with EVOO and EVOO-PC. Human plasma was incubated overnight with slight agitation at 4°C in the presence of EVOO (0.2 mg/mL of plasma) or EVOO-PC (1.76 mg/mL of plasma). The LDL and HDL were then isolated as described above.

2.5. Copper-Mediated Lipoprotein Oxidation. The lipoproteins were peroxidized as previously described using transition metal ions as oxidizing agents [22]. Briefly, control, EVOO, and EVOO-PC-enriched lipoproteins [(LDL 100 μg/mL) or (HDL 200 μg/mL)] were suspended in 10 mM sodium phosphate buffer (pH 7) and were incubated for 0 to 4 h at 37°C in the presence of 10 μM cupric sulfate. The oxidation reaction was stopped by adding EDTA. Lipid peroxide formation was assessed by monitoring conjugated diene formation at 234 nm.

2.6. Cell Cultures. Human THP-1 monocytes and J774 macrophages were cultured in RPMI 1640 and DMEM medium, respectively. The media were supplemented with 10% heat-inactivated FBS, 50 mM 2-β-mercaptoethanol (only for THP-1), 2 mM L-glutamine, 5 mg/mL of glucose, and 100 U/mL of penicillin. The differentiation of the THP-1 monocytes into macrophages was induced by culturing the monocytes in the presence of 100 μM PMA for 96 h.

2.7. Cholesterol Efflux Measurements. THP-1-derived macrophages and J774 macrophages were incubated in fresh growth medium containing 0.2 μCi/mL [^{3}H]-cholesterol for 48 h or 1 μCi/mL [^{3}H]-cholesterol for 24 h, respectively. The loaded cells were washed, equilibrated in serum-free medium containing 1% BSA for 12 h, washed again, and subjected to various treatments. The THP-1-derived macrophages were incubated for 24 h with (1) HDL-free medium, (2) HDL (50 μg/mL), (3) EVOO-enriched HDL (OO-HDL), or (4) EVOO-PC-enriched HDL (PC-HDL).

[^{3}H]-Cholesterol loaded THP-1-derived macrophages were subjected to oxidative stress by incubating them with 0.2 mM iron/ascorbate (Fe/Asc) in the absence or presence of EVOO-PC (320 μg/mL) for 6 h. They were then incubated with HDL for 24 h to assess cholesterol efflux under various conditions.

The effect of EVOO-PC on ABCA1-mediated cholesterol efflux was assessed using J774 macrophages. [^{3}H]-Cholesterol-loaded J774 macrophages were incubated for 12 h with 0 to 320 μg/mL of EVOO-PC to generate ABCA1-enriched cells

or with 300 μM 8-Br-cAMP (positive control) to stimulate ABCA1 gene transcription and surface protein expression. The J7774 macrophages were then incubated with 25 μg/mL of apo-AI for 4 h.

To better understand the mechanism of EVOO-PC-mediated cholesterol efflux, we studied the effect of the two major phenolic compounds in EVOO (tyrosol and hydroxytyrosol) on ABCA1-mediated cholesterol efflux. [^{3}H]-Cholesterol-loaded J774 macrophages were incubated for 12 h with 0 to 25 μM tyrosol or hydroxytyrosol to generate ABCA1-enriched cells and were then incubated with 25 μg/mL of apo-AI for 4 h. 8-Br-cAMP was used as a positive control.

Cholesterol efflux was determined by liquid scintillation counting, and the percent of radiolabeled cholesterol released (percent cholesterol efflux) was calculated using the following formula: (cpm in the medium/[cpm in the cells + medium]) × 100.

2.8. Western Blot Analyses. ABCA1 protein expression in J774 macrophages was studied by incubating them for 12 h with 0 to 320 mg/mL of EVOO-PC or 5 or 10 μM hydroxytyrosol or tyrosol. The proteins (20 μg) were separated by electrophoresis on 10% acrylamide gels and were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% milk in PBS/Tween 20 and were incubated with primary antibodies (anti-ABCA1) and then with specific IgG-HRP-conjugated secondary antibodies. β-actin was used as a control. The protein bands were detected using an enhanced chemiluminescence reagent (ECL) [10].

2.9. Statistical Analysis. Values are expressed as means ± SEM. A one-way analysis of variance (ANOVA) was used for multiple comparisons. A linear regression analysis was used to assess the association between two continuous variables. All statistical analyses were performed using GraphPad Prism-5 software.

3. Results

3.1. Effect of Extra Virgin Olive Oil and EVOO Phenolic Compound Extracts on Lipoprotein Oxidation. The concentration of total phenolic compounds (41.9 mM; gallic acid equivalent) was estimated using the Folin-Ciocalteu method.

The peroxidation by $CuSO_4$ of the polyunsaturated fatty acids (PUFA) in HDL and LDL was assessed by the formation of conjugated dienes. The peroxidation kinetics showed that the lag phase of LDL was longer than that of HDL. The lag phase was followed by the propagation and termination phases.

Our results showed that plasma LDL and HDL that had been pretreated with EVOO or EVOO-PC were less oxidizible and were much more resistant to lipid peroxidation than untreated (control) plasma LDL and HDL as shown by the significant increase in the lag phase and the decrease in conjugated diene formation in EVOO-PC- and EVOO-treated lipoproteins (Figures 1(b) and 1(e)). The enrichement of lipoproteins with EVOO-PC or EVOO increased the lag phase 1.42- ($p < 0.05$) and 2.39-fold ($p < 0.01$) for HDL and 1.51- and 1.50-fold ($p < 0.05$) for LDL, respectively, compared to the control (Figures 1(b) and 1(e)). On the other hand, the enrichment of HDL and LDL with EVOO or EVOO-PC reduced conjugated diene formation (OD_{max}) 4.53- ($p < 0.05$) and 7.71-fold ($p < 0.01$) for HDL and 1.75- ($p < 0.001$) and 14.58-fold ($p < 0.0001$) for LDL, respectively, compared to the control (Figures 1(c) and 1(f)).

3.2. Effect of Phenolic Compounds on Reverse Cholesterol Transport. To determine the effect of phenolic compounds on RCT, cholesterol efflux was measured. Incubating ^{3}H-cholesterol-loaded THP-1-derived macrophages for 24 h with EVOO or EVOO-PC enhanced cholesterol efflux by 41.5% and 39.93% ($p < 0.05$), respectively, compared to the control (Figure 2(a)).

Oxidative damage to macrophages impairs cholesterol efflux, as shown by the decrease in ABCA1 protein expression induced by Fe/Asc [23]. We thus investigated the effect of EVOO-PC on the capacity of HDL to mediate cholesterol efflux in THP-1-derived macrophages under oxidative stress induced by Fe/Asc. HDL-mediated cholesterol efflux was significantly impaired under oxidative stress conditions whereas the effect was much lower when the macrophages were pretreated with 320 μg/mL of EVOO-PC ($p < 0.001$) (Figure 2(b)).

To better understand the mechanism by which EVOO-PC enhances HDL-mediated cholesterol efflux, we investigated the effect of EVOO-PC on ABCA1-dependent cholesterol efflux from J774 macrophages. ^{3}H-Cholesterol-loaded J774 macrophages were incubated with apoA-1 in the absence of cAMP for 4 h (time range for measuring cholesterol efflux via the ABCA1 pathway). We observed little cholesterol efflux in the absence of cAMP. However, when the macrophages were preincubated overnight with 0 to 320 μg/mL of EVOO-PC or with a cAMP-analogue to induce ABCA1 protein expression and then with 25 μg/mL of apoA-1 for 4 h, we observed a significant EVOO-PC concentration-dependent increase in cholesterol efflux ($r^2 = 0.95$, $p < 0.01$) (Figure 3(a)).

To investigate the mechanism by which EVOO-PC induces the increase in cholesterol efflux from J774 macrophages to apoA-1, we performed Western blot analyses to measure ABCA1 protein expression on J774 macrophages incubated with EVOO-PC. We observed an EVOO-PC concentration-dependent increase in ABCA1 protein expression in J774 macrophages incubated with EVOO-PC (Figure 3(b)).

In light of these results, we then investigated the effect of two major phenolic compounds in EVOO-PC (purified tyrosol and hydroxytyrosol) on cholesterol efflux from and ABCA1 protein expression in J774 macrophages. Our results showed that tyrosol and hydroxytyrosol increase in concentration-dependent manner the ABCA1-dependent cholesterol efflux (Figures 4(a) and 4(b), resp.).

4. Discussion

Olive oil is the main source of fat in the Mediterranean diet. A large body of knowledge has provided evidence of the benefits of the Mediterranean diet and olive oil consumption on

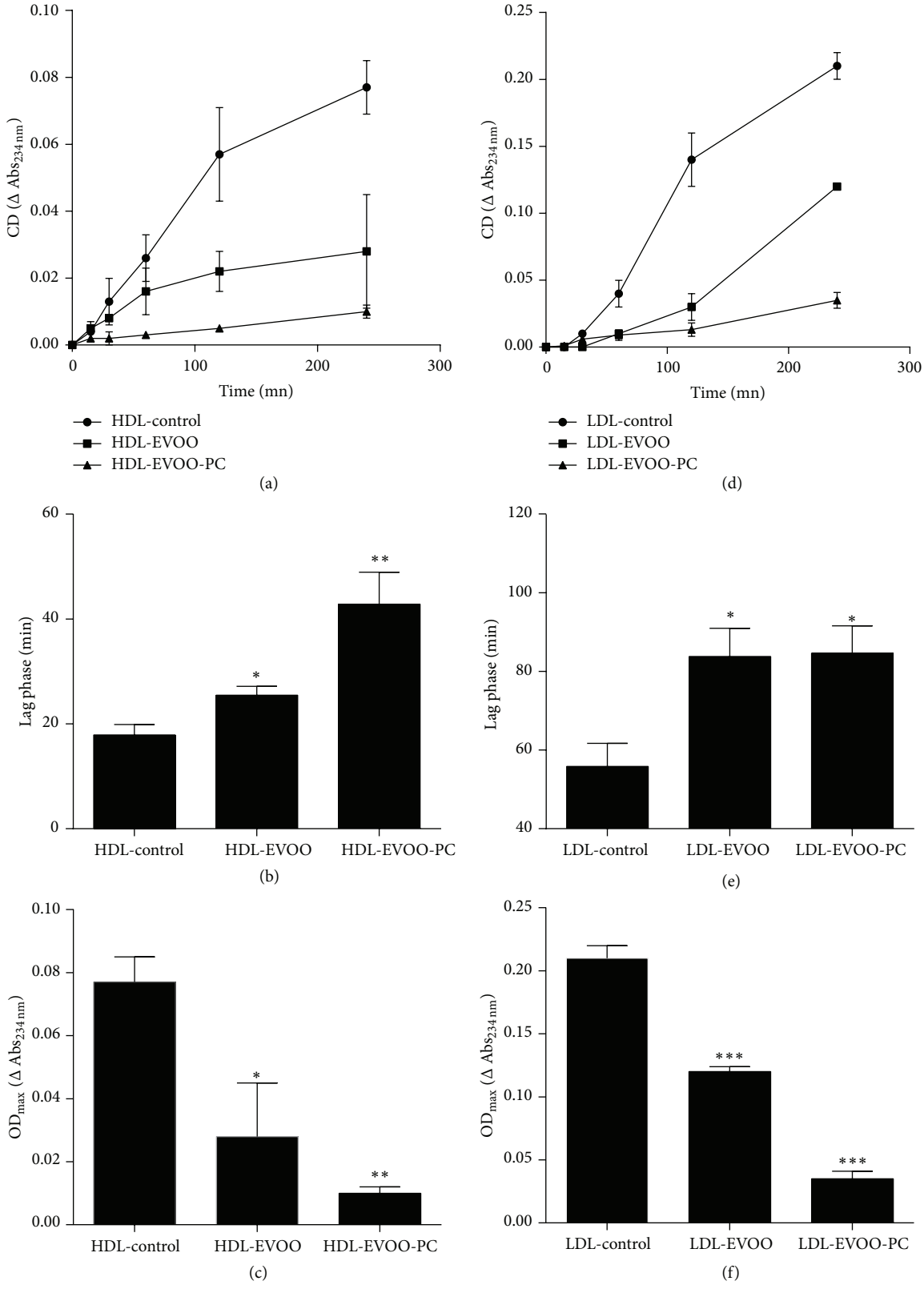

Figure 1: EVOO-PC enrichment decreases the oxidizability of lipoproteins. Plasma was incubated with EVOO or EVOO-PC prior to isolating HDL and LDL. HDL and LDL pretreated with EVOO or EVOO-PC as well as untreated controls were oxidized by incubation with copper ions for 4 h. The resistance to lipid peroxidation and the oxidizability of HDL and LDL were monitored by determining the lag phase (a, b, d, e) and by measuring conjugated diene formation (OD_{max}), respectively (c, f). Results are expressed as the means ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

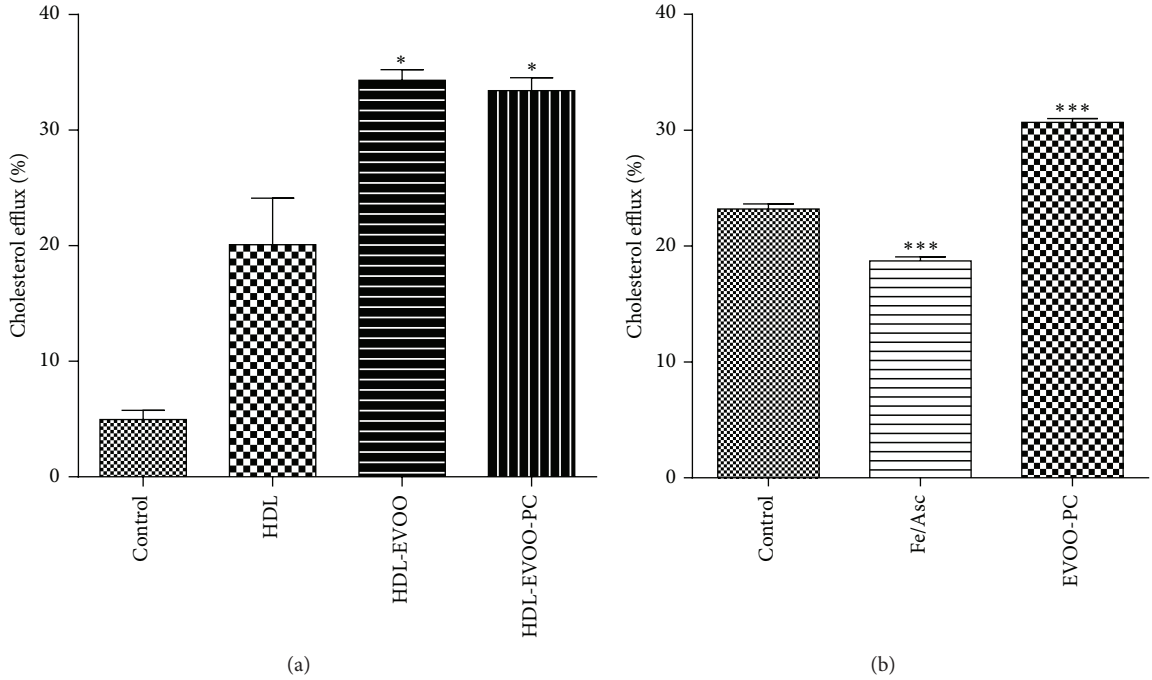

Figure 2: EVOO-PC protects macrophages against oxidation and promotes HDL-mediated cholesterol efflux. (a) THP-1-derived macrophages were loaded with [^{3}H]-cholesterol (2 μCi/mL) for 24 h. The cells were then washed, equilibrated, and incubated for a further 24 h with 50 μg/mL of HDL-free medium, HDL, EVOO-enriched HDL (EVOO-HDL), or EVOO-PC-enriched HDL (EVOO-PC-HDL). (b) The macrophages were stressed with 0.2 mM Fe/Asc, and cholesterol efflux was assessed using 50 μg/mL of HDL. Results are expressed as the means ± SEM of at least three independent experiments. $^*p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.001$.

the prevention of atherosclerosis and CHD [24–27]. Several studies have reported that the antiatherogenic effect of olive oil is related to the antioxidant and anti-inflammatory effects exerted by various components, especially monounsaturated fatty acids (MUFA) and polyphenols [11, 17, 28–30]. Phenolic compounds, especially hydroxytyrosol and oleuropein, dose-dependently inhibit LDL and HDL oxidation *in vitro* and *in vivo*, repress superoxide-driven reactions, and break the chain-like propagation of lipid peroxides [31–34]. Interestingly, a study by Covas et al. [17] showed that consuming EVOO increases the postprandial concentration of phenolic compounds in the plasma and in LDL and HDL, which may explain the protective effect of phenolic compounds.

