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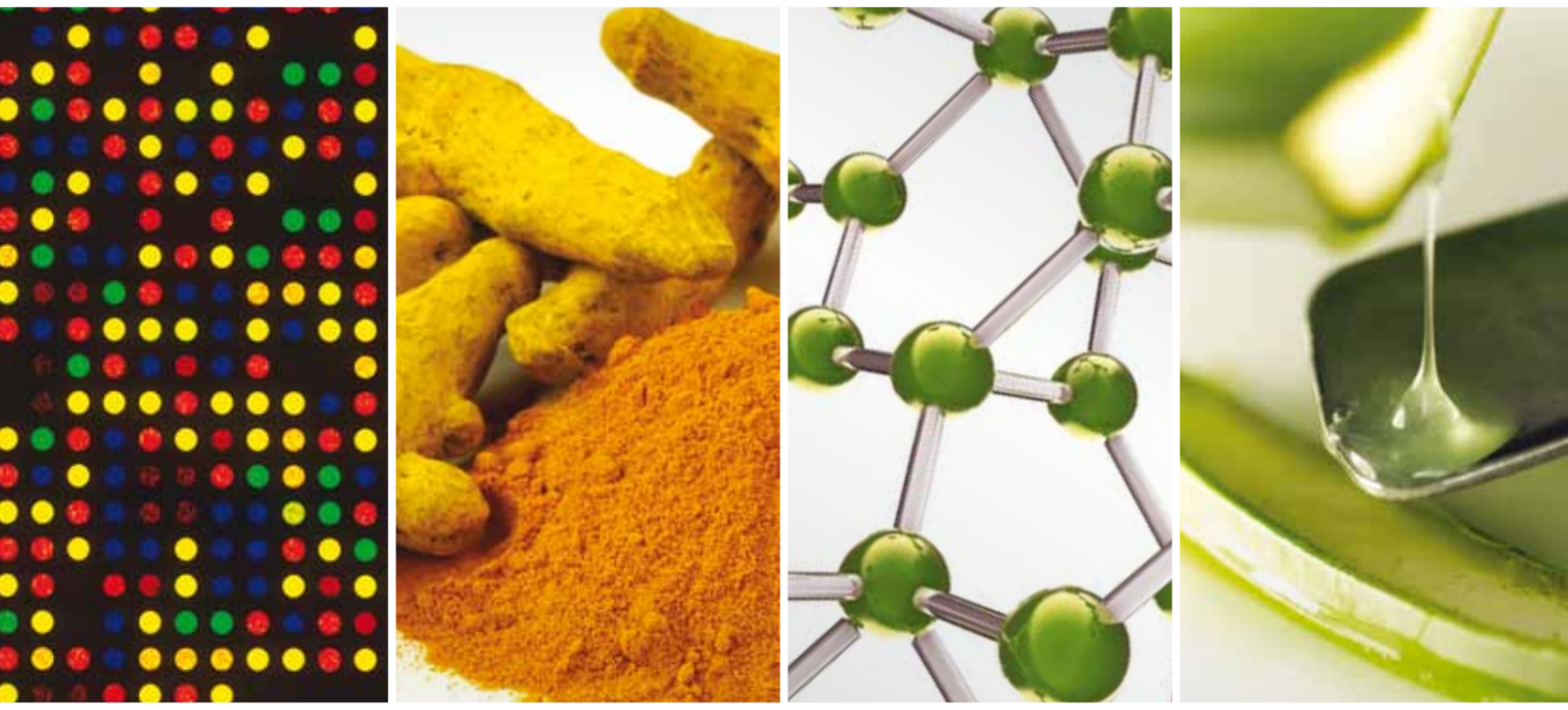
Role of Complementary and Alternative Medicine in Cardiovascular Diseases

Guest Editors: Waris Qidwai, Peng Nam Yeoh, Victor Inem, Kashmira Nanji, and Tabinda Ashfaq



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Editorial Board

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 142898, 2 pages
http://dx.doi.org/10.1155/2013/142898

Editorial

Role of Complementary and Alternative Medicine in Cardiovascular Diseases

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Received 4 April 2013; Accepted 4 April 2013

Introduction. This special issue focuses on the role of complementary and alternative medicine (CAM) in cardiovascular diseases (CVD). The role of CAM in healthcare specifically in cardiovascular diseases (CVDs) has been a contentious issue for centuries. With demographic shifts, urbanization, and changing lifestyles, disease burden of cardiovascular diseases (CVDs) has increased dramatically and can further increase in the future. Despite the growing popularity of CAM therapies, limited information is available regarding patterns of use of CAM therapies in cardiovascular diseases.

The definition of CAM has continued to evolve. As defined by the National Center of complementary and alternative medicine (NCCAM), "it is a group of diverse medical and healthcare systems, practices, and products that are not generally considered part of conventional medicine." The 5 domains of CAM as classified by the NCCAM are whole medical systems (e.g., homeopathy, and ayurvedic medicine), mind-body interventions (e.g., yoga, meditation, and hypnotherapy), biologically based therapies (e.g., herbal treatments, mega-dose vitamins), manipulative and body-based methods (e.g., chiropractic therapy), and energy therapies (e.g., Reiki, and magnetic therapy).

Over the last several decades, the use of CAM has become increasingly popular in both developed and the developing countries. A high proportion of patients using CAM believe CAM has remedial benefits and are safe compared to their prescribed treatments; this serves as a strong motivational factor for both present and future use of CAM. In addition, patients with CVD might be more likely to seek CAM treatments to decrease the psychological stress associated with this condition. Misconceptions regarding their efficacy have largely driven the popularity of these products whereas the adverse effects have been underreported. In disadvantaged societies where access to biomedical services is poor, the reliance on traditional/herbal medicines is more. In affluent population CAM is more used for disease prevention and health promotion. Data from the National Health Interview Surveys (NHIS) reported that 38% of adults in the USA were using CAM therapy in 2007 and among those 36% had CVD.

CVD patients are often unwilling to inform their medical practitioners of CAM use and the majority of attending physicians do not discuss CAM use with their patients. Since many commonly used CAM products have the potential to interfere with the intended action of concomitant prescription medications, this could lead to serious drug interactions. In addition, the use of CAM may have negative impact on the compliance with prescription medications.

A number of CAM therapies have purported cardiovascular effects; but most research on these products is either inconclusive, conflicting, or shows no benefit for their use. Several systematic reviews and meta-analysis on the effectiveness and possible side effects of CAM interventions suggest that some approaches may be beneficial as adjuncts to conventional management of cardiovascular disease, but no evidence exists to support their role as primary treatment.

Dietary supplements (fish oil, coenzyme Q10, garlic, etc.) are among the most commonly used treatment modalities in

patients with CVD. Fish oil supplements are accepted as a part of the treatment regimen for elevated serum triglycerides and the maintenance of vascular wall health. However, the efficacy of vitamin E has been questionable.

Another intervention of CAM, that is, mind-body therapies (relaxation, stress management, meditation, etc.) have minimal side effects. However, in some countries unavailability of trained professionals in the field poses hindrance in its usage. Several styles of meditation have been tested and found to reduce blood pressure, improve heart rate, and even provide survival benefit. Evidence-based trials have been supportive of the conclusion that yoga can lower blood pressure and improve physical fitness.

This one issue cannot answer all the questions regarding the safety, efficacy, and effectiveness of CAM therapies in CVDs. However, the main purpose of this issue is to open the communication line between patients and physicians on CAM use. It also illustrates the necessity of more rigorous researches to determine the precise pharmacological effects and long-term benefits on cardiovascular morbidity and mortality with CAM usage.

Altogether, 27 papers were submitted for publication, out of which 19 papers were accepted. The articles in this issue represent a wide range of therapeutic approaches of CAM in preventing cardiovascular diseases. There are papers on extracts of herbal plants such as *Nigella sativa*, extract of *black chokeberry*, *Salvia miltiorrhiza*, polyphenol and *Pueraria lobata*, and their cardioprotective role in treating hypertension. Ethanolic extract of black chokeberry fruits has a potential role as prophylactic agent but can also function as a nutritional supplement in the management of arterial hypertension. In addition, a study on the use of repeatedly heated oil (a common practice in Asian countries) concluded that it has the predisposing factor of atherosclerosis leading to cardiovascular diseases. Therefore, it is advisable to avoid the consumption of repeatedly heated palm oil.

This special issue also has a number of reviews on the role of CAM in preventing CVD. There is a review on the role of garlic in cardiovascular diseases: treatment and prevention which concluded that garlic can be used as an adjuvant with lipid lowering drugs for control of lipids. Moreover, another evidence-based review discusses CAM and CVDs; this review recommends that more rigorous researches are needed to determine the precise physiologic effects and long-term benefits on cardiovascular morbidity and mortality with CAM usage. In addition, there is a review on Chinese herbal medications (CHM) for hypertension. This review on 10 systematic reviews found that the majority of the RCTs (randomised controlled trials) do not include primary endpoints and therefore their conclusions remain uncertain. Another review on a traditional Chinese herbal formula, Zhen Gan Xi Feng Decoction, appears to be effective in improving blood pressure and symptoms in patients with essential hypertension.

The edition also includes a paper on protein kinase II signal transduction pathway that inhibits cardiac arrhythmia in rats with myocardial infarction. Another study on palm tocotrienol-rich fraction found that it was comparable to folate in reducing high-methionine diet-induced plasma hyperhomocysteinemia, aortic oxidative stress, and inflammatory changes in rats.

Conclusion. The articles presented in this issue represent the recognition of CAM's role in CVD patients. Nevertheless, better education of patients and medical practitioners is needed to improve the understanding of the risks and benefits of CAM use in CVD patients. Further pieces of evidence are required to determine the impacts of CAM use in CVD patients, particularly its clinical and prognostic impact when used in conjunction with prescription medicines. An open dialogue between healthcare professionals and patients regarding intended or present CAM use is also warranted.

Acknowledgments. We hope that this special issue informs and stimulates thinking about the rationale use of CAM in CVD patients. We also hope that readers will find the papers included in this issue a valuable contribution to the field and it reflects the recent trends. We would like to thank the contributors to this special issue for their insightful papers. We would also like to acknowledge the many reviewers for their detailed comments and constructive suggestions. I wish to express my gratitude to all the Guest Editors for encouraging this project throughout, and meticulously carrying out the numerous and often arduous tasks involved with this project.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 253479, 2 pages
http://dx.doi.org/10.1155/2013/253479

Erratum

Erratum to "*Nigella sativa* and Its Protective Role in Oxidative Stress and Hypertension"

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Received 21 May 2013; Accepted 22 May 2013

The part related to "patients with mild hypertension [24]" was incorrectly indicated as "100 mg/kg and 200 mg/kg" in Table 1; here it is corrected.

Table 1: Significant cardiovascular effects of NS and its constituents.

Reference	Study model	Constituents	Laboratory findings
[12]	Renovascular hypertensive rat	NS oil (i.p.) 0.2 mL/kg	↓ SBP, tissue MDA, luminol, and lucigenin CL ↑ tissue Na^+ and K^+-ATPase ↓ plasma CK, LDH, and ADMA ↑ plasma NO
[15]	Rat	(a) NS oil (i.v.) 4–32 μL/kg (b) TQ (i.v.) 0.2–1.6 mg/kg	↓ arterial BP and heart rate (dose dependent)
[16]	Guinea pig	NS oil (i.v.) 4–32 μL/kg	↓ arterial BP and heart rate (dose dependent)
[17]	Rat	(a) De-TQ volatile oil (i.v.) 2–16 μL/kg (b) α-pinene (i.v.) 1–4 μL/kg (c) p-cymene (i.v.) 2–16 μL/kg	↓ arterial BP and heart rate (dose dependent) *De-TQ volatile oil and p-cymene: 4, 8, and 16 μL/kg *α-pinene: 2 and 4 μL/kg
[18]	Rat	Thymol (*in vitro*)	↓ aortic contraction (dose dependent)
[19]	Canine and guinea pig	Thymol (*in vitro*)	Negative inotropic action (dose dependent)
[20]	Spontaneously hypertensive rat	NS seed extract (p.o.) 0.6 mL/kg	↑ diuresis ↓ arterial BP
[21]	Spontaneously hypertensive rat	NS extract (p.o.)	↓ SBP ↑ GFR, urinary, and electrolyte output
[22]	L-NAME-induced hypertensive rat	TQ (p.o.) 0.5 mg/kg and 1 mg/kg	↓ SBP and serum creatinine ↑ kidney GSH
[23]	L-NAME-induced hypertensive rat	NS seed extract (p.o.) 400 mg/kg	↓ arterial BP, SBP, DBP, and serum LDH ↑ serum NO
[24]	Patients with mild hypertension	NS seed extract (p.o.) 100 mg twice per day, 200 mg twice per day	↓ SBP and DBP (dose dependent) ↓ total and LDL cholesterol

NS: *Nigella sativa*; L-NAME: L-NG-nitroarginine methyl ester; i.p.: intraperitoneal; i.v.: intravenous; p.o.: per os; TQ: thymoquinone; De-TQ: de-thymoquinonated; SBP: systolic blood pressure; DBP: diastolic blood pressure; MDA: malondialdehyde; CL: chemiluminescence ; CK: creatine kinase; LDH: lactate dehydrogenase; ADMA: asymmetric dimethylarginine; NO: nitric oxide; GFR: glomerular filtration rate; GSH: glutathione; LDL: low-density lipoprotein.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 464508, 15 pages
http://dx.doi.org/10.1155/2013/464508

Research Article

Wenxin-Keli Regulates the Calcium/Calmodulin-Dependent Protein Kinase II Signal Transduction Pathway and Inhibits Cardiac Arrhythmia in Rats with Myocardial Infarction

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Received 1 November 2012; Accepted 21 February 2013

Academic Editor: Kashmira Nanji

Wenxin-Keli (WXKL) is a Chinese herbal compound reported to be of benefit in the treatment of cardiac arrhythmia, cardiac inflammation, and heart failure. Amiodarone is a noncompetitive inhibitor of the α- and β-adrenergic receptors and prevents calcium influx in the slow-response cells of the sinoatrial and atrioventricular nodes. Overexpression of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in transgenic mice results in heart failure and arrhythmias. We hypothesised that administration of WXKL and amiodarone can reduce the incidence of arrhythmias by regulating CaMKII signal transduction. A total of 100 healthy Sprague Dawley rats were used in the study. The rats were randomly divided into four groups (a sham group, a myocardial infarction (MI) group, a WXKL-treated group, and an amiodarone-treated group). A myocardial infarction model was established in these rats by ligating the left anterior descending coronary artery for 4 weeks. Western blotting was used to assess CaMKII, p-CaMKII (Thr-286), PLB, p-PLB (Thr-17), RYR2, and FK binding protein 12.6 (FKBP12.6) levels. The Ca^{2+} content in the sarcoplasmic reticulum (SR) and the calcium transient amplitude were studied by confocal imaging using the fluorescent indicator Fura-4. In conclusion, WXKL may inhibit heart failure and cardiac arrhythmias by regulating the CaMKII signal transduction pathway similar to amiodarone.

1. Introduction

Cardiovascular diseases are the most common threat to human health worldwide and are the leading cause of morbidity in human populations. Epidemiological data show that at present the number of patients with heart failure has increased to 22.5 million globally and is still increasing at a rate of 2 million patients per year. The 5-year survival rate of patients with heart failure is similar to that of patients with malignant tumours, and mortality continues to increase despite advances in our understanding of the underlying mechanisms of the disease and the development of new treatments. Currently, more than a quarter of a million patients die of heart failure annually in the United States [1]. The risk of sudden cardiac death in heart failure patients is six to nine times that of the general population, and approximately half of these patients die of ventricular arrhythmias. Although drugs that inhibit the adrenergic and renin-angiotensin aldosterone systems have improved the survival of individual patients receiving treatment, deaths

from heart failure increased by 28% between the years 1994 and 2004 [2–4]. Treating arrhythmias in patients with structural heart disease using ion channel antagonist drugs does not reduce mortality [5, 6]. These data highlight the importance of finding suitable agents for treating both heart failure and arrhythmias.

In the past, antiarrhythmic drug research mainly targeted the various types of ion channels in the cell membrane. With the rapid development of the biological sciences, finding new targets for anti-arrhythmic treatment at the level of cellular signal transduction has become a promising new avenue for research. The use of beta blockers for the management of heart failure is one example in which targeting a signalling pathway rather than an individual family of ion channels has proved effective. CaMKII has emerged as a key intracellular signalling molecule, that is, increasingly being recognised as a critical player in cardiac disease and arrhythmia. As we learn more about CaMKII and its effects in the heart, it appears that it also may be a potential therapeutic target [7]. Intracellular CaMKII signal transduction pathways play a central role in the regulation of intracellular calcium. CaMKII is a multifunctional serine/threonine protein kinase, and its expression is increased in both ischemic and dilated cardiomyopathies [8, 9]. CaMKII regulates a wide variety of downstream targets in the heart, including sarcolemmal ion channels (e.g., L-type Ca and Na channels), SR Ca release channels, and PLB, and therefore is important in regulating SR Ca release and Ca reuptake. Thus, CaMKII is critical for the fine tuning of cardiac excitation-contraction coupling (ECC) [10].

Under physiological conditions, CaMKII phosphorylation can keep the calcium channel open and keep intracellular calcium at a moderate level [11]. CaMKII mediates the activity of the L-type calcium channel (LTCC) and RyR via phosphorylation-dependent events, which are integral to normal ECC. When a cardiomyocyte is depolarised by a propagating action potential, calcium enters the cell via the LTCC. This initial calcium entry activates the ryanodine receptor, resulting in the release of calcium from the SR by a process termed calcium-induced calcium release. Release of calcium from the SR accounts for the majority of the intracellular calcium, that is, necessary for contraction and other functions of the cardiomyocyte. The majority of the cytosolic calcium is removed by sarcoplasmic reticulum calcium pump (SERCA), which is negatively regulated by PLB. The return of the cytosolic calcium to basal levels signals the beginning of diastole [4]. Heart failure is associated with excess CaMKII activity. This excess CaMKII activity results in hyperphosphorylation of the LTCC, RyR, SERCA, and PLB proteins, which impairs cardiac function and predisposes the cardiac myocytes to after depolarisations. Hyperphosphorylation of a subunit of the LTCC results in increased I_{Ca}, which is a factor in the predisposition of the cardiac myocytes to EADs (which occurs at phases 2 and 3 of the action potential). Hyperphosphorylation events at the SR lead to the depletion of SR calcium stores, which results in impaired cytosolic calcium transients that in turn cause systolic and diastolic dysfunction. Furthermore, hyperphosphorylation of RyR2 results in an SR calcium leak that can lead to a net inward Na current via sodium-calcium exchanger (NCX) resulting in delayed after depolarisations (i.e., occurring after the completion of repolarisation). However, recent studies have found that, under conditions of heart failure, the ability of protein kinase A (PKA) to regulate the phosphorylation of the RyR2 protein is dependent on CaMKII activity [12, 13]. FKBP12.6 is a regulator of RyR2 channel activity, and binding of FKBPl2.6 to RyR2 causes the channel to maintain its closed state. In heart failure, enhanced sympathetic nerve excitation leads to increased CaMKII activity, which results in hyperphosphorylation of RyR2. This hyperphosphorylation causes the dissociation of FKBP12.6 from RyR2, which opens the channel, and the resulting increase in Ca^{2+} leakage causes triggered beat activity to increase [14–17].

WXKL was developed at Guang'anmen Hospital, a facility of the Chinese Academy of Chinese Medical Sciences, and was the first Chinese-developed anti-arrhythmic medicine to be approved by the Chinese state. In clinical applications, WXKL has been shown to be effective in the treatment of chronic heart failure and arrhythmia. The main ingredients of WXKL consist of *Codonopsis*, *Polygonatum*, *Panax*, nard, and amber. A large number of clinical trials have confirmed that WXKL can increase coronary blood flow, reduce myocardial oxygen consumption, enhance myocardial compliance, improve myocardial hypoxia tolerance, relieve anterior and posterior cardiac loading, reduce myocardial tissue damage in patients with high blood pressure, and reduce the occurrence of arrhythmia [18]. Findings also indicate that WXKL produces atrial-selective depression of I_{Na}-dependent parameters in canine isolated coronary-perfused preparations via a unique mechanism and is effective in both suppressing AF and preventing its induction [19].

In the present study, the hypothesis that WXKL can reduce the incidence of arrhythmias by regulating the CaMKII signal transduction pathway was tested *in vitro* and *in vivo*, and its antiarrhythmic effects were compared to those of amiodarone. Amiodarone is a noncompetitive inhibitor of the α- and β-adrenergic receptors and prevents calcium influx in slow-response cells. Amiodarone can expand blood vessels and slow the heart rate to reduce myocardial ischemia. Amiodarone can also directly prolong the duration of the action potential, the repolarisation time, and the refractory period through inhibition of the outward potassium current, and it is categorised as a class III anti-arrhythmic drug. It has been reported that the ability of amiodarone to suppress the activity of CaMKII may be a component of its anti-arrhythmic therapeutic mechanism. However, the multisystem side effects associated with amiodarone limit its long-term use in patients.

2. Material and Methods

2.1. WXKL Compound. WXKL, consisting of *Rhizoma nardostachyos*, *Codonopsis*, *Notoginseng*, amber, and *Rhizoma Polygonati*, was provided by the BuChang Group, Xi'An, China. According to the national pharmacopoeia (National Pharmacopoeia Committee, 2005), the total amount of notoginseng saponin R1 ($C_{47}H_{80}O_{18}$), ginseng saponin Rg1 ($C_{42}H_{72}O_{14}$), and ginseng saponin Rb1 ($C_{54}H_{92}O_{23}$) should

not be less than 17 mg per bag (9 g). The powdered WXKL compound was dissolved in distilled water prior to use.

2.2. Animal Grouping and Administration of Drugs. One hundred male Sprague Dawley rats (160 ± 20 g), purchased from the animal laboratory of the Academy of Medical Sciences, Beijing, China, were initially divided into two groups: a sham group ($N = 25$) and an MI group ($N = 75$). MI and sham rats were fed normally for 2 weeks before being prescreened by twelve-lead electrocardiogram (ECG). The MI rats with 6–8 leads having q waves were included in the study. The 75 MI rats were randomly assigned to three treatment groups: the MI group ($N = 25$), in which the rats were treated with the vehicle alone (distilled water, 1 mL/kg/day) for oral administration; the WXKL group ($N = 25$), in which the rats were treated with the WXKL compound (4 g/kg/day) for oral administration; and the amiodarone group ($N = 25$), in which the rats were treated with amiodarone (30 mg/kg/day) for oral administration. All animals used in this study received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Establishment of the Myocardial Infarction Model and Sham-Operated Rats. The rats were anaesthetised by intraperitoneal injection of a 1% solution of sodium pentobarbital (50 mg/kg). The procedures performed consisted of endotracheal intubation, positive pressure ventilation, preoperative recording by twelve-lead ECG, one-lead monitoring, local skin disinfection, chest opening, thoracotomy device setup and opening of the pericardium, and ligation of the pulmonary cone and the left atrial appendage 2-3 mm from the bottom of the left anterior descending coronary artery ligation. In the sham group, the left anterior descending artery was not ligated. Additional twelve-lead ECG recordings were made postoperatively. The rats were fed normally for 4 weeks before being euthanised and dissected to isolate the heart for the subsequent experiments.

2.4. Histological Examination. Rat heart samples were cut into transverse sections and stained with haematoxylin and eosin (H&E).

2.5. Inhibitor of CaMKII. Treatment with KN93 (Sigma Inc.), a specific inhibitor of CaMKII, was also found to significantly reduce the Ca transient amplitudes in cardiac myocytes, while treatment with KN92 (Sigma Inc.), which has the same structure as KN93 but no CaMKII-inhibiting activity, had no effect.

2.6. Western Blot Analysis. All animals were euthanised after 4 weeks of drug administration, and their hearts were immediately harvested and stored in liquid nitrogen until Western blot analyses were performed. The following antibodies were used: rabbit polyclonal anti-CaMKII (1:500, Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-phospho-CaMKII (Ser-286) (1:1000, Cell Signaling Technology Inc.), antiphospholamban (1:1000, Cell Signaling Technology Inc.), polyclonal anti-phospho-phospholamban (Ser-17) (1:1000, Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-ryanodine receptor 2 (1:1000, Millipore Corporation.), and rabbit polyclonal anti-FKBP12.6 (1:500, Santa Cruz Biotechnology Inc.). Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes, which were then incubated with antibodies at 4°C. The membranes were further incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:15,000) for 2 hours at room temperature. ECL visualisation was performed, and the Gene Gnome Gel Imaging System (Syngene Co.) was used to capture the resulting images. Image J (NIH image, Bethesda, MD, USA) was used to analyse the gel images.

2.7. Rat Cardiac Myocyte Isolation. Excised rat hearts were mounted on a Langendorff perfusion apparatus and were retrogradely perfused with a nominally Ca-free Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 21.2 mM $MgCl_2$, 20 mM HEPES, 1.2 mM $NaH_2PO_4 \cdot 2H_2O$, 10 mM glucose, and 10 mM taurine) for 4 minutes at 37°C (pH 7.35). The hearts were then perfused with a digestion solution (25 μM $CaCl_2$, 10 mM BDM, 1 mg/mL taurine, 1 mg/mL BSA, and 22-23 mg/mL type II collagenase enzyme (USA, Worthington, 47K9848)) for 20 minutes, at which point the heart became flaccid. Ventricular tissue was removed and cut into small pieces. The tissue was then dispersed until no solid cardiac tissue was left by intensively mixing the myocardial tissue and the enzymatic digestion solution at 37°C for 3 minutes using a constant temperature shaker at 30–50 RPM. To ensure thorough digestion, Ca reintroduction was performed through stepwise increases in Ca concentration from 25 μM to 500 μM. Following their isolation, cardiac myocytes were plated onto superfusion chambers that were coated with laminin to allow cell adhesion. The plated cells were then immediately subjected to physiological analysis. The individual ventricular myocytes selected for study were rod-shaped and had clear striations and a smooth, glossy surface.

2.8. Confocal Imaging. To record single-cell calcium transients, myocardial cells were transferred to special laser confocal petri dishes (MstTek, P35G, 0.16 mm–0.19 mm thickness, P35G-1.5-14-C) with a 2 mL volume of extracellular fluid containing fluo-4 (F14201, Invitrogen). Measurements were made using a Zeiss LSM-510 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany. Lens: Plan-Neofluar 40x/1.3 oil, numerical aperture of 1.25). All image data were collected in the line-scanning mode along the long axis of the myocyte and with laser excitation at a wavelength of 488 nm. The Ca^{2+} level is reported as $F/F0$, where $F0$ is the resting or diastolic fluo-4 fluorescence. The stimulation frequency was set at 0.25 Hz, and the pulse width was 4 s. This type of electrical stimulation causes cell membrane depolarisation, which leads to opening of the LTCC and, therefore, inward calcium ion flow. This inward flow of calcium causes the SR to release large amounts of calcium ions, and this further induces myocardial cell shrinkage. At this point, the calcium concentration increases in the cell become calcium transients.

2.9. Determination of SR Ca^{2+} Content. Myocytes were field stimulated at 0.5 Hz, and their SR Ca^{2+} content was assessed by measurement of the amplitude of caffeine-induced Ca^{2+} transients [20].

2.10. Arrhythmia Induction and WXKL Treatment In Vivo. We randomly selected five rats from each experimental group (the sham group, the MI group, the WXKL group, and the amiodarone group). The rats were anaesthetised using 1% sodium pentobarbital as described previously [21], and an equivalent of lead I ECG recording was performed. After stabilisation of the subject, a control ECG was recorded for 5 minutes. This was followed by an intraperitoneal injection of ISO (3 mg/kg body weight) and a subsequent recording period of 10 minutes. During this period, the ECG was analysed for ISO-induced arrhythmias.

2.11. Statistical Methods. All experimental data were expressed as the mean ± SD. The data were statistically evaluated using one-way analysis of variance (ANOVA), and a post hoc analysis was performed using Fisher's least significant difference (LSD) test. The SPSS computer program (version 17.0) was used for the analyses. A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of WXKL on Survival Rate and Heart Weight/Body Weight Ratio at 4 Weeks after Treatment. After treatment for 4 weeks, deaths had occurred only among the MI group, and the survival rates of the MI group ($n = 23$), the sham group ($n = 25$), the WXKL group ($n = 25$), and the amiodarone group ($n = 25$) were therefore 92%, 100%, 100%, and 100%, respectively. No significant differences in survival were observed among the four groups. As shown in Figure 1, long-term treatment with either WXKL or amiodarone significantly reduced the heart weight/body weight ratio ($P < 0.05$).

3.2. Posttreatment Assessment of Cardiac Structure and Function by Echocardiography. We evaluated cardiac systolic function by a combination of ECG measurements that included the EF, the FS, EDV, ESV, the left ventricular end-diastolic dimension (LVDd), the LVDs, and the stroke volume (SV). Compared with the MI group ($n = 23$), the EF and FS measurements were elevated in the WXKL group ($n = 25$) and the amiodarone group ($n = 25$) ($P < 0.05$), while the EDV, ESV, and LVDs measurements were lowered ($P < 0.05$). Although the measurements obtained for LVDd displayed a decreasing trend in both the WXKL group and the amiodarone group versus the MI group, the difference was not statistically significant ($P > 0.05$). Compared with those of the sham group, the EF and FS measurements obtained from the MI group, the WXKL group, and the amiodarone group were reduced ($P < 0.05$), while the EDV, ESV, LVDd, and LVDs measurements were increased ($P < 0.05$) (Figure 2).

3.3. Effects of WXKL on Expression of CaMKII and Related Proteins. Western blotting analysis was performed to examine the expression of CaMKII, p-CaMKII (Ser-286), PLB, p-PLB (Thr-17), RYR2, and FKBP12.6 in different areas of the myocardium among the four experimental groups ($n = 5$ per group). Figure 3 shows the expression of these proteins in the interventricular septum and left ventricular areas (Figures 3(a) and 3(b)). As shown in Figure 3(c), the expression of CaMKII was increased in the MI group, the WXKL group, and the amiodarone group compared with the sham group ($P < 0.05$). Compared with the MI group, the expression of CaMKII was reduced in both the WXKL group and the amiodarone group ($P < 0.05$), while no significant differences were observed between the expression levels detected in the WXKL group and the amiodarone group ($P > 0.05$). No differences were detected in the expression levels of CaMKII between the interventricular septum and left ventricular areas ($P > 0.05$). As shown in Figure 3(d), phosphorylation of CaMKII at Thr-286 was increased in the MI group, the WXKL group, and the amiodarone group compared with the sham group ($P < 0.05$). However, when compared with that of the MI group, the expression level of Thr-286-phosphorylated CaMKII was significantly reduced in both the WXKL group and the amiodarone group ($P < 0.05$). Again, there were no statistically significant differences between the WXKL group and the amiodarone group in terms of their expression of Thr-286-phosphorylated CaMKII ($P > 0.05$). No statistically significant differences were observed in the expression of Thr286-phosphorylated CaMKII between the interventricular septum and the left ventricular areas ($P > 0.05$). The expression of PLB was significantly increased in the MI group and the amiodarone group compared with the sham group ($P < 0.05$), but the differences were not significant between the WXKL group and the sham group ($P > 0.05$) (Figure 3(e)). Compared with that seen in the amiodarone group, the PLB expression level in the WXKL group was significantly reduced ($P < 0.05$). In our analysis of the levels of phosphorylated PLB protein in the four experimental groups (Figure 3(f)), phosphorylation of PLB at Thr-17 was decreased in the MI group, the WXKL group, and the amiodarone group compared with the sham group ($P < 0.05$), but treatment with WXKL or amiodarone was found to significantly increase the level of Thr-17-phosphorylated PLB compared with that detected in the MI group ($P < 0.05$). As shown in Figure 3(f), the expression of RyR2 was significantly decreased in the MI group, the WXKL group, and the amiodarone group compared with the sham group ($P < 0.05$). Compared with that of the MI group, the RyR2 expression levels detected in rats treated with either WXKL or amiodarone were significantly increased ($P < 0.05$), but no significant differences in RyR2 levels were observed between the WXKL group and the amiodarone group ($P > 0.05$). When compared with that of the sham group, FKBP12.6 expression levels were significantly reduced in the MI group, the WXKL group, and the amiodarone group ($P < 0.05$). As was observed for RyR2 expression, treatment with either WXKL or amiodarone was found to significantly increase the expression of FKBP12.6, compared with levels detected in untreated MI group rats ($P < 0.05$), but

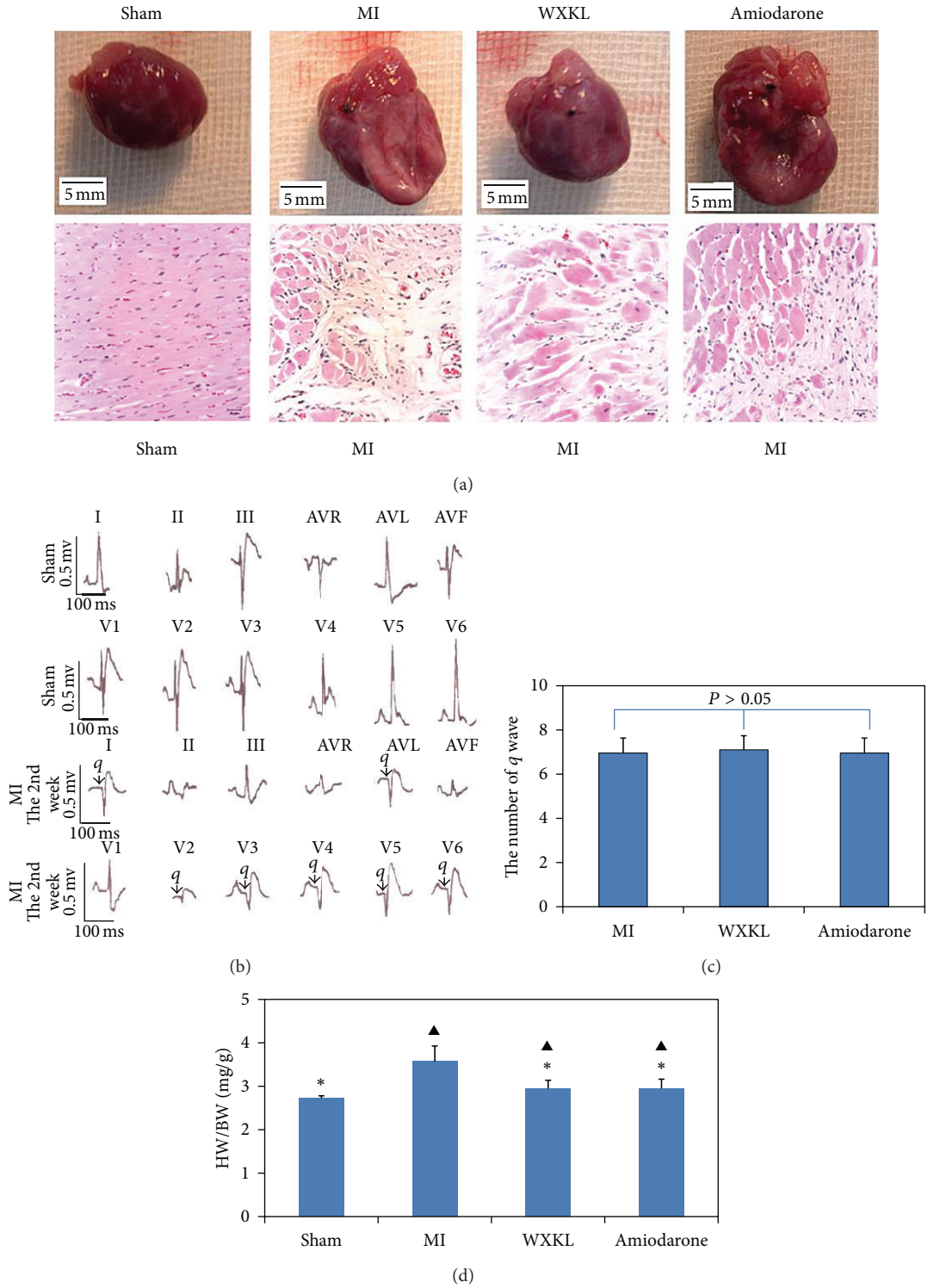

Figure 1: Heart preparation and pathological sections from normal and MI rats. (a) Heart preparations (top (1–4)) and pathological sections (bottom (5–8)) from the sham group, the MI group, the WXKL group, and the amiodarone group. (b) ECG recordings of the sham group and the MI group at 2 weeks after the operation. (c) The average number of q waves in rats from the MI group, the WXKL group, and the amiodarone group. (d) HW (heart weight): BW (body weight) ratios in the sham group ($n = 25$), the MI group ($n = 23$), the WXKL group ($n = 25$), and the amiodarone group ($n = 25$). ($^{*}P < 0.05$ versus the MI group, $^{\blacktriangle}P < 0.05$ versus the sham group).

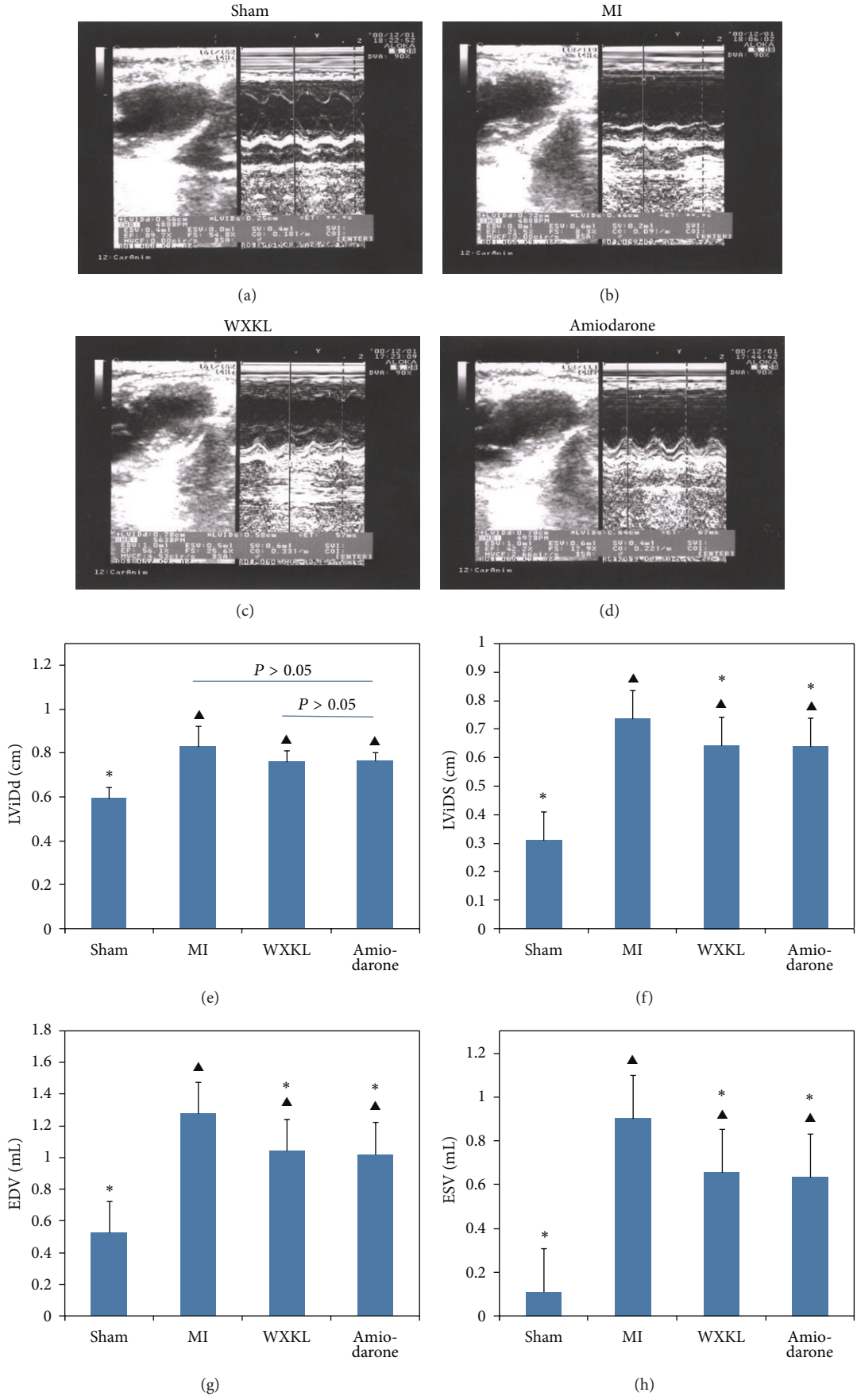

Figure 2: Continued.