Plasma HDL-cholesterol levels are markedly and inversely correlated to the risk of atherosclerotic cardiovascular diseases [35]. It has been suggested that HDL facilitates cholesterol efflux from peripheral tissues and transports it back to the liver in a process called RCT [36]. ABCA1 facilitates cholesterol efflux from cells to lipid-poor apo-AI but not to HDL [7, 37], whereas another ABC transporter, ABCG1, as well as the SR-BI receptor, is involved in cholesterol efflux from macrophages to HDL [38, 39]. Some studies have suggested that food nutrients and diet may play pivotal roles in the regulation of RCT [25, 40–42]. We have previously shown that EVOO consumption improves the RCT process by enhancing the capacity of HDL to mediate cholesterol efflux and of human monocyte-derived macrophages (HMDM) to excrete free cholesterol [43]. In the present study, we investigated how the consumption of EVOO may promote cholesterol efflux. We focused on the effect of EVOO-PC, especially essential phenols such as tyrosol and hydroxytyrosol.

Our results showed that enriching LDL and HDL with EVOO-PC results in an increase in the resistance of LDL and HDL to lipid peroxidation. This effect may be due to the antioxidant effect of the phenolic compounds, which may scavenge reactive oxygen species and thus inhibit lipoprotein oxidation [16, 44, 45]. Incubating plasma with EVOO-PC increased the binding of polyphenols to LDL and HDL lipoproteins, as previously reported by Covas et al. [17, 46] and Lamucla-Raventós et al. [47]. Moreover, in a recent study, Hernáez et al. [48] showed that olive oil polyphenols increase the size of HDL particles, enhance the stability of HDL by generating a triglyceride-poor core, and enhance the antioxidant status of HDL by increasing the olive oil polyphenol metabolite content of the lipoprotein. Olive oil polyphenols are highly bioavailable, which provides further support for their putative health-promoting effects (reviewed in [49, 50]). However, very few studies have been conducted on the effect of phenolic compounds on RCT. Our results showed that EVOO-PC-enriched HDL promotes RCT by enhancing cholesterol efflux from THP-1-derived macrophages. This effect may be related to an improvement in the physicochemical properties of HDL by increasing their phenol content, which protects HDL from oxidation, and by increasing the fluidity of the phospholipidic layer. Indeed, we previously

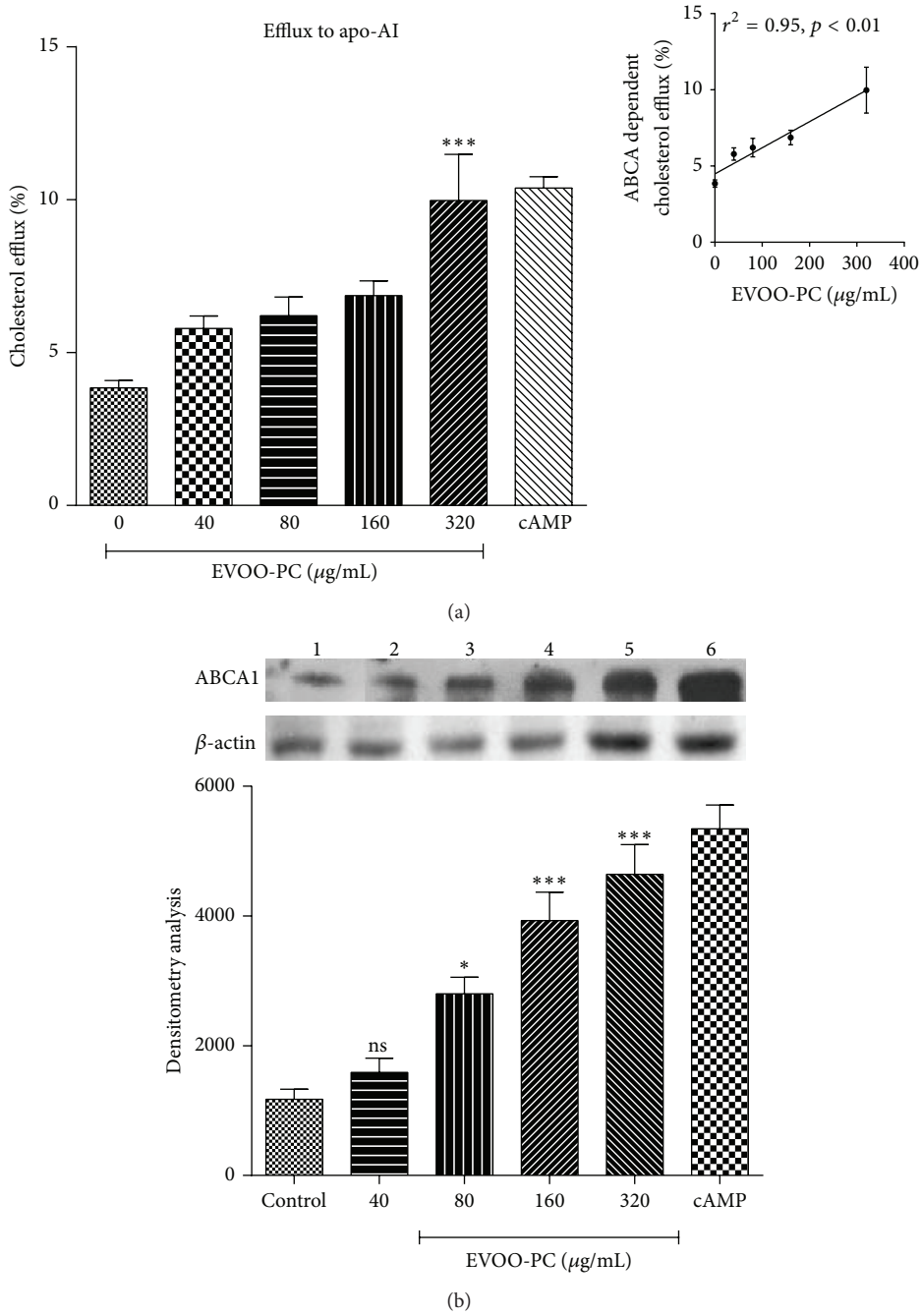

Figure 3: EVOO-PC increases ABCA1 protein expression and enhances apoA-I-mediated cholesterol efflux. (a) [^{3}H]-Cholesterol-loaded J774 macrophages were incubated for 12 h with various concentrations of EVOO-PC (0 to 320 μg/mL) or with cAMP (positive control) to generate ABCA1-enriched cells, which were incubated with 25 μg/mL of apo-AI for 4 h. The small upper panel shows the positive correlation between EVOO-PC concentrations and ABCA1-dependent cholesterol efflux. (b) ABCA1 protein expression after incubating J774 macrophages with increasing concentrations of EVOO-PC as determined by densitometric analyses of protein bands on PVDF membranes. Results are expressed as the means ± SEM of at least three independent experiments. $^*p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.001$.

showed that polyphenol compounds from argan oil (a polyphenol-rich vegetable oil) also enhance HDL-mediated cholesterol efflux by improving HDL fluidity and increasing HDL binding to cell membranes [16]. In the present study, we also investigated the effect of EVOO-PC on cholesterol efflux from THP-1-derived macrophages stressed by Fe/Asc, which induces lipid peroxidation [51] and reduces cholesterol efflux. Pretreating macrophages with EVOO-PC before incubating them with Fe/Asc significantly restored cholesterol efflux from macrophages to HDL, likely by suppressing the effect

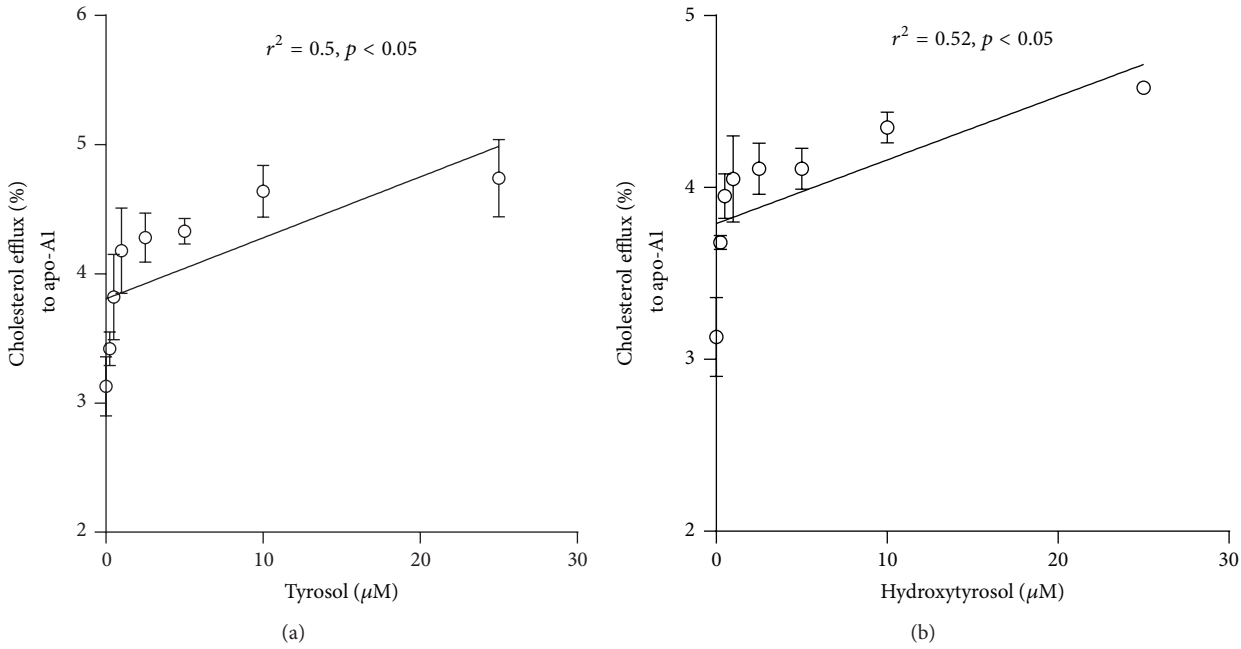

Figure 4: Tyrosol and hydroxytyrosol increase ABCA1 protein expression and enhance apoA-I-mediated cholesterol efflux. [^{3}H]-Cholesterol-loaded J774 macrophages were incubated for 12 h with different concentrations (0 to 25 μM) of tyrosol (a) or hydroxytyrosol (b) to generate ABCA1-enriched cells, which were then incubated with 25 μg/mL of apo-AI for 4 h. Results are expressed as the means ± SEM of at least three independent experiments.

of Fe/Asc on the cell surface receptors involved in this process. This effect has also been reported with vitamin E and butylhydroxytoluene (BHT), two other antioxidants [23].

However, little is known about the molecular mechanism by which phenolic compounds promote cholesterol efflux. To better understand the mechanism by which EVOO-PC enhances HDL-mediated cholesterol efflux, we investigated the effect of EVOO-PC on cell signaling pathways. Our results clearly showed that EVOO-PC, including tyrosol and hydroxytyrosol, stimulates ABCA1 protein expression in J774 macrophages, which may explain how these phenols promote cholesterol efflux to apoA-1. Uto-Kondo et al. [40] reported that coffee consumption by healthy humans enhances HDL-mediated cholesterol efflux by increasing ABCG1 and SR-BI but not ABCA1 expression and that this may be due to the phenolic acids in the coffee. This appears to be unlikely given that phenolic acids activate liver X receptor-α (LXRα) expression, which in turn transactivates both ABCA1 and ABCG1. However, other studies, including ours, have shown that resveratrol stimulates LXRα, ABCA1, and ABCG1 [52, 53]. It thus appears that different phenolic compounds may stimulate cholesterol efflux via different mechanisms.

In conclusion, our results showed that EVOO-PC enhances the antiatherogenic properties of HDL by reducing oxidative modifications to HDL and by maintaining the physicochemical properties of HDL, which in turn improve the functionality of HDL, especially the capacity to promote cholesterol efflux. EVOO-PC also protected cells from oxidative damage and stimulated ABCA1 protein expression, a key factor in cholesterol efflux and HDL genesis. Our results are in agreement with our previous findings showing that the consumption of olive oil polyphenols helps to reduce cardiovascular risk.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Luteolin Ameliorates Hypertensive Vascular Remodeling through Inhibiting the Proliferation and Migration of Vascular Smooth Muscle Cells

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Objectives. Preliminary researches showed that luteolin was used to treat hypertension. However, it is still unclear whether luteolin has effect on the hypertensive complication such as vascular remodeling. The present study was designed to investigate the effect of luteolin on the hypertensive vascular remodeling and its molecular mechanism. *Method and Results*. We evaluated the effect of luteolin on aorta thickening of hypertension in spontaneous hypertensive rats (SHRs) and found that luteolin could significantly decrease the blood pressure and media thickness of aorta *in vivo*. Luteolin could inhibit angiotensin II- (Ang II-) induced proliferation and migration of vascular smooth muscle cells (VSMCs). Dichlorofluorescein diacetate (DCFH-DA) staining result showed that luteolin reduced Ang II-stimulated ROS production in VSMCs. Furthermore, western blot and gelatin zymography results showed that luteolin treatment leaded to a decrease in ERK1/2, p-ERK1/2, p-p38, MMP2, and proliferating cell nuclear antigen (PCNA) protein level. *Conclusion*. These data support that luteolin can ameliorate hypertensive vascular remodeling by inhibiting the proliferation and migration of Ang II-induced VSMCs. Its mechanism is mediated by the regulation of MAPK signaling pathway and the production of ROS.

1. Introduction

Cardiovascular disease is generally regarded as the biggest cause of mortality in the world, and hypertension is mainly associated with increased risk of cardiovascular disease such as coronary artery disease, stroke, and heart failure [1]. An estimated 20% of Chinese adults (>18 years of age) population are in hypertension, exposing these individuals to an increased risk of mortality and cardiovascular events over their lifespan. Under the hypertensive condition, structural remodeling of blood vessels named vascular remodeling has closely participated in the development and maintenance of hypertension and its complications [2–4], which became one of the most serious hypertensive complications.

Vascular remodeling is structural changes of the arterial walls, such as increased intima-media thickness, arterial stiffening, and deteriorating endothelial function [5]. A reduction of lumen ratio and an increase of media-to-lumen ratio are found in almost all hypertensive subjects, as a result of abnormal proliferation, rearrangement of smooth muscle cells, and increased expressions of collagen and fibronectin [6]. Increased arterial wall to lumen diameter ratio may contribute to both enhanced vascular reactivity and vascular stiffness, two cardinal features of hypertension-associated vascular pathology [3, 7]. Furthermore, VSMCs are dynamic, multifunctional cells that act in arterial remodeling through numerous processes, such as cell growth (hyperplasia and hypertrophy), cell migration to the intima, cell apoptosis, reorganization of cells, and altered extracellular matrix composition [8, 9].

Ang II, one of the most important factors in the rennin angiotensin aldosterone system (RAAS), regulates blood

pressure and the volume of circulating blood. Dysregulation of Ang II is an important factor contributing to the pathogenesis of hypertension [10]. It has been regarded as a vasoconstrictor agent, which can directly elicit enhanced vasoconstrictor responses in essential hypertension [11]. More importantly, Ang II binds to angiotensin type 1 receptor (AT_1R) at the cell surface, which induces intracellular generation of reactive oxygen species (ROS) influencing redox-sensitive signaling molecules, such as p38 MAP kinase, ERK1/2, and matrix metalloproteinases (MMPs). The activation of pathways leads to the excessive proliferation and migration of VSMCs, which can cause vascular remodeling [6, 12].

Luteolin (3,4,5,7-tetrahydroxyflavone), a flavonoid, is an important plant compound postulated to be responsible for the biochemical benefits of eating vegetables and fruits and has been reported to possess a variety of biological and pharmacological activities, including antioxidant, anti-inflammatory, anticancer, antiplatelet, and other activities [13–15]. One of the most well-known benefits of luteolin is improving cardiovascular health [16].

Many studies have indicated that luteolin exerted an effect on cardiovascular protection, particularly on hypertension and its related diseases. Accumulating evidences suggested that the blood pressure of rat was directly decreased after oral administration of luteolin [17, 18]. In addition, some researchers found that treatment with luteolin markedly inhibited the impairment of phenylephrine-induced endothelium dependent contraction in aortic rings, which showed that luteolin may be a vascular protective agent [19–21]. Furthermore, a great number of recent advances in cellular biology had demonstrated that luteolin could suppress the proliferation and migration of VSMCs [22]. Endothelial dysfunction is also associated with the pathogenesis of vascular diseases [23, 24], which could be ameliorated by luteolin [25–27]. Hence, these studies showed that luteolin could exert effect on hypertensive vascular remodeling by protection of blood vessel.

In our preliminary experiment, we found that oral administration of luteolin at 25 mg/kg with buddleoside could significantly inhibit the blood pressure on SHR [28]. We further clarified that its effective dosage for antihypertensive treatment is about 75 mg/kg. Nevertheless, it was still uncertain whether luteolin was also effective in hypertensive complication such as vascular remodeling. Therefore, in this study, we employed SHR model to evaluate whether luteolin can inhibit the hypertensive vascular remodeling *in vivo*. Furthermore, we sought to clarify its molecular mechanism of action using Ang II-induced VSMCs model *in vitro*. The purpose of this study was to elucidate the mechanism of luteolin against cardiovascular complications caused by hypertension.

2. Materials and Methods

2.1. Cell Culture and Animals. VSMCs were isolated from thoracic aortas of Sprague-Dawley rats, which were purchased from Animal Supply Center of Zhejiang Academy of Medical Science (certificate number SCXK2008-0033, Hangzhou, China) following the tissue explants method as previously described [29]. VSMCs were allowed to grow from the explants for 7–10 days and grew in a typical "hill-and-valley" pattern. Cells were maintained in RPMI 1640 Medium supplemented with 20% FBS (Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. Early subcultured cells (from passage 2–5) were used in the experiments.

Male SHRs, age of 12 weeks, and Wistar-Kyoto (Wky) rats, age of 12 weeks, were obtained from Vital River Laboratories (certificate number SCXK2012-0001, Beijing, China) and acclimatized for at least two weeks. During this period, the rats were supplied with tap water and rodent laboratory chow ad libitum, as well as a daily health inspection under a controlled room with stable temperature, humidity, and light/dark cycle. All the procedures were in strict accordance following the guidelines for the Use and Care of Laboratory Animals published by the Zhejiang province (2009).

2.2. Drug and Chemicals Preparation. Luteolin (LUT, purity > 98%) was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). Valsartan (Val, purity > 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds for experiments *in vitro* were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions and were kept at −20°C as aliquots. The stock solution was diluted with serum-free medium before use. Valsartan Capsules, which from Beijing Novartis Pharma Co., Ltd. (Beijing, China, CHN Lot: x1542), were dissolved with distilled water into 0.8 mg/mL for positive control.

Luteolin enriched extracts (LUT50, purity > 50%) were extracted from peanut shells purchased from New Nongdu Co., Ltd. (Hangzhou, China) (see Figure 1(a)).

2.3. HPLC-DAD Analysis. All the crude extracts of peanut shells were analyzed with HPLC-DAD. The analysis was similar as described by Lv et al. [28]. Simply speaking, sample concentrations were 6.20 mg of extract in 25 mL of methanol and filtered through a 0.45 μm membrane filter before delivered into the system. The Agilent HPLC1200 (Agilent Technologies Inc., Palo Alto, USA) was used to determinate the content of luteolin in extracts by Kromasil 100-5 C18 (250 mm × 4.6 mm) column. The mobile phase was composed of methanol, water, and acetic acid (50 : 49 : 1, V : V : V). The solvent flow rate was 1 mL/min and the column temperature was set at 25°C. The injection volume was 5 μL. The photodiode array detector was set at 340 nm with a total runtime of 25 min. The HPLC chromatogram of extracts was shown in Figure 1(b).

2.4. Cytotoxicity Assay. MTT was used to measure the viability of VSMCs [30]. VSMCs in the logarithmic growth phase were digested and inoculated in 96-well plates. Each well contained 1.4×10^4 VSMCs in suspension. Cells were placed in serum-free media with various concentrations of LUT (0.1, 0.2, 0.3, 1, 2, 3, 5, 10, 20, and 30 μM) for 24 h. MTT solution (5 mg/mL) was added to each well. Following a 4 h incubation at 37°C, the cell culture medium was removed and 150 μL of DMSO was added to each well. The absorbance of each well was measured with a 96-well microplate reader with

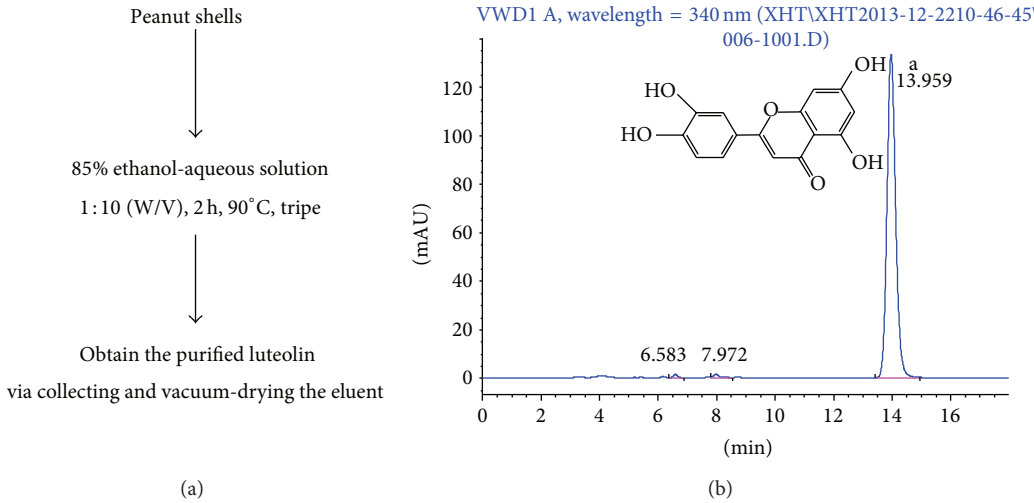

Figure 1: (a) The route diagram of preparing luteolin enriched extracts. (b) HPLC chromatogram of luteolin enriched extracts detected at 340 nm. Peak a was identified to be luteolin.

the detection wavelength set at 570 nm. The viability of cells in the experimental groups was expressed as a percentage of the viability of control cells (which was taken to be 100%).

2.5. Cell Proliferation Assay. The effect of luteolin on cell proliferation was estimated with a modified MTT assay as described in the paper. VSMCs (1.4×10^4 cells/well, 50–70% density) were counted and seeded in 96-well plates. Cells were stimulated with 1 μM Ang II (Merck KGaA, Darmstadt, Germany) in the absence or presence of LUT (1, 2, 3, 5, 10, 20, and 30 μM) during 24 h and followed the conventional procedure. The absorbance of 96-well culture plates was measured at 570 nm.

2.6. Crystal Violet Viability Assay. Experimentally, VSMCs (8.0×10^4 cells/well, 50–70% density) were seeded in 24-well plates. VSMCs were incubated with or without LUT (5, 10, and 20 μM) in the presence of Ang II (1 μM) for 24 h. At 24 h after treatment, cells were carefully washed with PBS and stained with 0.5% crystal violet formalin solution at room temperature for 20–30 min. The stained cells were washed with tap water and air-dried for taking macrographic images. For quantitative measurement, the stained cells were dissolved in 20% acetic acid at room temperature for 20 min with shaking. Absorbance at 570 nm was measured.

2.7. Monolayer-Wounding Cell Migration Assay. To evaluate the impact of luteolin on cell migration ability, a wound-healing model was used [31]. VSMCs (8.0×10^4 cells/well, 50–70% density) were seeded in 24-well plates and grew to be subjected to wounding. Then cell layers were wounded with a sterile 200 μL pipette tip. After washing away suspended cells, different concentrations of LUT (5, 10, and 20 μM) with Ang II (1 μM) were added into wells for 24 h. Images were photographed in each well at 100x magnification before and after 24 h drug treatment and analyzed with Image-Pro Plus 5.1 software. Average scraped width per well before and after 24 h drug treatment was measured. Migration distance was estimated based on the scraped width of well before and after drug treatment.

2.8. Boyden Chamber Transwell Migration Assay. The migration of the cultured cells was examined using a transwell chamber with a polycarbonate membrane (8 μm pores) [32]. The VSMCs were suspended in serum-free RPMI 1640 (2.4×10^5 cells/mL). Then a 250 μL cell suspension (containing different concentrations of LUT simulated by Ang II) was added to the upper chamber, with 10% FBS RPMI 1640 Medium (500 μL) placed in the lower chamber in the absence of cells. The transwell plate was incubated at 37°C in 5% CO_2 for 24 h. The cells migrated through the micropores, and the migrated cells attached to the lower surface of the transwell filter. After 24 h, the inserts were washed with PBS; upper surface cells were removed by cotton swabs and the lower side was fixed in 4% paraformaldehyde. The migrated cells were then stained with propidium iodide (PI). Three visual fields that were randomly selected from each of the transwell filters were captured at 200x magnification with an inverted fluorescence microscope, and the average number of cells that migrated through the transwell filters was counted under Image-Pro Plus 5.1 software.

2.9. Cell Cycle Analysis by Flow Cytometry. Cell cycle regulation was determined by flow cytometry [33]. Cells (4.0×10^5/well) were plated into dishes (60 × 15 mm) 1 day before treatment with LUT (5, 10, and 20 μM) in the presence of Ang II. After treatment for 24 h, cells were harvested, washed with PBS, fixed in cold 70% alcohol overnight at −20°C for at least 2 h, and stained with 50 ng/mL PI in the presence of 200 μg/mL RNase A by incubation at 37°C for at least 30 min. The stained cells were analyzed by flow cytometry (Millipore). Data were analyzed using FlowJo 7.6.1 software.

2.10. ROS Assay. ROS was detected under the manual's direction of ROS detection kit (Beyotime, Shanghai, China)

[34]. Experimentally, cells (1×10^5/well) were seeded in dishes (35×10 mm). And VSMCs were pretreated with the indicated concentration of LUT for 24 h. At 24 h after pretreatment, cells were stained with 40 μM DCFH-DA by incubation at 37°C for 20 min. Then Ang II (1 μM) was added to the dishes for 5 min. Finally, pictures were captured at 100x magnification with an inverted fluorescence microscope, and the intensity of fluorescence was analyzed and quantified using Image-Pro Plus 5.1 software.

2.11. Animal Treatments. Seven Wky rats and twenty-eight SHRs were randomly assigned to five groups. The first group (group 1, G1) and the second group (group 2, G2) were, respectively, set as Wky control group and SHR control group, both of which were given distilled water by oral administration. Valsartan (8 mg/kg, p.o.) was given to the third group (group 3, G3) for 6 weeks daily. The fourth group (group 4, G4) and the fifth group (group 5, G5) were received LUT50 (at the doses of 75 and 150 mg/kg, p.o., resp.) for 6 weeks. Throughout the experiment, body weight was evaluated.

2.12. Blood Pressure Measurement. In these five groups, doses were administered orally using an oral tube once daily for 6 consecutive weeks, and blood pressure was measured after administration at 6th weeks. Using a noninvasive method of tail-cuff plethysmography (Shanghai Alcott Biotech Co., Ltd., Shanghai China), the systolic, diastolic, and mean arterial blood pressures (SBP, DBP, and MAP for short) were measured at 2 h after administration. Each animal was placed in a 28°C warmer for several minutes. For each time point, four continuous blood pressure values were tested and averaged.

2.13. Enzyme-Linked Immunosorbent Assay (ELISA) for Ang II. At the end of the treatment, all rats were fasted overnight and the blood samples were collected via the rat ophthalmic venous plexus. All of the blood samples were centrifuged at 3500 rpm for 10 min, and the serum was separated to determine Ang II activity by the method of ELISA. All of the procedures were performed as described in the assay kit (Shanghai Xinfang Biological Pharmaceutical Technology Co., Ltd., Shanghai, China).

2.14. Histological Evaluation. The thoracic aortas were resected and placed in 4% neutral buffered formalin. After fixation, tissues were paraffin-embedded and cut into 4 μm sections. Then sections were stained with hematoxylin and eosin (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China) [35]. Images were captured with the microscope (40x). The thickness of the aorta was measured with Image-Pro Plus 5.1 software. The media thickness was determined by measuring the distance from the internal elastic lamina to the external elastic lamina. For each slide, measurements from 4 points (12, 3, 6, and 9 o'clock positions) were averaged. The lumen inner diameter was determined from 2 points (12 and 9 o'clock positions). The media-to-lumen ratio was calculated based on the measured lumen inner diameter and media data.

2.15. Gelatin Zymography. The thoracic aorta samples were homogenized in RIPA buffer (Solarbio, Beijing, China). After centrifugation, clear supernatant was collected. Tissue protein was mixed with 5× nonreducing sample buffer and loaded onto 7.5% polyacrylamide gels containing 0.1% gelatin gels (20 μL/sample), and electrophoresis was performed at 100 V for 4 h at 4°C [36]. After electrophoresis, the gel was rinsed with washing buffer for 1.5 h with shaking at room temperature. The buffer was then changed to incubation buffer and incubated for 48 h at 37°C. Gelatin gel was stained with coomassie blue and then destained with 10% acetic acid. The unstained bands correspond to the areas of gelatin digestion.