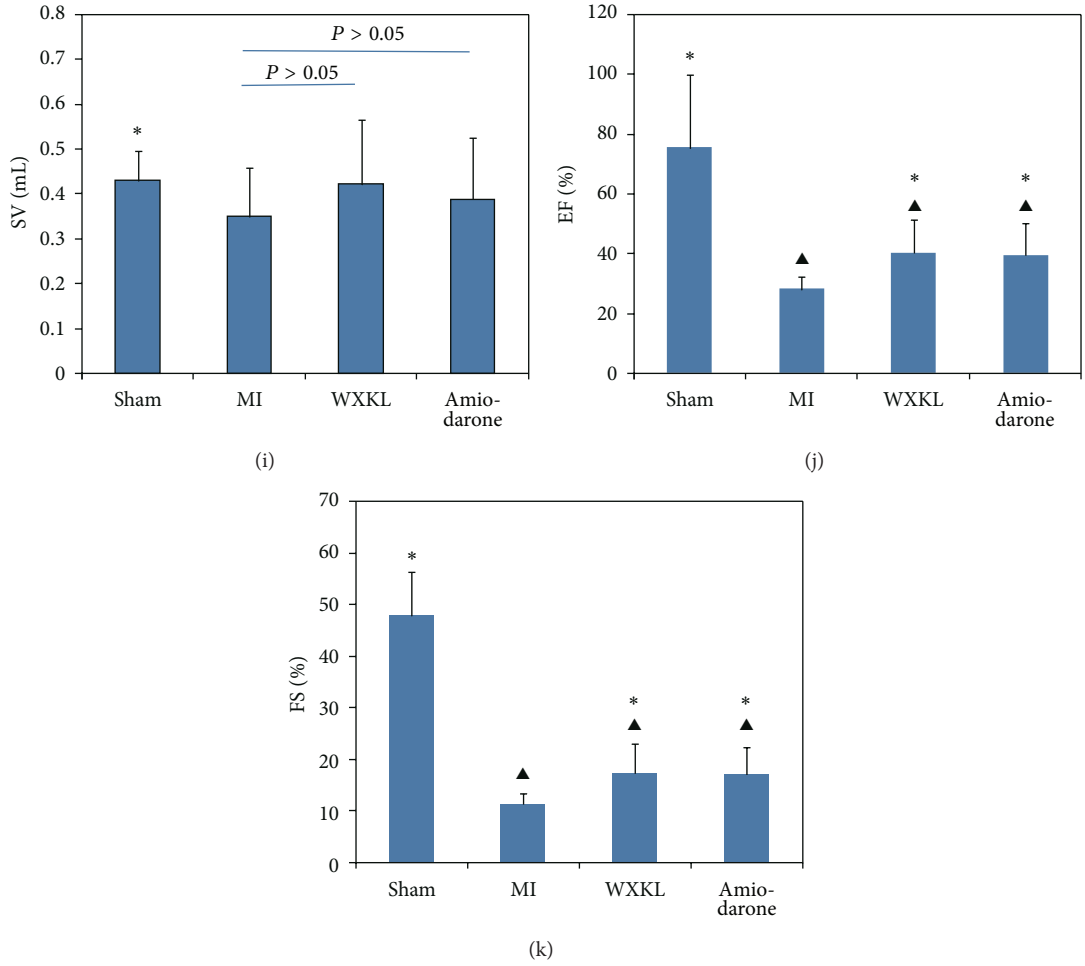

Figure 2: Typical echocardiography images from the sham group (a), the MI group (b), the WXKL group (c), and the amiodarone group (d). At the 4th week of WXKL and amiodarone administration, cardiac structure and function were measured in each group by echocardiography. We evaluated cardiac systolic and diastolic functions by measuring the following variables: left ventricular end-diastolic dimension (LViDd) (e), left ventricular end-systolic dimension (LViDs) (F), end-diastolic volume (EDV) (g), end-systolic volume (ESV) (h), stroke volume (SV) (i), ejection fraction (EF) (j), and fractional shortening (FS) (k). Treatment with either WXKL or amiodarone improved systolic function. The rats of the sham group ($n = 25$) and the MI group ($n = 23$) were treated with vehicle (distilled water) alone (1 mL/kg/day); the WXKL group ($n = 25$) were treated with 4 g/kg/day WXKL; and the amiodarone group ($n = 25$) were treated with 30 mg/kg/day amiodarone. ($^*P < 0.05$ versus the MI group, $^\blacktriangle P < 0.05$ versus the sham group).

no statistically significant differences were observed between the WXKL-treated group and the amiodarone-treated group ($P > 0.05$).

3.4. Effects of WXKL Treatment on Calcium Transients and SR Ca^{2+} Content In Vitro. To verify the pathological relevance of the changes in the expression of CaMKII and Ca^{2+}-handling proteins observed following myocardial infarction, we measured the calcium transient amplitude by stimulating cultured adult cardiomyocytes at a frequency of 0.5 Hz. The Ca^{2+} level data are reported here as $F/F0$, where $F0$ is the resting or diastolic fluo-4 fluorescence. As shown in Figures 4(a) and 4(b), the calcium transient amplitude was decreased in the MI group, the WXKL group, and the amiodarone group versus the sham group ($P < 0.05$; $n = 15$ per group). In comparison with the MI group, the WXKL group and the amiodarone group both exhibited significantly elevated calcium transient amplitudes of WXKL ($P < 0.05$; $n = 15$ per group), but there were no significant differences between the amplitudes measured in the WXKL-treated cardiac myocytes and those of the amiodarone-treated cells ($P > 0.05$; $n = 15$ per group).

We measured the Ca^{2+} content in the SR by assessing caffeine-induced calcium release in cultured adult cardiomyocytes. When Ca^{2+} release from the SR was triggered by the application of 20 mM caffeine, transient Ca^{2+} elevation was significantly decreased in the MI group versus the sham group ($P < 0.05$; $n = 12$ per group). As shown in Figures 4(c) and 4(d), the integrative volume of the Ca^{2+} transient was increased significantly in both the WXKL-treated group and the amiodarone-treated group compared with the MI group ($P < 0.05$; $n = 12$ per group).

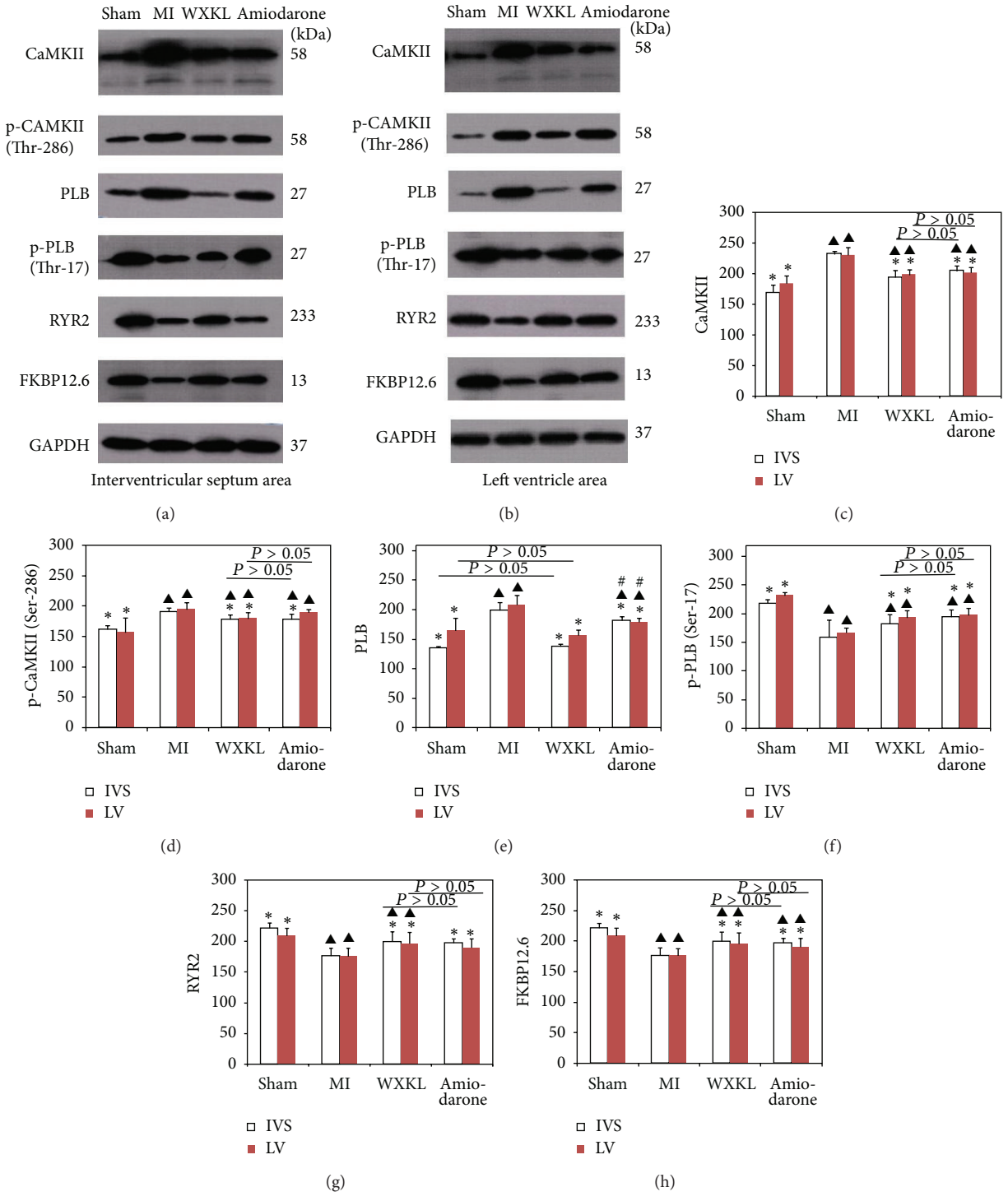

Figure 3: The expression levels of CaMKII and related proteins in the interventricular septum and left ventricle after the 4-week treatment period. (a) The expression levels of CaMKII, p-CaMKII (Thr-286), PLB, RyR2, p-PLB (Thr-17), and FKBP12.6 in the interventricular septum of the four groups. (b) The expression of CaMKII, p-CaMKII (Thr-286), PLB, RyR2, p-PLB (Thr-17), and FKBP12.6 in the left ventricle of the four groups. (c) The expression level of CaMKII of the four groups in the interventricular septum and the left ventricle. (d) The expression level of Thr-286-phosphorylated CaMKII of the four groups in the interventricular septum and the left ventricle. (e) The expression level of PLB of the four groups in the interventricular septum and the left ventricle. (f) The expression level of Thr-17-phosphorylated PLB of the four groups in the interventricular septum and the left ventricle. (g) The expression level of RyR2 of the four groups in the interventricular septum and the left ventricle. (h) The expression of FKBP12.6 of the four groups in the interventricular septum and the left ventricle. ($^{*}P < 0.05$ versus the MI group, $^{\blacktriangle}P < 0.05$ versus the sham group, and $^{\#}P < 0.05$ the amiodarone group versus the WXKL group).

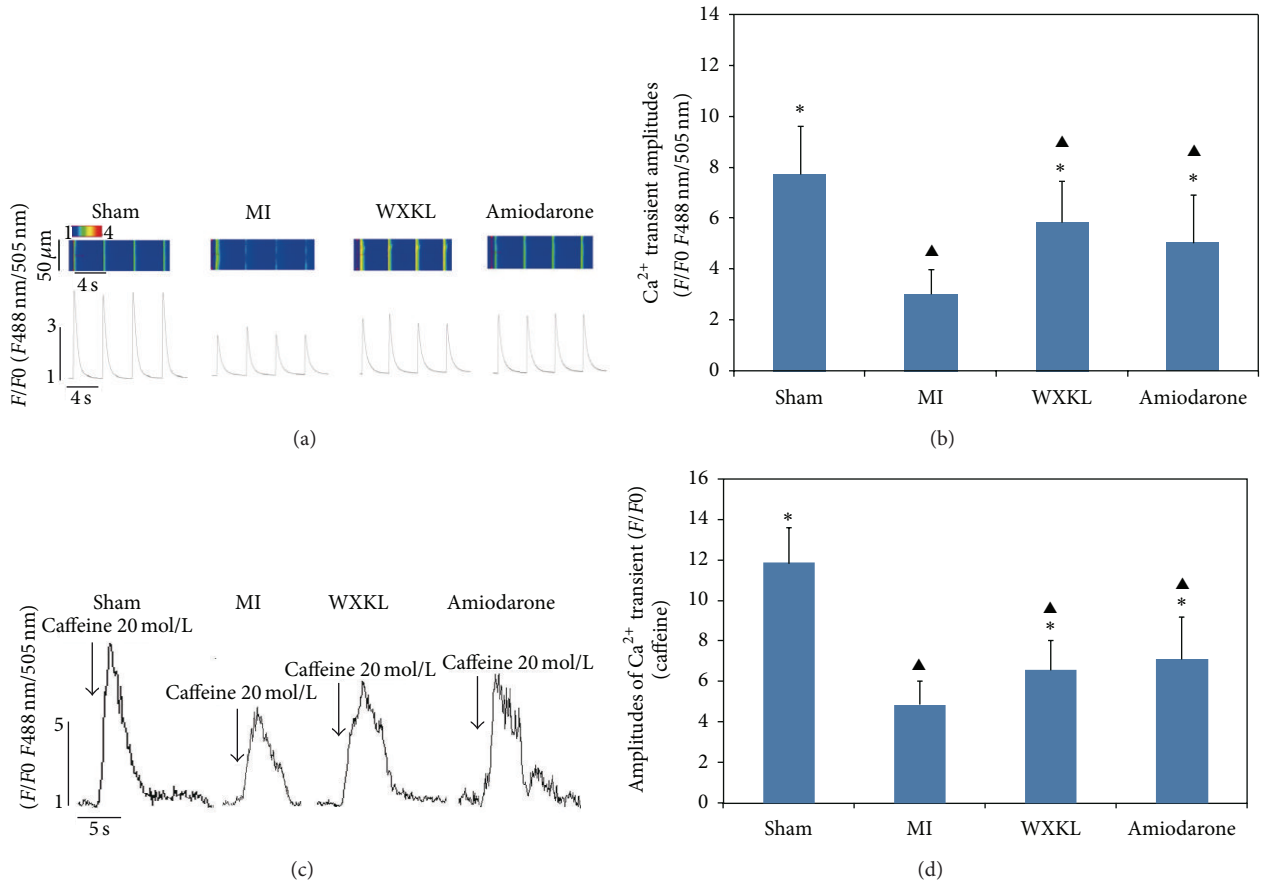

FIGURE 4: Effects of WXKL on the calcium transient and the Ca^{2+} content in the SR measured *in vitro* at 4 weeks after treatment. (a) The calcium transient amplitude was measured by stimulating the cultured adult cardiomyocytes at 0.5 Hz. (b) Compared with the sham group, the calcium transient amplitude was reduced in the MI group, the WXKL group, and the amiodarone group ($P < 0.05$, $n = 15$ cells per group). Compared with that of the MI group, the calcium transient amplitude was significantly elevated in the WXKL group and the amiodarone group ($P < 0.05$). (c) The Ca^{2+} content in the SR was measured by assessing caffeine-induced calcium release in cultured adult cardiomyocytes. (d) The integrative volumes of the Ca^{2+} transients in the WXKL-treated group and the amiodarone group were increased significantly compared with those of the MI group ($P < 0.05$, $n = 12$ cells per group). ($^*P < 0.05$ versus the MI group, $^▲P < 0.05$ versus the sham group).

3.5. Effects of WXKL on the Incidences of Early EADs and Delayed DADs In Vitro. We isolated rat myocardial cells from all four experimental groups using enzymatic hydrolysis and measured the incidences of early EADsand delayed DADs in the cultured cells. The cardiomyocytes ($n = 30$ per group) were stimulated for 1 min at a frequency of 0.5 Hz and a pulse width of 2 s WXKL. As shown in Figure 5(a), nonstimulated Ca transients (EAD and DAD) were frequently observed in MI myocytes. EAD was characterised by a non-stimulated Ca increase before the turning point of the Ca transient decay, and this was accompanied by a minor cell contraction. In contrast, DAD occurred after the turning point of the Ca transient decay and was accompanied by a major contraction of the myocyte. Cardiac myocytes from the MI group were found to have the highest incidence of EADs (Figure 5(b); 29.4%, 6.3%, 12.5%, and 17.6% in the cells from the MI, sham, WXKL-treated, and amiodarone-treated groups, respectively; $P < 0.05$ using Fisher's 2-sided exact test, $n = 30$ per group) and of DADs (Figure 5(c); 79.1%, 15.5%, 30.5%, and 39.7% in the cells from the MI, sham, WXKL-treated, and amiodarone-treated groups, respectively; $P < 0.05$ using Fisher's 2-sided exact test, $n = 30$ per group) of WXKL.

3.6. Effects of WXKL on the Incidence of Cardiac Arrhythmias In Vivo. Our observation, made at the cellular level, of the antiarrhythmic effects of CaMKII inhibition prompted us to test whether WXKL treatment is sufficient to reduce the incidence of cardiac arrhythmias *in vivo*. The results of ECG recordings made following an intraperitoneal injection of ISO (3 mg/kg body weight) into rats from each of the four experimental groups are shown in Figure 6(a), while Figure 6(b) shows detailed tracings for representative arrhythmic events that are likely to correspond to previously described bidirectional tachycardias. The data summarised in Figure 6(c) shows that the WXKL group exhibited a significantly reduced incidence of cardiac arrhythmias *in vivo* when compared with the MI group and the amiodarone group. Two of 6 rats in the WXKL-treated group exhibited arrhythmias in the first 8 minutes after ISO application, while 5 of 6 rats in MI group and 3 of 6 rats in the amiodarone

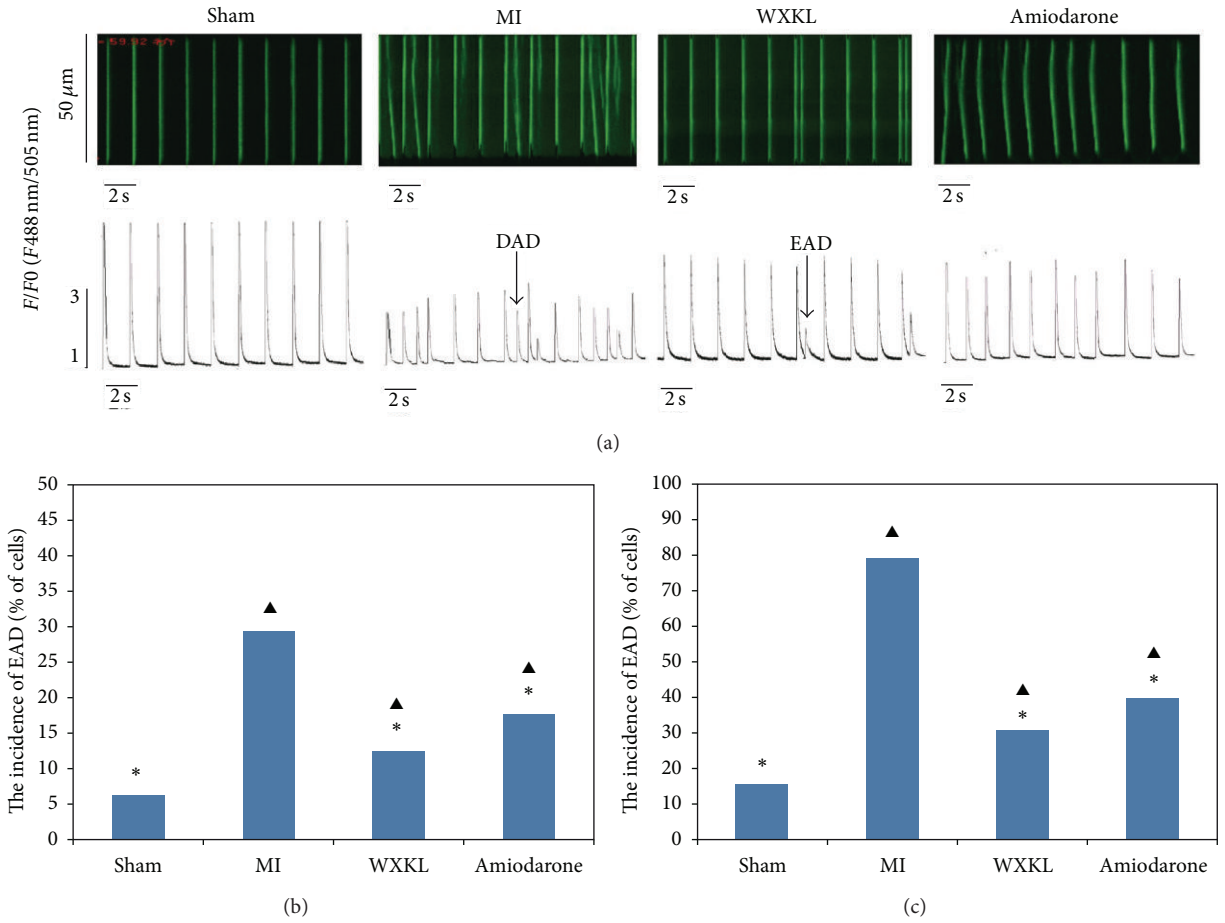

Figure 5: Effects of WXKL on the incidences of EADs and DADs *in vitro* at 4 weeks after treatment. (a) The incidences of EADs and DADs were recorded among the sham group, the MI group, the WXKL group, and the amiodarone group ($n = 30$ cells per group). (b) The incidence of EADs was significantly increased in the MI group compared with the sham group, the WXKL group, and the amiodarone group (29.4% versus 6.3%, 12.5% and 17.6%, respectively; $P < 0.05$ using Fisher's 2-sided exact test). (c) The incidence of DADs was significantly increased in the MI group compared with the sham group, the WXKL group, and the amiodarone group (79.1% versus 15.5%, 30.5% and 39.7%, respectively; $P < 0.05$ using Fisher's 2-sided exact test). ($^*P < 0.05$ versus the MI group, $^▲P < 0.05$ versus the sham group).

group exhibited arrhythmias during the same period. This difference was found to be statistically significant ($P < 0.05$ using Fisher's exact test).

3.7. Effects of WXKL on Ca Transient Amplitudes in ISO-Stimulated Cardiac Myocytes In Vitro. At high stimulation rates or in the presence of β-adrenergic stimulation, SR Ca load and [Ca]i increase substantially and may induce further arrhythmogenic triggers that could be a highly influential factor in the genesis of arrhythmias *in vivo*, and even under pathophysiological conditions such as in heart failure, catecholamine levels are known to be increased. Therefore, we decided to challenge the myocardial infarction cells using ISO (up to 10^{-6} M) to load their SR Ca stores and further unmask their potential to exhibit diastolic proarrhythmogenic events.

We first examined isolated normal myocytes using epi-fluorescence microscopy under basal and ISO-stimulated conditions (10^{-6} M ISO). As shown in Figures 7(a) and 7(b), the Ca transient amplitudes in cardiac myocytes under ISO-stimulated conditions were significantly increased compared with those of nonstimulated cells, but treatment with WXKL at doses of 1 g/L, 5 g/L, and 10 g/L reduced the Ca transient amplitudes in a dose-dependent manner. Treatment of cardiac myocytes with KN93 (1 mM) also significantly reduced the Ca transient amplitudes to a degree similar to that achieved by WXKL treatment, while treatment with KN92 (1 mM) did not produce a significant reduction in amplitudes.

4. Discussion

We can draw the following conclusions from the present study. (1) WXKL treatment significantly improves cardiac function and inhibits myocardial remodelling. (2) In rats with myocardial infarction, WXKL treatment can significantly reduce the expression of CaMKII, p-CaMKII (Thr-286), and PLB, while significantly increasing the expression of RyR2, p-PLB (Thr-17), and FKBP12.6. (3) WXKL can significantly increase both the Ca^{2+} content of the SR and the calcium transient amplitude in cultured cardiac myocytes from rats with myocardial infarction. (4) WXKL treatment can significantly

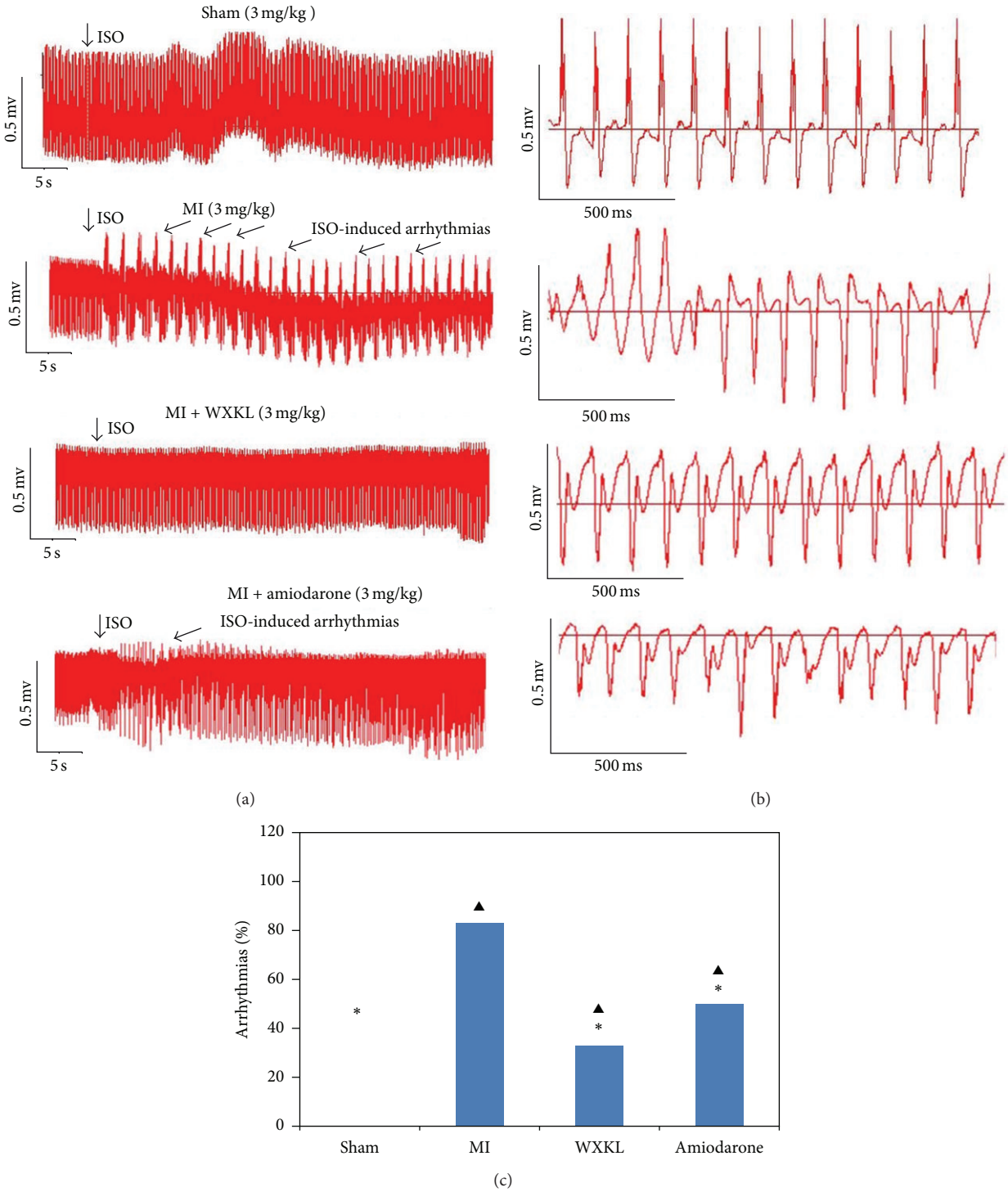

Figure 6: Effects of WXKL on the incidence of cardiac arrhythmias *in vivo*. (a) The ECG recordings from the four experimental groups following an intraperitoneal injection of ISO (3 mg/kg body weight). (b) Detailed tracings for the respective arrhythmic events. (c) WXKL treatment significantly reduced cardiac arrhythmias *in vivo* compared with the MI group and the amiodarone group ($^{*}P < 0.05$ versus the MI group, $^{\blacktriangle}P < 0.05$ versus the sham group, and $^{\#}P < 0.05$ the amiodarone group versus the WXKL group, using Fisher's 2-sided exact test, 6 rats were analysed per group).

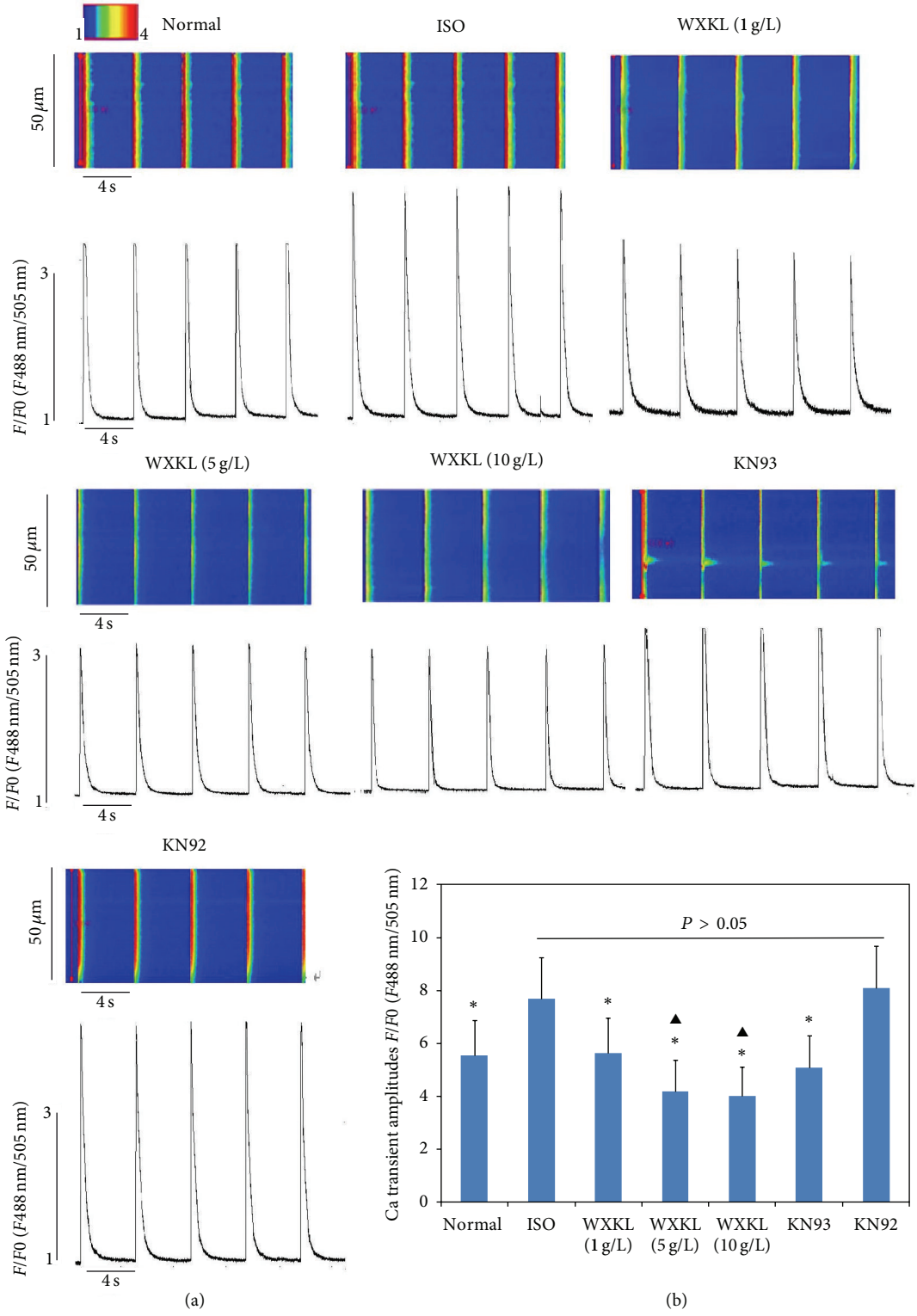

FIGURE 7: Effects of WXKL and KN93 on the Ca transient amplitudes in ISO-stimulated cardiac myocytes. (a) Detailed tracings for 7 treatment groups. (b) The Ca transient amplitudes in ISO-stimulated cardiac myocytes (10^{-6} M ISO) were significantly increased compared with those in non-ISO-stimulated cells, but WXKL treatment (1 g/L, 5 g/L and 10 g/L) reduced the Ca transient amplitudes in a dose-dependent manner. Treatment with KN93 (1 mM) also significantly reduced the Ca transient amplitudes, while treatment with KN92 (1 mM) did not reduce them. ($^{*}P < 0.05$ versus the ISO group, $^{\blacktriangle}P < 0.05$ versus the normal group).

decrease the incidences of EADs and DADs in myocardial infarction cardiomyocytes. (5) WXKL treatment can reduce the incidence of cardiac arrhythmias in rats with myocardial infarction. (6) WXKL can significantly suppress the Ca transient amplitudes in ISO-stimulated cardiac myocytes *in vitro*.

CaMKII is an appealing potential target for pharmacological inhibition. CaMKII activity is upregulated in hypertrophy and heart failure [22]. CaMKII overexpression in transgenic mice results in heart failure and arrhythmias [23, 24], whereas CaMKII inhibition protects the heart against the development of these conditions [25]. In the present study, we demonstrated that WXKL significantly decreased both the expression of CaMKII and its phosphorylation at Thr-286 in rats with myocardial infarction. When its functional effects were examined, WXKL was found to significantly improve cardiac function and inhibit myocardial remodelling. The observed decrease in both the expression of CaMKII and its phosphorylation at Thr-286 may be the primary mechanism by which WXKL inhibits heart failure and arrhythmia.

Dissociation of FKBP12.6 from RyR channels causes uncoupled channel gating, which results in defective closure of these channels [26, 27]. In a study of the mechanism underlying the partial loss of FKBP12.6 from RyR channels, Marx et al. [15] demonstrated that hyperphosphorylation of RyR causes the dissociation of FKBP12.6 from the ion channel and that this causes an increased sensitivity to Ca^{2+}-induced activation and defects. These findings suggest that failing hearts lack the normal FKBP12.6-mediated regulation of the RyR-family of ion channels and that this is the major cause of the serious abnormality in the regulation of intracellular Ca^{2+} and the consequent cardiac dysfunction. In line with our findings, Okuda et al. [28] demonstrated that hyperphosphorylation of the ryanodine receptor by PKA results in the channel exhibiting an abnormal Ca^{2+} leak and is associated with a decrease in the amount of ryanodine receptor-bound FKBP12.6. Treatment with WXKL or amiodarone significantly increased the expression of RyR2 and FKBP12.6 in rats with myocardial infarction, which may enhance the ability of FKBP12.6 to modulate the RyR2 ion channel. This may be the most important mechanism by which WXKL is able to inhibit arrhythmia.

Until recently, there was a general agreement that CaMKII phosphorylates PLB at Thr-17 and that this leads to an improved frequency-dependent acceleration of relaxation [29]. It was reported that the mRNA and protein expression levels of PLB were significantly upregulated by 55.5% ($P < 0.05$) and 84.8% ($P < 0.01$), respectively, in rats of the chronic heart failure (CHF) group following ligation of the coronary artery for 6 weeks [30]. In the present study, we demonstrated that WXKL significantly decreased the expression of PLB, but increased the level of Thr-17-phosphorylated PLB in the final stage of heart failure (Figure 3). By increasing the level of PLB, that is, phosphorylated at Thr-17, and decreasing the expression level of total PLB, WXKL may improve the frequency-dependent acceleration of relaxation in cardiac myocytes, thereby improving cardiac function and preventing arrhythmia.

In heart failure (HF), where CaMKII expression and activation are increased, RyR phosphorylation and the diastolic SR Ca leak are also increased [12], and this diastolic SR Ca leak can initiate DADs in which the depolarising current consists of an inward Na/Ca exchange. Studies in genetically modified animal models provide proofs of concept that this type of CaMKII-modified RyR behaviour can be a major arrhythmogenic factor that promotes HF and atrial fibrillation [31, 32]. In the present study, treatment with either WXKL or amiodarone clearly decreased the incidence of DADs and increased both the Ca^{2+} content in the SR and the calcium transient amplitude. It is possible, therefore, that the decreased incidence of DADs that followed treatment with WXKL or amiodarone was the result of a decrease in the diastolic SR Ca leak.

The results of this study demonstrate that myocardial infarction-induced overexpression of CaMKII increases the incidence of cellular proarrhythmogenic events. It has been reported elsewhere that CaMKII activity is associated with the generation of these systolic proarrhythmogenic events [24, 33]. CaMKII activity can contribute to L-type Ca current facilitation [23, 25] and may therefore favour EAD generation [24, 33]. However, the results of the present study demonstrate that, as shown in Figure 6, treatment with WXKL or amiodarone can reduce the incidence of EADs. By inhibiting the expression of CaMKII and p-CAMKII (Thr-286), WXKL and amiodarone may affect the function of LTCC and reduce systolic calcium influx into the cell, thereby inhibiting arrhythmia.

It should be noted that ISO may also activate CaMKII directly through exchanging protein directly activated by cAMP-dependent pathways and indirectly through increasing [Ca]i [34], thus resulting in more dramatic CaMKII-dependent cellular arrhythmias *in vitro*. The results of this study demonstrate that the Ca transient amplitudes in ISO-stimulated cardiac myocytes were significantly increased compared to those of non-ISO-stimulated cells. In our experiments examining the effect of WXKL on Ca transients, the Ca transient amplitudes decreased with increasing WXKL dose. Treatment with KN93, a specific inhibitor of CaMKII, was also found to significantly reduce the Ca transient amplitudes in cardiac myocytes, while treatment with KN92, which has the same structure as KN93 but no CaMKII-inhibiting activity, had no effect. It may be that WXKL by inhibiting CaMKII activity reduces [Ca]I and thereby prevents the occurrence of arrhythmia.

5. Conclusions

In summary, the present study shows that WXKL and amiodarone inhibit heart failure and cardiac arrhythmias via a mechanism that may involve the regulation of the CaMKII signal transduction pathway. WXKL treatment significantly reduced the expression of CaMKII, p-CaMKII (Thr-286), and PLB but significantly increased the expression of RYR2, p-PLB (Thr-17), and FKBP12.6 in rats with myocardial infarction to improve cardiac function and inhibit myocardial remodelling. While it was found to suppress the Ca transient amplitude in ISO-stimulated cardiac myocytes, WXKL

increased both the SR Ca^{2+} content and the calcium transient amplitude in isolated cardiac myocytes from rats with myocardial infarction, while also significantly decreasing the incidences of EADs and DADs in these cells. Furthermore, WXKL significantly reduced the incidence of cardiac arrhythmias in our *in vivo* rat myocardial infarction model.

Conflict of Interests

All authors declare that they have no conflict of interests.

Authors' Contribution

Yanwei Xing, Yonghong Gao, Jianxin Chen, and Haiyan Zhu contributed equally to this work.

Acknowledgments

This work was supported by the National Science Foundation of China under Grant no. 81001514 and Beijing Nova program under Grant no. 2011110.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 672097, 8 pages
http://dx.doi.org/10.1155/2013/672097

Review Article

Complementary and Alternative Medicine and Cardiovascular Disease: An Evidence-Based Review

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Received 11 December 2012; Accepted 21 March 2013

Academic Editor: Tabinda Ashfaq

Complementary and alternative medicine (CAM) plays a significant role in many aspects of healthcare worldwide, including cardiovascular disease (CVD). This review describes some of the challenges of CAM in terms of scientific research. Biologically-based therapies, mind-body therapies, manipulative and body-based therapies, whole medical systems, and energy medicine are reviewed in detail with regard to cardiovascular risk factors and mediation or modulation of cardiovascular disease pathogenesis. CAM use among patients with CVD is prevalent and in many instances provides positive and significant effects, with biologically-based and mind-body therapies being the most commonly used treatment modalities. More rigorous research to determine the precise physiologic effects and long-term benefits on cardiovascular morbidity and mortality with CAM usage, as well as more open lines of communication between patients and physicians regarding CAM use, is essential when determining optimal treatment plans.