2.16. Cell Protein Extraction and Western Blotting Analysis. Cells were collected and lysed in RAPI buffer (Solarbio, Beijing, China) with protease/phosphatase inhibitor (Cell Signaling Technology, Canada). After treatment on ice for 30 min, lysates were clarified by centrifugation at 12000 rpm for 15 min at 4°C and the protein content was measured using a BCA protein assay kit (Beyotime, Jiangsu, China). Sample protein was mixed with 5× Loading Buffer (Beyotime, Jiangsu, China). The western blot was similar as described by Gao et al. [36]. In brief, the samples were separated by SDS-PAGE and electrotransferred onto a polyvinylidene-difluoride membrane (Pall Corporation, Mexico). The membrane was blocked with BSA blocking buffer for two hours at room temperature, incubated overnight at 4°C with interest primary antibodies (Santa Cruz Biotechnology, USA, or Cell Signaling Technology, Canada) in PBST. After washing, the membrane was incubated with an appropriate secondary antibody (Santa Cruz Biotechnology, USA) for 30 min. The membrane was incubated with streptavidin HRP (Thermo, USA) for 30 min after washing. The blotted protein bands were detected by Chemiluminescent Substrate kit (BIO-RAD, USA).

2.17. Statistical Analysis. All values were expressed as mean ± standard deviation and subjected to one-way analysis of variance (ANOVA) by using SPSS 17.0 for windows. The LSD t-tests will be applied when homogeneity of variance assumptions is satisfied; otherwise, the Dunnet t-test will be used. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Luteolin Inhibits Ang II-Induced VSMC Proliferation. To clarify the effects of luteolin on vascular remodeling *in vitro*, rat aortic smooth muscle cells were explanted and subjected to examination. We examined the cytotoxicity of luteolin and its inhibitory effects on cell viability in VSMCs with stimulation of Ang II using MTT assay and crystal violet staining to assess the antiproliferation effect.

The cytotoxicity of luteolin was presented in Figure 2(a). The viability of cell administrated of LUT at 30 μM was markedly inhibited in comparison with control ($P < 0.05$). However, other groups' cell viability had no significant difference compared to control. The results suggested that a cytotoxic effect of luteolin was at a concentration of up to 30 μM. In addition, as shown in Figure 2(b), 1 μM Ang II significantly

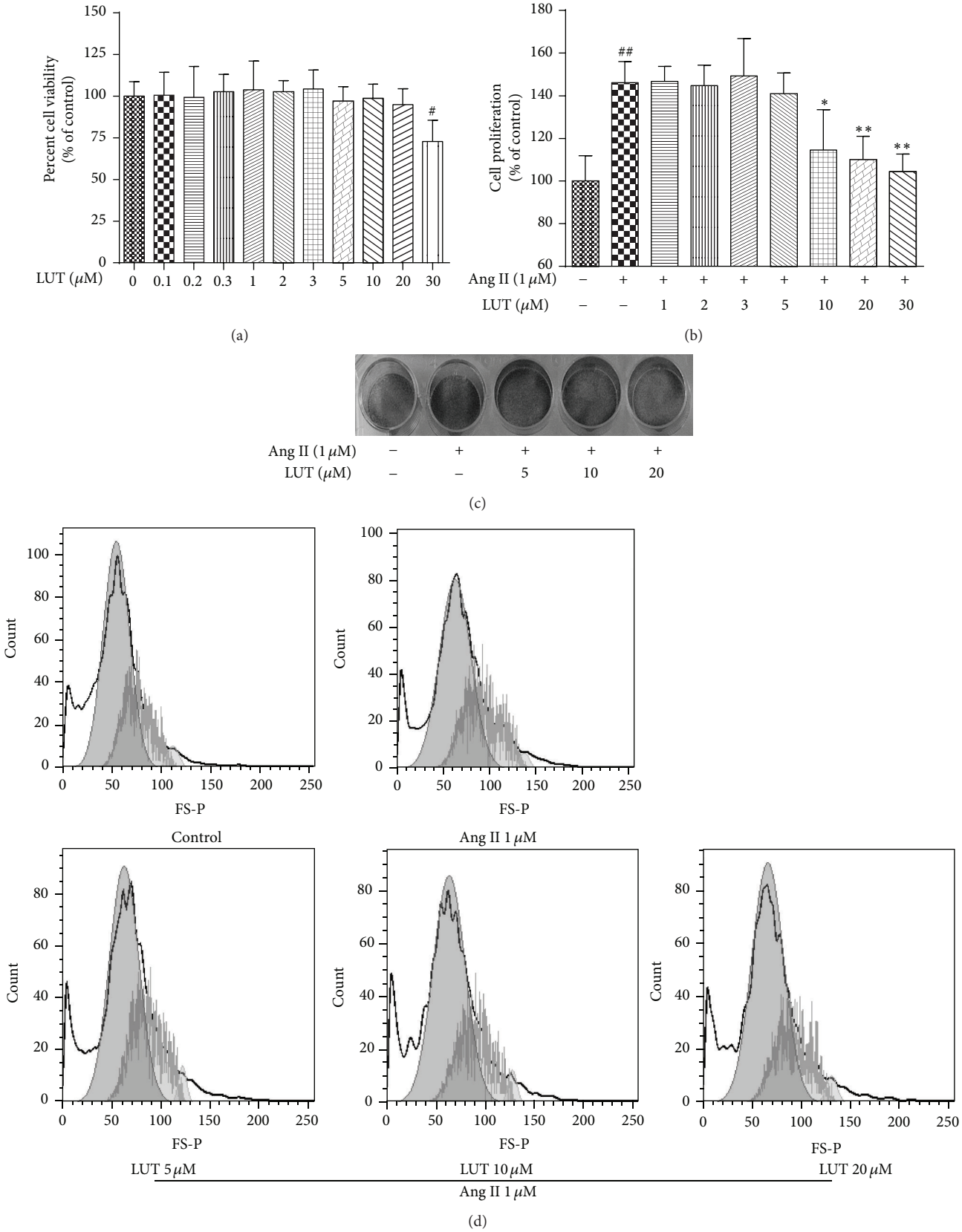

Figure 2: The inhibitory effects of luteolin on the Ang II-induced proliferation of VSMCs. (a) Cytotoxicity of luteolin on VSMCs. The data were expressed as mean ± SD ($n = 8$). (b) Antiproliferative activity of luteolin in VSMCs by MTT assay. The data were expressed as mean ± SD ($n = 8$). (c) Antiproliferative activity of luteolin in VSMCs by crystal violet viability assay. (d) Cell cycle phase analysis. $^{\#}P < 0.05$ versus control group; $^{\#\#}P < 0.01$ versus control group; $^{*}P < 0.05$ versus Ang II-treated group; $^{**}P < 0.01$ versus Ang II-treated group.

Table 1: The effect of luteolin on cell cycle of VSMCs stimulated by Ang II.

Distribution of cell cycle	Control	Ang II 1 μM	Ang II (1 μM)		
			LUT 5 μM	LUT 10 μM	LUT 20 μM
G_0/G_1	70.56%	68.09%	67.53%	69.79%	72.97%
G_2/M	3.15%	1.87%	2.83%	2.68%	2.79%
S	25.63%	30.22%	29.82%	24.72%	24.27%

stimulated VSMCs proliferation compared with the control group ($P < 0.01$). However, the action of Ang II was inhibited by LUT at the concentrations of 10 μM ($P < 0.05$), 20 μM, and 30 μM ($P < 0.01$). LUT (<10 μM) did not have any remarkable effect on VSMCs' proliferation. Taking into account the fact that LUT at 30 μM had a cytotoxic effect, LUT at 5 μM, 10 μM, and 20 μM was used in the following experiments. Furthermore, the result of crystal violet staining illustrated in Figure 2(c) proved that Ang II-induced VSMCs proliferation was suppressed by LUT.

To examine the possible mechanisms behind luteolin's inhibition effect on VSMCs' proliferation, we performed cell cycle analysis by FACS. As shown in Figure 2(d) and Table 1, 1 μM Ang II resulted in an accumulation of cells in the S phase, from 25.63% to 30.22%, and an attenuation of cells in G_2 phase, from 3.15% to 1.87% compared to control. When VSMCs stimulated by 1 μM Ang II were treated with LUT for 24 h, LUT at 10 μM induced a depletion of cells in the S phase, from 30.22% to 24.72%, and a concomitant accumulation of cells in G_2 phase, from 1.87% to 2.68%. LUT at 20 μM also induced a depletion of cells in the S phase, from 30.22% to 24.27%, and a concomitant accumulation of cells in G_2 phase, from 1.87% to 2.79%. LUT at 5 μM had not induced significant change. These data suggested that luteolin could inhibit VSMCs' proliferation.

3.2. Luteolin Suppresses Ang II-Induced VSMC Migration. Because VSMCs' migration plays an important role in vascular remodeling and hypertension-associated vascular changes, we determined whether luteolin could suppress Ang II-induced VSMCs migration.

As illustrated in Figures 3(a) and 3(c), VSMCs stimulated by 1 μM Ang II were markedly promoted to migrate from one side of the scratch to another side compared with the control group ($P < 0.01$). We discovered that after VSMCs were treated with LUT for 24 h, the Ang II-induced migration of VSMCs was significantly suppressed by 5 μM, 10 μM, and 20 μM LUT ($P < 0.01$). Meanwhile, the result of the Boyden chamber transwell assay illustrated in Figures 3(b) and 3(d) proved that luteolin could inhibit Ang II-induced VSMCs migration. 1 μM Ang II markedly promoted the migration of VSMCs from the upper chamber to the lower chamber in comparison with the control group ($P < 0.01$). When VSMCs were treated with 5 μM, 10 μM, and 20 μM LUT, the numbers of Ang II-induced migrated cells across the extracellular matrix protein-coated membranes were significantly decreased ($P < 0.01$).

MMPs are responsible for matrix degradation, necessary for efficient cell migration during vascular remodeling. To determine the effect of luteolin on the production of MMPs in rat aorta, gelatin zymography was used. As shown in Figure 3(e), pro-MMP 2 (Figure 3(e), top line) and MMP 2 (Figure 3(e), bottom line) enzyme activities in SHR control group were markedly increased in comparison with Wky control group. We found that the expression of pro-MMP 2 and MMP 2 was apparently reduced after SHRs were administrated by valsartan. Meanwhile, treatment with the dose of 75 mg/kg LUT50, the activation of pro-MMP 2 and MMP 2 were significantly suppressed.

3.3. Luteolin Inhibits Ang II-Induced Oxidative Stress in VSMCs. ROS are prime candidates in the etiology of vascular remodeling and ensuing cardiovascular disease. To elucidate whether Ang II increases ROS generation and whether luteolin ameliorates this effect, we determined ROS production in VSMCs by DCFH-DA staining.

As presented in Figure 4, compared with control group, the Ang II-stimulated VSMCs exhibited impressively increased DCF fluorescence intensity ($P < 0.01$). However, the effect of Ang II was markedly suppressed in VSMCs treated with 5 μM, 10 μM, and 20 μM LUT ($P < 0.01$). Hence, Ang II induced increase of ROS production, which resulted in oxidative stress. Ultimately, this effect of Ang II could be inhibited by luteolin.

3.4. Luteolin Attenuates Hypertension in SHR. To evaluate the direct effect of luteolin on blood pressure of SHR, we examine the blood pressure of SHR after 6 weeks of treatment with LUT50. We had reported that 25 mg/kg LUT50 did not lower the SBP or DBP of the SHRs significantly compared to SHR control group [28]. Hence, in this experiment, the dosage was enlarged to 75 mg/kg and 150 mg/kg.

As shown in Figures 5(a), 5(b), and 5(c), the SBP, DBP, and MBP of SHR control group were all obviously increased in comparison with Wky control group ($P < 0.01$). However, after administration of 8 mg/kg valsartan, the SBP, DBP, and MBP of SHR control group were all significantly decreased ($P < 0.01$). Both 75 mg/kg and 150 mg/kg LUT50 also showed a tendency to decrease the SBP, DBP, and MBP of SHRs ($P < 0.01$). Compared to SHR control group, the SBP of 75 mg/kg LUT50 group was decreased 19 mmHg, and the SBP of 150 mg/kg LUT50 group was reduced 16 mmHg. Furthermore, 75 mg/kg LUT50 markedly decreased DBP 8 mmHg of SHR, and 150 mg/kg LUT50 also significantly declined DBP 11 mmHg of SHR. These data suggested that lower dosage of

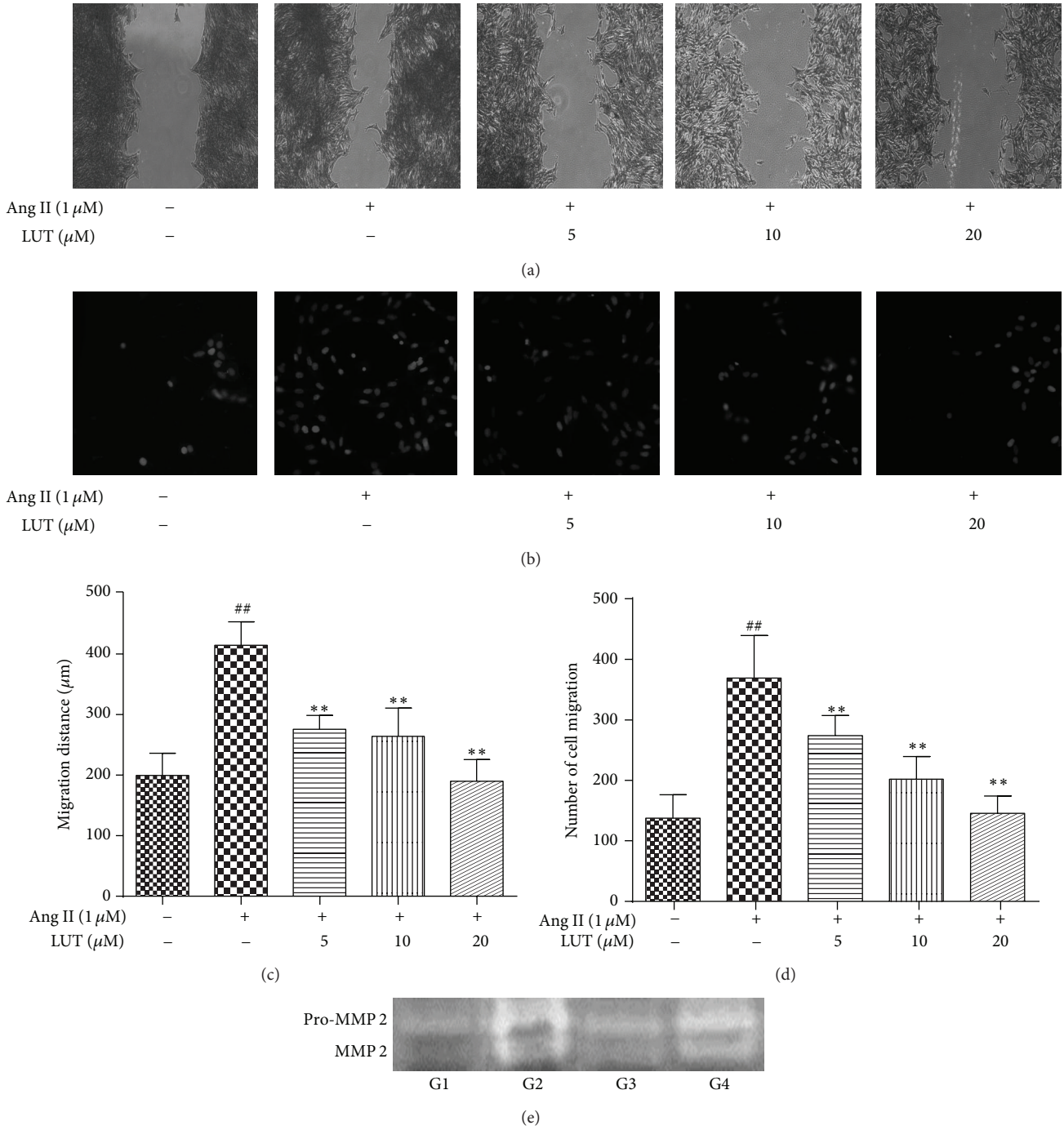

Figure 3: The inhibitory effects of luteolin on the Ang II-induced migration of VSMCs. (a) Monolayer-wounding cell migration assay. Views were photographed along the scraped line in the well at 100x magnification. (b) Boyden chamber transwell migration assay. Images were captured at 200x magnification. (c) The migration distance was tested by monolayer-wounding cell migration assay. The data were expressed as mean ± SD ($n = 9$). (d) The number of cell migration was tested by Boyden chamber transwell migration assay. The data were expressed as mean ± SD ($n = 9$). (e) Gelatin zymography. $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus Ang II-treated group. G1 = Wky control group, G2 = SHR control group, G3 = valsartan group, and G4 = 75 mg/kg LUT50 group.

luteolin was good at decreasing the SBP, while that higher dosage of luteolin was adept in declining the DBP. No matter what dose was more effective, it is clear that luteolin treatment attenuated hypertension of SHR.