1. Introduction

The National Center for Complementary and Alternative Medicine (NCCAM) defines complementary and alternative medicine (CAM) as "a group of diverse medical and health care systems, practices, and products that are not generally considered part of conventional medicine" [1]. Complementary medicine is used along with conventional medicine, whereas alternative medicine is used in place of conventional medicine. In the 2007 National Health Interview Survey (NHIS), approximately 38% of USA adults and 12% of children reported using CAM in the past 12 months, and lifetime prevalence of CAM use in the United States and worldwide has increased steadily since 1950 [2, 3]. A systematic search of the existing literature found that the prevalence of CAM use ranges between 5% and 74.8% [3]. The NHIS report also noted that 83,000,000 adults spent $33.9 billion out of pocket on CAM, constituting 11.2% of total out-of-pocket health care expenditures and approximately 1.5% of total health care expenditures [2].

The 2007 NHIS report demonstrated that the top five most frequently used CAM therapies (excluding prayer) were natural products, such as fish oil/omega 3, glucosamine, echinacea, and flaxseed (17.7%), deep breathing (12.7%), meditation (9.4%), chiropractic and osteopathic (8.6%), and massage (8.3%), followed by yoga, diet-based therapies, progressive relaxation, guided imagery, and homeopathic treatment [2]. Frass et al. report that the most used therapies are chiropractic manipulation, followed by phytotherapy/herbal medicine, massage, and homeopathy [3]. The conditions for which CAM is most frequently used according to the 2007 NHIS include back pain, neck pain, joint pain, arthritis, and anxiety, while Frass et al. report the top five conditions to be back pain/neck problems, depression, insomnia/trouble sleeping, severe headache/migraine, and stomach/intestinal illness [2, 3]. CAM use among adults is greatest among

women and those of middle age who are better educated and have higher incomes [2, 3].

The scientific study of CAM poses several unique challenges that must be taken into consideration in order to adequately produce and assess evidence-based data. Many forms of CAM are elements of broader healing systems based on unique theoretical constructs and systems of analysis, rather than stand-alone treatments—isolating a particular therapy (i.e., acupuncture) from its broader discipline (i.e., Chinese medicine) may not do it justice. There is the potential for disparity in disciplines: Chinese, Korean, and Japanese acupuncture styles are different, and adjunct therapies such as manual or electrical needle stimulation and use of herbal preparations orally or through moxibustion add to the disparity. Another potential confounder is that traditional outcome measures may not capture the full effect of treatment, since many such therapies do not have well-recognized and understood physiologic mechanisms of action (i.e., *qi* or *chi* in Chinese medicine). The placebo effect must also be considered. Because many of the popular CAM therapies are in fact physical methods (i.e., massage or acupuncture) of treatment, it is difficult to formulate a placebo that is both inert and indistinguishable from the real treatment. Additionally, the lack of a uniform definition of CAM and the huge diversity of the different methods, therapies, and dogmas of CAM make the studies difficult to compare [3]. Enthusiasm, individuality, and the specific nature of the doctor-patient relationships play a role. Finally, the quality of these trials is frequently lacking in that many have small sample sizes and are not prospective, randomized, rigorously conducted, placebo-controlled studies, and they often have poor methodological characteristics and high incidences of bias.

Cardiovascular disease (CVD) is the leading cause of mortality in the United States for both men and women [4]. Approximately 600,000 people die of heart disease in the United States every year, representing one in every four deaths [4]. The most common form of CVD is coronary heart disease, which kills more than 358,000 people and costs the United States $108.9 billion each year [4]. Risk factors for CVD include hypertension, high LDL-cholesterol, smoking, diabetes, overweight and obesity, poor diet, physical inactivity, and excessive alcohol use [4]. Despite growing interest in CAM for cardiovascular health, few data are available regarding patterns of use of CAM for cardiovascular disease in the United States [5]. One study used the 2002 NHIS to analyze data on CAM use among patients with CVD and found that 36% of patients with CVD had used CAM (excluding prayer) in the previous 12 months [5]. Herbal products (echinacea, garlic, ginseng, ginkgo biloba, and glucosamine) and mind-body therapies (deep-breathing exercises and meditation) were used by 18% and 17% of patients, respectively, and constituted the most commonly used therapies [5]. Overall, CAM use in this patient population mirrored CAM use in the general population, with the most common reasons for use being musculoskeletal complaints, anxiety/depression, and stress/emotional health and wellness [5]. According to this study, fewer respondents (10%) used CAM specifically for their cardiovascular conditions (5% for hypertension, 2% for coronary disease, 3% for vascular insufficiency, <1% for heart failure or stroke) [5].

Another study with an international basis of patient information found that the prevalence of CAM use among people with CVD ranged from 4% to 61% (biologically-based therapies 22%–68%, herbal medicine 2%–46%, vitamins/minerals/dietary supplements 3%–54%, and mind-body therapies 2–57%) [6]. This review found that the use of CAM specifically to treat CVD ranged from 7% to 82%, depending on the study [6]. Physician awareness of their patients' CAM therapy use ranged from 8% to 65% secondary to fear of physician disapproval and lack of inquiry on the subject by the physician [6]. Some reasons for CAM use cited by patients include that CAM was thought to be of greater benefit than conventional medications (15%), adverse drug reactions to conventional therapy (59%), and overall well-being and promotion of good health [6].

2. CAM Effects on the Cardiovascular System and CVD Risk Factors

CAM may be broadly divided into 5 separate categories: biologically-based therapies, mind-body therapies, manipulative and body-based therapies, whole medical systems, and energy medicine [3].

2.1. Biologically-Based Therapies. The biologically-based therapies include aromatherapy, chelation therapy, diet-based therapies, folk medicine, iridology, megavitamin therapy, neural therapy, and phytotherapy/herbal medicine [3]. A number of these therapies have purported cardiovascular effects, but most research on these products is either inconclusive, conflicting, or shows no benefit for their use. It is equally important for the advancement of the legitimate and rigorous study of CAM to report negative as well as positive results, and it illustrates the need for studies of higher quality in this area.

Marine-derived omega-3 polyunsaturated fatty acids (fish oil) are often touted as being preventative of major cardiovascular adverse outcomes by the postulated mechanisms of lowering triglyceride levels (for which they are approved by the United States Food and Drug Administration (FDA)), preventing arrhythmias, decreasing platelet aggregation, or lowering blood pressure [7]. And while experts agree that fish rich in omega-3 fatty acids should be included in a heart-healthy diet, there is no evidence that omega-3 fatty acids in supplement form protect against heart disease [1]. A recent meta-analysis found that omega-3 polyunsaturated fatty acid supplementation was not associated with a lower risk of all-cause mortality, cardiac death, sudden death, myocardial infarction, or stroke based on relative and absolute measures of association [7].

Garlic is used most frequently as a dietary supplement for treatment of hyperlipidemia, heart disease, and hypertension. A well-conducted, randomized trial demonstrated that there was no significant difference in LDL-cholesterol, HDL-cholesterol, triglycerides, or total cholesterol-HDL ratio after six months of treatment with three preparations of garlic

versus placebo [8]. There is evidence that garlic is associated with blood pressure reductions in patients with elevated systolic blood pressures (10–12 mm Hg systolic, 6–9 mm Hg diastolic), but not in normotensive patients [9–11]. However, there is insufficient evidence to determine whether garlic provides a therapeutic advantage versus placebo in terms of reducing the risk of cardiovascular morbidity and mortality [9].

There is some evidence that ginseng has a plethora of cardiovascular benefits, including cardioprotection, antihypertensive effects, and attenuation of myocardial hypertrophy and heart failure [12]. However, a randomized, double-blind, placebo-controlled study demonstrated that Korean red ginseng had no significant effect on blood pressure, lipid profile, oxidized low density lipoprotein, fasting blood glucose, or arterial stiffness in subjects with metabolic syndrome [13]. A systematic review and meta-analysis also failed to demonstrate superiority of red ginseng over placebo in regard to effectiveness for type 2 diabetes mellitus [14].

Ginkgo biloba is purported to have cardioprotective effects by several studies through its antioxidant, antiplatelet, antithrombotic, vasodilatory, and antihypertensive properties [15]. A double-blind, placebo-controlled, randomized clinical trial determined, however, that the herb does not reduce blood pressure or the incidence of hypertension in elderly men and women [15]. The trial also noted that there was no evidence that ginkgo biloba reduced total or CVD mortality or CVD events; there were, however, more peripheral vascular disease events in the placebo arm, suggesting that the herb may reduce the risk of developing peripheral arterial disease [16].

Hawthorn leaf and flower extracts are advocated as an oral treatment option for patients with chronic heart failure; in fact, the German Commission E approved the use of hawthorn extracts in patients with heart failure graded stage II [17]. The results of a Cochrane review suggest that there is a significant benefit in symptom control and physiologic outcomes from hawthorn extract as a treatment adjunct for chronic heart failure [17]. For the physiologic outcome of maximal workload, treatment with hawthorn extract was more beneficial than placebo [17]. Hawthorn extract increased exercise tolerance, beneficially decreased cardiac oxygen consumption, and improved symptoms such as shortness of breath and fatigue as compared with placebo [17]. However, no data on relevant mortality and morbidity were reported [17]. The SPICE trial, a large, randomized, placebo-controlled, double-blind study, specifically looked at morbidity and mortality as endpoints [18, 19]. The study concluded that the primary endpoints—reductions in cardiac death, nonfatal myocardial infarction, and hospitalization due to progressive heart failure—were not achieved [19]. The SPICE trial also found that the deaths of a sudden cardiac cause, deaths due to progressive heart failure, and fatal myocardial infarctions were all lower in the treatment group; these figures, however, did not reach statistical significance [19]. Finally, the study suggested that treatment with hawthorn may reduce sudden cardiac deaths specifically in patients with left ventricular ejection fractions between 25% and 35% [19].

A meta-analysis determined that overall, flaxseed supplementation was associated with a decrease in blood total and LDL-cholesterol concentrations but did not significantly affect HDL-cholesterol and triglycerides [20]. The study reported that whole flaxseed interventions resulted in significant reductions in total and LDL-cholesterol, while flaxseed oil did not [20]. Flaxseed contains a large amount of fiber, and dietary soluble fiber has been shown to have cholesterol-lowering effects [20]. Flaxseed is also a rich source of dietary lignans. Purified lignans have been shown to reduce cholesterol in animal studies, but human data are limited [20]. Importantly, the beneficial effects of flaxseed were observed only among those with relatively high initial cholesterol concentrations and were more apparent in females (particularly postmenopausal females) [20]. A multitude of other cardiovascular benefits have been proposed for flaxseed due to its high alpha-linolenic acid content [21–23], but not enough reliable data are available to determine whether flaxseed is effective for heart disease in humans.

Antioxidants, which include anthocyanins, beta-carotene, catechins, coenzyme Q10, flavonoids, lipoic acid, lutein, lycopene, selenium, and vitamins C and E, have shown promising results in laboratory and observational studies; however, systematic reviews of the literature and large, randomized, controlled trials have generally found no beneficial effects of antioxidant supplements for primary or secondary prevention. In fact, vitamin A, beta-carotene, and vitamin E may actually increase mortality [24]. The Physicians Health Study II concluded that neither vitamin E nor vitamin C reduced the risk of major cardiovascular events (nonfatal myocardial infarction, nonfatal stroke, and CVD death) or had a significant effect on total mortality [25]. The Women's Health Study concluded that, overall, vitamin E did not reduce the risk of death or major cardiovascular events (myocardial infarction, stroke, or death) in almost 40,000 healthy women; however, there was a significant 24% reduction in the secondary endpoint of cardiovascular deaths and a significant 26% reduction in major cardiovascular events among a subgroup of women aged at least 65 years [26]. The Women's Antioxidant Cardiovascular Study found that there were no overall effects of vitamins C, E, or beta-carotene on cardiovascular events among women at high risk for CVD [27]. Possible reasons for the "disconnect" between findings of laboratory and observational studies and results of clinical trials, according to one article, may be that trials are entirely too short to reverse the results of decades of oxidative stress contributing to atherosclerosis or that the antioxidants selected for study were chosen for their easy availability rather than proven efficacy (vitamin E) [28].

Red yeast rice contains monacolin K, which has the same chemical structure as lovastatin, an inhibitor of HMG-CoA reductase [29]. Monacolin K in substantial amounts lowers blood levels of total cholesterol and LDL-cholesterol [29–31]. One study reported, however, that there is marked variability of monacolin levels in commercial red yeast rice products, and several products had elevated levels of citrinin, a potentially nephrotoxic mycotoxin [32]. Products with very little monacolin K may have little to no effect on blood cholesterol levels. Although red yeast rice has been marketed

to patients intolerant of statin drugs due to those drugs' side effects, there have been case reports of myopathy and rhabdomyolysis associated with red yeast rice [29]. In 1998, the FDA ruled that a red yeast rice product that contained a substantial amount of monacolin K was no longer a dietary supplement but an unapproved new drug and that marketing the product as a dietary supplement would be illegal [1]. Despite FDA action, some products tested as recently as 2011 have been found to contain substantial amounts of monacolin K [1]. Consumers therefore have no way of knowing how much monacolin K is present in most red yeast rice products or whether a particular product is safe, effective, or legal [1].

Soy protein and isoflavones (phytoestrogens) have gained attention for their potential role in improving risk factors for CVD [33]. An American Heart Association scientific advisory concluded that isolated soy protein with isoflavones, as compared with milk or other proteins, decreased LDL-cholesterol concentrations by an average amount of approximately 3% when about half of the usual total daily protein intake was soy protein [33]. No significant effects on HDL-cholesterol, triglycerides, lipoproteins, or blood pressure were evident [33]. Earlier research indicating that soy protein has clinically important favorable effects has not been confirmed by the meager evidence from clinical trials [33].

L-carnitine is FDA approved for replacement therapy in primary (i.e., inborn errors of metabolism) and secondary (i.e., secondary to hemodialysis) L-carnitine deficiencies [34]. Many clinical trials have suggested acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC), two naturally occurring carnitine derivates, as potential strategies in the management of peripheral arterial disease (PAD), heart and cerebral ischemia, and congestive heart failure [35]. The beneficial effects of PLC on PAD, particularly in alleviating intermittent claudication, have been widely studied. It is generally agreed upon that PLC is able to improve exercise tolerance in terms of increasing the maximum walking distance in patients suffering from intermittent claudication as well as to improve most measures of quality of life (overall physical activity, pain while walking, and psychological activity) [35]. The recent Trans-Atlantic Inter-Society Consensus II update recommends the use of PLC in combination with physical training to improve the symptoms associated with PAD [35]. However, it has recently been reported that the long-term administration of PLC to patients with intermittent claudication did not result in a statistically significant improvement in peak treadmill performance or quality of life as compared with exercise alone [35]. The clinical effectiveness of L-carnitine in the treatment of other CVD entities is not well established [35].

Chelation therapy is used to rid the body of excess or toxic metals (i.e., in lead poisoning). Some physicians and CAM practitioners have recommended EDTA chelation as a treatment for coronary heart disease (CHD) [1]. The bulk of evidence supporting EDTA chelation therapy is from case reports and case series, and the available randomized clinical trials, although underpowered, have seen no significant difference in direct or indirect measurements of disease severity and subjective measures of improvements [34]. The National Institutes of Health, including the National Heart, Lung, and Blood Institute and NCCAM, sponsored the Trial to Assess Chelation Therapy (TACT), the first large-scale, multicenter study designed to determine the safety and efficacy of EDTA chelation for patients with CHD [1]. Preliminary results of the trial were shared at the American Heart Association Scientific Sessions on November 4, 2012; however, the results will not be reported until they are published in the literature [1].

The results of a systematic review indicate that supplement use is common in cardiac patients (26%–42%) and that the concomitant use of dietary supplements and prescription medication also appears to be frequent (16%–64%) [36]. These results are important for several reasons. Not only is the evidence regarding the efficacy of these products generally inconclusive or unfavorable, but also there is significant opportunity for danger in their use. Most patients believe that the government oversees the safety of CAM; however, the only requirement is for the manufacturer to send a copy of the product label to the FDA [37]. A new dietary supplement may be introduced and marketed rapidly despite containing new, experimental, or unregulated herbal ingredients, and many supplements contain ingredients or contaminants with adverse effects or interactions [37].

The general public regards biologically-based therapies as safe, natural, and as having fewer side effects than conventional medications (29%–60% of CAM users) [6, 37]. The lack of knowledge about herb-drug and herb-herb interactions and herb adverse effects by patients and health care providers is also problematic. A recent review determined that cardiovascular patients consumed on average seven prescribed medications and two herbal, vitamin, or mineral products daily [6]. One study identified 42 potential herb-drug interactions among these patients [6]. For instance, garlic may interact with aspirin, clopidogrel, warfarin, or heparinoids to increase bleeding risk [37]. Gingko biloba can increase hypoglycemia when taken with antidiabetes drugs and may increase bleeding when taken with aspirin or warfarin [37]. Ginseng also increases hypoglycemia with antidiabetes drugs, leads to falsely increased levels of digoxin, and decreases effectiveness of warfarin [37]. Hawthorn increases the effects of digoxin and increases coronary vasodilatory effects of calcium channel blockers or nitrates [37]. Echinacea increases QT interval when taken with amiodarone or ibutilide and increases the risk of hepatotoxic effects with statins, fibrates, or niacin [37]. St. John's wort decreases serum digoxin concentration, increases activity of clopidogrel, decreases warfarin effectiveness, decreases simvastatin effectiveness, and decreases the effectiveness of class IA and III antiarrhythmics [37]. Supplemental potassium was taken by 20% of patients in one study, which can result in adverse outcomes when used concomitantly with angiotensin converting enzyme inhibitors, aldosterone receptor antagonists, or angiotensin receptor blockers [6, 35]. One study demonstrated that 64% of the patients with a diagnosis of atrial fibrillation, CHF, or ischemic heart disease attending a cardiovascular clinic reported concomitant use of CAM and prescription drugs—58% took supplements that had potential interactions with warfarin, amiodarone, sotalol, or digoxin [37]. There are innumerable other interactions and side effects that must

be taken into consideration when using biologically-based therapies.

2.2. Mind-Body Therapies. The mind-body therapies (MBT) include anthroposophical medicine, autogenic training, biofeedback, bioresonance, cognitive-behavioral therapies, deep-breathing exercises, group support, hypnosis, imagery, meditation, prayer, relaxation, Qigong, tai chi, yoga, and shiatsu [3]. One review reported on the prevalence of MBT usage, which ranged from 2% to 57%, with deep breathing and meditation representing the most common therapies in the category [6]. In contrast to the complex and controversial body of research surrounding biologically-based therapies, there is a growing body of research suggesting that MBT are relatively safe and may have measurable benefits for cardiovascular health [5]. Cardiac patients used MBT most commonly for stress, emotional health, and general wellness—indeed, this use is supported by an established body of research on psychosocial support, stress management, and coping skills in cardiac rehabilitation and the influence of stress hormones, cortisol, and the hypothalamic-pituitary-adrenal (HPA) axis as mediators of cardiac risk [5]. Thus, the use of MBT for this purpose has become more widely accepted [5]. In fact, a systematic review suggested that MBT were cost-effective in patients with recent cardiac events and after cardiac surgery [5].

Relaxation techniques include practices such as progressive relaxation, guided imagery, biofeedback, self-hypnosis, and deep-breathing exercises [1]. The goal of these techniques is to consciously produce the body's natural relaxation response, characterized by slower breathing, lower blood pressure and oxygen consumption, and a feeling of calm and well-being [1]. A 2008 Cochrane review found that interventions to promote relaxation were associated with a small, but statistically significant, reduction in both systolic blood pressure (5.5 mm Hg) and diastolic blood pressure (3.5 mm Hg) [38]. However, when relaxation was compared with sham therapy, the mean reductions in blood pressure were smaller and not statistically significant [38]. The review noted that in light of the poor methodological quality of the included studies, it is difficult to draw any definitive conclusions regarding the efficacy of relaxation techniques for primary hypertension or for reducing morbidity (myocardial infarctions and stroke) and mortality [38]. A 2008 double-blind, randomized trial comparing relaxation versus lifestyle modification found that both groups had similar reductions in systolic blood pressure; however, significantly more participants in the relaxation response group eliminated an antihypertensive medication while maintaining adequate blood pressure control [39]. Although more studies are needed regarding the effect of relaxation on heart disease, one observational study did find that combining relaxation response training with cardiac rehabilitation resulted in significant reductions in blood pressure, decreases in blood lipid levels, and increases in psychological functioning [40].

Meditation refers to a group of techniques such as mantra meditation, mindfulness meditation, transcendental meditation, and Zen Buddhist meditation [1]. There is evidence that meditation is associated with potentially beneficial health effects. For instance, a meta-analysis found that transcendental meditation resulted in a reduction of 4.7 mm Hg in systolic blood pressure and 3.2 mm Hg in diastolic blood pressure [41]. Another review article suggested that transcendental meditation may reduce blood pressure as well as other risk factors for CVD such as cholesterol, oxidized lipids, and smoking [42]. However, most clinical trials on meditation practices are generally characterized by poor methodological quality with significant threats to validity in every major quality domain assessed [43]. Thus, future research must be more rigorous before firm conclusions may be drawn.

Yoga has many different styles, some more physically demanding than others. In general, practicing yoga, as well as other forms of regular exercise, leads to several cardiovascular benefits. Yoga typically causes increased heart rate during the act, but following prolonged training, a decrease occurs in exercise-induced heart rate [44]. One study that looked at the effects of yoga on heart rate and blood pressure in healthy men found that the men in the yoga group showed greater decreases in heart rate and blood pressure and greater aerobic performance after 3 months as compared to the control group (flexibility exercises and slow running) [44]. Numerous studies have also commented on positive findings regarding weight loss, control of blood glucose, control of blood lipids, reduction in number of anginal episodes in patients with advanced coronary artery disease, and improved general quality of life [44]. Some research indicates that there may be a difference between yoga and exercise. Different levels of intensity of exercise have been shown to affect the HPA axis response to acute stress differently—low-intensity exercise lowers cortisol levels and sympathetic stimulation, while intense exercise raises cortisol levels and stimulates the sympathetic nervous system, raising levels of epinephrine and norepinephrine [45]. Exactly how this influences cardiovascular morbidity and mortality requires further research.

Tai chi, sometimes referred to as "moving meditation," encompasses many styles, but all involve slow, relaxed, gentle movements [1]. A systematic review of the literature determined that in 22 of 26 studies, reductions in blood pressure (3–32 mm Hg systolic, 2–18 mm Hg diastolic) with tai chi were reported [46]. Another systematic review also concluded that tai chi appears to have physiological and psychosocial benefits and appears to be safe and effective in promoting balance control, flexibility, and cardiovascular fitness in older populations with chronic conditions [47]. However, limitations and biases exist in most studies analyzed; thus, drawing firm conclusions about the benefits reported is difficult [47]. A recent randomized clinical trial found that tai chi may improve quality of life, mood, and exercise self-efficacy in people with chronic heart failure, despite the absence of differential improvement in peak oxygen intake and 6-minute walk test compared with education only [48]. Given that tai chi practice is safe and has good rates of adherence, it may represent an important complement to standard medical care in the treatment of deconditioned patients with systolic heart failure [48]. Further research is needed to explore these possibilities.

2.3. Manipulative and Body-Based Therapies. The manipulative and body-based therapies include acupressure, Alexander technique, Bowen technique, chiropractic manipulation, Feldenkrais method, massage, osteopathic manipulation, reflexology, Rolfing, Trager bodywork, and Tui na [3].

Massage therapy encompasses many different techniques, such as Swedish massage, sports massage, deep tissue massage, and trigger point massage [1]. Our study found that deep tissue massage resulted in a systolic blood pressure reduction of 10.4 mm Hg, diastolic pressure reduction of 5.3 mm Hg, mean arterial pressure reduction of 7 mm Hg, and an average heart rate reduction of 10.8 beats per minute directly after the massage took place [49]. A review of the literature remarked that single treatment reductions in salivary cortisol and heart rate were consistently noted, but sustained reductions for these measures were not supported in the literature [50]. No change was seen in urinary catecholamines at any point [50]. More research on the long-term effects of repeated messages is necessary.

Spinal manipulation, as found in chiropractic and osteopathic manipulation, has been reported to successfully treat hypertension [51]. A systematic literature review however found that there is a lack of low bias evidence to support the use of spinal manipulation therapy to treat hypertension, as statistically significant decreases in blood pressure were not observed in trials with low bias [51].

2.4. Whole Medical Systems. The whole medical systems include acupuncture (as part of traditional Chinese medicine), Ayurveda, homeopathy, and naturopathy [3].

Acupuncture is a therapeutic modality anchored in traditional Chinese medicine (which also includes Chinese herbal medicine, moxibustion, cupping, Chinese massage, mind-body therapies such as Qigong and tai chi, and dietary therapy) [1]. A systematic review and meta-analysis that statistically pooled 3 sham-controlled trials out of 11 studies found that systolic blood pressure change was not statistically significant (−5 mm Hg) and acupuncture only marginally reduced diastolic blood pressure by 3 mm Hg, but substantial heterogeneity was observed [52]. When given with antihypertensive medication, acupuncture significantly reduced systolic blood pressure (−8 mm Hg) and diastolic blood pressure (−4 mm Hg) with no heterogeneity detected [52]. Given the poor methodological quality and small sample sizes of most acupuncture trials, the notion that acupuncture may lower high blood pressure is inconclusive [52]. A systematic review and meta-analysis of 29 randomized controlled trials found that acupuncture was associated with a significant reduction of average body weight of 1.72 kg compared to control of lifestyle and a significant reduction of body weight of 1.56 kg compared to placebo or sham treatment [53]. Again, given the poor methodological quality of the trials reviewed, it is difficult to say that the evidence is fully convincing [53]. There is also some evidence that acupuncture may help to correct various metabolic disorders such as hyperglycemia and hyperlipidemia, but further rigorous investigation in this area is warranted [54].

Most rigorous clinical trials and systematic analyses of the research on homeopathy have concluded that there is little evidence to support it as an effective treatment for any specific condition [1]. There are mixed results concerning the research for the efficacy of naturopathy, and there is little scientific evidence currently available on the overall effectiveness of this treatment system [1].

2.5. Energy Medicine. Energy medicine includes healing touch, light therapy, magnetic therapy, millimeter wave therapy, Qigong, Reiki, and sound energy therapy [3]. This category is reportedly the least utilized and least studied of the CAM modalities [3].

The biofield therapies of Reiki, therapeutic touch, and healing touch are known as "hand-mediated" therapies and are used to reduce pain and anxiety and to promote health through the direction of healing energy [55]. There are reports describing changes in the physiological parameters of heart rate, skin temperature, muscle tone, and skin conductance in response to biofield therapies [55]. Most reviews of the most commonly researched energy therapies conclude that more research is needed, despite potentially promising findings [56]. A 2007 review concluded that studies of biofield therapies can only suggest efficacy in reducing anxiety, improving muscle relaxation, aiding in stress reduction, relaxation, and sense of well-being, promoting wound healing, and reducing pain [56]. A 2010 randomized controlled study published in the Journal of the American College of Cardiology found in a study of immediate postacute coronary syndrome inpatients that reiki significantly increased vagal activity as measured by high-frequency heart rate variability compared with resting and music control conditions, with a decrease in negative and an increase in positive emotional states [57]. The magnitude of the effect on heart rate variability seen was similar to that of propranolol in the Beta Blocker Heart Attack Trial [57]. A randomized clinical trial measuring the efficacy of healing touch in coronary artery bypass surgery recovery found no significant decrease in the use of pain medication, antiemetic medication, or incidence of atrial fibrillation; however, significant differences were noted in anxiety scores and length of stay, and all healing touch patients showed a greater decrease in anxiety scores when compared to the visitor and control groups [58]. More rigorous research is needed to determine the physiologic mechanisms and long-term benefits of these therapies.

3. Conclusion

CAM use among patients with CVD is prevalent, with biologically-based and mind-body therapies being the most commonly used treatment modalities. This review illustrates the necessity of both more rigorous research to determine the precise physiologic effects and long-term benefits on cardiovascular morbidity and mortality with CAM usage as well as more open lines of communication between patients and physicians regarding CAM use. Finally, it is hoped that both physicians and patients gain an appreciation of what

the evidence does and does not say with respect to CAM use for CVD and take this into consideration when determining optimal treatment plans.

Disclosure

The authors have no relationships with pharmaceutical companies or products to disclose, nor do they discuss off-label or investigative products in this lesson.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 125649, 9 pages
http://dx.doi.org/10.1155/2013/125649

Review Article

Role of Garlic Usage in Cardiovascular Disease Prevention: An Evidence-Based Approach

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Received 20 February 2013; Accepted 23 March 2013

Academic Editor: Kashmira Nanji

Introduction. Rapidly growing prevalence of cardiovascular disease is a major threat for the developed as well as developing world warranting urgent need of intervention. Complementary and alternative medicines are gaining popularity among general population because of their safety profile and easy administration. Garlic, in particular, is considered to be one of the best disease-preventive foods because of its potent and widespread effects. This study was done to find out the role of garlic usage in cardiovascular disease prevention. *Methodology*. Major databases including Google, PubMed, MEDLINE, and Cochrane library view were used for the literature search. Clinical trials conducted on humans assessing role of garlic usage in cardiovascular disease prevention and the possible mechanisms responsible for such therapeutic actions were assessed. *Results*. Various clinical trials and meta-analyses conducted have shown positive impact of garlic in cardiovascular-disease prevention especially its effects on lipid levels; however, some contradictory results are also reported. Similarly, its effects on hypertension control, and platelet are also mild with limited data availability. The possible reason for these inconsistent results is the difference in preparations with diverse composition, variations in sulphur content present in different garlic preparations used, and methodological variations in subject recruitment, duration of study, dietary control and so forth. *Conclusion*. Garlic can be used as an adjuvant with lipid-lowering drugs for control of lipids, however, its role as a main therapeutic agent cannot be recommended and it is suggested that more meta-analyses using standardized preparations with a close watch on methodological shortfalls should be conducted to prove its role.

1. Introduction

The epidemic of cardiovascular disease is growing at an alarming pace throughout the world [1]. It is recognized as one of the leading causes of mortality worldwide causing more than 80% of deaths in low- and middle-income countries [2]. Cardiovascular disease refers to spectrum of illnesses that includes heart disease, vascular diseases of the brain, kidney, and peripheral arterial disease [3]. According to an estimate by World Health Organization, approximately 17.3 million people died from CVDs in 2008, representing 30% of all global deaths. Out of these deaths, 7.3 million occurred secondary to coronary heart disease and 6.2 million as a consequence of stroke [2]. It is anticipated that by 2020 cardiovascular diseases are predicted to be the major cause of morbidity and mortality in most developing nations around the globe [4]. Atherosclerosis and hypertension are measured as the major risk factor along with smoking, obesity, and sedentary life styles leading to increasing trend of this major threat [5].

Today, in this era of rapid advancement in medical technology, herbal or botanical preparations, commonly referred to as complementary and alternate medicine (CAM), approaches have gained lots of popularity in terms of health care maintenance, and a large number of population in the developing as well as developed world prefer to use (CAM) as a source of curative and preventive remedy for various illnesses [6, 7]. CAM is defined as a group of diverse medical and health systems practices and products that are not generally considered as part of conventional medicine [8]. According to 2007 National Health Interview Survey (NHIS) report, approximately 38% of US adults and 12% of children are using CAM in the past 12 months; lifetime prevalence of CAM use in the United States and worldwide has

increased steadily since 1950 (9.10). Most common types of CAM therapies used were natural products, such as fish oil/omega 3, glucosamine, *Echinacea*, and flaxseed (17.7%), deep breathing (12.7%), meditation (9.4%), chiropractic and osteopathic (8.6%), and massage (8.3%), followed by yoga, diet-based therapies, progressive relaxation, guided imagery, and homeopathic treatment [9].

Among all these remedies, plants-based functional foods have gained lots of recognition throughout the world and it is believed that these natural substances have the potential to reduce the detrimental effect of a number of cardiovascular diseases and associated risk factors [10]. The probable reason for this rising trend is skeptic approach of general public towards conventional medicine due to fear of more side effects and increasing cost. This fact was further driven by the belief of increased safety profile and easy availability of plant-based natural products in comparison to orthodox medicine. Garlic has been used as a therapeutic agent for many illnesses over centuries as evident from various studies; however, its role in cardiovascular disease prevention is still questionable. This review was done to find out the efficacy of garlic in cardiovascular disease prevention through evidence-based approach by analyzing clinical trials and systematic review in the above mentioned area.

2. Garlic as a Potential Herb

Garlic (*Allium sativum*) has played an important dietary as well as medicinal role in human history. The role of garlic (*Allium sativum*) as a potential herb has been acknowledged for over 5000 years. Garlic and its various preparations are being readily consumed as a food and spice by various cultures for centuries [11]. It was also documented as a choice of medical therapy to combat many diseases among Egyptians [12]. Similarly, it is also considered as an imperative part of Indian traditional medicine, that is, Ayurveda, Tibbi and Unani, and so forth. In addition, it is also claimed to possess beneficial effects for the prevention of various aspects of cardiovascular disease including hypertension and dyslipidemia [13].

3. Garlic Composition

Garlic is available in many forms among these raw garlic and aqueous extract preparation is used more frequently. Allicin is the principal bioactive compound present in garlic and primarily contains sulphur as a main constituent which on break down gives garlic its characteristic odor. It is produced as a result of activation of alliinase enzyme after crushing or chopping of raw garlic. The enzyme Allinase is inactivated by heat leaving behind alliin as the main constituent present in the water extract of heat-treated garlic. The composition of garlic powder which is produced after dehydration and crushing is the same as raw garlic and alliinase activity is preserved; however, caution needs to be taken regarding temperature regulation as Allinase is inactivated if temperature exceeds beyond 60°C. Apart from Alliin, other important sulfur-containing compounds present in garlic homogenate include allyl methyl thiosulfonate, 1-propenyl allyl thiosulfonate, and γ-L-glutamyl-S-alkyl-L-cysteine. On an average, a garlic bulb contains up to 0.9% g-glutamylcysteines and up to 1.8% alliin [14].

4. AGE Preparation

Another important and extensively studied garlic preparation is aged garlic extract (AGE). This form is produced by storage of sliced raw garlic in 15–20% ethanol for 20 months. This process of storage leads to alteration in composition of the garlic extract, the odorous, harsh, and irritating compounds in garlic are converted naturally into stable and safe sulfur compounds with substantial loss of allicin activity and increased activity of new compounds, like S-allylcysteine (SAC), S-allylmercaptocysteine, and allixin [15]. SAC can be used for standardization because of its bioavailability property.

5. Garlic Oil

Garlic oil another important preparation is produced as a result of distillation process of raw garlic. Garlic essential oil is obtained by steam distillation of garlic. The essential oil content of garlic cloves is 0.2–0.5% and consists of a variety of sulfides, such as diallyl disulphide (DADS) and diallyl trisulfide (DATS) [16, 17]. All the water soluble contents including allicin are completely eliminated from the oil. Oil macerates were originally developed for use as condiments. Oil macerate products are made of encapsulated mixtures of whole garlic cloves ground into vegetable oil. This preparation contains allicin-decomposed compounds such as dithiins, ajoene and sulfides, residual amounts of alliin, and other constituents in garlic [16].

6. Garlic Powder

Garlic powder is primarily used as a flavoring agent for condiments and processed foods. Garlic cloves are sliced or crushed, dried, and ground into powder. The composition of garlic powder is the same as that of raw garlic; however, the proportions and amounts of various constituents differ significantly; that is, average content of alliin present in garlic is 0.8% however, raw garlic contains around 3.7 mg/gm of alliin [18].

7. Garlic and Cardiovascular Disease Prevention

Cardiovascular disease is one of the leading cause of morbidity and mortality worldwide. Role of garlic in cardiovascular disease prevention has been a topic of concerns for many years. Various observational and experimental studies done on animals showed encouraging results. However, these claimed benefits were not supported by evidence-based clinical studies. This fact prompted many researchers to conduct clinical trials in order to explore and address the efficacy and association of garlic with various aspects of cardiovascular disease.

In recent years, garlic has been a focus of attention because of its potential role in the prevention of various

aspects of cardiovascular disease [19, 20]. Evidence from numerous studies suggests that garlic works through various mechanisms to achieve this favorable effect including reduction of serum lipids and blood pressure levels, inhibition of platelet aggregation, and increasing fibrinolytic antioxidant activity. Majority of the studies reported have shown positive impact; however, few numbers of contradictory studies have [21, 22] made the role of garlic questionable especially with regards to its effects on lipid levels and hypertension. This review will critically examine the current scientific literature concerning claims of cardiovascular benefits from regular consumption of garlic or its preparations and the possible mechanisms responsible for such therapeutic actions.

8. Methodology

This paper is based on a literature search of clinical trials and systematic reviews published from 1990–2012 to see the effect of Garlic on cardiovascular disease prevention. For this purpose multiple search engines including MEDLINE, PubMed, Google, and Cochrane library were used. Search was validated by other author.

9. Inclusion Criteria

All human studies (clinical trials) in English assessing the effect of garlic on cardiovascular disease prevention among patients with dyslipidemia, hypertension, or cardiovascular disease were included.

10. Exclusion Criteria

Studies conducted among animals were excluded. Theses, dissertations, unpublished data, and letter to editor were also excluded.

A number of keywords were used for data searching including garlic and cardiovascular disease clinical trial, garlic hypertension and dyslipidemia, platelet aggregation, and clinical trial.

11. Garlic Role in Dyslipidemia

Dyslipidemia is documented as a major risk factor responsible for the development of atherosclerosis and cardiovascular disease [23]. Lipid abnormalities include high LDL-C (low-density lipoprotein cholesterol), high triglycerides and low HDL-C (high-density lipoproteins cholesterol) levels. Cholesterol present in β-lipoprotein (LDL) and pre-B-lipoprotein gets deposited into the blood vessels, while α-lipoprotein (HDL) helps to reduce serum cholesterol [24]. Impact of garlic on elevated lipid level is the most widely studied outcome of interest as evident from Table 1. Considerable evidence from the literature supports the invaluable role of garlic in the treatment of hypercholesterolemia through inhibition of cholestrol biosynthesis in the liver and also by inhibition of oxidation of low-density lipoproteins [25]. Dietary approach is the initial step in the management of dyslipidemia, and many people with dyslipidemia are using garlic as an alternative medicine to normalize their raised lipid levels.

A number of randomized, controlled trials were carried out to see the effect of different preparation of garlic on lipid levels. In the early 1980s, a trial done [19] on human subject after ingestion of 40 gm garlic demonstrated significant reduction in total cholestrol and triglyceride levels. Similarly, one study conducted by Mader [20] in 1990 among patients suffering from dyslipidemia over a period of 16 weeks using 800 mg of garlic (standardized to 1.3% of Alliin) showed 12% reduction in serum cholestrol levels and 17% reduction in triglyceride levels in comparison to placebo; however, it was also noticed that the greatest cholesterol-lowering effects were seen in patients with initial total cholesterol values between 250 and 300 mg/dL. The results of this trial were somewhat contradicted by findings of a trial by Saradeth et al. [26] where 600 mg of dried garlic powder (Kwai, Lichwer standardized to 1.3% alliin) was given to healthy patients with normal lipid levels over a period of 10 weeks. There was a significant reduction in total cholestrol and triglycerides levels confirming the fact that it can induce changes in blood lipids, even if these variables had been normal to start with Similarly another trial by Gadkari and Joshi on healthy medical students after consumption of 10 gm raw garlic showed significant reduction in serum cholesterol and increase in clotting time and fibrinolytic activity [27].

Clinical trials using different types of garlic preparations in hypercholesterolemia patients have demonstrated debatable results, and it was assumed that these discrepancies may have resulted due to the differences of the composition of garlic preparations and the response they may induce. This fact was well proven by a study done by Sobenin et al. [28] in which patients with mildly raised lipid levels were given garlic powder tablets (allicor) containing 600 mg of garlic content. A moderate decrease in lipid levels was seen (7.6% decrease in cholesterol; 11.7% decrease in LDL levels); in addition, a substantial rise in HDL level 11.5% was also noticed. It was assumed that this hypocholesterolemic action of garlic preparations may be due to the use of a time-released form of garlic powder tablets. Similarly, a commonly used preparation of garlic in the form of AGE extract of 7.2 gm daily for 6 months also showed beneficial effects on the lipid profile of moderately hypercholesterolemia subjects. There was an overall 6.1% decrease in cholestrol levels and 4% decrease in LDL levels noticed thus confirming its efficacy [29].

Another randomized placebo control trial using 5 gm of raw garlic on patients with mildly raised lipids was used for 42 days and demonstrated significant reduction in cholestrol and triglyceride levels with a rise in HDL levels; however, these effects were not sustainable and returned to baseline levels as soon as the garlic use was withdrawn. This suggested that garlic consumption alone can decrease serum lipids in patients with mildly raised lipid levels; however, it cannot be used as the main therapeutic agent for hyperlipidemia [30].

Dyslipidemia refers to increase in cholestrol, triglycerides, and LDL levels with a decrease in HDL level (below 40 mg). It was expected that apart from decreasing cholestrol, LDL and triglycerides levels, garlic also has an impact on low HDL which was further established by a trial conducted on healthy individuals with a decreased HDL levels below 10 mg at baseline. They were given high-fat diet followed

Table 1: Effects of garlic on lipid levels.

Study	Type	Target	Duration of Rx	Dose	Case/control	Outcome
Mader, (1990) [20]	Randomized, placebo-controlled trial	Hyperlipidemic	12 weeks	800 mg garlic powder	130/131	Dec in T. chol level—12%, TG level—17%
Gadkari and Joshi, (1991) [27]	Randomized control trial	Normal individuals	2 months	10 gm of raw garlic	25/25	Dec T. chol, increase clotting time, and fibrinolytic activity
Rotzsch et al., (1992) [31]	Randomized, placebo-controlled, double-blind trial	Healthy individuals with low HDL	6 weeks	900 mg garlic powder	12/12	Dec TG levels and increase HDL levels
Saradeth et al., (1994) [26]	Randomized double-blind study, placebo-controlled trial	Healthy individuals with normal lipid levels	15 weeks	600 mg dried garlic powder	34/34	T. chol dec from 223 to 214 mg/dL TG dec from 124 to 118 mg/dL
Steiner et al., (1996) [29]	Double-blind crossover trial	Hyperlipidemic	11 months	7.2 g aged garlic	20/21	Dec T. chol 6.1%, dec LDL 4%, systolic BP 5.5% dec, and modest dec in diastolic Bp noticed
Isaacsohn et al., (1998) [38]	Randomized, double-blind, placebo-control trial	Hyperlipidemic	12 weeks	900 mg garlic powder (Kwai)	28/22	No change in lipid levels noticed
Berthold et al., (1998) [39]	Double-blind, randomized, placebo-controlled trial	Hyperlipidemic	12 weeks	10 mg garlic oil	12/13	No change in lipids or lipoproteins levels noticed
Satitvipawee et al., (2003) [37]	Randomized, double-blind, placebo-controlled trial	Hyperlipidemic	4 weeks/12 weeks	Garlic extract	70/76	No dec in T. chol, DL, TG, and HDL levels noticed
Mahmoodi et al., (2006) [30]	Clinical trial	Hyperlipidemic	42 days	Raw garlic 5 gm twice daily	30	Dec T. chol, dec LDL, dec TG, increase HDL level Reversed after stopping of garlic
Sobenin et al., (2008) [28]	Double blinded placebo controlled	Hyperlipidemic	12 weeks	Allicor (600 mg daily)	21/21	T. chol 7.6% dec, LDL 11.8%, and HDL inc 11.5%

T. chol: total cholesterol, HDL: high-density lipoprotein, TG: triglyceride, LDL: low-density lipoprotein, and VLDL: very low-density lipoprotein.

by garlic powder preparation (Sapec, Kwai) of 900 mg daily for 6 weeks. A significant decrease in triglyceride levels was observed in the treatment group in comparison to placebo group with a significant rise in HDL levels above baseline [31]. Similarly another study reported that 3 g of fresh garlic (1 clove) daily for 16 weeks had a 21% decrease in cholesterol levels [32].

Despite the existence of various clinical trials, the role of garlic in treating dyslipidemia is still debatable. In order to address this query a various meta-analyses were also conducted. A meta-analyses done by Silagy and Neil studied 16 trials among 952 patients using garlic, both in powder and nonpowder form. There was an overall reduction in cholestrol level seen that is, 8% with powdered form while 15% with nonpowder preparations. Significant lowering of serum triglyceride was also noticed, while HDL level remains unchanged [33].

Similarly, another meta-analyses by Warshafsky et al. among patients with cholestrol levels greater than 200 mg showed significant reduction in total cholesterol levels. It was suggested that garlic in an amount approximately one half to one clove per day is effective in reducing cholesterol levels by about 9% [34].

A recent meta-analyses conducted by Zeng et al. in 2012 clearly illustrated that garlic therapy is more effective if used for a long term with higher baseline total cholestrol levels; they also concluded that garlic powder and aged garlic extract were more effective in reducing serum TC levels, while garlic oil was more effective in lowering serum TG levels [35]. A trial comparing garlic with a commercial lipid-lowering drug (bezafibrate) found them to be equally effective in decreasing lipids to a statistically significant extent [36].

There were few clinical trials which did not show any effects on lipid levels. A trial done by Satitvipawee et al. for

Table 2: Effect of garlic on blood pressure levels.

Study	Type	Target	Duration of Rx	Dose	Case/control	Outcome
Zhang et al., (2001) [48]	Parallel-controlled trial	Hypertensives	16 weeks	Distilled garlic oil 12.3 mg/d	14/13	Garlic oil lowers SBP and DBP
Dhawan and Jain, (2004) [49]	Not placebo controlled	Hypertensives	2 months	Garlic pearls 250 mg	20/20	Dec Bp, dec ox-LDL, and 8-iso-PGF2alpha levels
Capraz et al., (2007) [55]	Randomized placebo control trial	Hypertensives	70 minutes	Rw garlic, Garlic tablets	25/25/25	No effects on BP levels
Duda et al., (2008) [51]	Prospective and uncontrolled clinical study	Hypertensives	30 days	Antihypertensive drug + Garlic capsules	38/32	Dec total lipids and lipid peroxidation noticed
Ried et al., (2010) [50]	Randomized placebo control trial	Hypertensives	12 weeks	960 mg AGE	25/25	SBP—10.2 ± 4.3 mmHg dec
Ried et al., (2013) [53]	Double-blind, randomized placebo-controlled, dose-response trial	Hypertensives	12 weeks	Aged garlic 240/480/960 mg	26/26/27	SBP—11.8 ± 5.4 mmHg, (2 capsule) 7.4 ± 4.1 mmHg (4 capsule)

T. chol: total cholesterol, HDL: high density lipoprotein, TG: triglyceride, LDL: low-density lipoprotein, VLDL: very low-density lipoprotein, 8-OHdG: (8-Hydroxy-2′-deoxyguanosine), and 8-iso-PGF2alpha: (8-iso-Prostaglandin F2alpha).

a period of 12 weeks using 5.6 mg/tablet garlic tablets showed no significant improvement in lipids levels [37].

An RCT in which 900 mg garlic in the form of tablets (Kwai) was given daily to patients with hypercholesterolemia showed no significant change in lipid levels in comparison to placebo group [38]. Similarly, in other trial steams distilled garlic oil in a quantity of 5 mg twice daily for 12 weeks showed no influence on lipid levels [39]. A trial with garlic usage in the form of dried form in a dose of 600 mg to 1500 mg did not show any effects suggesting that dried preparation in the dosage studied were ineffective in reducing lipid levels [40]. Similarly, a meta-analyses by Khoo and Aziz also showed insignificant outcomes [41].

One trial of garlic extract treatment in children with hypercholesterolemia found no adverse effects and no significant beneficial effect on lipid levels [42].

Clinical investigations exploring the effects of garlic and its various preparations in hypercholesterolemia have demonstrated somewhat contradictory results. The diverse composition and amount of active sulfur compounds of different garlic preparations used in various trials might be responsible for the above mentioned inconsistent findings. Other factors like subject recruitment, duration of study, dietary control, lifestyle, and methods of lipid analyses may also have an influence. These findings emphasize the need for standardization of garlic preparations in order to reach to a valid conclusion.

12. Effects of Garlic on Hypertension

Hypertension is an important risk factor for leading to cardiovascular disease. Currently, it affects 1 billion people worldwide, and this number is expected to rise to 1.6 billion by 2025 [43, 44]. Garlic regular consumption has shown some association with blood pressure control. Blood pressure reducing properties of garlic are related with the hydrogen sulphide production [45] and allicin content liberated from alliin and the enzyme alliinase [46] which is assumed to possess angiotensin II inhibiting and vasodilating effects. Garlic is used as a treatment remedy by many people worldwide to control blood pressure. According to one survey, approximately 29% of people are using garlic for their blood pressure control [47].

The antihypertensive effects of garlic have been studied, but the remaining controversial various studies done showed controversial results as evident from Table 2. Clinical trial done by Zhang et al. consuming garlic oil in hypertensive patients over the 16-week period showed significant results [48]. A trial using garlic pearls containing 250 mg of garlic among hypertensive patients for 2 months demonstrated decrease in blood pressure level and also showed decrease in biomarkers responsible for oxidative stress in blood (plasma-oxidized LDL, plasma, and urinary concentration of 8-iso-Prostaglandin F2alpha) ultimately decreasing the risk of cardiovascular disease [49].

Majority of patients used garlic as a remedy for prevention from dyslipidemia and hypertension various illnesses. An RCT conducted by Ried et al. on patients with uncontrolled blood pressure used AGE preparation of 900 mg garlic containing (2.4 mg salicystine) for 12 weeks and concluded that significant reduction in blood pressure level was noted only among patients who had blood pressure values of more than 140 mm Hg at baseline [50] suggesting that its role in primary prevention is questionable.

Another trial by Duda et al. assessed the role of garlic on blood pressure and lipids levels and concluded that garlic can be used as a tentative treatment along with antihypertensive drug because of its positive effect on lipid levels and antioxidant properties [51].

Few meta-analyses were also done to see the efficacy. In 1994, a meta-analyses assessed the effect of garlic on hypertension, among which three trials showed significant

Table 3: Effect of garlic on cardiovascular disease.

Study	Type	Target	Duration of treatment	Dose	Route	Case/control	Outcome
Bordia et al., (1998) [63]	Placebo control trial	Coronary artery disease patients	3 months	1 gm garlic (capsules)	Oral	30/30	Dec T. chol and TG, increase HDL level, and no effect on fibrinogen and glucose level
Sobenin et al., (2010) [71]	Randomized control trial	Coronary artery disease patients	1 year	Time-released garlic powder	Oral	26/25	Dec LDL—32.9 mg/dL in males, 27.3 mg/dL in females

T. chol: total cholesterol, HDL: high-density lipoprotein, TG triglyceride, LDL: low-density lipoprotein.

reductions in systolic blood pressure (7.7 mm Hg greater reduction), and four trials showed reductions in diastolic blood pressure (5 mm Hg greater reduction) in comparison to placebo [52].

A meta-analyses conducted by Ried et al. showed significant results with decrease in systolic blood pressure of about 16.3 mm Hg and diastolic blood pressure of about 9.3 mm Hg in comparison to placebo group; however, these effects were only observed in patient having systolic blood pressure values more than 140 mm Hg [53].

Another meta-analyses done concluded that garlic reduces mean supine systolic and diastolic blood pressure by approximately 10–12 mm Hg and 6–9 mm Hg, respectively, over and above the effect of placebo, but the confidence intervals for these effect estimates are not clear cut, and this difference in blood pressure reduction may be due to subjective variation in blood pressure measurements suggesting more clinical trials [54].

Few trials done by Capraz et al. and Pittler and Ernst showed insignificant results [55, 56]. Similarly a meta-analyses done by Simons et al. also showed insignificant results with no effects on blood pressure levels and concluded that the effect of garlic on blood pressure cannot be established [57].

To ascertain the effectiveness of garlic in blood pressure reduction, very few studies are available which have shown small positive effects, insufficient to draw any conclusions. Information gathered from the previous meta-analyses is also inconclusive due to methodological shortcomings. Therefore, in our view, use of garlic cannot be recommended as antihypertensive advice for hypertensive patients in daily practice. Further, meta-analyses are required to prove its efficacy.

13. Effects on Platelets and Fibrinolytic Activity

Garlic has a beneficial effect on platelet adhesion or aggregation, a potential risk factor for cardiovascular disease. The self-condensation products of allicin and ajeones are said to have antithrombotic action, in addition to its potential effect in the inhibition of platelet aggregation [58] 23. Dissolution of clots and thrombi through fibrinolysis is also improved by garlic.

A number of trials have been conducted to find out the usefulness of garlic or its preparation against platelets. A trial by Rahman and Billington reported that garlic causes inhibition of platelet aggregation by various mechanisms including inhibition of cyclooxygenase activity leading to thromboxane A2 formation, by suppressing mobilization of calcium into the platelets, and by increasing levels of messengers (cAMP and cGMP) with in the platelets. It also exhibits strong antioxidant property by increasing production of platelet-derived NO. Simultaneously, it also reduces the ability of platelets to bind to fibrinogen, thus overall resulting in inhibition of platelet aggregations and enhance fibrinolytic activity [59].

This fact was further confirmed by a trial by Allison et al. which showed that AGE extract modified raw preparation of garlic-inhibited platelet aggregation by suppressing the influx of calcium ions through their chelation within platelet cytosol or by altering other intracellular second messengers within the platelets [60].

A trial using AGE preparation of garlic recommended dose-dependent inhibition of platelet aggregation, that is, AGE inhibited platelet aggregation at dose of 7.2 gm however fibrinolytic activity was inhibited at all doses among hypercholesterolemia patients [61]. A trial on ischemic heart disease patients after using raw/fried garlic significantly increased fibrinolytic activity [62].

A study using garlic oil as an ingredient reported two important paraffinic polysulphides diallyl disulphide (DADS) and diallyl trisulphide (DATS) mainly responsible for causing antiplatelet inhibition. Action of DATS was found more potent as compared to DADS; however, it was seen that inhibition of platelet by DATS was reversible. The results of this trial conclude that garlic oil should not be used in patients with comorbid demanding necessary inhibition of platelets activity [63].

When discussing its efficacy in comparison to statins, its action was found comparable to compar to clopidogrel [64]. Similarly, it was also suggested that AGE preparation if taken as a dietary supplement by healthy individuals may be beneficial in protection against cardiovascular disease through inhibition of platelet aggregation [65].

All of the above results showed some beneficial effects; however, two studies done by [66] Legnani et al. and [67] Scharbert et al. on healthy individuals showed no effect on fibrinolysis and platelet activity.

It is concluded that garlic inhibits platelet aggregation by multiple mechanisms and may have a role in preventing cardiovascular disease. However, data is scarce, and further studies are required to prove this fact.

14. Garlic Role on Endothelium and Vascular Dilatation

Though garlic mainly protect against cardiovascular disease through reduction of lipid levels, however few studies suggest that it has some effects on endothelium and vascular dilatation through inhibition of oxidation process. Garlic contains allicin as the main active ingredient with prospect to provide beneficial effects on cardiovascular system. A study by Chan et al. [68] showed that allicin caused enhancement of antioxidant state by lowering of reactive oxygen species and increasing the production of glutathione. Similarly, garlic prevents from cardiovascular disease through inhibition of LDL oxidation thus inhibiting atherosclerosis of vessels, important risk factors for cardiovascular disease [69]. Budoff in 2006 conducted a pilot study in which patients who were already on statin therapy were given AGE extract of garlic and placebo and their degree of coronary artery calcification was assessed which slowed down in patient who were given Garlic therapy plus statin as compared to the other group [70].

Garlic role in primary and secondary prevention of cardiac disease was also questionable as few trials done showed positive results as demonstrated by Table 3. This fact was tested among patients with cardiovascular disease by giving garlic powder tablets allicor, and their 10-year prognostic risk of acute myocardial infarction and sudden death were assessed. It was seen that after 12-month treatment with allicor, there was significant decrease of cardiovascular risk. that is, 1.5 fold in men and 1.3 fold in women. The main influence that played a role in cardiovascular risk reduction was the decrease in LDL cholesterol by 32.9 mg/dL in men and by 27.3 mg/dL in women, thus proving the fact that it has effective role in secondary cardiovascular disease prevention [71].

15. Side Effects of Garlic

A couple of case reports have published the adverse effects of garlic ingestion, where one claimed allergic dermatitis observed in a patient taking raw garlic [72]. Another stated that the antithrombotic activity of garlic might interact with oral anticoagulants; therefore, caution must be taken when using in concordance with oral anticoagulants [73].

16. Conclusion

We conclude that the beneficial effect of garlic preparations on lipids and blood pressure extends also to platelet function, thus providing a wider potential protection of the cardiovascular system through its major effects on cholestrol reduction. However, its efficacy in blood pressure reduction is mild with some beneficial effects on platelet aggregation. This warrants the need for more meta-analyses using standardized preparations with a close watch on methodological shortfalls.

Disclosure

The authors have no relationships with pharmaceutical companies or products to disclose, and they do not discuss off-label or investigative products in this paper.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 976967, 10 pages
http://dx.doi.org/10.1155/2013/976967

Research Article

Palm Tocotrienol-Rich Fraction Improves Vascular Proatherosclerotic Changes in Hyperhomocysteinemic Rats

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Received 16 September 2012; Revised 23 January 2013; Accepted 18 February 2013

Academic Editor: Peng Nam Yeoh

This study investigated the effects of palm tocotrienol-rich fraction (TRF) on aortic proatherosclerotic changes in rats fed with a high methionine diet. Forty-two male Wistar rats were divided into six groups. The first group was the control (fed with a basal diet). Another five groups were fed with 1% methionine diet for 10 weeks. From week 6 onward, folate (8 mg/kg diet) or palm TRF (30, 60, and 150 mg/kg diets) was added into the diet of the last four rat groups, respectively. The high methionine diet raised the plasma total homocysteine and aortic lipid peroxidation, which were reduced by the palm TRF and folate supplementations. Plasma nitric oxide was reduced in the high methionine group compared to the control (3.72 ± 0.57 versus 6.65 ± 0.53 μmol/L, $P < 0.05$), which reduction was reversed by the palm TRF (60 and 150 mg/kg) and folate supplementations. The increased aortic vascular cell adhesion molecule-1 expression in the methionine group (2.58 ± 0.29) was significantly reduced by the folate (1.38 ± 0.18) and palm TRF at 150 mg/kg (1.19 ± 0.23). Palm TRF was comparable to folate in reducing high methionine diet-induced plasma hyperhomocysteinemia, aortic oxidative stress, and inflammatory changes in rats.

1. Introduction

Hyperhomocysteinemia is regarded as one of the important risk factors for cardiovascular diseases such as hypertension [1, 2], the most common cause of increased morbidity and mortality in many countries [3, 4]. Many studies have shown that hyperhomocysteinemia can be induced by feeding experimental animals with a high methionine diet [5, 6]. Hyperhomocysteinemia enhances production of a reactive oxygen species (ROS), leading to increased oxidative stress [7] which then reduces nitric oxide (NO) production [8]. It has been reported to impair vascular endothelial dysfunction [9, 10] and promote early changes of atherosclerosis [11].

Atherosclerosis is a chronic inflammatory process. The earliest changes involve recruitment of monocytes, which later differentiate to macrophages in subintimal layers. The recruitment and accumulation of these macrophages require the presence of adhesion molecules such as vascular cell adhesion molecules (VCAM-1) which is important for binding and adhesion of the monocytes in the blood stream [12, 13]. Previous studies have shown that raised homocysteine level lead to increased monocyte adhesion to the vessel wall [14, 15].

Many reports have been published regarding the role of antioxidants in cardiovascular diseases. Studies that investigated the effects of vitamin E on cardiovascular diseases

were mainly focused on α-tocopherol. Tocotrienol, another type of vitamin E has shown a promising beneficial effect on cardiovascular system in humans [16]. Several studies have demonstrated that it possesses antioxidant [17, 18], anti-inflammatory [19], and hypocholesterolemic [20] properties. We have previously shown that palm tocotrienol-rich fraction (TRF), a vitamin E extract from palm oil which contained both tocopherols and tocotrienols, reduced plasma homocysteine level and heart oxidative stress in rats fed with a high methionine diet [21].

Based on the previously mentioned reports, the objectives of this study were to determine the effects of palm TRF on hyperhomocysteinemia and vascular parameters in rats fed a high methionine diet. The effects of palm TRF on the parameters were also compared to folate, a standard intervention for hyperhomocysteinemia.

2. Materials and Methods

2.1. Animals and Chemicals. Forty-two male Wistar rats (180–200 gram) were obtained from the Laboratory Animal Resource Unit of Universiti Kebangsaan Malaysia. They were kept in polyethylene cages in a well-ventilated room at room temperature (28°C). Food and water were provided *ad libitum* based on their experimental groups. All chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The palm TRF used in this study was prepared by the Malaysian Palm Oil Board according to Gapor et al. [22], comprising 21% α-tocopherol, 17% α-tocotrienol, 4% γ-tocopherol, 33% γ-tocotrienol, and 24% δ-tocotrienol. The basal diet contained about 25.11 mg/kg total vitamin E, and its composition was as follows: α-tocopherol acetate, 40.62%, α-tocopherol 21.62%, α-tocotrienol, 10.71%, γ-tocopherol, 3.46%, γ-tocotrienol, 18.08%, and δ-tocotrienol, 5.49%. It also contained 4.1 g/kg methionine and 2.4 mg folate per tonne matric food.

2.2. Preparation of Experimental Diet. A high methionine diet (1% methionine) [23] was prepared by mixing 990 g of basal diet (Gold Coin Feedmills Malaysia Sdn Bhd) with 10 g methionine. Arabic gum (2%) solution was added to stick them together. For the palm TRF- or folate-supplemented diets, palm TRF at 30, 60, or 150 mg/kg diets [24] or folate (8 mg/kg diet) [25] was added. The diet was mixed for 30 minutes. Using a 10-cc syringe, it was remolded and left to dry overnight. The diets were then kept at −20°C before use.

2.3. Experimental Design. After one week of acclimatization, the rats were randomly divided equally into six groups ($n = 7$). The first group, the control, was fed basal rat chow throughout the ten-week study period. The second group was given a high methionine diet only. The other four groups were given the high methionine diet (from weeks 1 to 5) followed by supplementation with folate (M + Folate, 8 mg/kg) or palm TRF at 30 (M + TRF30), 60 (M + TRF60), or 150 (M + TRF150) mg/kg diets from the sixth week onwards. At the end of the treatment period, systolic blood pressure levels were measured using a noninvasive tail cuff method (Physiograph, Dess, USA). The rats were then deprived of food overnight and sacrificed. The aorta was removed and washed with ice-cold buffered saline. The abdominal part of the aorta was kept at −71°C until being used for biochemical determinations, while the thoracic part was processed for histological examination. The experimental procedure and humane animal handling were conducted in accordance with the national guidelines for the care and use of laboratory animal, and were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee. Rat food intake was recorded throughout the experiment duration and reported as total food intake.

2.4. Determination of Biochemical Parameters. Plasma total homocysteine level was determined at weeks 0, 5 and 10. The levels were quantified by the means of commercial diagnostic kit (Abbot Laboratories, IL, US) that utilized the fluorescence polarization immunoassay technique and expressed as μmol/L.

The abdominal aorta was used to measure the lipid peroxidation content [26, 27], expressed as TBARS (pmol MDA/mg protein). Plasma nitric oxide (NO_x) at weeks 0 and 10 was measured using Griess reagent.

2.5. Immunohistochemical Staining. The thoracic aorta was immersion-fixed in 10% buffered formalin overnight and then embedded longitudinally in paraffin. Sequential 5 μm paraffin-embedded sections were deparaffinized and incubated with H_2O_2 for 5 minutes to quench the endogenous peroxide. The sections were immunostained with primary antibody of rabbit monoclonal antibody (1 : 100) against rat VCAM-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for an hour at room temperature. After washing with trizma base solution (TBS), an anti-rabbit polymerized horseradish peroxidase labeled secondary antibody (DakoCytomation) was added, and the sections were incubated for 30 minutes at room temperature. Sections were then washed, incubated in liquid diaminobenzedine for 10 minutes, washed and counterstained with hematoxylin, cleared and mounted. Slides containing human tonsil were used as positive and negative controls. Positively expressing vascular cell adhesion molecule-1 was indicated by brown streaks in the intimal layer of the aorta. The immunohistochemical staining slides were manually assessed quantitatively by two experienced operators who were blinded to the study protocol, based on the following grading under ×400 magnification: 0 = no staining, 1 = staining less than 50% intimal surface, 2 = staining more than 50% intimal surface, 3 = complete surface staining but thin, 4 = complete and thick staining of intimal surface.

2.6. Histomorphometric Study. Verhoeff-stained and differentiated in 2% ferric chloride cross sections from thoracic aorta were checked microscopically until the nuclei and elastic fibers were stained black and washed again in water, followed by immersion in 95% alcohol to remove excess iodine. It was later counterstained in van Gieson for 30 seconds. The structure of the sections was observed under light microscope (Eclipse 80i, Nikon Corporation, Tokyo, Japan), and the images were captured using a camera (Qimaging

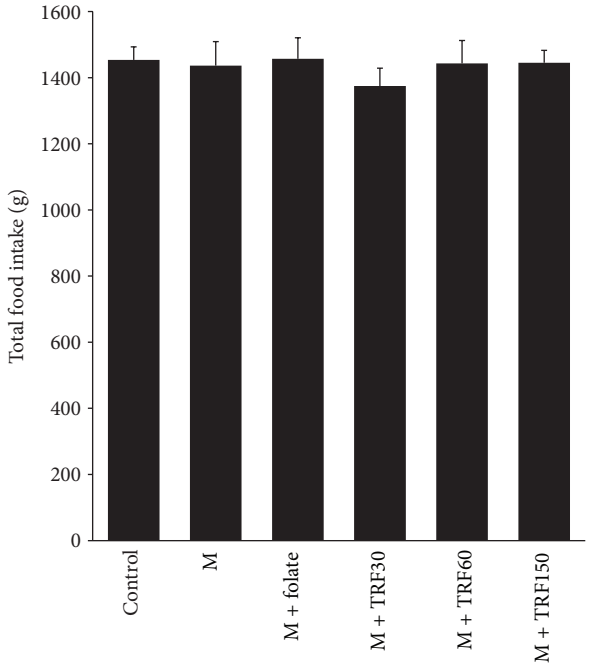

Figure 1: Total food intake in rats fed with a high methionine diet (M) (weeks 1–10) and supplemented with palm tocotrienol-rich fraction (TRF) at three different doses (30, 60, and 150 mg/kg diets) or folate (8 mg/kg diet) for 5 weeks (weeks 6–10). Bars represent means ± SEM ($n = 7$). No significant difference was seen between groups.

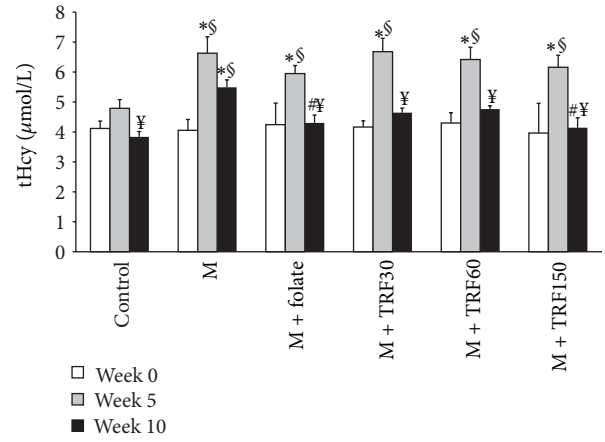

Figure 2: Total plasma homocysteine level at weeks 0, 5, and 10 in rats fed high methionine diet (M) (week 0–10) and supplemented with either folate (8 mg/kg diet) or palm tocotrienol-rich fraction (TRF) (30, 60, or 150 mg/kg diets) for 5 weeks (weeks 6–10). The result is expressed as means ± SEM ($n = 7$). *Significantly different from the control at the same duration of treatment ($P < 0.05$), #significantly different from the methionine group at week 10 ($P < 0.05$), §significantly different from week 0, and ¥significantly different from week 5, respectively ($P < 0.05$).

MicroPublisher 5.0, Surrey, Bc, Canada). The thickness of intima and media layer at 0°, 90°, 180°, and 270° of each section were quantitatively analyzed by using Image Pro Plus 5.0 (Media Cybernetics, Inc., Silver Spring, MD, USA) under ×200 magnification. The average readings of intima-media thickness and intima : media ratio were calculated.

2.7. Statistical Analysis. Statistical analyses were performed using Statistical Product for Social Science (SPSS) 11.5. Data are expressed as mean ± SEM. Kolmogorov Smirnov test was used to determine the normality of data distribution. As the data were normally distributed, analysis of variance (ANOVA) followed with post hoc Tukey test was used. The correlations between parameters were analyzed with Pearson correlation test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Food Intake. Mean total food intake in all experimental groups is shown in Figure 1. The mean total intake was about 1450 g for the duration of 10 weeks. It was similar in all groups, and no significant difference was observed amongst the groups.

3.2. Plasma Total Homocysteine. Plasma total homocysteine levels (Figure 2) at week 5 were significantly raised in all rat groups that were fed 1% methionine diet compared to the control group, and no difference was seen amongst the groups fed methionine diet. At week 10, the plasma total homocysteine levels in all supplemented groups (folate and palm TRF) were significantly lower compared to their respective groups at week 5. However, only reductions at week 10 in groups supplemented with folate and palm TRF at 150 mg kg diet were significantly lower than the high methionine group (at week 10). In the control group, a lower plasma total homocysteine was also observed. However, there was no significant difference seen in the homocysteine levels amongst the supplemented and control groups at week 10. No significant difference was observed in all groups at week 0.

3.3. Aortic TBARS Content. There was almost a 100% increase in the aortic TBARS content following the high methionine diet compared to the control (604.0 ± 51.0 versus 300.3 ± 58.7 pmol MDA/mg protein) (Figure 3). In rats that received folate (300.3 ± 96.2 pmol MDA/mg protein) and palm TRF supplementation (30 mg/kg, 334.0 ± 75.4, 60 mg/kg, 337.8 ± 72.3, and 150 mg/kg, 222.3 ± 41.2 pmol MDA/mg protein) for 5 weeks in addition to the high methionine diet, the TBARS contents were significantly lower compared to the methionine group. No difference amongst the palm TRF- and folate-supplemented groups was observed.

3.4. Plasma Nitric Oxide. Intake of the high methionine diet for 10 weeks significantly decreased plasma nitric oxide compared to the control (3.72±0.57 versus 6.65±0.53 μmol/L, $P < 0.05$) and its own group at week 0 (Figure 4). Groups supplemented with folate and palm TRF at 60 and 150 mg/kg diets increased the plasma level significantly compared to the methionine group. Both groups supplemented with 60 and 150 mg/kg palm TRF had significantly higher plasma

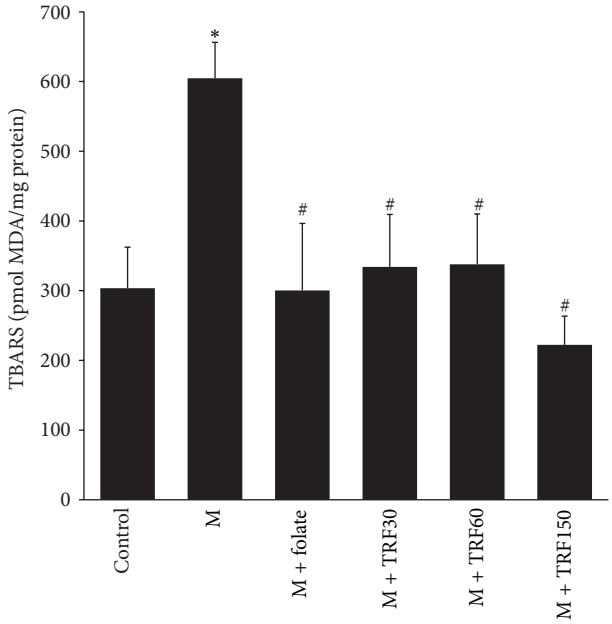

Figure 3: The thiobarbituric acid reactive substance (TBARS) content (pmol MDA/mg protein) in aorta of rat fed either basal (control), high methionine (M, 1%) (week 0–10), methionine + folate (8 mg/kg diet) or palm tocotrienol rich fraction (TRF) (30, 60 or 150 mg/kg) diets (week 6–10). Bars represent means ± SEM ($n = 7$). *Significantly different from the control ($P < 0.05$), #Significantly different from the M group ($P < 0.05$).

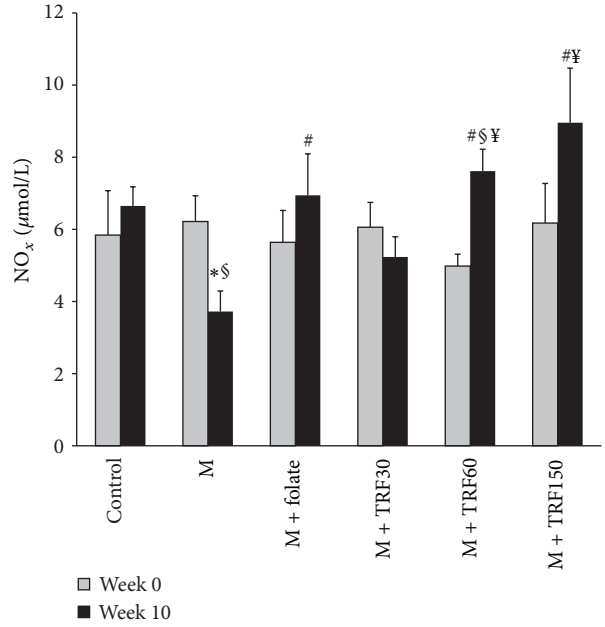

Figure 4: Plasma nitric oxide in rats given high methionine (M) (1%) diet for 10 weeks and supplemented with palm tocotrienol rich fraction (TRF, 30, 60 and 150 mg/kg diet from weeks 6 to 10) or folate (8 mg/kg diet). Bars represent means ± SEM ($n = 7$). *Significantly different from the control ($P < 0.05$), #significantly different from the M group ($P < 0.05$), §significantly different from week 0 respectively and ¥significantly different from the TRF30-treated group ($P < 0.05$).

nitric oxide than the group supplemented palm TRF at low dose (30 mg/kg) (7.62 ± 0.61 and 8.96 ± 1.51 versus 5.23 ± 0.56 μmol/L, $P < 0.05$). The plasma levels in these two groups were not significantly different. No significant difference was seen amongst the groups at week 0.

3.5. Aortic VCAM-1 Expression. The representatives of the aortic intimal layer positively expressing VCAM-1 (indicated by brown stained streak) from each group are demonstrated in Figure 5. The positive-stained area was significantly observed in the methionine group. While in the folate- and palm TRF-supplemented groups, the area were less intensified.

The high methionine diet (2.58 ± 0.29 versus 1.09 ± 0.29, $P < 0.05$) caused a significant increase in the area of aortic intimal layer that was positively expressing VCAM-1 (Figure 6). The supplementations of folate (1.38 ± 0.18) and palm TRF at 150 mg/kg diet (1.19±0.23) significantly reduced the expression of VCAM-1. Palm TRF at 30 or 60 mg/kg however, did not influence the expression of VCAM-1 significantly.

3.6. Aortic Histomorphometric Study. The Verhoeff van Gieson-stained cross-sections showing the intima and media of the aorta from each group were shown in Figure 7. The aortic intima-media thickness and intima : media ratio were not influenced by the dietary high methionine. Supplementations of neither folate nor palm TRF at all doses significantly affected these parameters (Table 1).

Table 1: The aortic intima and media ratio in rats fed 1% methionine diet (M) (week 0–10), and supplemented with folate (8 mg/kg diet) or palm tocotrienol rich fraction (TRF) (30, 60 or 150 mg/kg diet) for 5 weeks (week 6–10).

Groups	Intima-media thickness (μm)	Intima : media ratio
Control	95.78 ± 7.19	0.0163 ± 0.0017
Methionine (M)	89.02 ± 7.98	0.0160 ± 0.0011
M + Folate	92.11 ± 8.33	0.0157 ± 0.0016
M + TRF30	98.05 ± 8.26	0.0170 ± 0.0020
M + TRF60	84.41 ± 7.95	0.0154 ± 0.0023
M + TRF150	82.74 ± 9.88	0.0146 ± 0.0018

Values are means ± SEM ($n = 7$). No significant difference amongst groups.

3.7. Systolic Blood Pressure. The high methionine diet for 10 weeks did not significantly influence the systolic blood pressure in rats (101.37 ± 1.92 versus 92.15 ± 2.98 mmHg). Supplementations of palm TRF (30 mg/kg, 93.65 ± 1.15, 60 mg/kg, 99.48 ± 2.22, and 150 mg/kg, 100.58 ± 1.79 mmHg) and folate (99.23 ± 1.24 mmHg) also had no significant effect on this parameter (Figure 8).

3.8. Relationships between Parameters. Plasma total homocysteine level was positively correlated to the aortic VCAM-1 ($r = 0.339$, $P < 0.05$) and TBARS ($r = 0.369$, $P < 0.05$). Aortic TBARS was also found to be positively correlated to

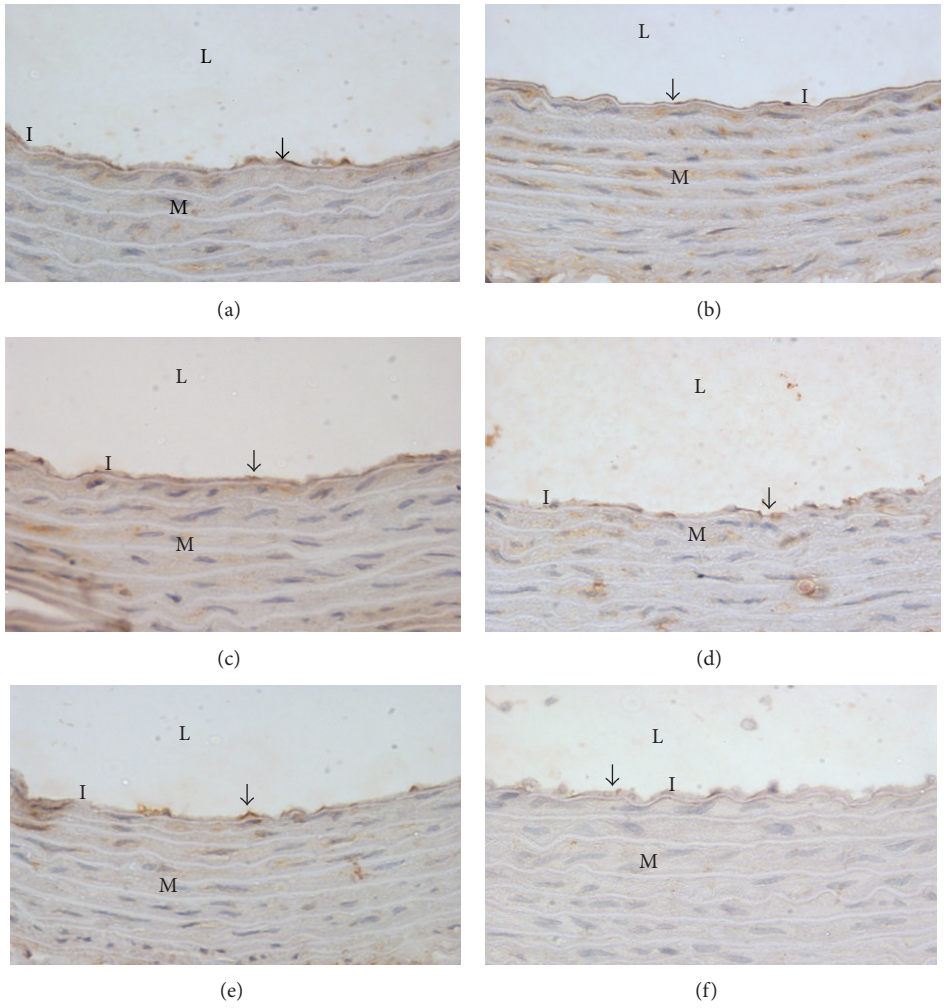

Figure 5: Intimal layer of aorta positively expressing vascular cell adhesion molecule-1 (VCAM-1, brown streaks indicated by arrows) (×400 magnification) in rats fed basal (control, (a)), high methionine (1%, (b)) (weeks 0–10) diets and supplemented with folate (8 mg/kg, (c)) or palm tocotrienol-rich fraction (TRF) at 30 (d), 60 (e), or 150 (f) mg/kg for 5 weeks (weeks 6–10), in addition to high methionine diet. L: lumen, I: intima, and M: media.