3.5. Luteolin Improves Rat Aorta Vascular Remodeling. The media-to-lumen ratio was used as an index of aortic vascular remodeling. Hence, to evaluate the effect of luteolin on vascular remodeling of SHR, we determined the media thickness

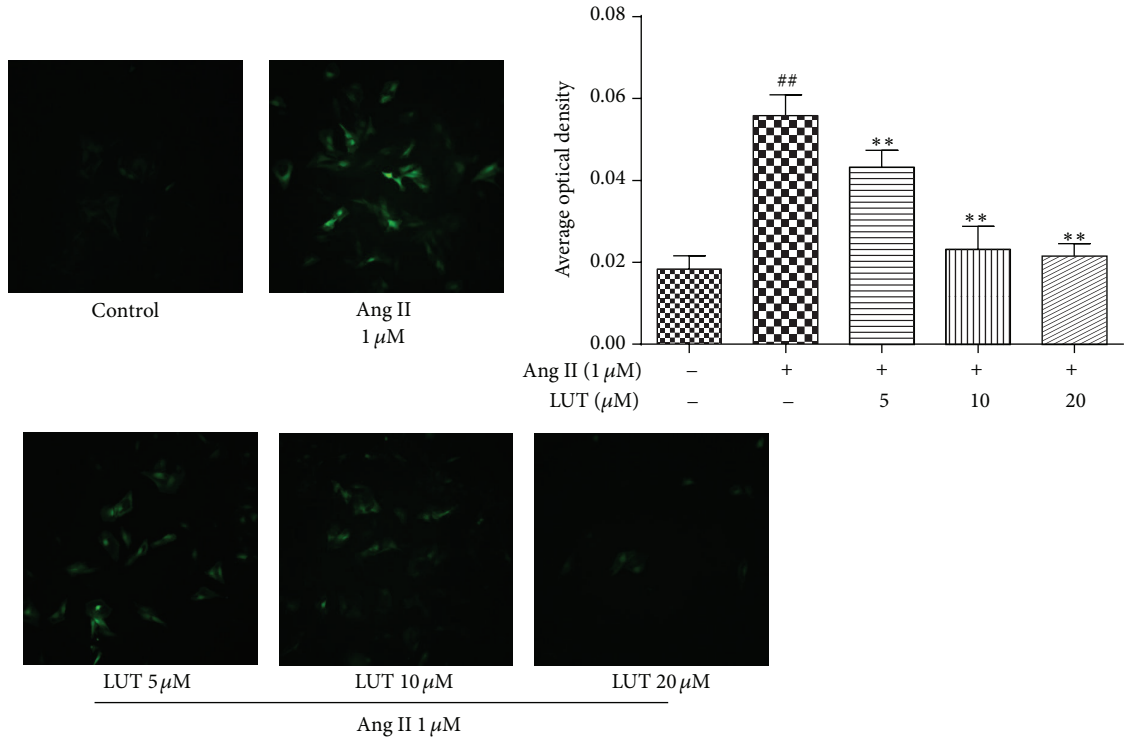

Figure 4: The protective effect of luteolin on Ang II-induced oxidative stress in VSMCs. Images were captured at 100x magnification. The data were expressed as mean ± SD ($n = 9$). $^{\#\#}P < 0.01$ versus control group; $^{**}P < 0.01$ versus Ang II-treated group.

of vascular wall and lumen inner diameter in rat aorta using the HE staining.

The HE staining for rat aorta tissues was presented in Figure 6. As shown in Figure 6(b), the media thickness of vascular wall in SHR was significantly increased compared to Wky control group ($P < 0.01$). The increased media thickness in SHR indicated the progressive worsening of thoracic aorta vascular remodeling by hypertension. However, 8 mg/kg valsartan treatment markedly decreased the media thickness in SHR ($P < 0.01$). Meanwhile, it is pleasant that the media thickness in SHR was apparently decreased after administration of 75 mg/kg LUT50 ($P < 0.01$). Of note, as illustrated in Figure 6(c), there was no significant difference in the lumen inner diameter between SHR and Wky control group with or without treatment with LUT50. Furthermore, as shown in Figure 6(d), there were significant changes in the media-to-lumen ratio of SHR control group in comparison with Wky control group ($P < 0.01$). As excepted, the media-to-lumen ratio was markedly declined in the 75 mg/kg LUT50 or 8 mg/kg valsartan group compared to the SHR control group ($P < 0.01$).

3.6. The Potential Drug Targets for Antiremodeling by Luteolin. Ang II, a well-known activator of this signaling pathway, plays a critical role during hypertensive vascular remodeling. In addition, the mitogen-activated protein kinase (MAPK) cascade, particularly the p38 MAP kinase, may play a role in mediating responses that are related to vascular remodeling. To explore the antiremodeling mechanisms of luteolin, we examined the expression of related factors using western blot and ELISA.

Firstly, we detected the Ang II level in serum by ELISA. As shown in Figure 5(d), compared to Wky control group, Ang II level in serum of SHR was apparently increased ($P < 0.05$). Conversely, Ang II level of 8 mg/kg valsartan group was significantly decreased in comparison with SHR control group ($P < 0.01$). As expected, 75 mg/kg LUT50 markedly diminished Ang II level in serum of SHR ($P < 0.01$).

Consequently, we examined the expression of related factors in VSMCs stimulated with Ang II for 5 min by western blot, including ERK1/2, p38, p-ERK1/2, p-p38, and PCNA. The results of western blot were presented in Figure 7. The protein expressions of ERK1/2, p38, p-ERK1/2, and p-p38 were found to be significantly higher in Ang II-treated groups compared to control. Furthermore, PCNA, a downstream factor of MAPK, was markedly upregulated. However, LUT treatment significantly inhibited the phosphorylation of ERK1/2 and p38 and decreased their activation, especially in the high concentration of LUT at 20 μM. These results demonstrated that luteolin could effectively attenuate vascular remodeling through the mechanisms of downregulating the expression of Ang II, as well as suppressing the phosphorylation of ERK1/2 and p38.

4. Discussion

Luteolin has been shown to exhibit antihypertension activity in many experiments. Ichimura and coworkers had reported that orally administered luteolin (50 mg/kg), which is one of

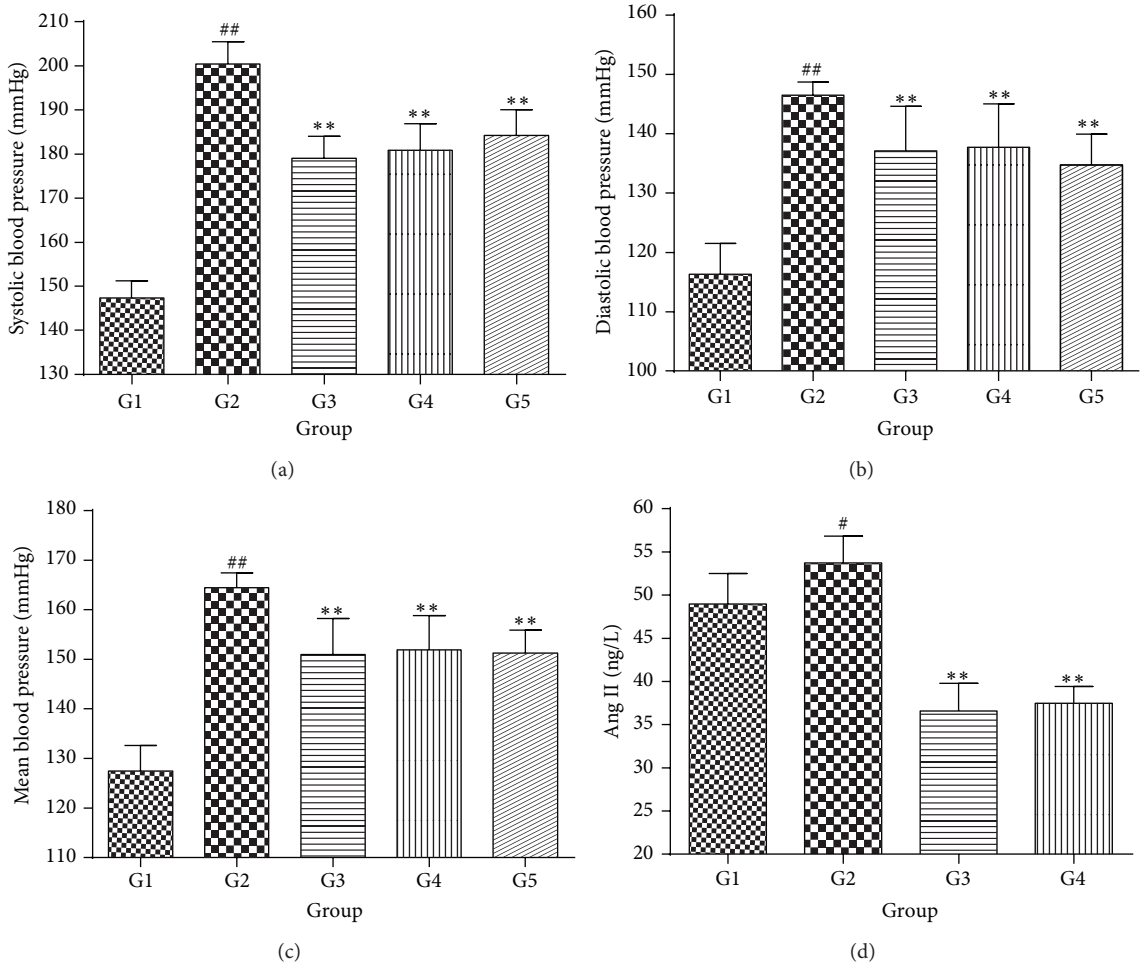

Figure 5: The antihypertensive effect of luteolin extracts in SHRs. (a) The effect on SBP. The data were expressed as mean ± SD ($n = 7$). (b) The effect on DBP. The data were expressed as mean ± SD ($n = 7$). (c) The effect on MBP. The data were expressed as mean ± SD ($n = 7$). (d) The Ang II level in serum. The data were expressed as mean ± SD ($n = 6$). $^{\#}P < 0.05$ versus Wky control group; $^{\#\#}P < 0.01$ versus Wky control group; $^{**}P < 0.01$ versus SHR control group. G1 = Wky control group, G2 = SHR control group, G3 = valsartan group, G4 = 75 mg/kg LUT50 group, and G5 = 150 mg/kg LUT50 group.

consistent polyphenols of the extract, significantly lowered systolic blood pressure in SHRs [18]. In some experiments, aortic rings were precontracted with phenylephrine (PE) to investigate the vasoactive effects of luteolin and its mechanisms of action on the rat thoracic aorta. They all founded that treatment with luteolin markedly inhibited the impairment of PE-induced endothelium dependent contraction in aortic rings [19–21]. Furthermore, a great number of evidences had demonstrated that luteolin suppressed the proliferation and migration of VSMCs [22]. Kim et al. tested the effects of luteolin on rat VSMCs in culture and found the antiproliferation of luteolin could act through downregulation of ERK1/2 cascade [37, 38].

As mentioned above, luteolin exhibits significant antihypertension activity as well as its inhibitory effect on the proliferation and migration of VSMCs. However, the effect of luteolin on hypertensive complication especially vascular remodeling and the molecular mechanisms are not fully understood. In the present study, we used SHR model to examine the blood pressure and the thickness of the aorta to evaluate whether luteolin could ameliorate the hypertensive vascular remodeling *in vivo*. Meanwhile, to identify these molecule's mechanisms of hypertensive vascular remodeling, we carried out the VSMCs' proliferation, migration, and oxidative stress by Ang II stimulation.

Results from the present study demonstrated that luteolin could decline the blood pressure and media thickness of vascular wall of SHR. It is pleasant that treatment with higher dosage of luteolin, the blood pressure of SHR was significantly decreased. Interestingly, the blood pressure of SHR was also markedly diminished, administration of lower dosage of luteolin. Of note, there was no significant difference among these four groups in inner diameter. However, the media thickness and media-to-lumen ratio were markedly declined by luteolin treatment. The media-to-lumen ratio was used as an index of aortic vascular remodeling [7]. Hence, luteolin can ameliorate hypertension and hypertensive vascular remodeling.