Table 2: Correlation (r) between parameters in rats fed high methionine diet.

	Aortic VCAM-1	Plasma NO_x	Aortic TBARS
Plasma total homocysteine	0.339*	−0.049	0.369*
Aortic TBARS	0.364*	−0.321*	
Plasma NO_x	−0.300		

*Significant correlation ($P < 0.05$).

the aortic VCAM-1 ($r = 0.364$, $P < 0.05$). However, it demonstrated a negative correlation to plasma nitric oxide ($r = -0.321$, $P < 0.05$) (Table 2).

4. Discussion

A high methionine diet model is an established experimental model for the induction of hyperhomocysteinemia. In the present study, the plasma total homocysteine level was increased following ingestion of 1% methionine diet, which confirms previous data demonstrating that hyperhomocysteinemia could be induced by a high methionine diet [5, 6]. No difference seen in the mean total food intake in all groups suggests that the intake of methionine in all groups except in the control group was similar. The average daily food intake was about 20 g per rat, which means that in the methionine fed groups, each rat consumed approximately 250–280 mg of methionine daily. While in the control group, the methionine consumption was only 80–85 mg. The basal methionine content in the rat chow did not significantly increase the plasma homocysteine, as seen in the present study.

In the body, methionine from the diet is converted to S-adenosyl-methionine and S-adenosyl-homocysteine and finally to homocysteine. The homocysteine is then remethylated to methionine by the action of methionine synthase, or it undergoes transsulfuration pathway through which

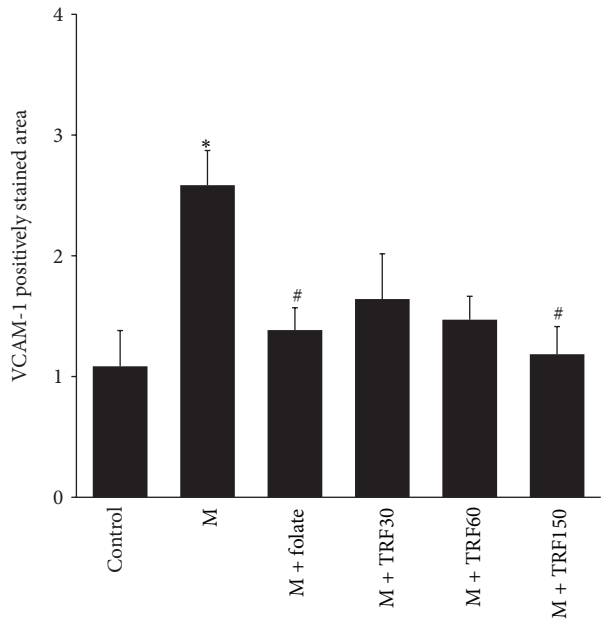

Figure 6: The effects of folate (F; 8 mg/kg diet) and palm tocotrienol-rich fraction (TRF; 30, 60, and 150 mg/kg diets) for 5 weeks (From weeks 6 to 10) on aortic vascular cell adhesion molecule 1 (VCAM-1) expression in rats fed high methionine diet (M) (From weeks 0 to 10). Bars represent means ± SEM ($n = 7$). *Significantly different from the control ($P < 0.05$) and #significantly different from the methionine group ($P < 0.05$).

it is converted into cysteine by cystathionine-β-synthase [28]. Vitamin B12 and folate act as important coenzymes for homocysteine remethylation in the folate cycle, which results in a reduced level of homocysteine [29]. This explains the reduction of the plasma total homocysteine level seen in the rats that were supplemented with folate in the present study. Folate supplementation has been reported by other researchers to significantly reduce plasma homocysteine level in experimental animals [14, 30] and humans [31, 32].

Recent epidemiological studies reported that folic acid supplementation to lower homocysteine levels had no significant beneficial effects on vascular outcomes [33, 34]. However, there were other epidemiological data that had shown otherwise. The epidemiological review had found that homocysteine lowering effect of B vitamins supplementation showed a significant protective effect on stroke [35] and in preventing vascular events as well as cardiovascular diseases mortality in high risk individuals [36].

Reductions in the plasma total homocysteine level in all palm TRF-supplemented groups, were comparable to that observed in the folate-supplemented group and its effect was not dose dependent. It was shown that α-tocopherol supplementation for two months had failed to reduce plasma homocysteine in highly trained athletes [37]. Similarly, a combination of antioxidants which consisted of α-tocopherol, β-carotene, vitamin C, troxerutin, and selenium did not influence the plasma homocysteine level in patients with hyperhomocysteinemia [38]. The discrepancy in the effect of both antioxidants, tocopherol and tocotrienol, on the blood parameter is not well understood. It could be that palm TRF which contained more than 70% tocotrienol reduced plasma total homocysteine by affecting the enzymes and cofactors involved in the homocysteine metabolism. This probable effect might be demonstrated by a different mechanism, not via its antioxidant effect. This postulation warrants further investigation. The reduction seen in the control group at week 10 compared to the level at week 5 was also not understood. However, the level was comparable to the levels in the supplemented groups at week 10.

Hyperhomocysteinemia induced by high methionine diet increased vascular oxidative stress, as shown by increased aortic TBARS. The increase in the plasma total homocysteine level was positively correlated with the increase in oxidative stress. A similar finding was also reported by Yu et al. [39]. Homocysteine increased the reactive oxygen species generations, namely, superoxide and peroxynitrite mediated by NADPH oxidase in vascular cells, leading to endothelial dysfunction [40]. The supplementations of folate and all doses of palm TRF prevented the increase in aortic TBARS induced by high methionine diet, at similar efficacy. The protective effect of palm TRF to reduce oxidative stress has been previously reported [17, 18]. It was also reported to prevent the increase in heart oxidative stress in hyperhomocysteinemic rats [21]. The ability of α-tocopherol to inhibit the increased reactive oxygen species generation induced by homocysteine was also seen in vascular smooth muscle cells [41]. Folate was also reported to possess a good antioxidant property [42, 43].

In rats that were fed high methionine diet, a significant reduction in the plasma nitric oxide was observed. Homocysteine was directly shown to inhibit nitric oxide synthase activity which later resulted in decreased nitric oxide level [44]. A significant association between the homocysteine and nitric oxide levels was not shown in our study, even though He et al. [45] had previously reported their significant negative correlation in human subjects. Nitric oxide is important to blood vessels due to its vasorelaxant effect. Diminished nitric oxide bioavailability is accompanied by an increase in oxidative stress [46, 47], and the significant negative correlation between these two parameters was demonstrated in this study. The palm TRF supplementations at 60 and 150 mg/kg diets and folate managed to significantly prevent the loss of plasma nitric oxide.

In our study, the high methionine diet-induced hyperhomocysteinemia increased the expression of aortic VCAM-1, an inflammatory biomarker, and was in agreement with the study of Li et al. [14]. They had shown that high methionine diet raised plasma homocysteine level and subsequently augmented the expression of VCAM-1 in rat thoracic aorta. Indeed, VCAM-1 plays a more important role than intracellular adhesion molecule (ICAM-1) in the early progression of atherosclerosis [48]. Its significant association with hyperhomocysteinemia was confirmed in the present study which suggests that in hyperhomocysteinemia, the formation of proatherosclerotic marker in the blood vessels is a part of the pathological changes. It has a crucial role in leukocytes recruitment into the inflamed sites in the vascular tissues

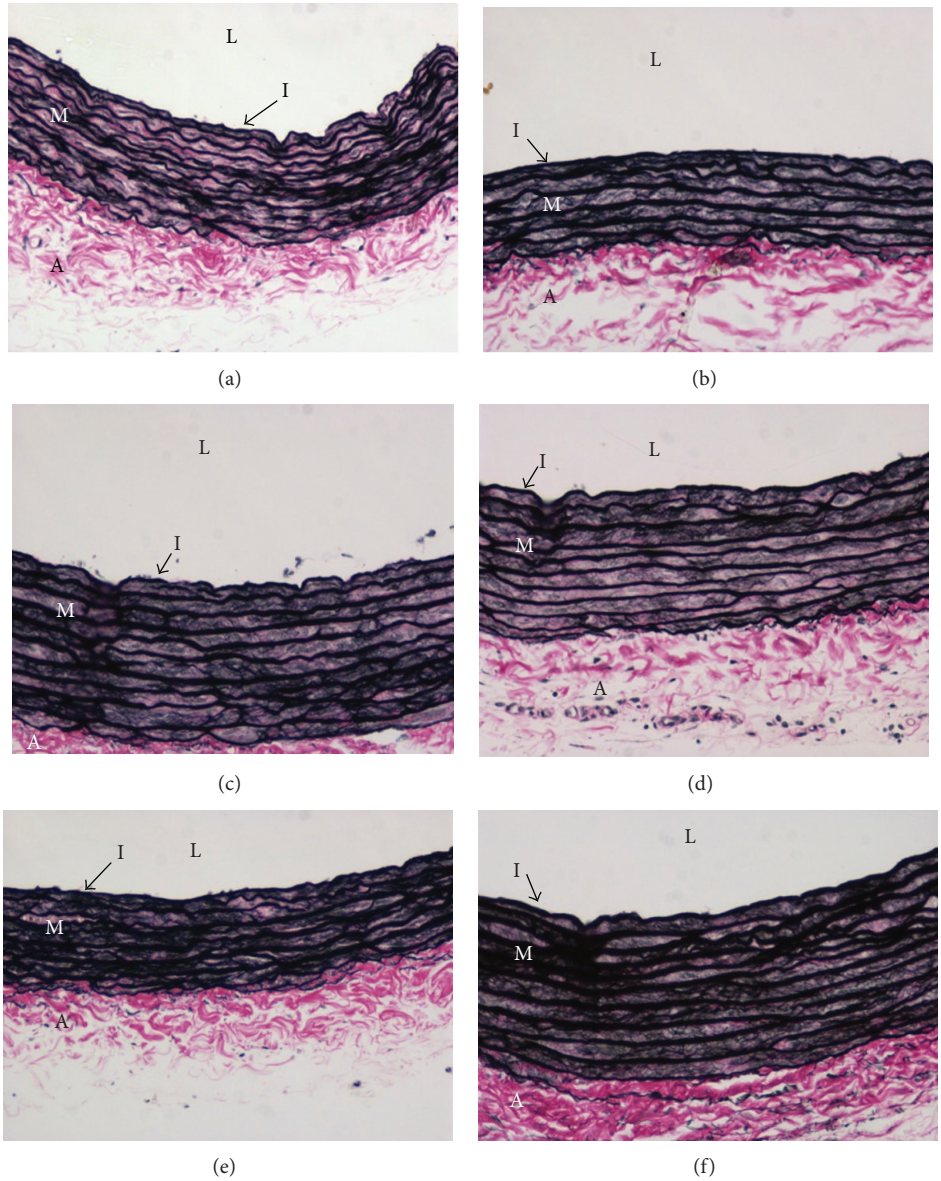

Figure 7: Verhoeff van Gieson staining of the aorta (×200 magnification) in rats fed basal (control, (a)), high methionine (weeks 0–10, (b)) diets and supplemented with folate (8 mg/kg diet, (c)) or palm tocotrienol-rich fraction (TRF) at 30 (d), 60 (e), or 150 (f) mg/kg diets) for 5 weeks (week 6–10) in addition to high methionine diet. L: lumen, I: intima, M: media, and A: adventitia.

[49], by mediating the adhesion of leukocytes to the surface of endothelial cells, thus promoting smooth muscle cell migration through the endothelium into the intima during atherogenesis [50]. The VCAM-1 increased expression was shown to be related to increased oxidative stress in fructose-fed rats [51] and their positive correlation was demonstrated in our study. However at this stage, hyperhomocysteinemia did not affect aortic histomorphometric changes, calculated as intima-media thickness and intima : media ratio, as well as no effect on systolic blood pressure.

Clinically, indirect measurement of intimal thickness of arteries, like carotid and femoral, can be used to study disease progression and effects of treatment in cardiovascular diseases. Previous reported studies had exhibited no relationship between hyperhomocysteinemia and carotid intima-media thickness [52, 53]. However, Sprague-Dawley rats fed a 1% methionine diet for a month showed a significant increase in the intima : media ratio which was associated with a four-fold increase in homocysteine level [54], but we only found an approximately two-fold increment in our study.

A study carried out by Robin et al. [55] showed that the systolic blood pressure was elevated in normotensive rats supplemented with 1.45% methionine after 7 weeks, a dose higher than the present study. Their study also confirmed that the positive association between plasma homocysteine and blood pressure was unlikely to be causal. The increased plasma homocysteine seen in the hypertensive rats was accompanied with a significant decrease in blood pressure

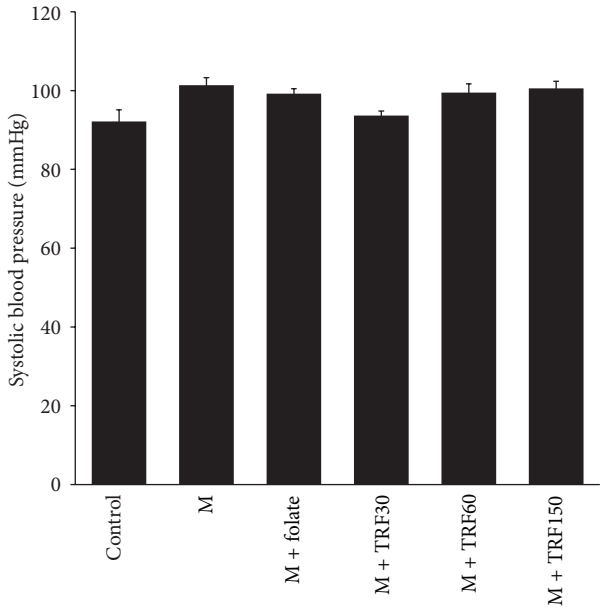

Figure 8: Systolic blood pressure in rats given high methionine (M) (1%) diet (From weeks 1 to 10) and supplemented with palm tocotrienol-rich fraction (TRF; 30, 60, and 150 mg/kg diets from weeks 6 to 10) or folate (8 mg/kg diet). Bars represent means ± SEM ($n = 7$). No significant different was seen amongst groups.

instead. It can be postulated that in our study the early atherosclerotic changes induced by hyperhomocysteinemia had already taken place in the blood vessels before the manifestation of hypertension.

Similar to the high methionine group, both palm TRF and folate did not significantly affect the vascular histomorphometry and systolic blood pressure. However, palm TRF at the highest dose (150 mg/kg diet) and folate suppressed vascular proatherosclerotic changes in the hyperhomocysteinemic rats. This finding was indicated by normalized expression of VCAM-1. Tocopherols and tocotrienols were demonstrated to inhibit inflammatory biomarker *in vitro* which resulted in a reduced endothelial invasiveness and leukocyte attachment [56, 57]. For tocotrienols, the suppression was the highest in cells treated with δ-tocotrienol (77%) followed by β-tocotrienol (63%), γ-tocotrienol (54%), α-tocotrienol (38%), and the least with α-tocopherol (29%). The difference in efficacy of the vitamin E isomers could be related to the higher antioxidant function of the tocotrienol compared to the tocopherol, due to its higher mobility in cell membrane and better recycling of its chromanoxyl radical [57]. Similarly, the suppressive effect by the tocopherols was the highest with δ-tocopherol, followed by γ-tocopherol, and the least being α-tocopherol [56]. Collectively, it can be concluded that the inhibitory effect of palm TRF on VCAM-1 expression is primarily mediated by its tocotrienol content. As for folate, it has been shown to reduce VCAM-1 expression in hyperhomocysteinemic rat aorta [14]. This study has confirmed the anti-inflammatory property of both folate and palm TRF, in addition to their antioxidant effect.

5. Conclusions

This study demonstrated that a high methionine diet-induced hyperhomocysteinemia was associated with increased aortic oxidative stress and inflammatory changes in male adult rats. Dietary supplementation of palm TRF particularly at the highest dose (150 mg/kg) was shown to be effective in reducing the plasma total homocysteine, aortic oxidative stress, and inflammatory changes, comparable to that of folate.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This work was supported by Grant FF-013-2006 from the Faculty of Medicine, Universiti Kebangsaan Malaysia. The authors would also like to thank Puan Azizah Osman (Department of Pharmacology, UKMMC) for technical assistance.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 982380, 9 pages
http://dx.doi.org/10.1155/2013/982380

Research Article

Zhen Gan Xi Feng Decoction, a Traditional Chinese Herbal Formula, for the Treatment of Essential Hypertension: A Systematic Review of Randomized Controlled Trials

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Received 21 August 2012; Accepted 22 February 2013

Academic Editor: Tabinda Ashfaq

Objectives. To assess the clinical effectiveness and adverse effects of Zhen Gan Xi Feng Decoction (ZGXFD) for essential hypertension (EH). *Methods*. Five major electronic databases were searched up to August 2012 to retrieve any potential randomized controlled trials designed to evaluate the clinical effectiveness of ZGXFD for EH reported in any language, with main outcome measure as blood pressure (BP). *Results*. Six randomized trials were included. Methodological quality of the trials was evaluated as generally low. Four trials compared prescriptions based on ZGXFD with antihypertensive drugs. Meta-analysis showed that ZGXFD was more effective in BP control and TCM syndrome and symptom differentiation (TCM-SSD) scores than antihypertensive drugs. Two trials compared the combination of modified ZGXFD plus antihypertensive drugs with antihypertensive drugs. Meta-analysis showed that there is significant beneficial effect on TCM-SSD scores. However, no significant effect on BP was found. The safety of ZGXFD is still uncertain. *Conclusions*. ZGXFD appears to be effective in improving blood pressure and hypertension-related symptoms for EH. However, the evidence remains weak due to poor methodological quality of the included studies. More rigorous trials are warranted to support their clinical use.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality all over the world. Despite advances in prevention and treatment over the past 20 years, CVD remains a leading cause of death and disability. The emergence of CVD as a leading cause of morbidity and mortality in China, in large part, is a result of the rapid economic growth and associated sociodemographic change that has occurred over the past few decades [1]. Hypertension is the most common CVD in the world, with a prevalence above 20 percent in the general population [2]. It is the most powerful predictor of stroke, myocardial infarction, heart failure, and renal failure. Prior clinical trials have consistently shown that reductions in blood pressure reduce the incidence of stroke and myocardial infarction [3].

Complementary and alternative medicine (CAM) refers to a series of medical and health care practices and products

that are not an integral part of conventional medicine due to insufficient proof of their safety and effectiveness [4, 5]. The number of patients who utilize CAM as a treatment of CVDs continues to grow [6, 7]. Traditional Chinese medicine (TCM) is one of the most important parts in CAM [8]. Many studies have shown that TCM, either herbal medicine or acupuncture, could contribute to blood pressure control [9–12]. Eugene Braunwald, a world leader in cardiology for more than half a century, pointed out that current cardiology practice is evidence-based and global in scope [13]. Thus, it is important to investigate the beneficial and harmful effects of Chinese herbs and formulas in the treatment of hypertension under the guidance of scientific assessment methods.

Zhen Gan Xi Feng Decoction (ZGXFD), a traditional Chinese herbal formula containing twelve commonly used herbs (achyranthes root, ruddle, dragon bone, oyster shell, plastrum testudinis, white peony root, radix scrophulariae, radix asparagi, fructus toosendan, raw malt, artemisia capillaris Thunb, and glycyrrhiza), is widely used to treat hypertension-related symptoms in clinical practice for centuries in China. Recent researches showed that ZGXFD could contribute to blood pressure control. The mechanism of the prescription maybe related to calming liver, suppressing liver yang hyperactivity, and nourishing kidney yin in Chinese medicine. Biochemically, ZGXFD also showed good effect in decreasing the concentrations of angiotensin in plasma and myocardium, reducing the endothelin content in brain and improving PPARγ mRNA expression in rats with essential hypertension [20, 21].

Currently, ZGXFD used alone or combined with antihypertensive drugs has been widely used as an alternative and effective method for essential hypertension treatment in China. And until now a number of clinical studies of ZGXFD reported the effectiveness ranging from case reports and case series to controlled observational studies and randomized clinical trials. However, there is no critically appraised evidence such as systematic reviews or meta-analyses on potential benefit and safety of ZGXFD for essential hypertension to justify their clinical use and their recommendation. Understanding the effect of ZGXFD on blood pressure, quality of life (QOL) and cardiovascular risk factors could be valuable for the management of essential hypertension. The present paper aims to evaluate the beneficial and harmful effects of ZGXFD for treatment of essential hypertension in randomized trials.

2. Methods

2.1. Database and Search Strategies. The literature searches were conducted in the Cochrane Central Register of Controlled Trials (CENTRAL) in the Cochrane Library (August, 2012), PubMed, Chinese Biomedical Literature Database (CBM), Chinese National Knowledge Infrastructure (CNKI), Chinese Scientific Journal Database (VIP), and searched the reference list of retrieved papers. All of those searches ended on August 15, 2012. Ongoing registered clinical trials were searched in the website of Chinese clinical trial registry (http://www.chictr.org/) and international clinical trial registry by US National Institutes of Health (http://clinicaltrials.gov/). The following search terms were used individually or combined: "essential hypertension," "hypertension," "Zhen Gan Xi Feng Decoction," "clinical trial," and "randomized controlled trial." The bibliographies of included studies were searched for additional references.

2.2. Inclusion Criteria. All the randomized controlled trials (RCTs) of all the prescriptions based on "Zhen Gan Xi Feng Decoction" compared with antihypertensive drugs in patients with hypertension were included. RCTs combined ZGXFD with antihypertensive drugs compared with antihypertensive drugs, and all the modified ZGXFD were included as well. There were no restrictions on population characteristics, language, and publication type.

The primary outcome measure was blood pressure (BP), and the secondary outcome measure was TCM syndrome and symptom differentiation (TCM-SSD) scores. The criteria "significant effective, effective, or not effective" were also included in the outcome measurement. Duplicated publications reporting the same groups of participants were excluded.

2.3. Data Extraction and Quality Assessment. Two authors conducted the literature searching (X. J. Xiong and X. C. Yang), study selection (X. J. Xiong and W. Liu), and data extraction (X. J. Xiong and X. Du) independently. The extracted data included authors and title of study, year of publication, study size, age and sex of the participants, details of methodological information, name and component of Chinese herbs, treatment process, details of the control interventions, outcomes (e.g., blood pressure), and adverse effects for each study. Disagreement was resolved by discussion and reached consensus through a third party (J. Wang).

Methodological quality of trials was assessed using 7 criteria from the Cochrane Handbook for Systematic Review of Interventions, Version 5.1.0 (X. J. Xiong and B. Feng) [22]. The items included random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias. The quality of all trials was categorized to low/unclear/high risk of bias ("Yes" for a low of bias, "No" for a high risk of bias, or "Unclear" otherwise). Three levels were used to evaluate the trials: low risk of bias (all the items were in low risk of bias), high risk of bias (at least one item was in high risk of bias), and unclear risk of bias (at least one item was in unclear).

2.4. Data Synthesis. RevMan 5.1 software provided by Cochrane Collaboration was used for data analyses. Dichotomous data were expressed as relative risk (RR) and continuous outcomes as weighted mean difference (WMD), both with 95% confidence intervals (CI). Meta-analysis was performed if the intervention, control, and outcome were the same or similar. The statistical heterogeneity was presented

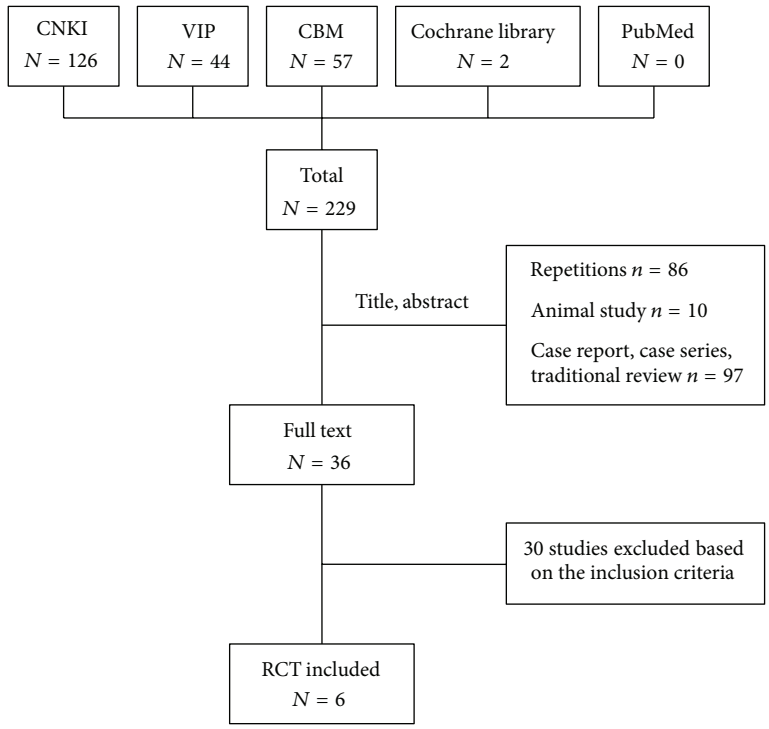

Figure 1: Study selection process.

as significant when I square (I^2) is over 50% or $P < 0.1$. In the absence of significant heterogeneity, we pooled data using a fixed-effect model ($I^2 < 50\%$), and otherwise we used random effects model ($I^2 > 50\%$) [22].

3. Result

3.1. Description of Included Trials. After primary search of 5 databases, 229 trials were screened out from electronic and manual searches (Figure 1), and the majority were excluded due to obvious ineligibility which included irrelevant titles and abstract (some papers being found from more than one database). After reading the titles and abstracts, a majority of them was excluded. Eighty-six trials were excluded because of duplicated publication, 10 trials were excluded due to the animal studies, and the rest 97 trials were noncontrolled clinical trials including case report, case series traditional review. Thirty out of the rest of 36 articles were excluded based on the inclusion criteria. In the end, 6 RCTs were reviewed [14–19]. All the RCTs were conducted in China and published in Chinese. The characteristics of 6 randomized trials are summarized in Table 1.

The 6 RCTs involved 830 patients with essential hypertension. There was a wide variation in the age of subjects (18–87 years). Six (6) trials specified three diagnostic criteria of hypertension, one trial [15] used 1999 WHO-ISH guidelines for the management of hypertension (1999 WHO-ISH GMH), one trial [16] used Chinese Guidelines for the Management of Hypertension-1999 (CGMH-1999), one trial [17] used China Guidelines on Prevention and Management of High Blood Pressure-2004 (CGPMHBP-2004), and three trials [14, 18, 19] only demonstrated patients with essential hypertension. Six (6) trials have reported TCM diagnostic criteria with yin-deficiency and excessive yang syndrome, four trials [14–17] used Guidelines of Clinical Research of New Drugs of Traditional Chinese Medicine (GCRNDTCM), and two trials [18, 19] only demonstrated patients with yin-deficiency and excessive yang syndrome in TCM.

The interventions included all the prescriptions based on "Zhen Gan Xi Feng Decoction" alone and ZGXFD with antihypertensive drugs. The controls included antihypertensive drugs alone. Four trials [14–16, 18] investigated the prescriptions based on "Zhen Gan Xi Feng Decoction" using alone versus antihypertensive drugs, and the remainig two trials [17, 19] compared the prescriptions based on "Zhen Gan Xi Feng Decoction" plus antihypertensive drugs versus antihypertensive drugs.

The total treatment duration ranged from 2 weeks to 4 weeks. The variable prescriptions are presented in Table 1. The different compositions of formula ZGXFD are presented in Table 2. All of the 6 trials used the blood pressure (BP) as the main outcome measure. Other outcome measures include the scale for TCM syndrome and symptom differentiation (TCM-SSD). Adverse effect was described in details. Three classes were used to evaluate treatment effects, including significant effective, effective, and ineffective according to BP and TCM-SSD.

Table 1: Characteristics and methodological quality of included studies.

Study ID	Sample	Diagnosis standard	Intervention	Control	Course (week)	Outcome measure
Guo et al. 2002 [14]	129	Hypertension diagnostic criteria (unclear); GCRNDTCM	ZGXFD	Captopril	4	BP; TCM-SSD; side effect
Mao 2005 [15]	70	1999 WHO -ISH GMH; GCRNDTCM	Modified ZGXFD	Indapamide	3	BP; TCM-SSD
Luo 2008 [16]	45	CGMH-1999; GCRNDTCM	ZGXFD	Captopril	2	BP; TCM-SSD
Liu and Zhong 2008 [17]	120	CGPMHBP-2004; GCRNDTCM	Modified ZGXFD plus benazepril	Benazepril	3	BP; TCM-SSD
Li and Zheng 2012 [18]	166	Hypertension diagnostic criteria (unclear); TCM diagnostic criteria (unclear)	Modified ZGXFD	Captopril	4	BP; side effect
Zhou 2000 [19]	300	Hypertension diagnostic criteria (unclear); TCM diagnostic criteria (unclear)	Modified ZGXFD plus nitrendipine	Nitrendipine	4	BP; side effect

Table 2: Composition of formula.

Study ID	Formula	Composition of formula
Guo et al. 2002 [14]	ZGXFD	Achyranthes root, ruddle, dragon bone, oyster shell, plastrum testudinis, white peony root, radix scrophulariae, radix asparagi, fructus toosendan, raw malt, artemisia capillaris thunb, and glycyrrhiza.
Mao 2005 [15]	Modified ZGXFD	Achyranthes root 30 g, ruddle 30 g, uncaria 30 g (put in later), dragon bone 15 g, oyster shell 15 g, plastrum testudinis 15 g, white peony root 12 g, radix scrophulariae 12 g, radix asparagi 12 g, fructus toosendan 9 g, raw malt 20 g, artemisia capillaris thumb 9 g, and glycyrrhiza 6 g.
Luo 2008 [16]	ZGXFD	White peony root 30 g, radix asparagi 15 g, achyranthes root 30 g, ruddle 30 g, dragon bone 30 g, oyster shell 30 g, plastrum testudinis 25 g, radix scrophulariae 15 g, fructus toosendan 10 g, raw malt 10 g, artemisia capillaris thumb 15 g, and glycyrrhiza 10 g
Liu and Zhong 2008 [17]	Modified ZGXFD plus benazapril	White peony root 15 g, radix asparagi 15 g, plastrum testudinis 15 g, oyster shell 15 g, fructus toosendan 6 g, ruddle 30 g, achyranthes root 30 g, radix scrophulariae 15 g, dragon bone 15 g, artemisia capillaris thumb 6 g, raw malt 6 g, and glycyrrhiza 5 g. Severe headache plus chrysanthemum 10 g; insomnia plus pearl shell 15 g and caulis polygoni multiflori 15 g; vexation plus gardenia 10 g and scutellaria baicalensis georgi 10 g; severe phlegm-heat plus pinellia pedatisecta schott 6 g and fritillaria cirrhosa 10 g.
Li and Zheng 2012 [18]	Modified ZGXFD	Radix scrophulariae 15 g, ruddle 30 g, white peony root 15 g, achyranthes root 30 g, radix asparagi 15 g, dragon bone 15 g, oyster shell 15 g, raw malt 6 g, artemisia capillaris thumb 6 g, plastrum testudinis 15 g, fructus toosendan 6 g, and glycyrrhiza 3 g. Vexation plus plaster stone; abundant sputum plus pinellia pedatisecta schott and bamboo bark; slow-weak pulse plus prepared radix rehmanniae and pulp of cornus; diarrhea remove plastrum testudinis and ruddle, plus halloysitum rubrum; insomnia plus coptis chinensis, rehmanniae radix and caulis polygoni multiflori; severe headache plus abalone shell; severe dizziness plus gastrodia elata.
Zhou 2000 [19]	Modified ZGXFD plus nitrendipine	White peony root 20 g, radix asparagi 10 g, plastrum testudinis 5 g, oyster shell 30 g, abalone shell 20 g, ruddle 80 g, magnetite 30 g, achyranthes root 10 g, radix scrophulariae 15 g, salvia miltiorrhira 30 g, and panpax notoginseng 10 g. Severe headache plus antelope horn; insomnia plus pearl shell and caulis polygoni multiflori; vexation plus gardenia and scutellaria baicalensis georgi; severe phlegm-heat plus pinellia pedatisecta schott and fritillaria cirrhosa.

3.2. Methodological Quality of Included Trials. The methodological quality of the included trials was assessed to be generally low according to the predefined quality assessment criteria in Table 3. The randomized allocation of participants was mentioned in all the included trials [14–19], and only 2 trials [14, 15] stated the methods for sequence generation including random number table. The other 4 trials [16–19] have not reported the randomized allocation of participants with detailed information. However, insufficient information was provided to judge whether it was conducted properly or not. Allocation concealment and double-blind were not mentioned in all trials. None of trials reported dropout or withdraw. None of trials had a pretrial estimation of sample size, which indicated the lack of statistical power to ensure appropriate estimation of the therapeutic effect. Selective reporting was generally unclear in the RCTs due to the inaccessibility to the trial protocol. All the trials did not mention followup. We contacted the authors for further information but regrettably no information could be gotten.

3.3. Effect of the Interventions

3.3.1. "Zhen Gan Xi Feng Decoction" versus Antihypertensive Drugs (Western Medicine). Four trials [14–16, 18] compared prescriptions based on "Zhen Gan Xi Feng Decoction" using alone with antihypertensive drugs.

Blood Pressure. Four trials [14–16, 18] used blood pressure decrease to measure the outcome: significant effective (diastolic blood pressure decreased by 10 mmHg reaching the normal range, or diastolic blood pressure has not yet returned to normal but has been reduced ≥ 20 mmHg), effective (diastolic blood pressure decreased to less than 10 mmHg reaching the normal range, or diastolic blood pressure decreased by 10–19 mmHg but did not reach the normal range, or systolic blood pressure decreased ≥ 30 mmHg), and ineffective (not to meet the previous standards). The trial showed significant difference between treatment and control group on the three criteria outcome measurement (RR: 1.93 [1.14, 3.25]; $P = 0.01$). Two trials [14, 15] compared the effectiveness using the blood pressure value, and significant difference was found between treatment and control group in systolic blood pressure (WMD: −7.05 [−10.74, −3.35]; $P = 0.0002$) and diastolic blood pressure (WMD: −6.24 [−8.42, −4.07]; $P < 0.00001$) (Tables 4, 5, and 6).

TCM-SSD Scores. Three trials [14–16] used the TCM-SSD scores to measure the outcome: significant effective (the main symptoms such as headache, dizziness, palpitations, insomnia, tinnitus, and irritability disappear, or TCM-SSD scores reduced rate ≥70%), effective (the main symptoms relieved, or 70% > TCM-SSD scores reduced rate ≥30%), and ineffective (The main symptoms do not change, or TCM-SSD scores reduced rate <30%). Significant difference was found between treatment and control group after treatment. Meta-analysis of three trials showed significant difference in favor of modified ZGXFD compare to antihypertensive drugs (RR: 3.78 [1.82, 7.85]; $P = 0.0004$) (Table 7).

3.3.2. "Zhen Gan Xi Feng Decoction" Plus Antihypertensive Drugs versus Antihypertensive Drugs. Two trials [17, 19] compared the combination of modified ZGXFD plus antihypertensive drugs with antihypertensive drugs.

Blood Pressure. Meta-analysis of two trials [17, 19] showed no significant difference on blood pressure (RR: 1.03 [0.47, 2.25]; $P = 0.93$) (Table 4).

TCM-SSD Scores. There is only one trial [17] who reported the TCM-SSD scores decrease. The meta-analysis showed that there is significant beneficial effect on the combination group compare to the antihypertensive drugs using alone (RR: 3.87 [1.18, 12.68]; $P = 0.03$) (Table 7). We cannot obtain more details of the TCM-SSD scores. So, we cannot get the analysis of comparison between groups.

3.4. Sensitivity Analysis, Subgroup Analysis, and Publication Bias. The number of trials was too small to conduct any sufficient additional analysis of sensitivity, subgroup, and publication bias.

3.5. Adverse Effect. Three out of six trials mentioned the adverse effect [14, 18, 19]. Three trials reported nine specific symptoms including headache, dry cough, diarrhea, palpitations, neutropenia, nausea, dizziness, sleepiness, and itchy skin. One trial reported adverse effect in captopril group including headache and dry cough [14]. One trial mentioned adverse effect both groups, with diarrhea in modified ZGXFD group and dry cough, palpitations, and neutropenia in captopril group [18]. One trial mentioned adverse effect both groups, with gastrointestinal discomfort, dizziness, sleepiness, and itchy skin in modified ZGXFD plus nitrendipine group and nausea, dizziness, and itchy skin in nitrendipine group [19].

4. Discussion

Currently, more and more systematic reviews (SRs) and meta-analysis have been conducted to assess the efficiency of Chinese herbal medicine for essential hypertension [23–31]. It is demonstrated that Chinese herbal medicine could not only contribute to low BP smoothly, recover the circadian rhythm of BP, but also improve symptoms and signs especially [32–36]. As an adjunctive treatment to antihypertensive drugs, ZGXFD is a popular TCM formula for the treatment of essential hypertension. And until now, more and more RCTs have been published in Chinese language but have not been evaluated according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standard [37]. This study aims to assess the current clinical evidence of ZGXFD for essential hypertension. Our systematic review suggested that ZGXFD may be effective on blood pressure or improvement of TCM-SSD scores (symptoms and signs). However, according to potential publication bias and low-quality trials, available data are not adequate to draw a definite conclusion of ZGXFD in treating essential hypertension.

Table 3: Quality assessment of included randomized controlled trials.

Included trials	Sequence generation	Allocation concealment	Blinding of participants personnel and outcome assessors	Incomplete outcome data	Selective outcome reporting	Other sources of bias	Risk of bias
Guo et al. 2002 [14]	Table of random number	Unclear	Unclear	No	No	Unclear	Unclear
Mao 2005 [15]	Table of random number	Unclear	Unclear	Yes	No	Unclear	Unclear
Luo 2008 [16]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Liu and Zhong 2008 [17]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Li and Zheng 2012 [18]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Zhou 2000 [19]	Unclear	Unclear	Unclear	Yes	No	Unclear	High

Table 4: Analyses of blood pressure.

Trials		Intervention (n/N)	Control (n/N)	RR [95% CI]	P value
ZGXFD versus antihypertensive drugs					
ZGXFD versus captopril	1	54/68	40/61	2.02 [0.92, 4.46]	0.08
Modified ZGXFD versus indapamide	1	40/47	19/23	1.20 [0.31, 4.61]	0.79
ZGXFD versus captopril	1	22/24	18/21	1.83 [0.28, 12.19]	0.53
Modified ZGXFD versus captopril	1	78/86	65/80	2.25 [0.90, 5.64]	0.08
Meta-analysis	4	194/225	142/185	1.93 [1.14, 3.25]	0.01
ZGXFD plus antihypertensive drugs versus antihypertensive drugs					
Modified ZGXFD plus benazapril versus benazapril	1	56/60	47/60	3.87 [1.18, 12.68]	0.03
Modified ZGXFD plus nitrendipine versus nitrendipine	1	183/200	72/100	0.07 [0.00, 1.22]	0.07
Meta-analysis	2	239/260	119/132	1.03 [0.47, 2.25]	0.93

Table 5: Analyses of systolic blood pressure.

Trials		MD [95% CI]	P value
ZGXFD versus antihypertensive drugs			
ZGXFD versus captopril	1	−10.94 [−15.64, −6.24]	<0.00001
Modified ZGXFD versus indapamide	1	−0.74 [−6.72, 5.24]	0.81
Meta-analysis	2	−7.05 [−10.74, −3.35]	0.0002

Table 6: Analyses of diastolic blood pressure.

Trials		MD [95% CI]	P value
ZGXFD versus antihypertensive drugs			
ZGXFD versus captopril	1	−8.42 [−10.98, −5.86]	<0.00001
Modified ZGXFD versus indapamide	1	−0.52 [−4.67, 3.63]	0.81
Meta-analysis	2	−6.24 [−8.42, −4.07]	<0.00001

Table 7: Analyses of TCM-SSD Scores.

Trials		Intervention (n/N)	Control (n/N)	RR [95% CI]	P value
ZGXFD versus antihypertensive drugs					
ZGXFD versus captopril	1	59/68	44/61	2.53 [1.03, 6.21]	0.04
Modified ZGXFD versus indapamide	1	45/47	17/23	7.94 [1.46, 43.24]	0.02
ZGXFD versus captopril	1	23/24	15/21	9.20 [1.00, 84.26]	0.05
Meta-analysis	3	127/139	76/105	3.78 [1.82, 7.85]	0.0004
BBTD plus antihypertensive drugs versus antihypertensive drugs					
modified ZGXFD plus benazapril versus benazapril	1	56/60	47/60	3.87 [1.18, 12.68]	0.03
Meta-analysis	1	56/60	47/60	3.87 [1.18, 12.68]	0.03

More specifically, the positive findings should be interpreted conservatively due to the following facts.

Firstly, all the six trials included in this paper had risk of bias in terms of design, reporting, and methodology. Only two randomized controlled trials (RCTs) [14, 15] stated randomization procedure with table of random number. However, limited information was provided to judge whether randomization was conducted properly and really. For the other 4 trials [16–19], they just mentioned that "patients were randomized into two groups" with no detailed information. All RCTs did not mention allocation concealment. Therefore, we could not exclude the possibility that some of the claimed RCTs are not real RCTs. What is more, these trials, including Mao 2005, Luo 2008, Zhou 2000, Liu and Zhong 2008, and Li and Zheng 2012 [15–19], only have one author or two authors. It is impossible for an RCT to be done properly in terms of randomization procedure and the allocation concealment. It is noteworthy that all the trials did not describe the blinding in details. It directly led to performance bias and detection bias due to patients and researchers being aware of the therapeutic interventions for the subjective outcome measures. All the included RCTs did not report presample size estimation and were not multicenter, large scale RCTs. Therefore, it directly prohibited us to perform meaningful analysis between groups. It is well known that, if poorly designed, all the trials would show larger differences between experimental and control groups than those conducted rigorously [38].

Second, there was lack of knowledge for the final indicator at endpoint. As we know, the primary goal of essential hypertension treatment is to reduce mortality or prevent progression to severe complications. However, all the included trials only reported the outcomes such as blood pressure and symptom improvement. None of the trials reported the mortality rate or the incidence of complications. Future RCTs of ZGXFD with appropriate design need to be carried out to measure the mortality and morbidity of hypertension.

Third, our review found inadequate reporting on adverse events in the included trials. Only two of the six trials reported the adverse effect of ZGXFD or modified ZGXFD briefly, providing limited information. One trial [18] mentioned diarrhea, and the other [19] mentioned gastrointestinal discomfort, dizziness, sleepiness, and itchy skin. The remaining four trials did not mention whether they had monitored adverse effects at all. Therefore, conclusions about the safety of ZGXFD cannot be made from this review due to the limited, inadequate recording and reporting of adverse events. There is a widely accepted perception that it is safe to use herbal medicines for various diseases in China. However, with the increasing reports of liver toxicity and other adverse events associated with Chinese herbal medicines [39–44], the safety of ZGXFD needs to be monitored rigorously and reported appropriately in the future clinical trials.

Fourth, publication and other biases may play an important role in the review. Only trials published in China could be identified and included after conducting comprehensive searches. We tried to avoid language bias and location bias; however, potential publication bias could not be excluded totally. Almost all the RCTs claimed positive effect of ZGXFD though some of them turned out to be negative when analyzed by standard statistical techniques using risk ratios or mean differences. We have conducted extensive searches for unpublished material, but no unpublished "negative" studies were found.

In summary, the reported effectiveness and safety of ZGXFD for essential hypertension cannot be taken as confirmative conclusion. Due to poorly designed and low-quality methodology, the evidence is still inconclusive. We hope that further RCTs with better research methods as a good approach to evaluate the effectiveness will be needed in ZGXFD for essential hypertension clinical study.

Conflict of Interests

All authors declare that they have no conflict of interests.

Authors' Contributions

X. Xiong, X. Yang, B. Feng, W. Liu, H. Li, J. Ma, X. Du, P. Wang, K. Su, F. Chu, G. Zhang, and X. Li contributed equally to this paper.

Acknowledgments

The current work was partially supported by the National Basic Research Program of China (973 Program, no. 2003CB517103) and the National Natural Science Foundation Project of China (no. 90209011). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 364519, 16 pages
http://dx.doi.org/10.1155/2013/364519

Research Article

Cardioprotective Effects of Quercetin in Cardiomyocyte under Ischemia/Reperfusion Injury

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Received 10 August 2012; Revised 22 November 2012; Accepted 7 February 2013

Academic Editor: Peng Nam Yeoh

Quercetin, a polyphenolic compound existing in many vegetables, fruits, has antiinflammatory, antiproliferation, and antioxidant effect on mammalian cells. Quercetin was evaluated for protecting cardiomyocytes from ischemia/reperfusion injury, but its protective mechanism remains unclear in the current study. The cardioprotective effects of quercetin are achieved by reducing the activity of Src kinase, signal transducer and activator of transcription 3 (STAT3), caspase 9, Bax, intracellular reactive oxygen species production, and inflammatory factor and inducible MnSOD expression. Fluorescence two-dimensional differential gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) can reveal the differentially expressed proteins of H9C2 cells treated with H_2O_2 or quercetin. Although 17 identified proteins were altered in H_2O_2-induced cells, these proteins such as alpha-soluble NSF attachment protein (α-SNAP), Ena/VASP-like protein (Evl), and isopentenyl-diphosphate delta-isomerase 1 (Idi-1) were reverted by pretreatment with quercetin, which correlates with kinase activation, DNA repair, lipid, and protein metabolism. Quercetin dephosphorylates Src kinase in H_2O_2-induced H9C2 cells and likely blocks the H_2O_2-induced inflammatory response through STAT3 kinase modulation. This probably contributes to prevent ischemia/reperfusion injury in cardiomyocytes.

1. Introduction

Because of their high incidence and mortality rate, cardiovascular diseases have recently become a primary health concern worldwide. Myocardial ischemia/reperfusion injury, which causes excess reactive oxygen species (ROS) production that can lead to cardiac hypertrophy or dysfunction, is the most acute cardiovascular disease [1, 2]. In 2010, Chou et al. showed that ROS may affect intercellular connections and cytoskeleton resulting in cell detachment, morphology change, or death. Src kinase also plays a key role in ROS-induced phosphorylation and cell damage in cardiomyocytes [2].

The ROS in this study includes hydrogen peroxide (H_2O_2), singlet oxygen ($O^{\bullet}$), superoxide (O^{2-}), and the hydroxyl radical ($OH^{\bullet}$). Among these ROS species, H_2O_2 is the most stable and the most abundant in human cells. Although the optimal amount of ROS plays an important role in signal transduction, excess ROS causes cell damage [3]. H_2O_2 regulates signal transduction-related proteins by phosphorylating or modifying the active sites of proteins but also inhibits phosphatase activity [4].

Quercetin, a type of polyphenolic compound, has anti-inflammatory, antiproliferation, anti-histamine, and antioxidant effects. Quercetin exists in many types of vegetables and fruits. Several reports have shown that quercetin has protective effects on different types of cells, including myocytes, testis, renal cells, and liver cells in ischemia/reperfusion injury [5]. A study conducted in 1992 showed that quercetin

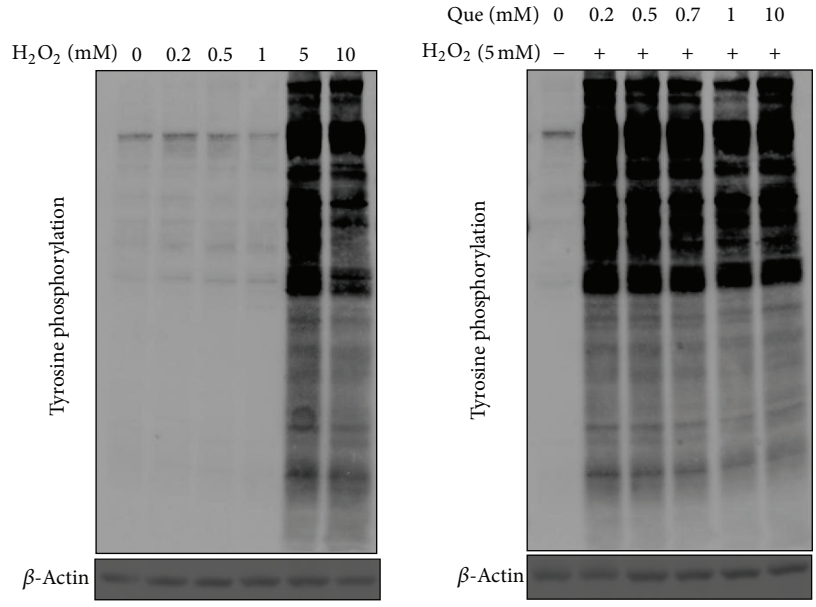


Figure 1: Hydrogen peroxide treatment induces tyrosine phosphorylation in H9C2 cells. (a) Total cell lysates were prepared from H9C2 cells treated with a range of H_2O_2 concentrations (0, 0.2, 0.5, 1, 5, and 10 mM) for 20 min. H9C2 total cell lysate proteins were separated by 1D SDS-PAGE, transferred onto a PVDF membrane (Pall) electrophoretically, and then probed with specific primary antibodies antiphosphotyrosine and β-actin. (b) Effects of quercetin on H_2O_2-induced tyrosine phosphorylation in H9C2 cells. The total cell lysates were prepared from H9C2 cells pretreated with different quercetin concentrations (0, 0.2, 0.5, 0.7, 1, and 10 mM) for 1 h and then treated with 5 mM H_2O_2 for 20 min. Cells were immunoblotted with phosphotyrosine and β-actin (upper image). β-Actin is a loading control for this experiment.

reduces the oxidative stress caused by ischemia/reperfusion in cardiomyocytes by inhibiting the xanthine dehydrogenase/xanthine oxidase system [6]. Several reports have also indicated that quercetin and isorhamnetin can scavenge ROS and inhibit the activation of ERK or MAP kinase in ROS-induced cardiomyopathy [7, 8]. In cancer therapy, combining quercetin with doxorubicin augmented the effects of doxorubicin in highly invasive breast cancer cells [9] and can protect cardiomyocytes from doxorubicin-induced toxicity by chelating iron, inducing antioxidant activity, and inhibiting carbonyl reductase [10]. Although quercetin has been reported to play a role in protecting myocardial cells from ischemia/reperfusion injury, its protective mechanism remains unclear in current knowledge.

Ischemia/reperfusion injury in cardiomyocytes is the result of myocardial inflammation [11]. Muthian and Bright showed that quercetin blocks the IL-12-induced inflammatory response through a signal transducer and activator of transcription 3 (STAT3) activation in T lymphocytes [12]. However, previous research failed to show a direct relationship between quercetin and STAT3-activated inflammation in cardiomyocytes. STAT3 is a transcription factor that plays an important role in numerous cytokine signaling transductions including cell survival, proliferation, cell cycle progression, and cell growth. STAT3 has two important phosphorylated and activate sites: Tyr705 and Ser727. STAT3 activation was phosphorylated at tyrosine 705 induced by various factors, including cardiotrophin-1, IL-6, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) [5, 13]. pY705-STAT3 is also essential for the dimerization of STAT3 and the translocation of STAT3 into the nucleus. In addition, STAT3 has been observed to be phosphorylated at serine 727 under oxidative stress to enhance the transcription activity of STAT3 in previous cerebral ischemia preconditioning study [14]. Moreover, The JAK2/STAT3 signaling pathways participate in an oxidative stress-induced immune response [3, 15].

Two-dimensional gel electrophoresis (2-DE) is a common tool for analyzing thousands of proteins in different biological samples and is complementary to LC-MS results. However, varying quantification between gels remains the primary challenge in 2-DE. Therefore, 2D-DIGE reduces the variation between gels and gels, which codetected the sample abundances on the same gel by using differential fluorescent labeling [2].

This study investigates the potential protective role of quercetin in H_2O_2-induced H9C2 cell injury. We focus on the correlation between quercetin in cardiomyocytes and the cardioprotective role of Src kinase inhibition and inflammatory response of STAT3 using 2D-DIGE combined with MALDI-TOF MS and immunoblotting.

2. Materials and Methods

2.1. Chemicals and Reagents. Quercetin was purchased from Sigma-Aldrich (St. Louis, USA). The primary antibody

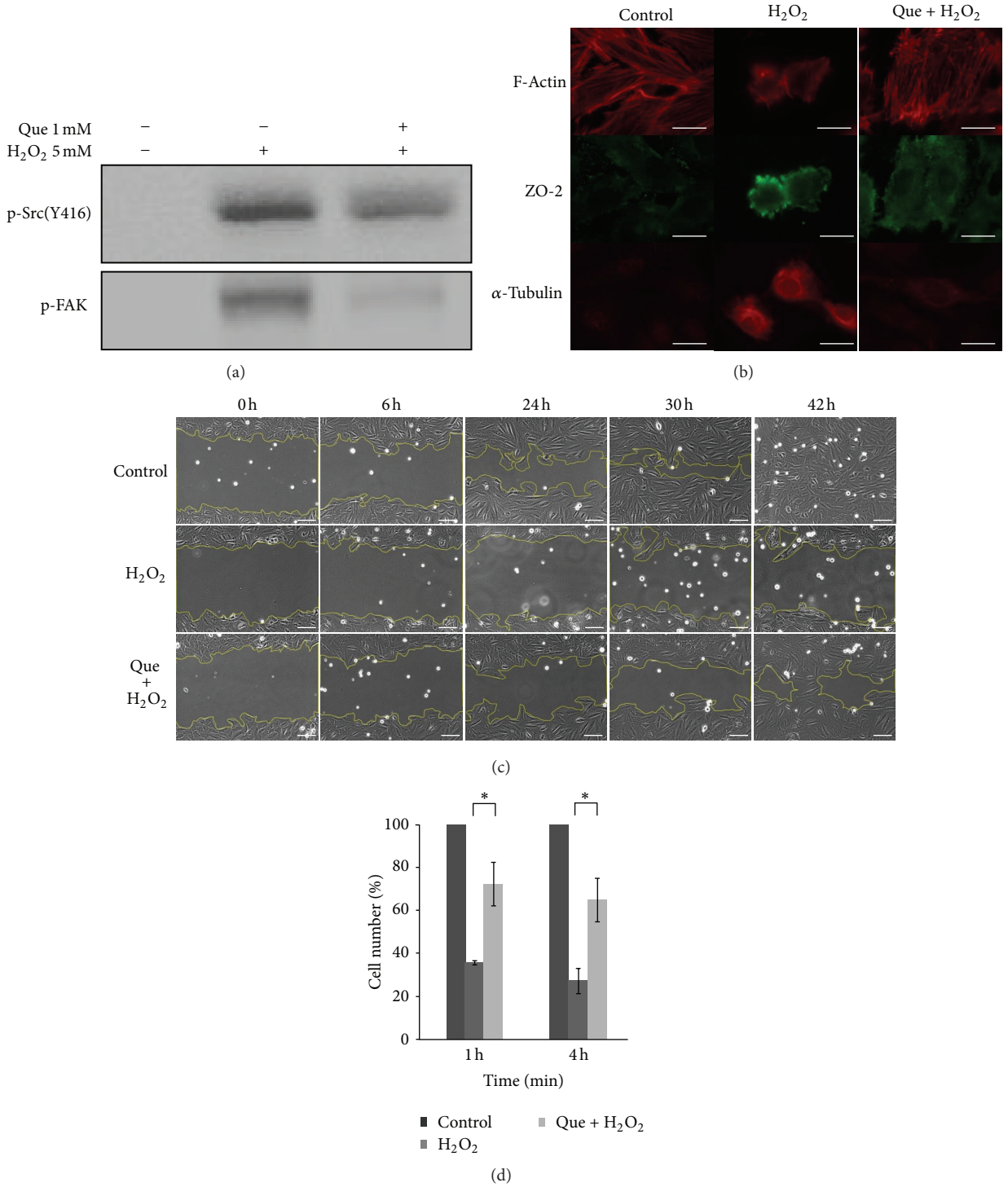


Figure 2: Effects of quercetin on the cell morphology, migration, and adhesion of H_2O_2-treated H9C2 cells. (a) The expressions of phospho-Src (Tyr-416) and phospho-FAK (Tyr-576/577) in H9C2 cells were detected using immunoblotting. (b) The cell morphology and protein location of proteins in H9C2 cells were analyzed by immunostaining. Each set of fluorescence images was taken at the same exposure time. Scale bar = 20 μm. (c) The wound healing images were captured at different culture times (0 h, 6 h, 24 h, 30 h, and 42 h) using a fluorescence microscope (Zeiss) after H9C2 cells were treated with H_2O_2 for 20 min or pretreated with quercetin for 1 h. Scale bar = 100 μm. (d) Adhesion assays in which H9C2 cells were treated with H_2O_2 for 20 min or pretreated with quercetin for 1 h and then incubated for 1 h and 4 h in a serum-free medium. After incubation, H9C2 cells were trypsinized, and the cell number was counted using a hemocytometer. Data represent the mean ± standard deviation for 3 independent experiments and are represented as a percentage of the control. The control contains only serum-free DMEM. ($^*P < 0.05$).

Table 1: Differentially expressed proteins were listed alphabetically after 2D-DIGE and MALDI-TOF mass spectrometry analysis in H9C2 cells in response to H_2O_2 treated and pretreated with quercetin. The average ratios of these 44 spots are differentially expressed between untreated (control), H_2O_2-treated, and quercetin-pretreated followed by treatment with H_2O_2, calculated from triplicate gels. Boldface numbers represent proteins for which the changes between the H_2O_2 treatment and the control are significantly greater than changes between quercetin pretreatment followed by H_2O_2 treatment and control.

Spot no.	Swissprot no.	Protein name	Pred. MW	Pred. PI	Cov. %	MASCOT score	No. of peptides match/ supplied	H_2O_2/Ctrl	Quercetin + H_2O_2/Ctrl	Peptide sequence	Function
1420	P63324	40S ribosomal protein S12	14858	6.82	42%	55/51	6/30	−1.25	−2.77	QAHLCVLASNCDEPMYVK, TALIHDGLAR	Protein synthesis
691	Q9JLJ3	4-Trimethylaminobutyraldehyde dehydrogenase	54530	6.57	44%	109/56	16/66	1.68	1.9	AFEPATGR, AGAPNGLFNVVQGGAATGQFLCQHR	Protein synthesis
473	P63039	60 kDa heat shock protein (mitochondrial)	61088	5.91	20%	61/56	8/30	1.37	1.54	AAVEEGIVLGGGCALLR, ISSVQSIVPALEIANAHR	Chaperon
1010	P60711	Actin (cytoplasmic)	42052	5.29	23%	54/51	6/37	**−1.51**	**−1.27**	GYSFTTTAER, SYELPDGQVITIGNER	Cytoskeleton
704	P11884	Aldehyde dehydrogenase, mitochondria	56966	6.63	41%	143/56	16/54	1.12	1.56	RVTLELGGK, SGQQEGAK	Redox regulation
990	P54921	Alpha-soluble NSF attachment protein	33627	5.3	50%	120/56	13/39	**9.85**	**7.69**	QAEAMALLAEAER, IEEACEIYAR	Transport
1002	P55260	Annexin A4	36168	5.31	39%	145/51	13/28	−1.92	−2.35	GDTSGDYR, WGTDEVK	Transport/Ca^{2+}
396	P48037	Annexin A6	76106	5.39	52%	253/56	36/74	−1.35	−1.73	YELTGKFER, AINEAYKEDYHK	Transport/Ca^{2+}
1116	P35426	Cell division protein kinase 4	34006	6.09	54%	198/56	15/32	**3.5**	**3.22**	VTLVFEHIDQDLR, VPNGGAAGGGLPVSTVR	Cell cycle
1319	P47875	Cysteine and glycine-rich protein 1	21455	8.9	51%	58/56	6/44	−7.38	−11.9	NLDSTTVAVHGEEIYCK, GLESTTLADKDGEIYCK	Cytoskeleton regulation
1323	P47875	Cysteine and glycine-rich protein 1	21455	8.9	54%	91/56	7/40	**13.77**	**12.77**	TVYFAEEVQCEGNSFHK, HEEAPGHRPTTNPNASK	Cytoskeleton regulation
416	Q5XI50	E3 ubiquitin-protein ligase MARCH7	76932	7.64	16%	53/51	9/44	−2.86	−3.98	MVSGNRGTSLNDSYHSR, CTGSLQYVHQECMK	Protein degradation
709	P62630	Elongation factor 1-alpha 1	50424	9.1	29%	87/56	11/35	**−2.01**	**−1.1**	EHALLAYTLGVK, STTTGHLIYK	Protein synthesis
1244	O08719	Ena/VASP-like protein	42183	8.74	20%	59/51	6/43	**2.27**	**1.63**	WVPIKPGQQGFSR, VKPAGSVNDVGLDALDLDRMK	Cytoskeleton regulation
305	Q99PF5	Far upstream element-binding protein 2	74466	6.38	41%	169/56	20/43	−2.09	−2.25	ERDQGGFGDR. IGQQPQQPGAPPQQDYTK	Gene expression
886	P97590	Galectin-7	15333	6.43	29%	52/51	6/43	−1.41	−1.58	MPSSNVRSVEVGGDVQLHSVK, MSATHHK	Apoptosis
1251	Q9Z1B2	Glutathione S-transferase Mu 5	27067	6.33	50%	76/56	15/54	−4.85	−9.44	ITQSNAILR, VDIMENQIMDFR	Redox regulation

Table 1: Continued.

Spot no.	Swissprot no.	Protein name	Pred. MW	Pred. PI	Cov. %	MASCOT score	No. of peptides match/ supplied	H_2O_2/Ctrl	Quercetin + H_2O_2/Ctrl	Peptide sequence	Function
1255	P42930	Heat shock protein beta-1	22936	6.12	56%	147/56	12/65	−3.9	−4.49	KYTLPPGVDPTLVSSSLSPEGTLTVEA, VPFSLLR	Chaperon
954	Q6RUG5	Islet cell autoantigen 1-like protein	49298	5.23	31%	54/51	9/53	**−1.64**	**−1.6**	MDSFEHLRPEDSQSVVSRMQK, DASQELDPDTFK	Unknown
1240	O35760	Isopentenyl-diphosphate Delta-isomerase 1	26721	5.57	38%	61/56	6/58	**−1.8**	**−1.31**	MPEINASNLDEK, AELGIPLEEVDLNEMNYLTR	Lipid synthesis
1237	Q63279	Keratin, type I cytoskeletal 19	44609	5.21	25%	51/51	10/73	**4.02**	**3.7**	QGPGPFRDYSQYFK, MSVEADINGLRR	Cytoskeleton
1219	Q6QLM7	Kinesin heavy chain isoform 5A	117642	5.56	15%	54/51	12/43	−2	−2.3	SLTEYMQTVELKK, MAETNNECSIKVLCR	Transport
418	P56536	Kinesin heavy chain isoform 5C (Fragment)	27376	5.87	25%	63/51	6/40	−2.7	−3.79	FVSSPEEVMDVIDEGK, NRHVAVTNMNEHSSR	Transport
349	P48679	Lamin-A	74564	6.54	48%	221/56	32/68	−2.04	−2.28	LQDEMLRR, LESSESR	Cytoskeleton
1263	Q6AYP2	Microfibrillar-associated protein 3-like	45804	4.9	13%	55/51	7/30	1	2.22	DEVYTIPNSLKR, VTQFKTMEFAR	Sperm development
1355	P13832	Myosin regulatory light chain RLC-A	19940	4.67	62%	102/56	13/52	**2.18**	**1.82**	DGFIDKEDLHDMLASMGK, GNFNYIEFTR	Muscle contraction
1357	Q64122	Myosin regulatory light polypeptide 9	19765	4.8	42%	68/51	10/48	**2.12**	**1.5**	EAFNMIDQNR, KGNFNYVEFTR	Muscle contraction
1276	Q63716	Peroxiredoxin-1	22323	8.27	41%	77/56	7/44	−1.84	−2.56	ADEGISFR, MSSGNAKIGHPAPSFK	Redox regulation
1213	P97562	Peroxisomal acyl-coenzyme A oxidase 2	77548	7.64	17%	53/51	10/41	**2.19**	**1.96**	HGMHAFIVPIR, LAWSLGWSEDGPER	lipid metabolism
1198	P25113	Phosphoglycerate mutase 1	28928	6.67	40%	72/51	8/28	−1.66	−5.8	YADLTEDQLPSCESLKDTIAR, VLIAAHGNSLR	Glycolysis
1202	P25113	Phosphoglycerate mutase 1	28928	6.67	60%	169/51	22/59	1.41	1.56	HGESAWNLENR, FSGWYDADLSPAGHEEAK	Glycolysis
1405	P62963	Profilin-1	15119	8.46	75%	94/56	11/44	**−3.01**	**1.12**	EGVHGGLINK, EGVHGGLINKK	Cytoskeleton regulation
1120	P18420	Proteasome subunit alpha type-1	29784	6.15	44%	115/56	12/28	−1.37	−1.53	NQYDNDVTVWSPQGR QECLDSR	Protein degradation
1242	P40112	Proteasome subunit beta type-3	23235	6.15	43%	60/51	9/50	**−3.09**	**−1.49**	LNLYELKEGR, NCVAIAADRR	Protein degradation
1300	P34067	Proteasome subunit beta type-4	29349	6.45	34%	70/56	12/62	6.23	6.45	FDCGVVIAADMLGSYGSLAR, VNDSTMLGASGDYADFQYLK	Protein degradation
1321	P28075	Proteasome subunit beta type-5	28738	6.52	35%	75/56	8/48	**15.04**	**13.13**	GMGLSMGTMICGWDKR, RGPGLYYVDSEGNR	Protein degradation
522	P11598	Protein disulfide-isomerase A3	57044	5.88	26%	77/56	11/38	1.31	1.69	GFPTIYFSPANK, IFRDGEEAGAYDGPR	Redox regulation
1301	Q6IML7	Rab and DnaJ domain-containing protein	31329	8.72	26%	54/51	6/30	**−3.01**	**−2.61**	EPLKSLR, CIDESEGRLWAESR	Signal transduction

Table 1: Continued.

Spot no.	Swissprot no.	Protein name	Pred. MW	Pred. PI	Cov. %	MASCOT score	No. of peptides match/ supplied	H_2O_2/Ctrl	Quercetin + H_2O_2/Ctrl	Peptide sequence	Function
781	P29315	Ribonuclease inhibitor	51653	4.67	57%	174/56	18/64	**−1.56**	**−1.42**	LSLQNCSLTEAGCGVLPDVLR, LQLEYCNLTATSCEPLASVLR	Gene expression
952	P62138	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	38229	5.94	53%	163/56	16/49	−1.44	−1.52	TFTDCFNCLPIAAIVDEK, IYGFYDECK	Signal transduction
397	P48721	Stress-70 protein (mitochondrial)	74097	5.97	33%	103/56	21/79	−2.12	−4.1	VCQGER, DNMALQR	Chaperon
437	O35814	Stress-induced-phosphoprotein 1	63158	6.4	20%	88/51	12/62	1.26	1.56	AAALEFLNR, TLLSDPTYR	Transport
1300	P83941	Transcription elongation factor B polypeptide 1	12636	4.74	45%	56/56	5/62	6.23	6.45	AMLSGPGQFAENETNEVNFR, EIPSHVLSKVCMYFTYK	Gene expression
1208	Q91Y78	Ubiquitin carboxyl-terminal hydrolase isozyme L3	26278	5.01	63%	124/56	13/43	−1.64	−1.62	HLENYDAIR, VDLHFIALVHVDGHLYELDGR	Protein degradation

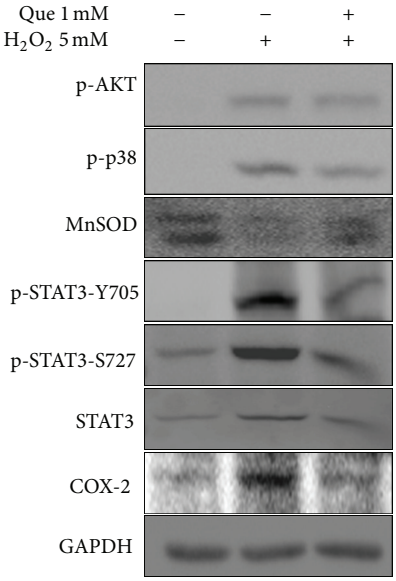


Figure 3: Effects of quercetin on the inflammatory response in H_2O_2-treated H9C2 cells. The expressed levels of phospho-Akt (Ser-473), phospho-p38 (Tyr-180/182), Mn-SOD, phospho-STAT3 (Tyr-705), phospho-STAT3 (Ser-727), COX-2, and STAT3 in H9C2 cell were detected by immunoblotting. GAPDH served as a loading control.

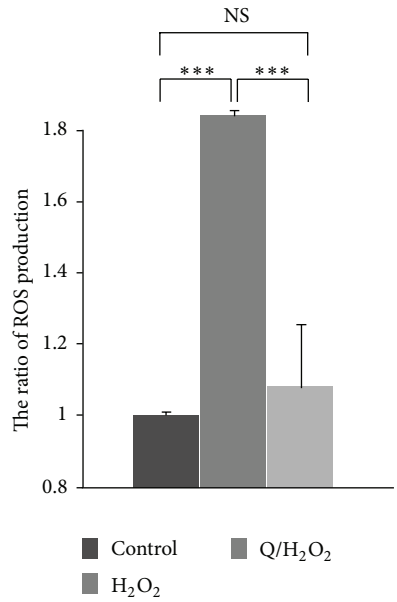


Figure 4: Effects of quercetin on ROS production in H_2O_2-treated H9C2 cells. The level of ROS in H9C2 cells was analyzed using a DCFH-DA assay. Values represent the mean ± standard variation for 3 independent experiments performed in triplicate and are represented as a ratio of the control. The control contains only serum-free DMEM. ($^{***}P < 0.001$).

phopho-FAK, Bax, caspase9, Bcl-2, GAPDH, and STIP1 were purchased from Genetex (Hsinchu, Taiwan). Horseradish peroxidase and fluorescence conjugated secondary antibodies against mouse and rabbit were purchased from Sigma-Aldrich (St. Louis, USA). Annexin V-FITC and a propidium iodide (PI) labeling kit were purchased from Invitrogen. We purchased 2,7-dichlorofluorescein diacetate (DCFH-DA) from Molecular Probes. The chemicals and reagents of 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden).

2.2. Cell Lines, Cell Culture, and Cell Treatment. The H9C2 rat cardiomyocyte cell line purchased from American Type Culture Collection (Manassas, VA, USA) was chosen as a cellular model for this study as this cell line retains the characteristics of isolated primary cardiomyocytes and has been used as a model in ischemia and reperfusion studies [2]. The H9C2 was cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS) at 37°C. Cells cultured in normal growth medium were treated with various concentrations of H_2O_2 for 20 min. H9C2 cells were pretreated with quercetin (Sigma) for 1 h followed by treatment with H_2O_2 for 20 min.

2.3. Immunoblotting. The methods of quantifying and separating cell lysates for immunoblotting were similar to our previous paper [2]. The primary antibodies used in this study included Src-phospho-Y416, phospho-FAK, phosphor-Y99, phospho-AKT, p38, Bax, caspase9, Bcl-2, GAPDH, CDK4, and STIP1.

2.4. Immunostaining and Fluorescence Microscopy. For completing immunofluorescence staining, H9C2 cells grown on coverslips (12 mm) were treated with 5 mM H_2O_2 for 20 min alone, 1 mM quercetin for 1 h prior to treatment with 5 mM H_2O_2 for 20 min, or left untreated [2]. The cell fixing, immunostaining, and fluorescence image analysis methods in this study were similar to our previous paper [2].

2.5. Wound Healing Assay. H9C2 cells (10^5 cells/well) were incubated in 24-well plate at 37°C for 12 h and then scraped with a 10 μL tip and treated with H_2O_2 for 20 min, pretreated with quercetin, or left untreated. H9C2 cells were incubated with medium containing 10% FBS, and a fluorescence microscope captured images at different incubation times (0 h, 6 h, 24 h, 30 h, and 42 h).

2.6. Adhesion Assays. H9C2 cells (8×10^4 cells/well) were incubated in a 3 cm dish containing DMEM containing 10% FBS and treated with 1 mM quercetin for 1 h followed by 5 mM H_2O_2 for 20 min. After treatment, H9C2 cells were incubated with serum-free medium for 1 h and 4 h and then were counted. The cell culture environment and cell counting were similar to our previous study [2]. All conditions have been performed in duplicate-independent experiments.

2.7. Apoptosis Assay Using Flow Cytometry. H9C2 cells (10^6 cells) were labeled with annexin V-FITC and PI at room temperature for 15 min and treated with H_2O_2, pretreated with quercetin, or left untreated. The FITC and PI fluorescence

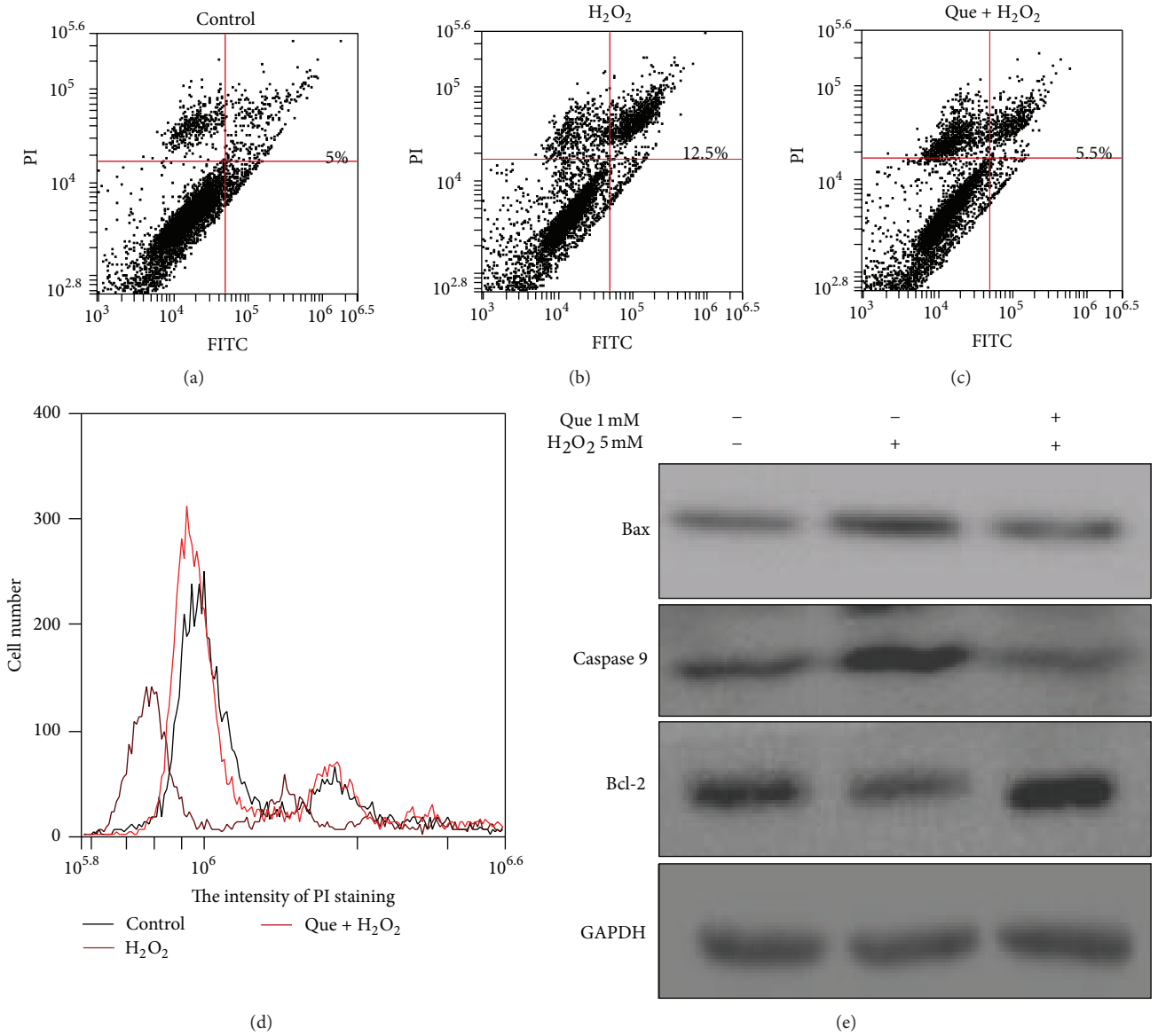


FIGURE 5: Effects of quercetin on cell apoptosis in H_2O_2-treated H9C2 cells. ((a), (b), and (c)) Typical dot plots of annexin V-FITC and PI are cells untreated, H_2O_2 treated, and quercetin pretreated followed by H_2O_2 treatment. The x-axis and y-axis represent the intensity of annexin V-FITC and PI, respectively. The lower left area of (a), (b), and (c) presented background staining by annexin V-FITC and PI in normal cells and apoptosis signals located in the right area. This figure is representative of 3 replicates. (d) The full lengths of DNA in H9C2 cells were detected by FACS. The x-axis shows the intensity of PI, and the y-axis shows the number of cells. (e) The levels of Bax, BCL-2, and caspase 9 in H9C2 cells were detected by immunoblotting. GAPDH served as a sample loading control.

signals were recorded by fluorescence-activated cell sorting FACS (Accuri 6) and analyzed using CFlow plus software [2].

2.8. Reactive Oxygen Species in Cells Were Detected Using DCFH-DA Assay. H9C2 cells (10^5 cells/well) were grown on a 24-well plate, treated with H_2O_2 for 20 min, pretreated with quercetin for 1 h, or left untreated. After washing, H9C2 cells were incubated with 10 μM of 2,7-dichlorofluorescin diacetate (DCFH-DA) at 37°C for 20 min. Fluorescence was recorded by Spectra Max Gemini EM (Molecular Device) at an excitation wavelength of 488 nm and an emission wavelength of 504 nm [16, 17].

2.9. 2D-DIGE and Gel Image Analysis in Gel Digestion and Protein Identification by MALDI-TOF MS. The experiments in this study used Cy-Dye labeling and comparative quantification methods to perform lysine-2D-DIGE analysis. Proteins were identified through MALDI-TOF MS with peptide mass fingerprinting (PMF) in our previous paper [2].