We investigated the molecular mechanisms underlying the antiremodeling activity of luteolin. The data showed that luteolin could inhibit VSMCs' proliferation and migration

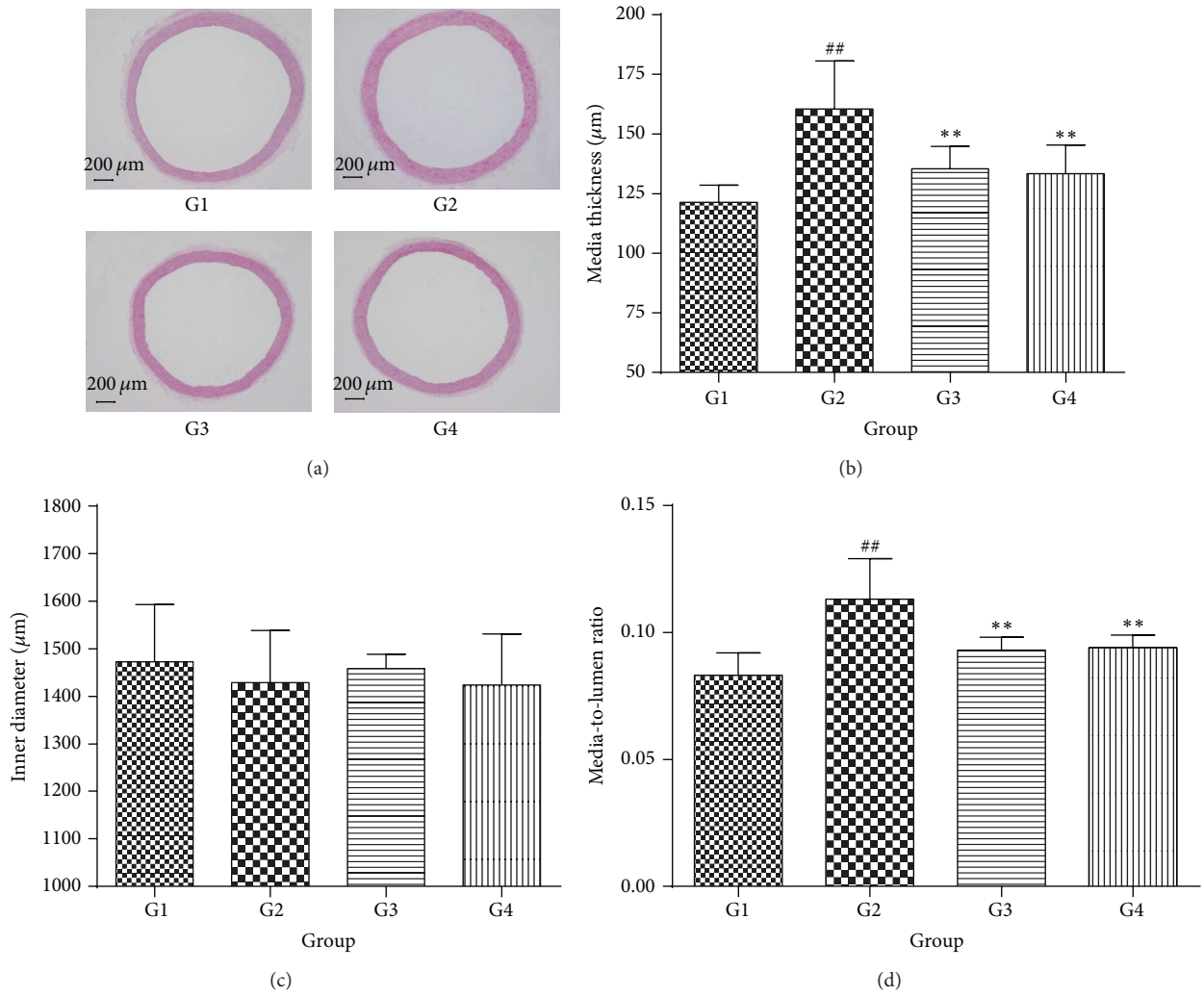

FIGURE 6: Effect of luteolin on rat aortic remodeling. The data were expressed as mean ± SD ($n = 6$). (a) Representative images of vessel sections stained with HE staining at 40x magnification. (b) The media thickness. (c) The inner diameter. (d) The media-to-lumen ratio. $^{\#\#}P < 0.01$ versus Wky control group; $^{*}P < 0.05$ versus SHR control group; $^{**}P < 0.01$ versus SHR control group. G1 = Wky control group, G2 = SHR control group, G3 = valsartan group, and G4 = 75 mg/kg LUT50 group.

induced by Ang II. Meanwhile, luteolin treatment group exhibited a decreased level of ERK1/2, p-ERK1/2, p-p38, MMP-2, and PCNA protein and reduced ROS generation. These results strongly suggest that the inhibitory effect of luteolin on vascular remodeling may be at least in part mediated by inhibiting VSMCs proliferation through depressing the activation of ERK1/2, p38 and downregulating the expression of PCNA, or regulating VSMCs migration factors including ROS and MMP-2, although further investigation is required.

One of the key mechanisms involved in hypertensive vascular remodeling is the proliferation and migration of VSMCs. Ang II is a potent promoter of VSMCs proliferation and migration and has been implicated in vascular remodeling [34, 39]. As known, Ang II binds to AT_1R at the cell surface and induces intracellular ROS generation influencing redox-sensitive signaling molecules, such as p38, ERK1/2, and MMPs [6]. On the other hand, Ang II binding to AT_1R can directly activate all four of the major MAP kinases, including ERK1/2, p38, c-Jun NH2-terminal kinases (JNK), and ERK5 [12]. The activation of pathways leads to the excessive proliferation and migration of VSMCs, which can cause vascular remodeling [40].

In our studies, treatment with Ang II significantly increased the proliferation of VSMCs and promoted the migration of VSMCs from the upper chamber to the lower chamber. However, the effect of Ang II was suppressed by luteolin treatment. As a consequence, luteolin inhibited the Ang II-stimulated proliferation and migration of VSMCs, which was thought to be the major reason that the vascular remodeling was suppressed by luteolin administration.

It is well known that VSMCs proliferation plays major roles in vascular remodeling [41]. MAPK family is best characterized of the many growth-signaling pathways [42]. ERK1/2 is a key growth signaling kinase, which has been implicated in proliferation and migration of VSMCs [43]. Activated p38

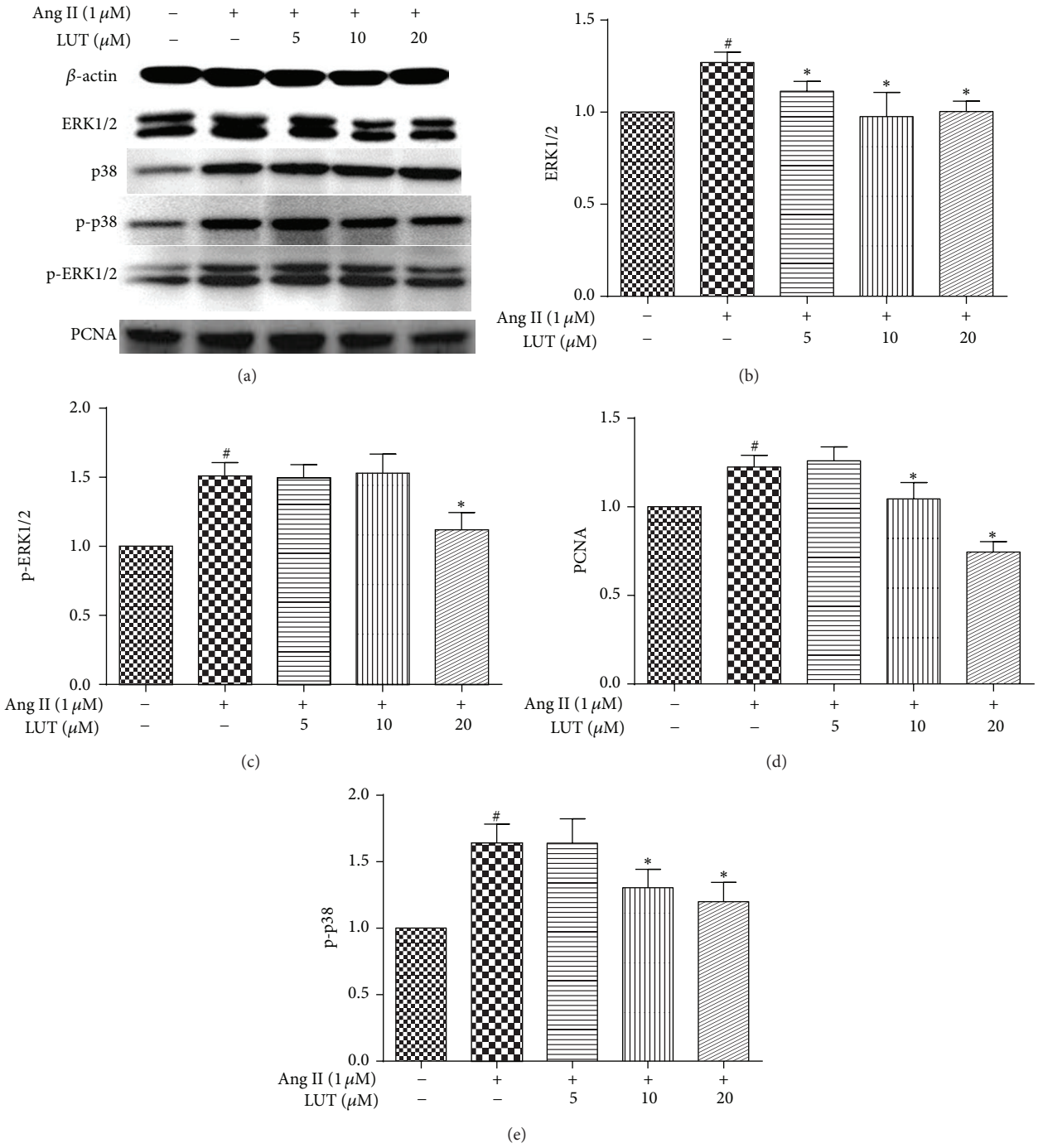

Figure 7: The potential drug targets for antiremodeling by luteolin. The data were expressed as mean ± SD ($n = 3$). $^{\#}P < 0.05$ versus control group; $^{*}P < 0.05$ versus Ang II-treated group.

can also affect cell proliferation, differentiation, and cytokine synthesis by upregulating the expression of transcription factors [44]. Ang II-stimulated activation of ERK1/2 and p38 is augmented [45]. These processes have been associated with enhanced vascular smooth muscle cell growth, inflammation, and fibrosis, as well as increased vascular contractility [6]. Pleasantly, we found that luteolin could significantly inhibit Ang II-induced activation of ERK1/2, p38.

PCNA is an intranuclear 36 kD polypeptide whose expression and synthesis are linked with cell proliferation [46]. PCNA expression is widely used as a marker of cell proliferation. In our study, we demonstrated that Ang II increased the expression of PCNA, which was suppressed by luteolin administration. Taken together, luteolin suppressed the activation of ERK1/2 and p38 and then declined the PCNA expression and regulated cellular proliferation in VSMCs.

Moreover, ROS plays a crucial role in Ang II-induced proliferation and migration of VSMCs [47, 48]. In the present study, we confirmed the increase in oxidative stress on cultured VSMCs stimulated by Ang II. Luteolin administration inhibited the Ang II-induced oxidative stress detected by DCFH-DA staining. As known, ROS production leads to oxidative stress, which can activate MMPs [49]. MMPs are a family of structurally related, zinc-containing enzymes that degrade the extracellular matrix and connective tissue proteins. The proteolytic effects of MMPs play an important role in cellular migration and vascular remodeling [50, 51]. We found the increase in the activity of both Pro-MMP 2 and MMP-2 in SHR. However, luteolin administration decreased Pro-MMP 2 and MMP-2 enzyme activities detected by gelatin zymography.

Another important mechanism of attenuating hypertensive vascular remodeling of luteolin is that it can suppress RAAS system, directly resulting in the decrease of Ang II expression. The renin-angiotensin system plays an important role in regulating pathophysiological processes of cardiovascular disease. Ang II, one of the most important factors in the RAAS, is a potent vasoactive peptide that causes blood vessels to constrict, resulting in increased blood pressure [28]. Meanwhile, Ang II plays a crucial role in promoting vascular remodeling [52]. In our studies, administration of luteolin, increased Ang II level in serum of SHR, was apparently declined.

Taken together, as shown in Figure 8, our studies indicate that luteolin is a potential inhibitor of hypertensive vascular remodeling by the inhibitory effect on the proliferation and migration of VSMCs.

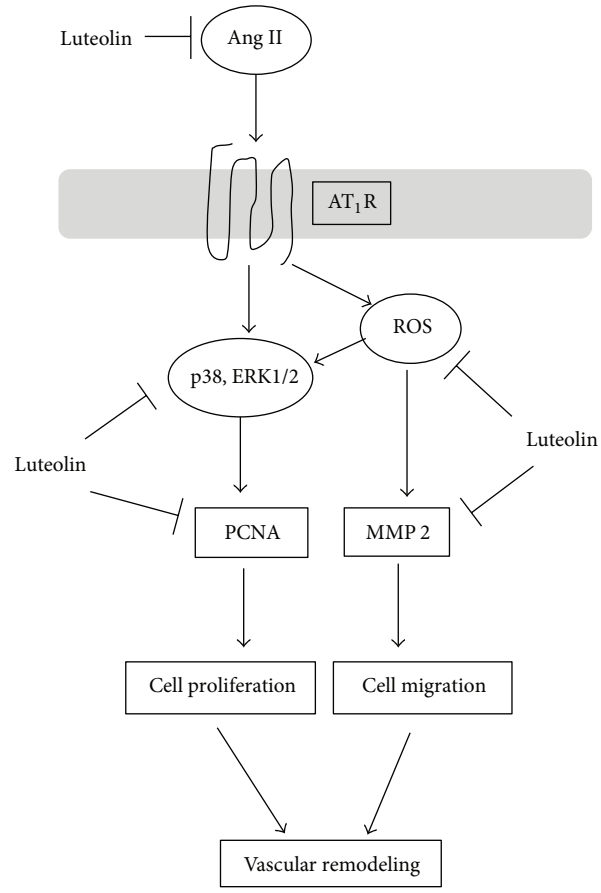

Figure 8: The influence of luteolin on antiremodeling related factors.

5. Conclusion

In the present study, the potential antiremodeling and antihypertensive effect of luteolin are investigated. The results suggest that luteolin is capable of directly lowering arterial blood pressure of SHR. Furthermore, luteolin can decline media thickness of vascular wall in SHR and attenuate hypertensive vascular remodeling. The antiremodeling effect of luteolin is partially associated with the suppression of VSMCs' proliferation and migration, which owes to the depression of RAAS system, directly resulting in the decrease of Ang II expression, as well as the regulation of ROS production and MAPK pathway. In short, luteolin attenuates hypertensive vascular remodeling and has the potential to be an antihypertensive drug.

Conflict of Interests

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

Authors' Contribution

Jie Su and Han-Ting Xu contributed equally to the work.

Acknowledgments

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Research Article

Danhong Promotes Angiogenesis in Diabetic Mice after Critical Limb Ischemia by Activation of CSE-H_2S-VEGF Axis

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The aim of this paper is to investigate effect and mechanism of Danhong injection (DH) on angiogenesis in the diabetic hind limb ischemia mouse model. Thirty diabetic hind limb ischemic model mice and ten normal mice, established by intraperitoneal (i.p.) injection of streptozotocin (STZ) or PBS and ligation/excision of femoral artery, and then twenty diabetic hind limb ischemic model mice of all were evenly randomized to saline (control, $n = 10$) and DH i.p. injection (2 mL/kg weight for 7 days, $n = 10$) groups. Limb perfusion recovery and femoral blood hydrogen sulfide (H_2S) and vessel regeneration and lower limb vascular endothelial growth factor (VEGF)/cystathionine γ-lyase (CSE) expression were evaluated during intervention and after euthanasia, respectively. DH i.p. increased ischemic limb perfusion and promoted collateral circulation generation without decreasing blood glucose level. Increased local CSE-H_2S-VEGF expression contributed to beneficial effects of DH injection. In conclusion, activation of local CSE-H_2S-VEGF axis might participate in proangiogenesis effects of DH injection in diabetic hind limb ischemia model mice, suggesting a potential therapy for diabetic patients with critical limb ischemia.

1. Introduction

Current standard of care for critical limb ischemia (CLI), annually affecting estimated 500 to 1000 per million people worldwide [1] and particularly diabetics, includes lifestyle modification, pharmacotherapy to reduce blood cholesterol, glucose, and hypertension, and revascularization by angioplasty or bypass surgery. However, revascularization is associated with high long-term restenosis rate, does not address underlying pathology, and is not an option for all patients [2], who often undergo major limb amputation with 30% second amputation and 25% death rates within one year [3] prompting continued search for clinical and cost-effective treatments. Among traditional Chinese medicine (TCM) drugs potentially useful for therapeutic angiogenesis, Danhong injection (DH), a Chinese Materia Medica standardized product extracted from *Radix Salviae miltiorrhizae* and *Flos Carthamus tinctorius*, [4], has been shown in animal and clinical studies to improve angina and decrease acute and chronic cardiovascular event occurrence by modulating angiogenesis, inflammation, immunity, and oxidative stress [5–10]. This study therefore examined proangiogenesis effects and underlying mechanisms of Danhong injection in the diabetic hind limb ischemia mouse model to gain insight for potential clinical use in diabetics with CLI.

2. Materials and Methods

2.1. Preparation of DH and Quality Control. According to the production protocol of DH injection provided by Shanxi Buchang Pharmaceutical Co. (Shanxi, China), powdered *Radix et Rhizoma Salviae miltiorrhizae* (750 g) is twice immersed in 7.5 L of 30% ethanol in order to maximally dissolve active ingredient, and then both of the immersions are extracted for 1 h at 50°C, after filtration, and mixed with

Flos Carthamus (250 g). The mixture is then twice immersed in 2.5 L of water for 1 h at 35°C and vacuum evaporated to relative density of 1.10–1.20 (65°C). The solution is filtered and stored at 4°C for 24 h. Water is added to the solution of 1.0 L; sodium chloride and sodium hydroxide are added to achieve an isotonic solution with pH of 6-7 for injection, again filtered, and then sterilized and encapsulated into ampoules (10 mL per ampoule). DH was approved over 5 years ago by the Chinese Food and Drug Administration (CFDA) as Chinese herbal patented product for coronary heart disease patients and listed in the Chinese Pharmacopoeia (Heze Buchang Pharmaceutical Co., Ltd., drug approval number Z20026866).

DH contains two herbal medicinal components, *Salvia miltiorrhiza BUNGE* and *Carthamus tinctorius L*, authenticated and standardized based on marker compounds according to Chinese Pharmacopoeia 2005. DH dose variability was minimized by strict standardization of batches, species, origin, harvest time, medicinal components, and preparation methods, which was confirmed by high performance liquid chromatography (HPLC) according to established protocol [11].

2.2. Chemicals and Reagents. Purified rat anti-mouse CD31 monoclonal IgG2a antibody was purchased from BD Bioscience (San Diego, CA, USA). Rabbit anti-mouse vascular endothelial growth factor (VEGF) polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anticystathionine γ-lyase (CSE), beta-actin antibodies, and horseradish peroxidase-linked secondary antibodies were obtained from Abcam (San Francisco, CA, USA). Mouse VEGF ELISA kits were purchased from eBioscience (San Diego, CA, USA). Sodium sulfide standard was from Alfa Aesar (cat. number 65122, Ward Hill, MA, USA). Streptozotocin (STZ) and other chemicals frequently used in our laboratory were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

2.3. Diabetic Hind Limb Ischemia Model with BALB/c Mice. Study protocol (Figure 1) was approved by Second Military Medical University's Animal Care and Use Committee. After intraperitoneal (i.p.) injection of STZ (150 mg/kg) in BALB/c mice, blood glucose levels were continuously monitored for 2 weeks with diabetes modeling success verified when two random blood glucose levels >16 mmol/L were confirmed. At 2 weeks, hind limb ischemia model was established by ligation and excision of left femoral artery, saphenous arteries, circumflex branch of external iliac artery, and muscular branches of femoral artery [12]. Lower limb perfusion was assessed by laser Doppler perfusion imaging (LDPI) as described below. Immediately after femoral artery ligation, blood flow in the ischemic hind limb was equally reduced in both control and diabetic mice. Consistent with previous studies [13, 14], LDPI showed significantly attenuated perfusion recovery in diabetic compared to control mice on postoperative weeks 4 and 6 (Figures 2(a) and 2(b)), confirming successful STZ-based diabetes and hind limb ischemia modeling of nude mice.

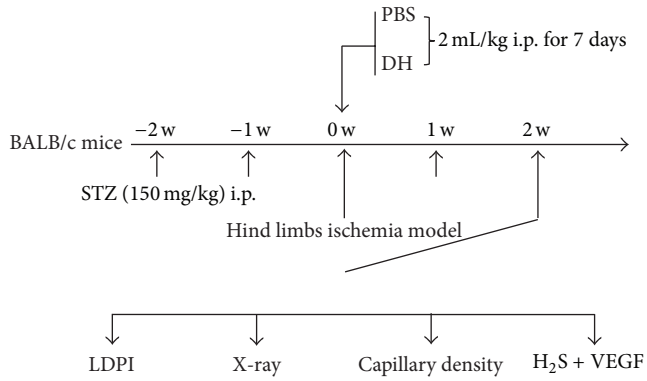

Figure 1: Study protocol. BALB/c mice in hind limb ischemia diabetic model were divided into saline (control, $n = 10$) and DH i.p. injection (2 mL/kg body weight for 7 days, $n = 10$) groups. Diabetic model was established with STZ (150 mg/kg) i.p., and blood glucose levels were monitored for 2 weeks. Diabetes modeling success was identified by twice random blood glucose levels >16 mmol/L. Hind limb ischemia model was established by ligation and excision of femoral artery, saphenous arteries, circumflex of external iliac artery, and muscular branches of femoral artery. Lower limb perfusion was assessed using LDPI. Vein blood was sampled for H_2S analysis by HPLC-FLD, mice were euthanized, and collateral circulation was assessed by angiography, digital X-ray imaging, and immunohistochemical staining. CSE and VEGF expression (key in proangiogenic axis) were investigated with qPCR assay.

2.4. In Vivo Assessment of Limb Function. Semiquantitative assessment of ischemic limb function was performed serially using the following scoring system: 6, full and fast walking; 5, normal but slow walking; 4, walking with only mild deficit; 3, supporting weight, probability of taking 1 or 2 steps; 2, frequent and vigorous movement, no weight bearing; 1, barely perceptible movement, no weight bearing; and 0, no movement [15, 16]. Two independent observers blinded to the study evaluated scores.

2.5. Laser Doppler Perfusion Imaging. Mice were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). Serial, noninvasive assessment of ischemic limb microvascular perfusion was performed in triplicate and in a blinded manner using a LDPI system (PeriScan PIM 3, Perimed, Sweden) after placing mice on a homoeothermic heating pad maintained at 37°C. Using LDPI image processing software (v5.0), perfusion was quantified in regions, equal in area, encompassing the distal leg (entire foot) of both ischemic and contralateral nonischemic limbs. All perfusion data were expressed as a ratio of operated ischemic to nonoperated control limb perfusion, which could minimize data variation possibly secondary to ambient temperature changes.

2.6. HPLC-FLD Analysis of H_2S in Femoral Artery Blood. Animals were anesthetized with sodium pentobarbital (40 mg/kg body weight intraperitoneal injection), and a 30 G insulin syringe was inserted into the femoral vein to draw blood for H_2S analysis. H_2S detection was performed in duplicate for each blood sample following a modified

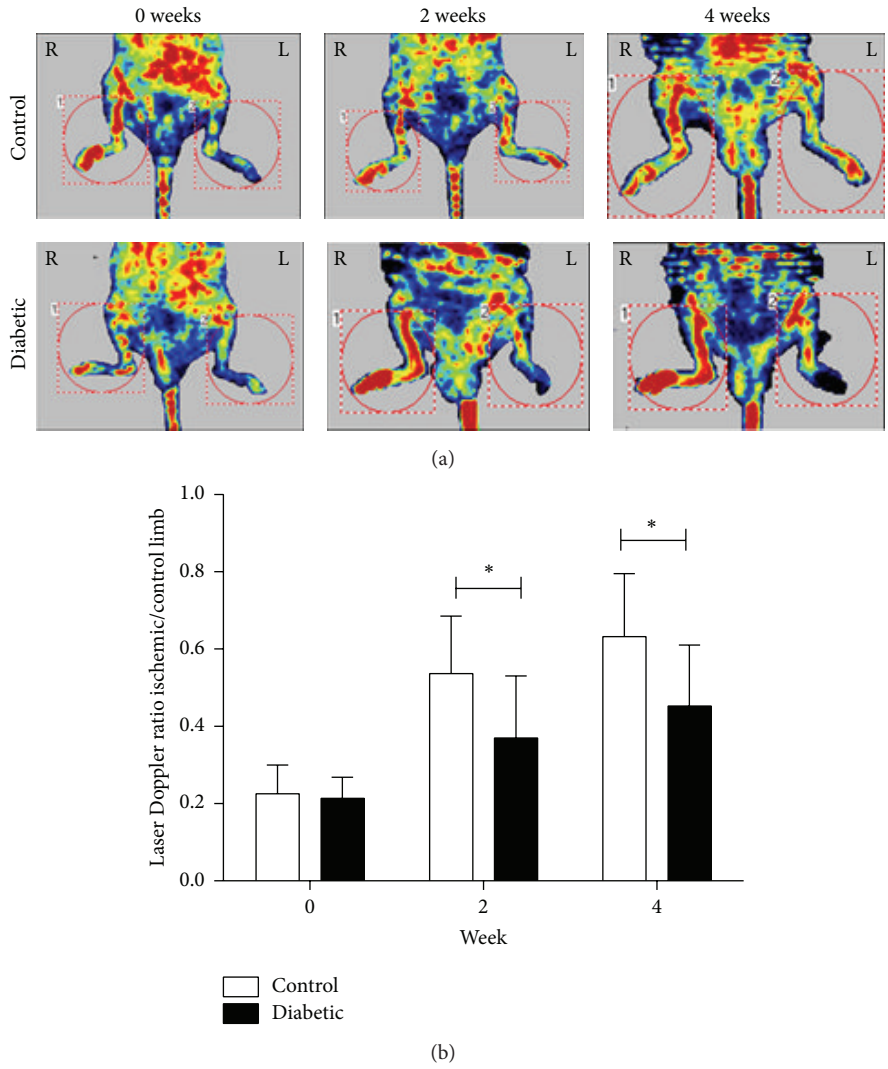

FIGURE 2: Recovery of blood flow in control and diabetic mice monitored by LPDI. Representative evaluations of ischemic (right) and nonischemic (left) hind limbs, immediately after femoral artery ligation and at weeks 0, 2, and 4. Red and blue indicate normal perfusion and marked blood flow reduction, respectively, in ischemic hind limb. Blood flow recovery (ischemic-to-contralateral hind limb perfusion ratio) is impaired in diabetic versus control mice (nondiabetic mice). Data are expressed as mean ± SD. ∗ indicates $p < 0.05$ versus control.

protocol from a previous study based on the fluorescence derivation between monobromobimane (MBB) and hydrogen sulfide (H_2S) contained in plasma [17]. Briefly, 30 μL plasma was collected after centrifuging and incubated with excess MBB in 100 mM Tris-HCl buffer (pH 9.5, 0.1 mM diethylenetriamine pentaacetic acid) for 30 min in 1% oxygen at room temperature to form a stable derivation, and then the fluorescent product sulfide-dibimane (SDB) was analyzed by RP-HPLC using a Dikma-C18 Leapsil column (2.7 μm × 4.6 mm × 100 mm) with gradient elution by 0.1% (v/v) trifluoroacetic acid in acetonitrile. Using the modified protocol with new HPLC column suitable for both HPLC and UPLC system, detection time could be decreased from 12 to 5 minutes without compromising sensitivity and specificity. Standard curve was established based on different concentrations of sodium sulfide solutions prepared in a strict-control hypoxic chamber by purging with nitrogen gas to 1% O_2. Retention time of SDB was 3.3 min, and detection limit was 0.5 pM.

2.7. Angiographic Assessment of Collateral Circulation. Animals were anesthetized as described above; the hearts were rapidly excised and retrogradely perfused via abdominal aorta with heparin saline (0.1% heparin in 0.9% saline). Post-mortem angiography was performed using Omnipaque (Amersham), hand infused angiographic contrast at 0.5 mL/s for 20 seconds, and a high-definition digital X-ray system (MX-20, Faxitron, USA). Recorded images of the pelvis and both hind limbs were analyzed using Image J software (NIH) to calculate angiographic score of thigh-hip joint to knee area. Specifically, a grid was laid over an image of the arterial filling phase vasculature and number of collateral vessel intersections with the grid counted over a defined, bilaterally equal area. Angiographic score was expressed as ratio of numbers

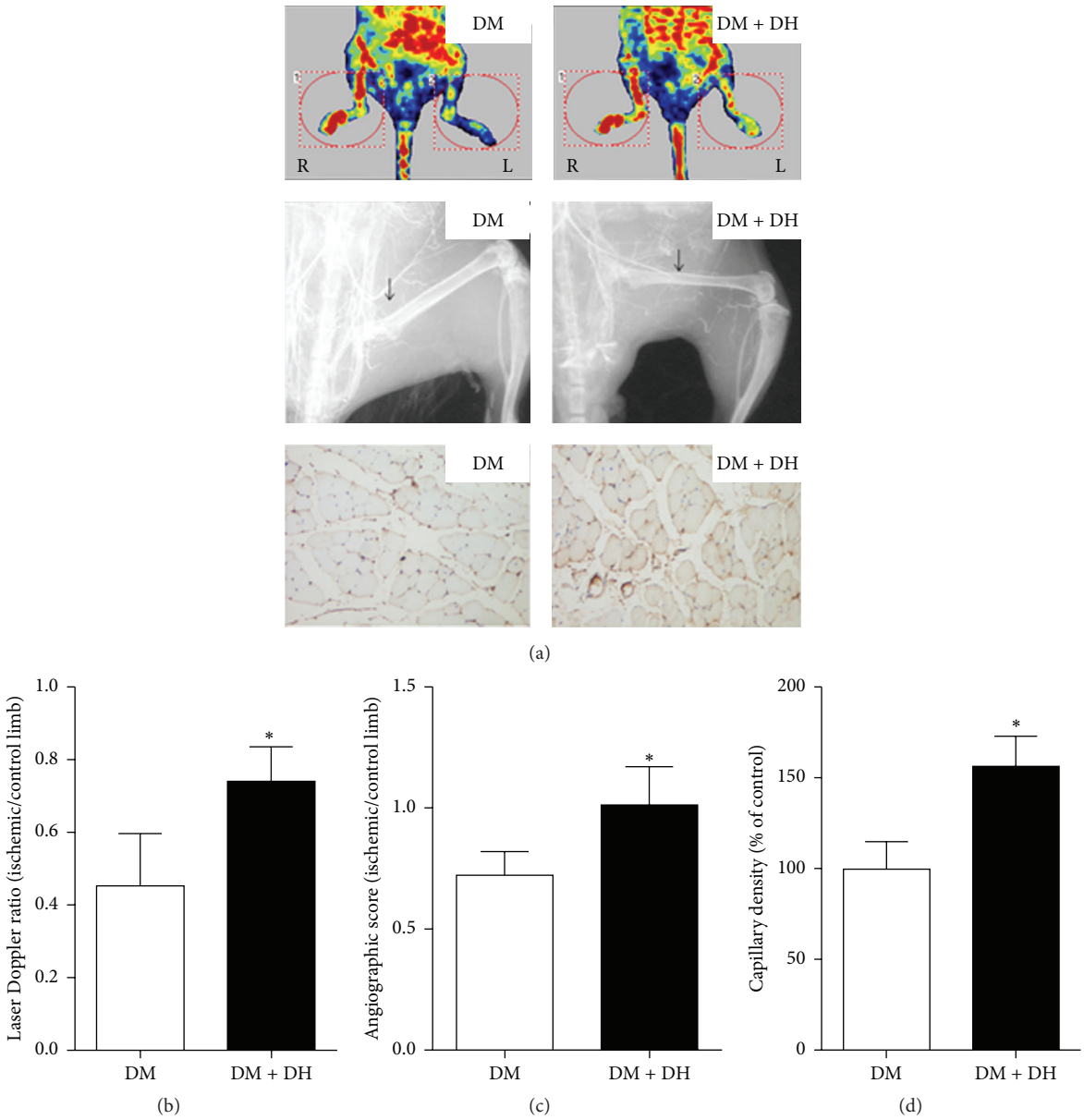

Figure 3: Effect of DH on recovery of blood flow monitored by LDPI, X-ray, and CD31 staining in diabetic mice. Representative evaluation of ischemic (right) and control (left) hind limbs on week 4 after operation. Blood flow perfusion was measured by LDPI, collateral circulation was assessed by angiography in X-ray, and CD31 staining was used to observe microvessel producing. DH significantly increased angiogenesis in diabetic hind limb ischemia versus DM model group (equal dosage PBS treatment). Data are expressed as mean ± SD. $*$ indicates $p < 0.05$ versus DM model group.

of collateral vessels within operated ischemic-to-contralateral leg.