3. Results

3.1. Quercetin Pretreatment Suppresses Hydrogen Peroxide-Induced Tyrosine Phosphorylation in Cardiomyocytes. H_2O_2,

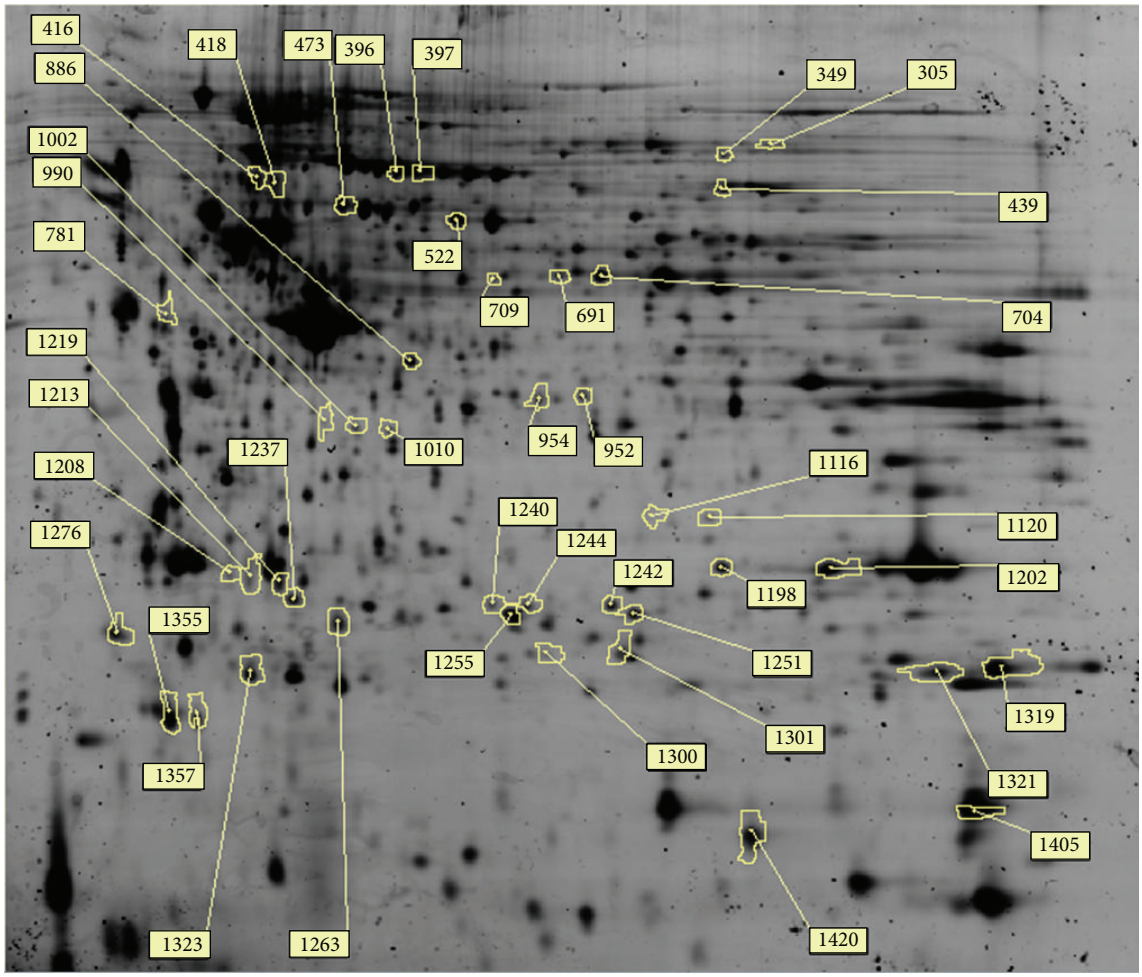
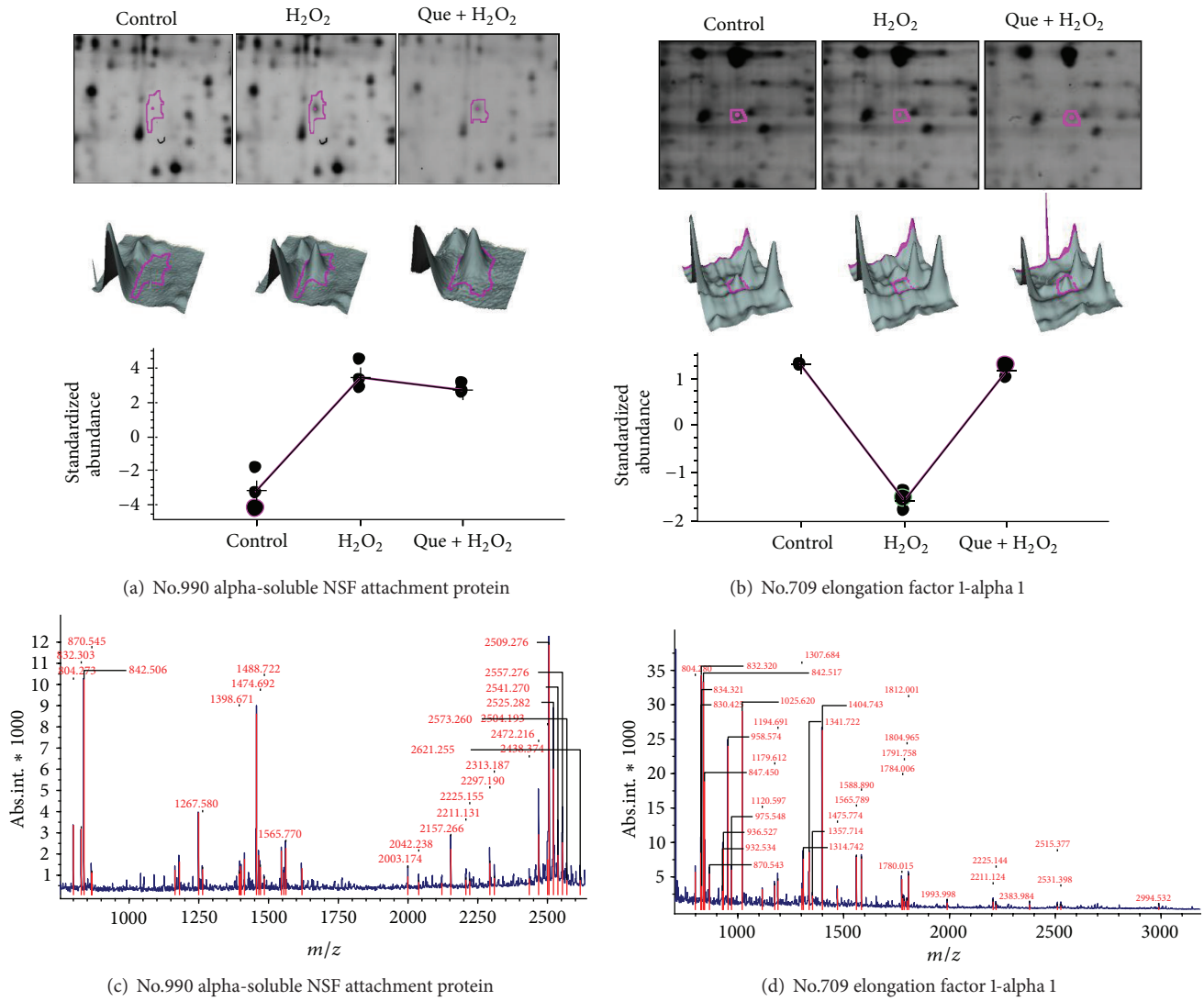


Figure 6: 2D-DIGE analysis of H9C2 cell proteome in response to H_2O_2 and quercetin treatment. H9C2 cells were lysed and arranged for a triplicate electrophoresis using pH 3 to 10 nonlinear, 24 cm IPG strips, and SDS-PAGE after treatment. 2D-DIGE image of protein sample (Cy2) is shown here. The spot numbers represent differentially expressed proteins.

an important signal mediator, induces large scale of protein phosphorylation and protein modification resulting in cellular physiology alteration including cell morphology, adhesion, and viability. Because heart ischemia/reperfusion injury stimulates H_2O_2 production, H9C2 cells were treated with varying H_2O_2 doses to find the optimal phosphotyrosine response. The optimal response represents the maximal ratio of phosphotyrosine intensity to H_2O_2 concentration by immunoblotting (Figure 1(a)). Results show that 5 mM H_2O_2 treatment led to a robust phosphotyrosine response, but the phosphotyrosine response decreased in 10 mM H_2O_2 cells. Quercetin may also play an important role in oxidative stress-damaged cells, and phosphotyrosine signals were detected with a range of quercetin followed by treatment with 5 mM H_2O_2 (Figure 1(b)). These results reveal that H9C2 pretreated with 1 mM quercetin and subsequently treated with 5 mM H_2O_2 induced a lesser phosphotyrosine response than that of H9C2 cells treated with 5 mM H_2O_2. Subsequent experiments were carried out based on these H_2O_2 and quercetin treatment concentrations.

3.2. Quercetin Inhibits Hydrogen Peroxide-Induced Changes in Cell Morphology and Loss of Cell Adhesion. H_2O_2 stimulates the activation of Src kinase that regulates cytoskeleton, cell adhesion, and cell motility. Previous report indicated that PP1, a Src kinase inhibitor, inhibits H_2O_2-induced Src kinase activation [2]. In this study, quercetin pretreatment reduces the tyrosine phosphorylation of Src kinase and FAK in H_2O_2-treated H9C2 cells (Figure 2(a)). The immunostained images represent the H9C2 proteins against specific antibodies, including cytoskeleton protein (F-actin and α-tubulin) and cell-cell interaction protein (ZO-2). Oxidative damage affects cytoskeleton proteins and ZO-2, effectively altering cell morphology (Figure 2(b)). Quercetin pretreatment improved changes in ROS-induced cell morphology.

In the wound healing assay, H9C2 cell images were captured at different time points (0 h, 6 h, 24 h, 30 h, and 42 h) using a microscope (Zeiss). Cells were untreated, H_2O_2 treated, and quercetin pretreated followed by hydrogen peroxide treatment (Figure 2(c)). After incubation, the closure areas of H_2O_2-treated H9C2 cells were larger than those



(a) No.990 alpha-soluble NSF attachment protein

(b) No.709 elongation factor 1-alpha 1

(c) No.990 alpha-soluble NSF attachment protein

(d) No.709 elongation factor 1-alpha 1

FIGURE 7: Representative images of identified proteins spots and MALDI-TOF MS analysis of (a) alpha-soluble NSF attachment protein (α-SNAP); (b) elongation factor 1-alpha 1 display differentially expressed proteins among untreated, H_2O_2 treated, and quercetin-pretreated followed by H_2O_2 treatment. The differentially expressed levels of these proteins appear as 2D patterns (top images), 3D spot images (middle images), and protein abundance levels (bottom images). The PMF patterns were ((c) and (d)) from MALDI-TOF MS.

of untreated and quercetin pretreatment followed by H_2O_2 treatment.

An adhesion assay was also performed to analyze the effects of quercetin on ROS-damaged cardiomyocytes. H9C2 cells untreated, treated with H_2O_2 alone, or pretreated with quercetin were followed by treatment with H_2O_2. Cells were then incubated in a serum-free medium for 1 h or 4 h. The adherent cells were counted after incubation. Results show that H_2O_2-treated cells had reduced adhesive ability; yet, this could be significantly improved by pretreatment with quercetin (Figure 2(d)). Thus, quercetin can stimulate cell migration and maintain cell adhesion in H_2O_2-damaged H9C2 cell.

3.3. Quercetin Inhibits Phosphorylation of STAT3, PI3K/Akt, and p38 Kinase and the Expression of COX-2 in H_2O_2-Induced H9C2 Cells. To determine whether quercetin affects cell signalings associated with inflammatory response and cell proliferation, we examined the activation of AKT, p38, and STAT3 and the expression of COX-2 and MnSOD in ROS-induced cardiomyocytes. Results show that excess ROS increased the phosphorylation of Akt, p38, and STAT3 (Tyr-705 and Ser-727) and the level of COX-2 but repressed the expression of MnSOD in H9C2 cells. Quercetin significantly reduces the phosphorylation of STAT3 and level of COX-2 and increases the expression of MnSOD in H_2O_2-treated cells (Figure 3). These results show that quercetin suppresses inflammation in H_2O_2-induced H9C2 cells.

3.4. Pretreatment with Quercetin Suppresses ROS Production in H_2O_2-Treated H9C2 Cells. DCF fluorescence revealed ROS production in H9C2 cells induced by oxidative damage.

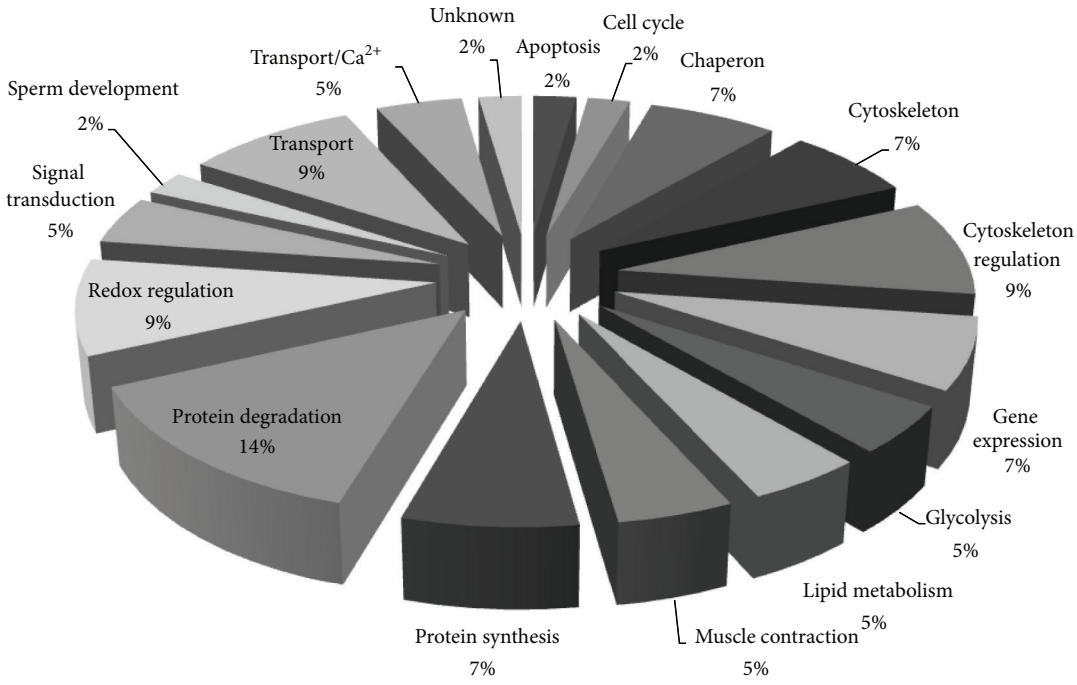


FIGURE 8: Percentage of functional distribution of differentially expressed proteins in H9C2 cell responses to H_2O_2 and quercetin treatment based on proteomic analysis.

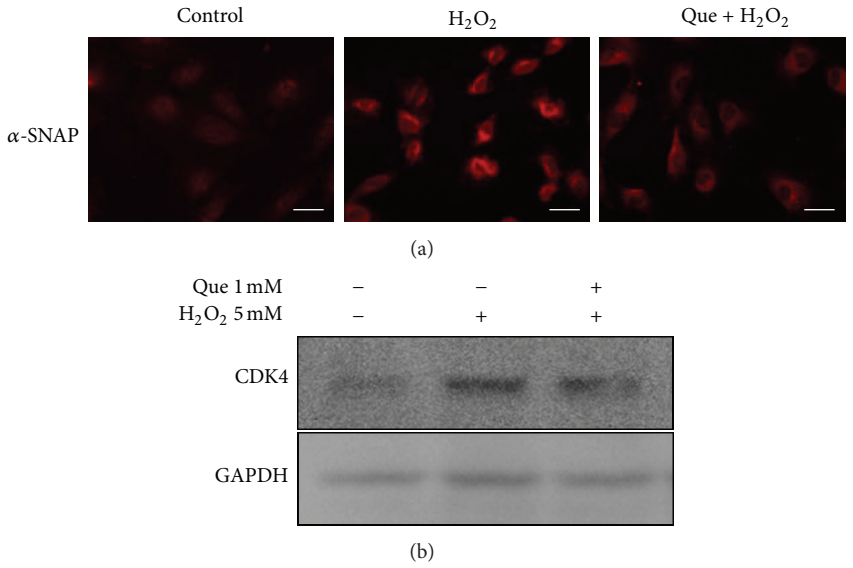


FIGURE 9: Comprehensive immunofluorescence images and immunoblotting analysis of the differentially expressed proteins identified by MALDI-TOF MS. (a) The differential expression and distribution of α-SNAP in H9C2 cells responded to H_2O_2 and quercetin (Que) were analyzed using immunofluorescence. (b) Immunoblotting was performed to validate CDK4 and STIP1 in the H9C2 cell lysate. α-Tubulin served as a loading control. Scale bar = 20 μm.

Excess ROS accumulated in H_2O_2-induced H9C2 cells, but quercetin significantly inhibited H_2O_2-induced ROS production in cardiomyocytes (Figure 4).

3.5. Quercetin Reduces Hydrogen Peroxide-Induced H9C2 Cell Apoptosis. Excess ROS production from ischemia/reperfusion-injured cardiomyocyte [18] alters redox homeostasis and induces cell apoptosis. During cell apoptosis, the asymmetric distribution of phospholipids of the plasma membrane gets lost and phosphatidylserine is translocated to the outer surface of the plasma membrane which has a high affinity to annexin V-FITC. PI can penetrate the cell

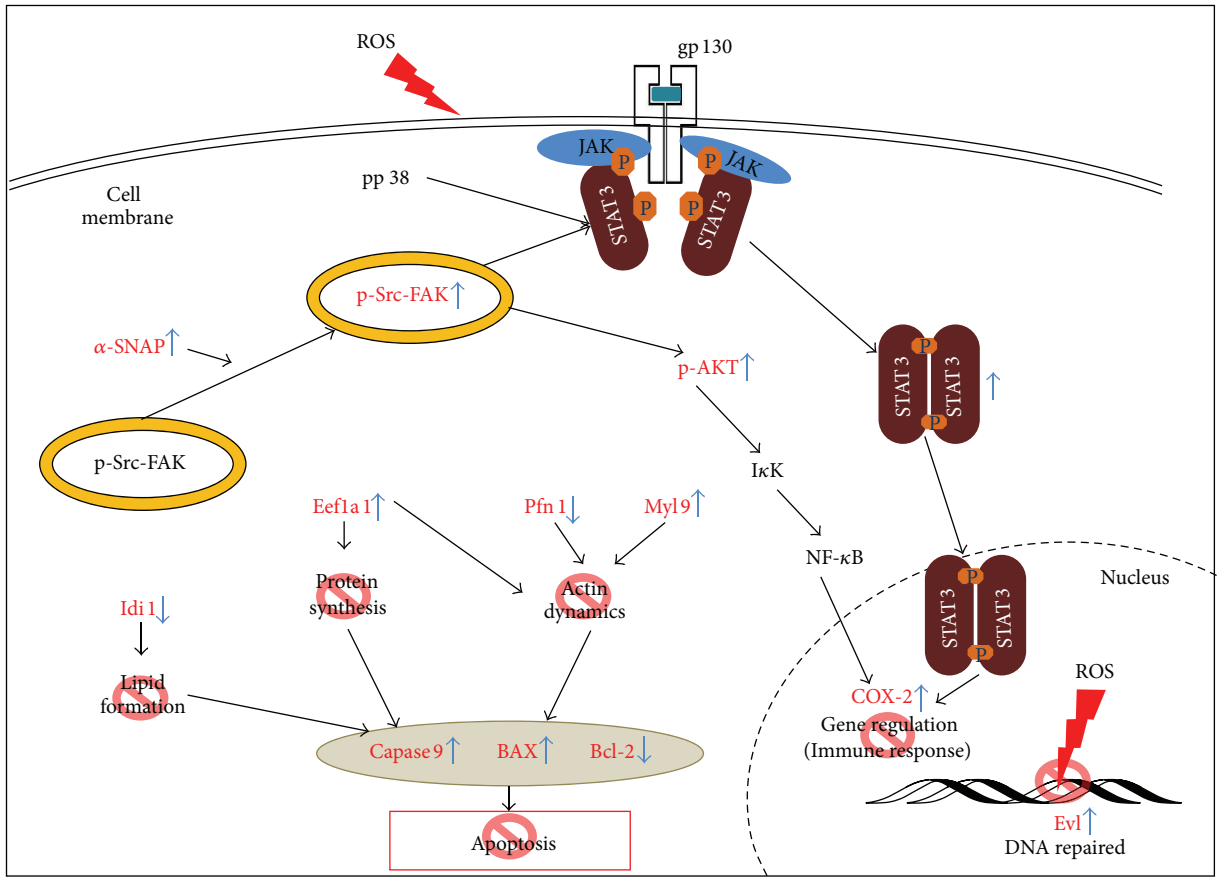


Figure 10: Model illustrating how quercetin protects cardiomyocytes from ROS treatment. ROS activates Src kinase and the overexpression of α-SNAP. ROS-induced α-SNAP causes phospho-Src-FAK complexes to move from the cytosol to a nearby inner cell membrane. ROS-activated p-Src and p-p38 stimulate the phosphorylation of STAT3 at tyrosine 705 and serine 727. After p-Src kinase and p-p38 activated STAT3, the p-STAT3 dimerized to translocate into nucleus. The dimerization of p-STAT3 induces the proinflammatory response gene expression (i.e., COX-2) in oxidative stress. Ena/VASP-like protein (Evl), which participates in actin binding and homologous recombination, is upregulated in ROS-induced cells and can repair ROS-damaged DNA. Elongation factor 1-alpha 1 (Eef1a1) decreased in oxidative stress resulting in cell death. Myosin regulatory light polypeptide 9 (Myl9), profilin-1 (Pfn1), and Eef1a1 are correlated with cytoskeleton, which may induce cell deadhesion and apoptosis. ROS inhibited the expression of isopentenyl-diphosphate delta-isomerase 1 (Idi1) in cells that may block the formation of lipophilic molecular such as sterols, ubiquinones, and terpenoids. Quercetin may protect ROS-damaged cardiomyocytes via these routes (stop sign in red). The proteins (red) were altered in a H_2O_2-dependant manner but reverted by pretreatment with quercetin.

nucleus when cells undergo apoptosis. Cell apoptosis was detected using FACS. The dot plots of annexin V and PI staining are analyzed using FACS, appearing in Figures 5(a), 5(b), and 5(c). The cell apoptosis rate increased from 5% to 12.5% upon H_2O_2 treatment, whereas the cell apoptosis rate decreased to 5.5% after H9C2 cells were pretreated with quercetin before H_2O_2 treatment. In addition, the PI staining signal of H_2O_2-treated H9C2 shifted forward, compared to that of untreated cells and cells pretreated with quercetin followed by H_2O_2 (Figure 5(d)). The levels of Bax, Caspase 9, and Bcl-2 were detected using immunoblotting for untreated H9C2 cells, treated H_2O_2, and pretreated quercetin followed by H_2O_2 treatment. ROS increased the expression level of apoptosis factors caspase 9 and Bax and reduced anti-apoptosis marker Bcl-2 expression (Figure 5(e)). According to the data, quercetin can protect and stabilize the total chromosome of DNA in H9C2 cells from oxidative damage by inhibiting cell apoptosis and chromosome attrition.

3.6. 2D-DIGE Analysis of Untreated and H_2O_2-Treated H9C2 Cells and Quercetin Pretreatment Followed by Treatment with H_2O_2. Three types of cell lysates were analyzed using 2D-DIGE. The results of 2D-DIGE analysis and DeCyder processing identified 1535 proteins spots, and 44 proteins showed differential expression ($\geqq$1.5-fold or $\leqq$ −1.5-fold; $P < 0.05$) among these 3 conditions (Figure 6). Table 1 shows that 44 proteins were identified using MALDI-TOF MS and 17 protein spots of the 44 identified protein spots that displayed H_2O_2-dependent alteration could be reversed by pretreatment with quercetin (Table 1, boldface numbers). For example, the alpha-soluble NSF attachment protein (α-SNAP) (No.990) was upregulated (9.85-fold) in H_2O_2-treated cells, whereas quercetin reduced the overexpression of H_2O_2-treated α-SNAP (7.69-fold). Protein spot number 1405, which was identified as profilin-1, was downregulated in H_2O_2 treatment only (−3.01-fold) but showed no significant expression after quercetin pretreatment followed by

H_2O_2 treatment (1.12-fold). These results suggest that the protective mechanisms of quercetin significantly reduced H_2O_2-induced damage in cardiomyocytes. Figure 7 shows the 2D-gel images, 3D images, and protein abundances from untreated, H_2O_2 treated, and quercetin pretreated followed by H_2O_2 cells.

Figure 8 shows the functional distribution of identified proteins from 2D-DIGE results. Most of proteins identified using MALDI-TOF MS are related to the cytoskeleton (9%), redox regulation (9%), and protein degradation (14%), implying that quercetin can reverse ROS damage to the cytoskeleton and redox homeostasis in cardiomyocytes.

3.7. Verification by Immunoblotting and Immunostaining. The levels of the alpha-soluble NSF attachment protein (α-SNAP) and cell division protein kinase 4 (CDK4) were examined by immunoblotting or immunostaining to validate the results of 2D-DIGE analysis. These results indicate that α-SNAP and CDK4 were overexpressed in response to H_2O_2. However, quercetin suppressed ROS-induced α-SNAP and CDK4 protein expression in H9C2 cells (Figures 9(a) and 9(b)). These data are consistent with 2D-DIGE results.

4. Discussions

Cardiovascular diseases have become a primary health concern worldwide in recent years. Ischemia/reperfusion injury in cardiomyocytes, which leads to excess ROS generation, is a particularly serious result of cardiovascular diseases. Many studies have focused on how to alleviate ischemia reperfusion-induced ROS in cardiomyocytes. For example, many plant molecules, including resveratrol, quercetin, sasanquasaponin, proanthocyanidin, safflower, and orientin, function as protectors in ischemia/reperfusion-damaged cardiomyocytes [19–23]. However, the role of quercetin in the ischemia/reperfusion injury of cardiomyocytes remains unclear.

According to previous reports, Src kinase regulates many cell signals, including cell adhesion, migration, proliferation, and apoptosis [24, 25]. During oxidative stress, Src kinase induces cell death by inactivating PI-3 K, cell migration, and spreading [26]. PP1, a Src kinase inhibitor, can rescue ROS-damaged H9C2 cells by inhibiting cell apoptosis and enhancing cell adhesion/viability [2]. However, the inhibition of Src kinase activity with PP1 is generally unsuitable for mammalian cells. Alternatively, in our findings, H9C2 cells pretreated with quercetin for 1 h are protected against H_2O_2-induced apoptosis in this study. The role of quercetin in H_2O_2-treated cardiomyocytes is to inhibit inflammatory response and maintain cell physiology, including morphology, redox status, and metabolism, by regulating Src kinase, FAK, and STAT3.

The results of this study indicate that H_2O_2 stimulates the tyrosine phosphorylation of Src kinase and FAK, which affect cell morphology and tight junction proteins, leading to cell detachment [27]. Quercetin, however, inhibits the tyrosine phosphorylation of Src kinase and FAK which maintain cell-cell interaction and morphology. Many studies have shown that quercetin protects retina, testis, neuron, cerebral, and cardiovascular cells from ischemia/reperfusion injury [28–31]. This study further demonstrates that quercetin increases migration and survival in H_2O_2-treated cardiomyocytes (Figure 2).

α-SNAP is a component of the soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptors (SNAREs) complex required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus. The major function of α-SNAP is to recycle the SNARE complex. Several reports have shown that SNARE-dependent trafficking is required for integrin signaling through a FAK/Src/PI3 K-dependent pathway [32], and the inhibition of SNARE-mediated exocytosis attenuates ischemia/reperfusion injury [33]. α-SNAP may play a critical role in regulating Src kinase signaling and inducing ischemia/reperfusion injury in cardiomyocytes. This study shows that α-SNAP was robust to overexpression (9.85-fold) in 5 mM H_2O_2-treated H9C2 cells. However, pretreatment with quercetin reduced H_2O_2-induced α-SNAP expression. Quercetin inhibits ROS-induced α-SNAP overexpression in cardiomyocytes, which could be effectively applied for protecting cardiomyocytes from oxidative stress (Figure 10).

The major functions of the Ena/VASP-like (Evl) protein include the regulation of cytoskeletal dynamics and organization axon guidance, platelet aggregation, cell motility, and cell adhesion [34, 35]. However, several studies have shown that the Evl protein has another function in homologous pairing and strand exchange through interaction with RAD51 and RAD51B [36]. Because H_2O_2 targets DNA, oxidative stress causes base damage such as strand breaking in DNA. At this moment, the repaired mechanisms, including base excision repair (BER), transcription-coupled repair (TCR), mismatch repair (MMR), nonhomologous end-joining (NHEJ), translesion synthesis (TLS), global genome repair (GGR), and homologous recombination (HR), will been turned on [37]. ROS-treated cells exhibited DNA damage, stimulating homologous recombination. In this case, Evl expression increased in cardiomyocytes, but quercetin pretreatment reduced the expression of ROS-induced Evl (Figure 10). This suggests that quercetin may stabilize the DNA structure of ROS-damaged cardiomyocytes.

Isopentenyl-diphosphate delta-isomerase 1, which is located in peroxisomes, catalyzes the isomerization of 1,3-allylic rearrangement of the homoallylic substrate isopentenyl (IPP) to dimethylallyl diphosphate (DMAPP), which is a strong electrophile allylic isomer. DMAPP is also an important product in the synthesis of many lipophilic molecules such as sterols, ubiquinones, and terpenoids. Yochem et al. demonstrated that losing idi-1 gene is lethal in *Caenorhabditis elegans*, leading to accumulated and enlarged lysosomes and autophagosomes [38]. This study shows that ROS-treated block isopentenyl-diphosphate delta-isomerase 1 expression may induce cell death; however, quercetin pretreatment reversed isopentenyl-diphosphate delta-isomerase 1 expression in H9C2 cell (Figure 10).

Elongation factor 1-alpha (EF-1 alpha) is a multifunctions protein that promotes peptide synthesis through GTP-dependent binding of aminoacyl-tRNA to the A-site of

ribosomes and binds to filamentous actin [39] and severs microtubules, leading to abnormal tetraploid cells and cell death [40]. In 1996, Gałasiński demonstrated that quercetin prevents the peptide elongation by interacting with EF-1 alpha in plant [41]. The present data show that H_2O_2 downregulates the expression of EF-1 alpha in H9C2 cells, whereas quercetin pretreatment reverses the expression of EF-1 alpha. Quercetin can prevent ROS-induced cytoskeleton damage and promote protein synthesis in cardiomyocytes (Figure 10).

Cellular antioxidant enzymes including superoxide dismutases (Mn-SOD and CuZn-SOD), catalase (CAT), peroxidases, and glutathione S-transferases regulate redox homeostasis in mammalian cells. Catalase and peroxidases scavenge H_2O_2 or convert it to hydroxyl radicals. Superoxide dismutases convert superoxide anions (O^{2-}) to H_2O_2. The observation of the oxidative state in this study demonstrates that ROS inhibits the MnSOD expression that leads to O^{2-} accumulation in cell. However, quercetin pretreatment not only reduces ROS production, but also prevents MnSOD expression in H_2O_2-treated H9C2 cells (Figure 3).

Inflammation contributes to the pathophysiology of cardiac ischemia/reperfusion injury. Myocardial ischemia and reperfusion, sepsis, viral myocarditis, and immune rejection induce the inflammatory response [11]. Cardiac ischemia/reperfusion is an acute inflammatory response that may activate phospholipase A2, metabolizing arachidonic acid into inflammatory factors by cyclooxygenases (COX-1 and COX-2), cytochrome P450, and lipoxygenase. These enzymes increase ROS production in the mitochondria. Xanthine oxidase and NADPH oxidase, which produce ROS in cells, result in inflammatory gene expression. These results suggest that quercetin blocks ROS-induced inflammatory responses such as COX-2, which converts arachidonic acid to prostaglandin (Figure 10).

STAT3, which belongs to the STAT protein family, is a protein transcription factor regulating many downstream signals for cell survival, apoptosis, proliferation, angiogenesis, and metabolic and anti-oxidative pathways [42]. Previous reports mentioned that oxidative stress activates the JAK2/STAT3/IL6 signal pathway in obese Zucker rats' fatty livers [15]. Furthermore, ROS production in hepatoma cells infected with Hepatitis C virus (HCV) activated STAT3 through JAK, Src kinase, and p38 MAP kinase pathways [43], and the decreased phosphorylation of p38 MAPK blocks the oxidative stress-induced senescence of myeloid leukemic cells [44]. PI-3K/AKT pathways, playing an important role in cell survival, proliferation, and growth, were activated by IL-1, leading to the proinflammatory gene activation of NF-κB regulation. This study shows that H_2O_2 induced the phosphorylation of Src kinase, AKT, p38, and STAT3 (pY-705 and pS-727) in cardiomyocytes inhibited by pretreatment with quercetin. Quercetin protects H9C2 cells from ROS-induced hyperinflammatory responses that inhibit the activation of Src, p38, and STAT3 (Figure 10).

In summary, this study shows that quercetin inhibits Src kinase, a potential therapeutic target in vitro, and kinases such as FAK, p38, and STAT3. Thus, quercetin has comprehensive effects on cardiomyocyte. The inhibition of an inflammatory response through STAT3 inactivation in cardiomyocyte may be beneficial for an ischemia/reperfusion injury model. Hence, quercetin should be tested in an animal model to verify its therapeutic role.

Abbreviations

2D-DIGE:	Two-dimensional differential gel electrophoresis
FBS:	Fetal bovine serum
H_2O_2:	Hydrogen peroxide
MS:	Mass spectrometry
MALDI-TOF MS:	Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
ROS:	Reactive oxygen species
STAT3:	Signal transducer and activator of transcription 3
DCFH-DA:	2,7-Dichlorofluorescein diacetate
FACS:	Fluorescence activated cell sorting
FAK:	Focal adhesion kinase.

Conflict of Interests

The authors confirm that there is no conflict of interests.

Acknowledgments

This work was supported by NSC Grants (99-2311-B-007-002, 100-2311-B-007-005, and 101–2311-B-007-011) from National Science Council, Taiwan, NTHU and CGH Grant (100N2723E1) from National Tsing Hua University, NTHU Booster Grant (99N2908E1) from National Tsing Hua University, Toward Word-Class University project from National Tsing Hua University (100N2051E1), and VGHUST Grant (99-P5-22) from Veteran General Hospitals University System of Taiwan.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 132912, 8 pages
http://dx.doi.org/10.1155/2013/132912

Research Article

Cardiovascular Protective Effects of Adjunctive Alternative Medicine (*Salvia miltiorrhiza* and *Pueraria lobata*) in High-Risk Hypertension

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Received 11 December 2012; Accepted 29 January 2013

Academic Editor: Kashmira Nanji

Introduction. Hypertension in association with diabetes (DM), renal impairment (RI), and left ventricular hypertrophy (LVH) increases the risk of future cardiovascular events. We hypothesize, traditional herbal medicines Danshen and Gegen (D&G) have beneficial effects on atherogenesis in these high-risk hypertensive subjects. *Subjects and Methods*. 90 asymptomatic hypertensive subjects associated with LVH (63.3%), DM (62.2%), or RI (30%) were randomized to receive D&G herbal capsules 1 gm/day, 2 gm/day, or identical placebo capsules in double-blind and parallel fashion for 12 months. Brachial flow-mediated dilation (endothelium-dependent dilation, FMD) and carotid intima-media thickness (IMT) were measured by ultrasound. All data were analyzed using the Statistical Package for Social Sciences in Windows 16.0. *Results*. Their mean age was 55 ± 8 years, and 74.4% were male. After 12 months of adjunctive therapies and compared with baseline, there were no significant changes in blood pressure, heart rate, hematological, glucose, and creatinine profiles in both placebo and D&G groups. FMD improved significantly during D&G ($P = 0.0001$) and less so after placebo treatment ($P = 0.001$). There was a mild but significant decrease in carotid IMT after D&G ($P < 0.001$) but no significant changes after placebo. A trend of better improvement in FMD after higher versus lower D&G dosages was seen. D&G were well tolerated, with no significant adverse events or blood biochemistry changes. *Conclusion*. D&G adjunctive treatment was well tolerated and significantly improved atherogenesis in high-risk hypertensive patients, with potential in primary atherosclerosis prevention.

1. Introduction

Atherosclerosis disease (particularly stroke and coronary artery disease) is the most important health issue in modernized society, and hypertension is an important predisposing factor [1, 2]. Hypertensive subjects with subclinical target organ damage or cardiovascular risk factors, including left ventricular hypertrophy (LVH), diabetes mellitus, or impaired renal function, are particularly vulnerable to these atherosclerotic complications in spite of best available antihypertensive therapies [3–6]. Accordingly, adjunctive primary preventive strategies are mandatory to improve their long-term natural history.

"Danshen" (丹參) and "gegen" (葛根) (D&G) are commonly used herbal materia medica in treatment of cardiac symptoms and atherosclerosis-related disorders [7]. "Danshen" consists of the dried root and rhizome of the perennial herb *Salvia miltiorrhiza* Bge. "Gegen" is the dried roots of

Pueraria lobata (willd.) [8]. Our preliminary works have confirmed the favorable effects of "Danshen and Gegen" on some metabolic indices and atherogenic process, which can be ascribed to their antithrombotic, lipid-modulating, antioxidant, anti-inflammatory, and phytoestrogenic properties [9–12]. A pilot double-blind placebo control secondary prevention study, on patients with coronary artery disease, has documented a significant improvement in brachial arterial endothelial function and carotid intima-media thickness, as surrogate atherosclerosis endpoints, after combination formula of D&G compared with placebo treatment [8]. Adjunctive treatment with D&G on top of standard cardiac drugs including statins, β blockers, aspirin, ACE inhibitors, and diuretics has been well tolerated, with no significant adverse events [8].

While the evidence base for long-term benefit of D&G treatment on hard clinical data for coronary patients is awaited with much interest, the extension of this novel but promising cardiovascular protective adjunctive regimen for primary atherosclerosis prevention in high- and very high-risk hypertensive cohort will be of tremendous clinical interests. This study proposed to evaluate the impact of D&G adjunctive therapy on top of antihypertensive treatment on improving surrogate atherosclerosis endpoints as a primary preventive strategy in high- and very high-risk hypertensive subject.

2. Patients and Methods

This proposed study is a double-blind, randomized, parallel, and placebo-controlled trial.

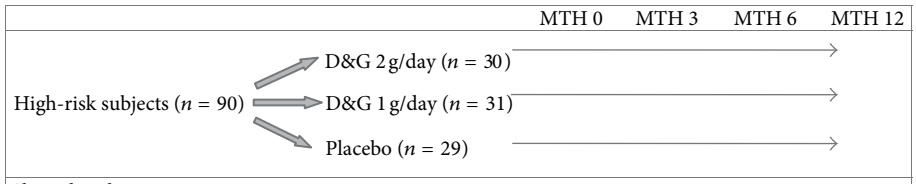
2.1. Subjects. 90 patients with essential hypertension (SBP 160/90 mmHg before treatment) attending the hypertension clinics of the Prince of Wales Hospital, Yan Chai Hospital, and Alice Ho Miu Ling Nethersole Hospital were studied. They were associated with left ventricular hypertrophy on electrographic (ECG) or echocardiographic criteria in 57 (63.3%) patients, diabetes mellitus (fasting blood glucose >7.0 mmol/L or receiving diabetic drugs) in 56 (62.2%), patients and mild-to-moderate renal impairment (serum creatinine 120–250 μmol/L) in 27 (30%) patients. Their blood pressures were currently under good control (BP 110/60 to 140/90 mmHg), and those patients with secondary hypertension, bleeding disorders, significant coexisting hepatic or gastrointestinal diseases, or on long-term anticoagulants were excluded. None has had any coronary, stroke, or other vascular events, and none was taking regular vitamins or other herbal medicines.

2.2. Study Protocol. After written informed consent and successful completion of screening, all subjects were randomized by computer using a stratified block method to receive either oral D&G capsules 1 g/day, D&G capsules 2 g/day, or image-matched placebos in double-blind parallel fashion (Figure 1). Subjects, clinical staff and investigators were masked regarding the assigned treatments. Subjects were reviewed at baseline, 6, and 12 months. On each occasion, all subjects attended after 14 hours fast (except for their usual study medications) for a blood test (routine hematological, fasting glucose, lipid profile, renal, and liver functions), measurement of resting sitting blood pressure, and ultrasonic vascular study. On the assumption of baseline FMD being 7.0 ± 1.5%, enrolment of 90 patients would be adequately powered to detect a 10% relative change in FMD in post-D&G treatment (power = 80%, $\alpha = 0.05$).

The study protocol was approved by the Institutional Ethics Committee on routine hematological human research of The Chinese University of Hong Kong, in compliance with the Declaration of Helsinki (1964). The experiment was conducted with the full understanding and consents of the subjects.

2.3. Vascular Studies. The ultrasound method for measuring brachial flow-mediated dilation (FMD) and nitroglycerin-induced dilation (NTG) was performed as described by Celermajer and Deanfield [13, 14]. In brief, the diameter of the brachial artery was measured (digital caliper manually) by high-resolution B-mode ultrasound (7.5 MHz median frequency linear array L10-5 transducer and standard Advanced Technology Laboratories 5000 system) at rest, in response to reactive hyperemia, and again after sublingual nitroglycerin (200 μg) administered 15 minutes after reactive hyperemia. Reactive hyperemia was induced by inflation of a pneumatic tourniquet placed around the forearm (distal to the segment of the artery being scanned) to a pressure of 220–240 mmHg for 4.5 min, followed by a release. Vessel diameter during systole was measured at a single time point 50–60 seconds post cuff deflation. FMD was expressed as vessel diameter during reactive hyperemia minus vessel diameter at baseline, over vessel diameter at baseline ×100%. Doppler-derived arterial flow (Doppler velocity time integral × vessel diameter × heart rate) was measured at rest, during reactive hyperemia, and 5 minutes after 200 μg sublingual nitroglycerin. Physiologically, increased blood flow stimulates the release of vasodilators from the endothelium, such as nitric oxide, which in turn causes arterial dilation FMD. By contrast, NTG acts directly on the arterial smooth muscle and induces endothelium-independent dilation. The experiments were conducted in quiet environment, and no significant changes in their heart rate and blood pressure were observed. All scans were recorded on super-VHS videotape for subsequent offline analysis, by the same investigator (CP), blinded to subjects' identity and stage of experiment. FMD correlates significantly well with both the coronary endothelial function in the same patient [15, 16] and with extent of coronary atherosclerosis [17]. The accuracy, reproducibility, and low interobserver error for this measurement of arterial physiology have been demonstrated previously [18], which we have achieved in our previous experiments (a mean relative difference of 3% in FMD over time) [19, 20].