2.8. Histological Assays. After euthanasia, thigh muscles were isolated from limbs and routinely fixed overnight in 4% buffered formalin and embedded in paraffin. Four-micrometer tissue sections were subjected to immune-peroxidase biotin-avidin reaction using the labeled streptavidin biotin method to determine CD31 and VEGF expression. Sections for immunohistochemical analysis were cut and mounted on 3-aminopropyltriethoxysilane-coated (Sigma) slides, allowed to dry overnight at 37°C to ensure optimal adhesion, dewaxed, rehydrated, and treated with 0.3% H_2O_2 in methanol for 10 min to block endogenous peroxidase. For antigen retrieval, sections were microwave-treated in 1 mmol/L EDTA at pH 8 for 10 min and then allowed to cool for 20 min. Endogenous biotin was saturated using a biotin blocking kit (Vector Laboratories). Sections were incubated at 37°C for 30 min with the following antibodies: purified rat anti-mouse CD31 (dilution 1 : 30; monoclonal IgG2a, BD Bioscience) and rabbit anti-mouse VEGF (dilution 1 : 100, polyclonal, Santa Cruz Biotechnology). Binding was visualized using

biotinylated secondary antibody (1 h incubation) and streptavidin-biotin peroxidase complex developed with diaminobenzidine. Finally, slides were counterstained with hematoxylin. Capillary density and leukocyte infiltration expressed as number of $CD31^+$ cells per square millimeter were measured by counting six random high-power (magnification ×200) fields for a minimum of 200 fibers from each ischemic and contralateral limb. The area was measured with Image J software. Two operators analyzed the results independently.

2.9. qPCR Assay of CSE-VEGF Axis. RNA was isolated from ischemia hind limbs using the RNeasy kit (Qiagen, Hilden, Germany). Total RNA was analyzed by Nanodrop (Thermo, USA). Reverse transcription was performed with PrimeScript 1st Strand cDNA Synthesis kit (Takara, Japan) and cDNA amplified by iQ SYBR Green Real-Time PCR Supermix (Bio-rad, USA) using primers for VEGF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in an CFX Connect real-time PCR detection system from Bio-rad. All primers were obtained from Life Technology (USA). Data were analyzed based on relative expression method with the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ (sample) $- \Delta CT$ (calibrator = average CT values of all samples within each group), with ΔCT being CT of housekeeping gene (GAPDH) subtracted from CT of target gene.

2.10. Statistical Analysis. Data are presented as mean ± standard deviation and were compared using paired t-test with GraphPad Prism 5.01 (La Jolla, CA, USA) and SPSS for Windows 7.0. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Protective Effects of DH Injection on Blood Flow Recovery, Collateral Vessel Formation, and Limb Function in Hind Limb Ischemia Diabetic Mouse Model. Perfusion recovery was significantly improved in mice receiving intraperitoneal DH administration at 2 mL/kg weight for 7 days as compared to control receiving equal amount of phosphate buffered saline as scheduled (Figures 3(a) and 3(b)).

Formation of collateral vessels below ligation site was examined using X-ray to ascertain whether improvement in tissue perfusion originated from increased blood flow or collateral vessel formation. At 2 weeks after ischemia induction, significantly more bridging collaterals with some degree of distal filling and originating from the internal iliac artery were visible in the thighs of mice treated with DH, in contrast to no apparent collateral vessel in the same area of controls (Figures 3(a) and 3(c)). In assessment of ischemic hind limbs using anti-mouse CD31 immunohistochemical staining, capillary density was significantly higher in DH i.p. than control group (Figures 3(a) and 3(d)).

Survival analysis showed that after femoral artery ligation DH group mice recovered not only better but also faster than controls (Figure 4).

3.2. Activation of CSE-H_2S-VEGF Axis in Association with DH Protective Effects. An antihyperglycemic effect of DH was excluded by lack of significant differences in weight (time 0: 30.61 ± 1.64 versus 30.39 ± 1.83 g, week 1: 31.23 ± 2.39 versus 31.02 ± 2.24 g, and week 2: 30.69 ± 2.43 versus 32.33 ± 3.18 g) and blood glucose levels (time 0: 6.66 ± 0.68 versus 6.26 ± 0.50 mmol/L, week 1: 11.32 ± 1.78 versus 10.05 ± 2.07 mmol/L, and week 2: 21.05 ± 1.64 versus 19.19 ± 1.67 mmol/L) between saline control and DH groups, respectively, during experimental period. CD31 levels in local muscular tissue increased after DH treatment, as did VEGF mRNA and protein levels compared with controls (Figures 5(a) and 5(b)). Several studies [18, 19] suggested a key role for muscular tissue H_2S system in modulating VEGF expression after different stimuli, and DH was associated with increased venous H_2S levels and CSE mRNA expression (Figures 5(c)–5(e)).

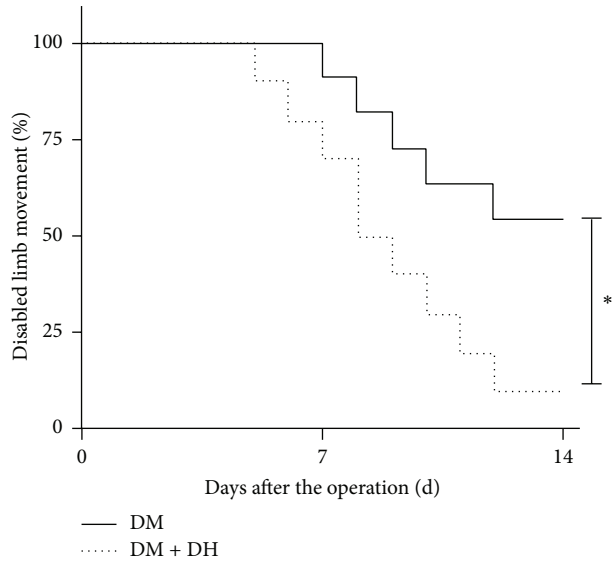

Figure 4: Effect of DH on ischemic limb function recovery. Representative serial evaluation of limb function in control DM model (full line) and DM + DH (dotted line) mice. Function was scored from 0 to 6, that is, from no movement to full and fast walking, and expressed as percentage of disabled limb movement (scores ≤ 3, supports weight, and may take 1 or 2 steps) for each day of 2-week experimental period. ∗ in vertical bar indicates $p < 0.05$ versus DM model group.

4. Discussion

This study showed that the traditional Chinese medicine herbal DH preparation improves blood flow in association with collateral vessel formation and activation of the cystathionine γ-lyase- (CSE-) hydrogen sulfide- (H_2S-) vascular endothelial growth factor (VEGF) axis in a diabetic hind limb ischemia mouse model.

Ischemic vascular diseases remain a leading cause of mortality and morbidity worldwide [19] despite significant advances in medical and surgical intervention. Restoration of blood flow to ischemic organs is vital to prevent tissue death after arterial occlusion, and current treatment modalities are only partially efficacious. Discovery of angiogenic growth factors opened up the possibility of therapeutic angiogenesis

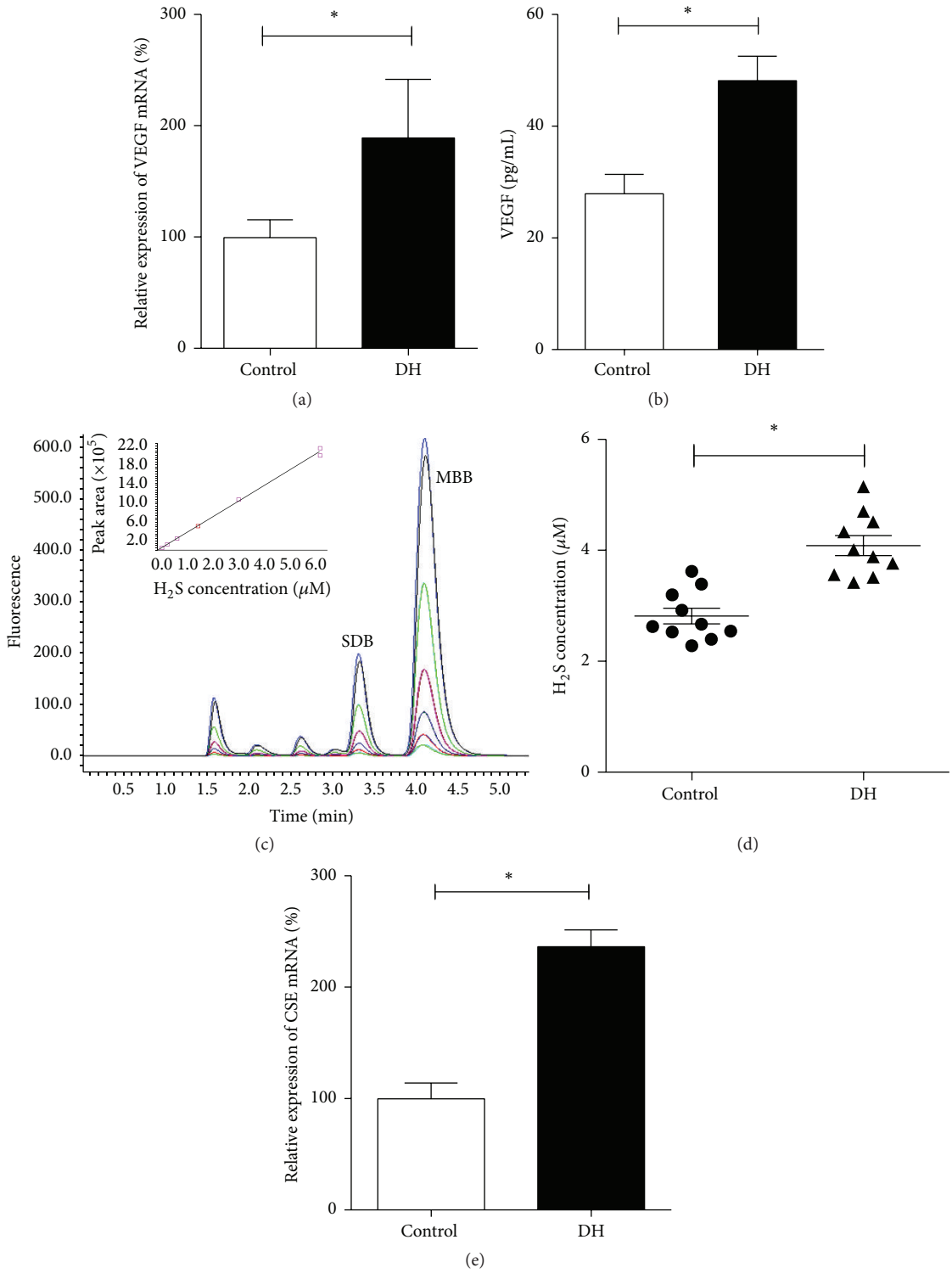

Figure 5: Activation by DH of CSE-hydrogen sulfide- (H_2S-) VEGF axis in local tissue of diabetic mice. VEGF mRNA (a) and protein expression (b) in control and DH injected mice. H_2S was analyzed by HPLC-FLD, and standard dilutions were used to plot the standard curve (Y (AUC) = 354000X (H_2S, μmol/L) + 7640; R^2 = 0.9996) (c). DH (triangles) versus control (circles) significantly increased H_2S (d) and CSE (e) levels in femoral vein blood. Data are expressed as mean ± SD. $*$ indicates $p < 0.05$ versus control.

for acute and chronic ischemia. In preclinical studies, three main approaches have been tested to deliver angiogenic agents: protein, gene, and cell therapy. In protein therapy, recombinant proteins are used directly to induce therapeutic effects [20], which are only transient because of the very short half-life of exogenous proteins in target tissues [21]. In contrast, gene therapy uses nonviral or viral vectors to carry a gene construct encoding a therapeutic protein into target tissues, where it is abundantly expressed [22]; however, this approach is hurdled by an inability to deliver genes efficiently and to obtain sustained expression. The premise of cell therapy in its present form is that transplanted cells by functioning as factories of multiple endogenous growth factors will induce vascular growth mainly in a paracrine manner rather than directly replacing damaged cells [23]. However, at least at the clinical level, efficacy of cell therapies has not been very satisfactory owing to poor in vivo viability of transfused cells, possibly reflecting abnormal cell microenvironment in pathological conditions.

A large body of evidence has shown the above limitations to current and developing therapeutic strategies apply to ischemic diseases associated with diabetes. Traditional Chinese medicine (TCM), widely used for centuries for ischemic diseases, has been the subject of numerous research reports elucidating effective components and their underlying mechanisms [24–28]. Exploring the effects of TCM on improvement of vascular microenvironment and functional recovery has significant clinical implications. Based on results from the present study, DH not only improved angiogenesis but also promoted ischemic organs' function recovery [29, 30], while our data are consistent with clinical experience and encourage clinical studies in the vast spectrum of diabetic patients. Association between local CSE-H_2S-VEGF system and DH protective effects opens up the possibility that DH might act similar to H_2S-donor drugs, which warrants further research.

DH treatment might have potential for use in other fields of medicine. However, proper assessment of therapeutic potential warrants further studies on mechanisms underlying DH protective effects such as the role of local tissue or bone marrow stem cells and in particular intracellular signaling pathways among others.

Conflict of Interests

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

Authors' Contribution

Feng Wu, Zhiqing He, and Ru Ding contributed equally to this work.

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