All carotid scans were performed by a single operator (CP) after a predetermined and standardized scanning protocol for the right and left carotid arteries as described by Salonen and Bots et al. [21, 22], using images of the far wall of the distal 10 mm of the common carotid arteries. All scans



	MTH 0	MTH 3	MTH 6	MTH 12
Clinical evaluation	+	+	+	+
Routine biochemistry	+	0	+	+
Lipids	+	0	+	+
Vascular study (ultrasound)	+	0	+	+
Drug dispensary	+	+	+	0

Figure 1: Danshen and Gegen Supplementation Protocol.

were recorded on super-VHS videotape for subsequent offline measurement of intima-media thickness (IMT) by a blinded investigator, using a verified automatic edge-detecting and measurement software package as we described previously [23]. The intraobserver variability for mean carotid IMT was 0.03 + 0.01 mm (coefficient of variation 1.0%).

2.4. Statistical Analysis. We used the Statistical Package for Social Sciences (SPSS) 16.0 for Windows in the statistical analysis of the data. Descriptive data were expressed as mean ± SD. Collected data were evaluated using an analysis of covariance (ANCOVA) model, with baseline parameter included as a covariate. The primary efficacy endpoints were brachial FMD and carotid IMT. Differences in the clinical and vascular parameters among the 3 periods were determined using repeated measures analysis of variance (ANOVA) and compared after the Bonferroni adjustment for multiple comparison [24]. Backward stepwise multivariate analysis of variance (MANOVA) was carried out to assess the major determinants of the pooled brachial FMD and carotid IMT data at all time frames, including age, systolic and diastolic blood pressure, heart rate, hemoglobin A1-C and LDL cholesterol and creatinine levels. Statistical significance was inferred at a two-tailed P value of <0.05.

3. Results

Their mean age was 55 + 8 years, and 67 patients (74.4%) were male. The 3 groups were fairly identical in their baseline demographic, clinical, and vascular parameters (Table 1), except there was a slightly higher diastolic blood pressure in combined D&G group ($P = 0.017$). After 12 months and compared with baseline, there were no significant changes in their blood pressures, heart rate, blood lipids, hemoglobin A1-C, and creatinine levels in all 3 groups (Table 2).

Improvement in brachial FMD but not NTG was witnessed in both D&G ($P < 0.0001$) and placebo groups ($P = 0.001$) (Tables 2 and 3) but was more impressive after D&G treatment at 6 months (increased by 23.2%), and at 12 months (increased by 30.2%) compared with placebo treatment at 6 months (increased by 10.0%, $P = 0.031$) and 12 months, respectively (increased by 15.5%, $P = 0.028$) (Table 4). This improvement in FMD was greater after higher dosage (2 gm) D&G at 6 months (increased by 24.7%) and 12 months (increased by 32.2%) compared with lower (1 gm) D&G treatment (increased by 21.1%, $P = 0.394$ and 27.8%, $P = 0.507$, resp.) and placebo treatment (increased by 10% at 6 months $P = 0.063$ and by 15.5% at 12 months, $P = 0.05$) (Table 4).

Carotid IMT improved significantly after both D&G (1 gm) (0.79 + 0.20 to 0.74 + 0.16 mm, decrease of 3.4%, $P = 0.001$) and D&G (2 gm) treatment (0.85 + 0.16 to 0.82 + 0.15 mm, decrease of 4.1%, $P < 0.0001$) but not after placebo (0.80 + 0.18 mm to 0.81 + 0.17 mm, increase of 1.3%, $P = 0.510$) (Table 3). On multivariate backward stepwise regression, improvement in carotid IMT was related to D&G treatment ($\beta = 0.379$, $P = 0.006$) and baseline LDL cholesterol ($\beta = 0.276$, P value =0.04), after adjustment for age, gender, blood pressure, hemoglobin A1-C, and creatinine level ($R^2 = 0.161$, F value =5.815, $P < 0.005$).

D&G herbal drugs were well tolerated in 3 groups, with no significant adverse events, nor any significant changes in their liver enzymes and hematological profiles (Table 5).

4. Discussion

Randomized trials on adjunctive traditional Chinese medicine versus placebo on cardiovascular disease are scarce. The present study is among the first ones comparing head-to-head with placebo on top of standard antihypertensive drugs. Our results of D&G adjunctive therapy on surrogate vascular endpoints in high-risk but asymptomatic hypertensive patients are very encouraging, confirming higher dose of D&G treatment is well tolerated and perhaps better improves brachial FMD, as well as regression of carotid IMT compared with placebo. Our present study however has not been adequately powered for the differential effects on FMD and IMT between the 2 D&G dosages. Both brachial FMD and carotid IMT are surrogate atherosclerotic markers predictive of stroke and cardiovascular outcome [17, 25–27]. A 0.1 mm difference in carotid IMT is associated with 1.15 (1.12 to 1.87) relative risk of myocardial infarction and 1.18 (1.16 to 1.21) relative risk of stroke [26]. These findings concur

Table 1: Baseline characteristics of 90 hypertensive subjects.

Item	Placebo[1] ($n = 29$)	D&G (1 gm) ($n = 31$)	D&G (2 gm) ($n = 30$)	D&G (1 gm + 2 gm)[2] ($n = 61$)	P value	P value (1 versus 2)
Age	55.6 ± 8.2	51.7 ± 7.7	56.9 ± 7.4	54.3 ± 8.0	0.031	0.484
Gender						
Male	20	27	20	47	0.134	0.445
Female	9	4	10	14		
DM	18	11	17	28	0.091	0.152
LVH	12	13	10	23	0.749	0.738
CRF	10	12	12	24	0.901	0.657
Body weight (kg)	70.8 ± 12.9	70.0 ± 11.0	69.5 ± 14.7	69.7 ± 12.8	0.920	0.703
BMI (kg/m^2)	26.8 ± 4.8	25.8 ± 3.0	25.9 ± 4.9	25.9 ± 4.0	0.641	0.346
SBP (mmHg)	132.6 ± 18.5	128.8 ± 11.1	133.6 ± 15.9	131.2 ± 13.8	0.449	0.694
DBP (mmHg)	77.4 ± 11.2	82.4 ± 9.3	83.5 ± 9.8	82.9 ± 9.5	0.053	0.017
TC (mmol/L)	4.81 ± 0.92	5.36 ± 1.06	5.16 ± 0.94	5.26 ± 1.00	0.113	0.051
TG (mmol/L)	1.99 ± 1.37	2.53 ± 2.41	1.69 ± 0.75	1.94 ± 1.34	0.159	0.886
HBA1C (%)	7.46 ± 2.62	6.81 ± 2.04	6.41 ± 0.99	6.60 ± 1.57	0.251	0.180
Creatinine (umol/L)	110.0 ± 40.6	127.9 ± 57.9	110.1 ± 49.3	119.1 ± 54.2	0.278	0.421
FMD (%)	5.46 ± 1.27	5.38 ± 1.89	4.97 ± 1.52	5.17 ± 1.71	0.490	0.465
IMT (mm)	0.80 ± 0.18	0.79 ± 0.20	0.85 ± 0.16	0.82 ± 0.18	0.372	0.607

BMI: body mass index, CRF: chronic renal failure, DM: diabetis mellitus, DBP: diastolic blood pressure, FMD: flow-mediated dilation, HBA1-C: hemoglobin A1-C, IMT: intima-media thickness, LVH: left ventricular hypertrophy, SBP: systolic blood pressure, TC: total cholesterol, TG: triglyceride, 1: placebo, and 2: D&G (1 gm + 2 gm).

Table 2: Changes in clinical and vascular parameters after 12 months.

	Placebo ($n = 29$)		D&G (1 gm/day) ($n = 31$)		D&G (2 gm/day) ($n = 30$)	
	Baseline	12 months	Baseline	12 months	Baseline	12 months
SBP (mmHg)	124.4 ± 17.8	136.0 ± 24.0	132.3 ± 13.5	127.3 ± 11.0	134.9 ± 18.8	140.3 ± 14.6
DBP (mmHg)	78.7 ± 13.7	81.90 ± 12.50	88.0 ± 7.6	84.1 ± 8.3	82.9 ± 11.0	84.4 ± 7.1
TC (mmol/L)	4.8 ± 0.9	4.7 ± 1.0	5.4 ± 1.1	5.1 ± 1.0	5.2 ± 0.9	5.0 ± 0.9
TG (mmol/L)	2.0 ± 1.4	2.0 ± 1.5	2.2 ± 1.7	1.8 ± 1.3	1.7 ± 0.8	1.6 ± 0.9
HBA1C (%)	7.5 ± 2.6	7.3 ± 2.1	6.8 ± 2.0	7.0 ± 2.4	6.4 ± 1.0	6.6 ± 0.9
Creatinine (mol/L)	110.0 ± 40.6	109.4 ± 50.3	127.9 ± 58.0	130.0 ± 64.0	110.1 ± 49.3	110.4 ± 83.2
Reactive hyperemia (%)	543 ± 192	485 ± 105	615 ± 208	606 ± 154	535 ± 206	505 ± 133
FMD (%)	5.5 ± 1.3	6.2 ± 1.1*	5.4 ± 1.9	7.0 ± 2.1**	5.0 ± 1.5	6.2 ± 1.6**
NTG (%)	15.2 ± 2.5	15.9 ± 3.0	18.3 ± 11.9	16.7 ± 3.3	15.4 ± 3.3	16.1 ± 3.1
IMT (mm)	0.80 ± 0.18	0.81 ± 0.17	0.79 ± 0.20	0.74 ± 0.16**	0.85 ± 0.16	0.82 ± 0.15**

Compared with baseline within group: $^*P = 0.001$; $^{**}P < 0.0001$. SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: total cholesterol, TG: triglyceride, HBA1-C: hemoglobin A1-C, FMD: flow-mediated dilation, NTG: nitroglycerin-induced dilation, and IMT: intima-media thickness, Reactive hyperemia = (velocity − time integral$_2$ × heart rate$_2$)/(velocity − time integral$_1$ × heart rate$_1$) × 100.

with the vascular protecting effect of D&G in patients with coronary artery disease previously reported [8].

Endothelial dysfunction (impaired FMD), oxidation of circulating LDL-cholesterol and the inward migration of oxidized LDL-cholesterol-lasden monocytes, other inflammatory infiltration, in the blood vessel wall, and subsequently intima-media thickening are the critical processes in the development of atherosclerosis [28]. Several mechanisms may explain the improvement in vascular function and structure after D&G therapy, including their lipid-lowering, antioxidant and nitric oxide production or facilitating effects, as well as their phytoestrogenic properties [8, 29–33]. The potent antioxidation property of *Pueraria lobata* isoflavones has been proposed from previous *in vitro* studies, lending support to the present findings [9, 34]. An *in vitro* endothelial and monocyte cell line experiment has revealed that D&G combination inhibits dose-dependently macrophage/foam cell transformation from fat-fed monocytes [35]. This cell-modulating mechanism underlines the possible scientific basis of our favourable vascular protective effect in primary atherosclerosis prevention in hypertensive subjects. Recently endothelial progenitor (stem) cell (EPC) activity has emerged as an innovative basic scientific concept and key process in the wear and healing of arterial endothelial cells and henceforth

Table 3: Changes in vascular parameters at 6 and 12 months.

Vascular parameters	Baseline	6 months	12 months	P value[1]	P value[2]
FMD (%)					
Placebo	5.5 ± 1.3	5.9 ± 1.2	6.2 ± 1.1	0.001	0.0001
D&G (1 gm)	5.4 ± 1.9	6.3 ± 2.1	7.0 ± 2.1	<0.0001	<0.0001
D&G (2 gm)	5.0 ± 1.5	5.9 ± 1.5	6.2 ± 1.6	<0.0001	<0.0001
P value[3]	0.850	0.411	0.088		
P value[4]	0.275	0.917	0.933		
P value[5]	0.465	0.693	0.347		
IMT (mm)					
Placebo	0.80 ± 0.18	0.80 ± 0.17	0.81 ± 0.17	0.385	0.510
D&G (1 gm)	0.79 ± 0.20	0.77 ± 0.18	0.74 ± 0.16	0.001	0.001
D&G (2 gm)	0.85 ± 0.16	0.83 ± 0.15	0.82 ± 0.15	0.006	<0.0001
D&G (2 gm + 1 gm)	0.82 ± 0.18	0.80 ± 0.17	0.78 ± 0.16	<0.0001	<0.0001
P value[3]	0.850	0.457	0.130		
P value[4]	0.272	0.637	0.796		
P value[5]	0.607	0.037	0.001		
NTG (%)					
Placebo	15.2 ± 2.5	15.6 ± 2.4	15.9 ± 3.1	0.276	0.510
D&G (1 gm)	16.0 ± 2.8	16.7 ± 3.0	16.7 ± 3.3	0.376	0.914
D&G (2 gm)	15.4 ± 3.3	15.9 ± 3.2	16.1 ± 3.1	0.198	0.301
P value[3]	0.154	0.203	0.437		
P value[4]	0.901	0.684	0.882		

1: 6 months versus baseline (paired t-test); 2: 12 months (paired t-test) versus baseline; 3: D&G (1 gm) versus placebo (ANOVA); 4: D&G (2 gm) versus placebo (ANOVA); 5: combined D + G (1 gm +2 gm) versus placebo (ANOVA).
D&G: Danshen + Gegen.
FMD: flow-mediated dilation.
IMT: intima-media thickness.
NTG: nitroglycerin-induced dilation.

Table 4: Percent change of FMD from baseline.

Group	Baseline (mm)	6 months (%)	12 months (%)	P value[1]	P value[2]
D&G (1 gm)	5.38 ± 1.89	21.6	27.8	0.001	0.001
D&G (2 gm)	4.97 ± 1.52	24.7	32.2	0.001	0.001
D&G (2 gm + 1 gm)	5.17 ± 1.71	23.2	30.2	0.001	0.001
Placebo	5.46 ± 1.27	10.0	15.5	0.001	0.001
P value[3]	0.867	0.147	0.171		
P value[4]	0.275	0.063	0.05		
P value[5]	0.465	0.031	0.028		

P value[1]: comparison of 6 months versus baseline.
P value[2]: comparison of 12 months versus baseline.
P value[3]: comparison between D&G (1 gm) with placebo (Kruskal-Wallis test).
P value[4]: comparison between D&G (2 gm) with placebo (Kruskal-Wallis test).
P value[5]: comparison between D&G (2 gm + 1 gm) with placebo (Mann-Whitney test).

in vascular protection [36, 37]. However, our preliminary substudy, to evaluate the impact of D&G treatment adjunctive to traditional antihypertensive therapies, failed to support such novel mechanism of D&G treatment in vascular protection [38].

Earlier studies of Danshen (*Salvia miltiorrhiza*) have focused on the alcohol soluble constituents, but more recent studies targeted on hydrophilic compounds [31, 32]. Over 50 components have been isolated and identified from the extracts of danshen, including diterpene compounds, Danshensu, tanshinone 1, tanshinone IIA, sodium-tanshinone. IIA sulfonate salvianolic acid, and other phenolic acids, baicalin, β-sitosterol ursolic acid, daucosterol, and dimethoxy flavanone [31]. Extraction of *Pueraria lobata* (gegen) compounds, commonly called Yega, yields over 15 compounds, including puerarin, daidzein, daidzin, and

Table 5: Changes in hematological and biochemical parameters.

Item	Placebo		D&G (1 gm)		D&G (2 gm)	
	Baseline	12 months	Baseline	12 months	Baseline	12 months
WBC (10^9/L)	7.34 ± 2.00	7.60 ± 1.70	7.0 ± 2.20	5.90 ± 7.0*	7.10 ± 1.80	6.70 ± 1.90
Haemoglobin (%)	14.30 ± 1.20	14.0 ± 1.40	14.50 ± 1.60	14.40 ± 1.40	14.30 ± 1.30	14.20 ± 1.60
Platelet (10^9/L)	252 ± 54	272 ± 49	277 ± 62	271 ± 62	257 ± 65	251 ± 63
Glucose (mmol/L)	6.80 ± 2.40	6.90 ± 2.90	7.20 ± 3.10	7.20 ± 3.30	6.60 ± 1.30	6.50 ± 1.30
ALP (u/L)	74.80 ± 21.3	67.90 ± 13.80	74.60 ± 24.0	67.90 ± 18.50	75.0 ± 15.0	73.80 ± 17.80
ALT (u/L)	28.30 ± 15.20	31.30 ± 18.20	27.80 ± 14.40	24.50 ± 18.50	30.50 ± 15.80	29.90 ± 15.60

*Compared with placebo $P = 0.017$.
ALP: alkaline phosphatase.
ALT: alanine aminotransferase.
WBC: white blood cells.

other phytoestrogenic compound [33, 34]. Many of these compounds have shown antiatherogenic and favorable hemodynamic effects, either alone or in combination in tissue models experiments [9, 10, 27, 31–34].

Ischemia-reperfusion tissue model experiment has confirmed the efficacy of "Danshen and Gegen" combination in the ratio of 7 : 3 of the raw herbs [11, 12]. On this basis, our group has successfully produced a quality preparation of this combination (in 500 mg capsule), through several tedious processes, including the use of DNA finger printing and chemical assays for quality control and authentication, bacterial, heavy metal, and chemical toxicology monitoring, processing and aqueous extraction of raw herbs according to the guidelines of Good Manufacturing Practice (GMP) [8].

For over 50 years, the use of Danshen products has been associated with extreme safety, with no major adverse effects reported [31, 32]. The present study reiterates the high tolerability profile of combination D&G, even on top of standard drugs in coronary patients and as adjunct to standard antihypertensive therapies in high-risk hypertensive. There have been reports of interactions with warfarin, salicylate, diazepam, and ginseng [31, 32, 39–44]. Caution should be executed in clinical practice until these issues could be further clarified with wider utilization. Therapeutic (*in vitro* and *in vivo*) efficacy of individual Danshen component has been reported over the past 5 decades [45–48]. For long time, empirical clinical observations with herbal medicine suspect that these therapeutic effects are better with multiple drugs combination versus single drug. The unique and strong point of the present study is the application of a Danshen-Gegen combination formula rather than single herbal drug component, which was found to be safe and effective in improving early stage of atherogenesis.

The present study documents an improvement in brachial FMD and carotid IMT after a moderate dosage (1-2 g/day) of D&G therapy, independent of blood pressure or lipid lowering. It is quite possible that lower doses of D&G therapy and over longer period might result in similar or better benefit. Further study will be required to address specifically the dose response and different combination formulae issues, as well as drug interaction with standard antihypertensive drugs, which conceivably will require another prospectively planned study and much bigger numbers of subjects. The possible improvement in carotid IMT (4%) and FMD (30%), as surrogate atherosclerosis markers observed over 12 months intervention in this pilot project, although statistically significant, is quite subtle and may be of borderline biological significance. Nevertheless, together with our previous work in coronary patients, the present study with encouraging vascular protecting findings and safety profiles will provide the much needed evidence base for the application of adjunctive complementary medicine for both primary and secondary atherosclerosis prevention. Longer intervention studies focusing on hard clinical endpoints, including stroke, heart attacks, and total mortality, are awaited with enthusiasm and great interest.

5. Conclusion

Asymptomatic essential hypertensive subjects with LVH, DM, or renal impairment have a greater atherosclerosis burden than subjects with hypertension only. Danshen and Gegen adjunctive treatment has been well tolerated and significantly improved atherogenic process in these high-risk hypertensive patients, with potential in primary prevention of atherosclerosis.

Conflict of Interests

No competing financial interests exist.

Acknowledgment

The work reported in this paper was partially supported by an Area of Excellence Grant from the University Grants Committee of the Hong Kong Special Administration Region, China (Project No. AoE/B-10/01).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 985746, 7 pages
http://dx.doi.org/10.1155/2013/985746

Research Article

Correlation between Platelet Gelsolin and Platelet Activation Level in Acute Myocardial Infarction Rats and Intervention Effect of Effective Components of Chuanxiong Rhizome and Red Peony Root

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Received 21 August 2012; Revised 2 December 2012; Accepted 7 February 2013

Academic Editor: Peng Nam Yeoh

The biological role of platelet gelsolin in platelet activation of acute myocardial infarction is not defined. In order to provide a potential new antiplatelet target for Chinese medicine and to elucidate the contribution of Xiongshao capsule, the effective components of Chuanxiong rhizome and red peony root, in this study, we randomly allocated Sprague Dawley rats to left anterior descending coronary artery ligation or sham surgery and different drug prophylaxis as control. We found that gelsolin is highly expressed in platelet rich plasma and lowly expressed in platelet poor plasma, accompanied by the high platelet activation level in model rats; plasma actin filaments and mean fluorescence intensity (MFI) of platelet calcium ion increased and plasma vitamin D binding protein decreased in model rats. Xiongshao capsule could inhibit the gelsolin expression in platelet rich plasma and ischemic heart tissue simultaneously and reduce the level of plasma F-actin and MFI of platelet calcium ion. Our study concludes that platelet gelsolin is an important contributor to platelet activation, and platelet gelsolin inhibition may form a novel target for antiplatelet therapy. Xiongshao capsule may be a promising Chinese medicine drug for antiplatelet and aspirin-like cardioprotection effect.

1. Introduction

Despite recent medical advances, cardiovascular diseases remain the predominant cause of morbidity and mortality all over the world [1, 2]. Rupture of atherosclerotic plaque and the ensuing thrombotic changes are the triggers for acute coronary event. Platelet activation and aggregation play crucial roles in this process of atherothrombosis. The emergence of antiplatelet drug is the milestone of prevention and therapy of cardiovascular disease and provides the primary therapeutic strategy to combat cardiovascular diseases. The proper application of antiplatelet drug in reducing the mortality and morbidity of acute myocardial infarction successfully has been verified by a large number of large-scale clinical trials [3]. Antiplatelet drug, such as aspirin, now is recommended for the secondary prevention of cardiovascular disease (CVD) in patients with CVD because it decreases the risk of CVD events and mortality in clinical trials of men and women with CVD [4]. But many clinical problems arose along with the wide range of application of antiplatelet drugs (such as aspirin and clopidogrel, etc.) during the past 10 years [5, 6]. Despite their proven benefits, recurrent cardiovascular events still occur in those taking antiplatelet drugs. This has led to the concept of "antiplatelet drug resistance," most commonly aspirin or clopidogrel resistance. The latest research shows that aspirin prophylaxis in people without prior CVD does not lead to reductions in cardiovascular death, for the benefits are further offset by clinically important bleeding events [7], which limit the clinical practice of antiplatelet drugs widely. These phenomena suggest that other pathways capable of stimulating platelet activation may exist and provide an impetus for developing new antiplatelet drugs which possess higher efficacy and fewer adverse effects.

Proteomics technology has been successfully applied to platelet research during the past 5 years, contributing to the emerging field of platelet proteomics which led to

the identification of a considerable amount of novel platelet proteins, many of which have been further studied at their functional level [8]. Our previous work [9] indicated that platelet gelsolin is the main platelet differential functional protein between patients of coronary heart disease and healthy people by platelet proteomics. Studies have also shown that platelet gelsolin is highly expressed in patients with acute coronary syndrome (ACS) and the blood-stasis syndrome (BSS) of traditional Chinese medicine (TCM) [10, 11]. Gelsolin is known to have one of the key roles in extracellular actin-scavenger system (EASS) [12], but the biological role of platelet gelsolin in platelet activation of acute myocardial infarction (AMI) is unclear. On the prevention of atherosclerosis or vulnerable plaque, Chinese medicine and Western medicine agree on stabling plaque and promoting blood circulation. Based on the agreed thoughts of the Eastern and Western worlds, the application of Chinese herbs for activating blood circulation (ABC herbs) has valuable significance in the exploration of reducing the risk of cardiovascular event [13, 14]. Chuanxiong rhizome and Red peony root are the two classical ABC herbs in China and have been used for thousands of years in the prevention and treatment of CVD. Xiongshao capsule (XSC) is a patent drug in China and is composed of effective components of Chuanxiong rhizome and Red peony root. Our previous studies have showed that paeoniflorin, ferulic acid and total phenolic acid are the major active principles of the water extract from Xiongshao capsule [15, 16]. Clinical studies indicated that XSC can effectively prevent restenosis after percutaneous coronary intervention (PCI) [17], but the antiplatelet target of XSC is not defined.

In the present study, we used AMI as a disease model to investigate the correlation between platelet gelsolin and platelet activation level in rat model of AMI and the prophylaxis mechanism of XSC *in vivo*.

2. Materials and Methods

2.1. Drug and Reagents. Xiongshao Capsule (XSC), which contained paeoniflorin (more than or equal to 28 mg each capsule), ferulate (more than or equal to 3.5 mg each capsule), and total phenolic acid (more than or equal to 34 mg each capsule), 0.25 g per capsule, were provided by Beijing International Institute of Biological Products (batch no. 200091, Beijing, China); aspirin, 0.1 g per capsule, was purchased from Bayer HealthCare Manufacturing (batch no. BJ01653, Beijing, China); verapamil, 0.04 g per tablet, was purchased from the Central Pharmaceutical Co., Ltd (batch no. 100402, Tianjing, China). All the drugs were dissolved in pure water before use.

Fluo-3AM was purchased from Sigma (St Louis, MO, USA); rabbit anti-gelsolin polyclonal antibody was purchased from Abcam (San Francisco, USA); mouse anti-β-actin monoclonal antibody was purchased from Sigma (St Louis, MO, USA); FITC-Phalloidin was purchased from Sigma (St Louis, MO, USA); enzyme-linked immunosorbent assay (ELISA) kit of P-selectin, gelsolin, F-actin, vitamin D binding protein (VDBP), CK-MB, cTnI, TXB_2, and COX-1 were purchased from Huamei Biological Technology Company (Wuhan, Hubei province, China).

2.2. Animal Grouping and Treatment. Sprague Dawley (SD) rats (male, weight 220–250 g, $n = 90$) were obtained from Beijing University Laboratory Animal Center (the animal certificate No: SCXK (Jing) 2006–0009). The rats were housed in humidity-controlled (55 ± 5)% rooms at (22 ± 2)°C with a 12 h on/12 h off light cycle. The animals were maintained with free access to standard diet and tap water.

After one week of adaptive feeding, we randomly allocated the SD rats into six groups of 15 rats each as follows: Model group, Sham group, Aspirin group, Xscd group (the high dose group), Xscx group (the low doses group), and Verapamil group. Aspirin 40 mg/kg/day, verapamil 4 $mg{\cdot}kg^{-1}\,d^{-1}$, and XSC 390 mg/kg/day, 195 mg/kg/day per gavage for 3 consecutive weeks were administrated to the aspirin, verapamil, Xscd and Xscx groups respectively. Rats in the Sham and Model groups received the same volume of distilled water, per gavage for 3 weeks. After 3 weeks, myocardial infarction (MI) model was created in rats by ligating the left anterior descending coronary artery (LAD) as described before [18]. The Animal Care and Use Committee of Xiyuan hospital approved the experimental protocol.

2.3. Sample Preparation. After 3 hours of ligation, all the rats were killed after anesthesia by intraperitoneal injection of 20% urethane (0.5 mL/100 g). Fresh blood (10 mL) was drawn from the abdominal aorta and collected into vacutainer tubes containing acid citrate dextrose (ACD) 9% v/v (trisodium citrate 22.0 g/L, citric acid 8.0 g/L, dextrose 24.5 g/L) as anticoagulant. The initial 2 mL of blood was discarded to avoid spontaneous platelet activation. The blood was centrifuged for 10 min at 150 ×g at room temperature to obtain platelet-rich plasma (PRP) and the remaining blood centrifuged for 20 min at 800 ×g to obtain platelet poor plasma (PPP).

Ischemic heart tissue was taken after blood collection and preserved at −80°C for detection of gelsolin expression by western blotting.

2.4. Enzyme-Linked Immunosorbent Assay Analysis. The concentration of PRP and PPP of gelsolin, plasma F-actin, VDBP, CK-MB, cTnI, TXB_2, COX-1 were determined by enzyme-linked immunoadsorbent assay (ELISA), as per the manufacturer's instructions. The absorbance was measured at 450 nm in an ELISA reader.

2.5. Western Blotting Analysis. The level of gelsolin in ischemic heart tissues was determined by Western blot analysis according to the standard procedure as described previously [19]. β-actin was used as a loading control.

2.6. Detection of MFI of Platelet Calcium Ion. Platelet-rich plasma was prepared and incubated with 4 μmol/L Fluo-3-AM (Sigma, Saint Louis, MO, USA) at 37°C for 40 min. The calcium concentration of platelets was determined using flow cytometry to measure the mean fluorescence intensity (MFI), as previously described [20].

2.7. Statistical Analysis. Data are presented as mean ± SD. The SPSS Statistics 11.0 package was utilized to analyze the data.

Table 1: The outcome among the different groups after ligation of LAD.

Group	N	Dead rats (n)	Surviving rats (n)
Sham	15	6	9
Model	15	6	9
Aspirin	15	5	10
Xscd	15	6	9
Xscx	15	7	8
Verapamil	15	7	8

Differences among groups were analyzed using the one-way analysis of variance (ANOVA), followed by multiple comparisons by Least-Significant Difference (LSD) test. Spearman's correlation coefficients were calculated to study the relations between gelsolin concentration in PRP and plasma P-selectin level. Differences between groups were at $P < 0.05$.

3. Results

3.1. General Condition. All the rats in the different groups survived and exhibited normal physical appearance and behavior during the gavage period of different drugs. The outcome among the different groups after ligation of LAD is presented in Table 1.

3.2. XSC Reduces the Concentration of Myocardial Injury Markers. We chose CK-MB and cTnI as the myocardial injury markers in rats with acute myocardial infarction (AMI). Compared with the Sham group, the concentration of CK-MB and cTnI of Model group increased significantly after ligation of LAD for 3 hours ($P < 0.01$). The high dose of XSC (390 mg/kg/day) can reduce the concentration of CK-MB and cTnI markedly ($P < 0.05$); this has similar effect with aspirin *in vivo* (see Table 2).

3.3. XSC Inhibits the Platelet Activation Level. We choose the plasma P-selectin as the marker of platelet activation level. Compared with Sham group, the plasma P-selectin concentration of the Model group increased significantly after ligation of LAD for 3 hours ($P < 0.01$). The high dose of XSC can inhibit P-selection level markedly ($P < 0.05$), this has similar effect with the Aspirin group (see Figure 1).

3.4. XSC Reduces the Platelet Gelsolin Level and Enhances the Activity of Extracellular Actin-Scavenger System (EASS). Plasma gelsolin and VDBP are the main components of the EASS which undertake the responsibility as scavenger of the abnormal increased extracellular filament actin (F-actin). Compared with the Sham group, the plasma gelsolin and VDBP of the Model group was reduced significantly ($P < 0.05$) and F-actin increased markedly ($P < 0.01$), while platelet gelsolin it increased markedly ($P < 0.01$). High dose of XSC can reduce platelet gelsolin and F-actin level ($P < 0.05$), while it increased plasma gelsolin and VDBP significantly ($P < 0.05$) (see Figures 2, 3, and 4).

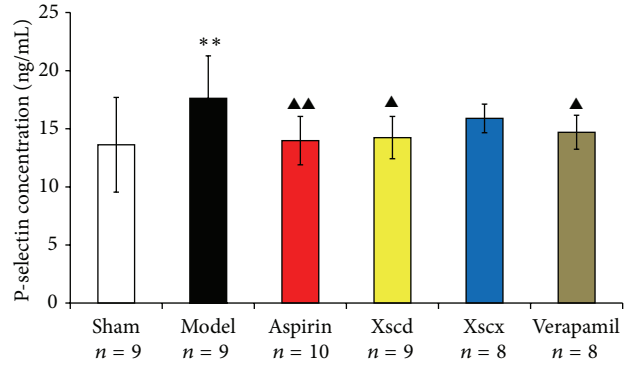

Figure 1: Effect of Xiongshao Capsule (XSC) on P-selectin concentration of AMI rats. $^{**}P < 0.01$ compared to Sham group, and $^{\blacktriangle}P < 0.05$ or $^{\blacktriangle\blacktriangle}P < 0.01$ compared to Model group.

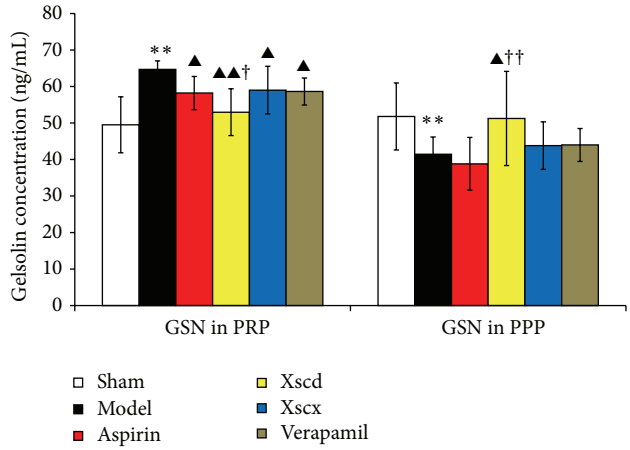

Figure 2: Effect of Xiongshao Capsule (XSC) on Gelsolin concentration among PRP and PPP of AMI rats. $^{**}P < 0.01$ compared to Sham group, $^{\blacktriangle}P < 0.05$ or $^{\blacktriangle\blacktriangle}P < 0.01$ compared to Model group, and $^{\dagger}P < 0.05$ or $^{\dagger\dagger}P < 0.01$ compared to Aspirin group.

3.5. XSC Inhibits the Activation of TXB_2 and COX-1. Compared with Sham group, the concentration of TXB_2 and COX-1 of Model group increased significantly after ligation of LAD for 3 hours ($P < 0.01$). High dose of XSC can reduce COX-1 and TXB_2 level significantly ($P < 0.05$); this has similar effect with the Aspirin group (see Figure 5).

3.6. XSC Inhibits the MFI of Calcium. Compared with Sham group, the MFI of calcium of the Model group increased markedly ($P < 0.01$), High dose of XSC can inhibit platelet calcium increase ($P < 0.05$). This has similar effect to the Verapamil group ($P < 0.05$) (see Figure 6).

3.7. XSC Attenuates the Expression of Gelsolin in Infarcted Myocardium. Compared with Sham group, the gelsolin expression of infarcted myocardium of Model group increased markedly, and XSC can inhibit gelsolin expression of infarcted myocardium, but verapamil has no such effect (see Figure 7).

Table 2: Effect of Xiongshao Capsule (XSC) on the concentration of myocardial injury markers of AMI rats.

Group	N	CK-MB (ng/mL)	cTnI (pg/mL)
Sham	9	0.279 ± 0.074	9.81 ± 2.62
Model	9	0.386 ± 0.043**	15.18 ± 4.3**
Aspirin	10	0.340 ± 0.024†	12.04 ± 1.19†
Xscd	9	0.336 ± 0.027†	12.23 ± 1.41†
Xscx	8	0.351 ± 0.013	13.85 ± 3.02
Verapamil	8	0.358 ± 0.017	14.43 ± 2.98

$^{**}P < 0.01$ compared to Sham group and $^{\dagger}P < 0.05$ compared to Model group.

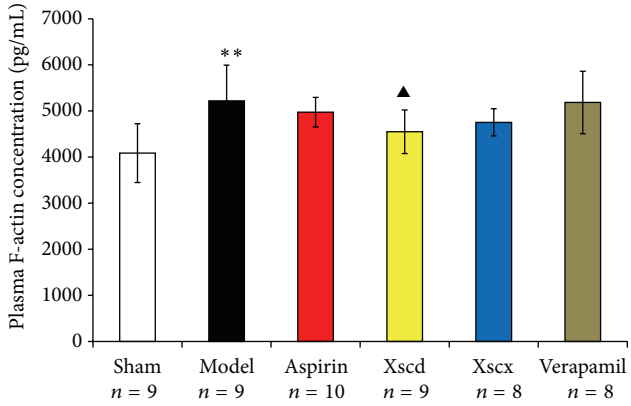

Figure 3: Effect of Xiongshao Capsule (XSC) on Plasma F-actin concentration of AMI rats. $^{**}P < 0.01$ compared to Sham group, and $^{\blacktriangle}P < 0.05$ compared to Model group.

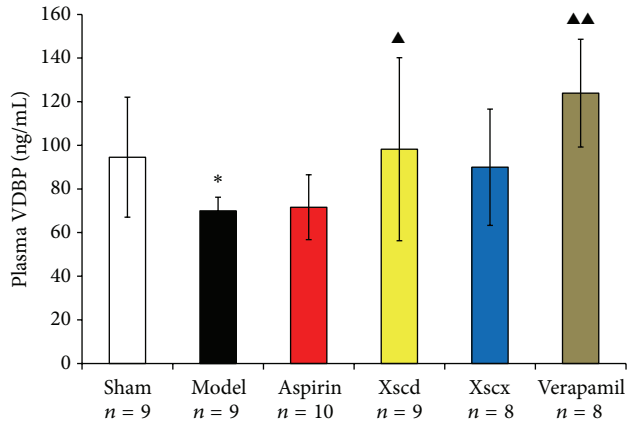

Figure 4: Effect of Xiongshao Capsule (XSC) on Plasma VDBP concentration of AMI rats. $^{*}P < 0.05$ compared to Sham group, and $^{\blacktriangle}P < 0.05$ or $^{\blacktriangle\blacktriangle}P < 0.01$ compared to Model group.

3.8. Analyses of Correlation between Platelet Gelsolin Concentration and Plasma P-Selectin Level. Next we investigated any potential correlation between the platelet gelsolin concentration and plasma P-selectin levels that may exist in the Model group and Xscd group. Correlation analysis showed that platelet gelsolin concentrations were high positively correlated with plasma P-selectin levels in the Model group (see Figure 8(a)) and Xscd group (see Figure 8(b)).

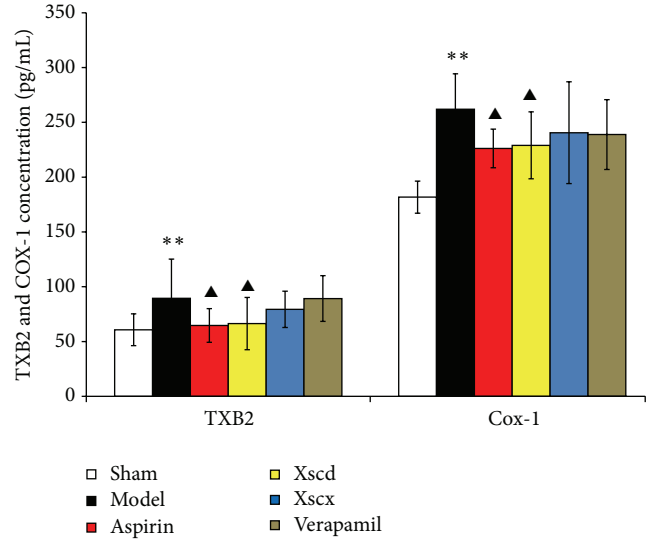

Figure 5: Effect of Xiongshao Capsule (XSC) on Plasma TXB_2 and COX-1 concentration of AMI rats. $^{**}P < 0.01$ compared to Sham group, and $^{\blacktriangle}P < 0.05$ compared to Model group.

4. Discussion

Gelsolin is a calcium-regulated actin filament (F-actin) severing and capping protein, which is expressed as both cytoplasmic and plasma isoforms. The functions of extracellular gelsolin are less well defined. Gelsolin is also an important cytoskeletal protein, which is a key actin binding protein (ABPs) as well. Increasingly evidence has shown that gelsolin has close relationship with many diseases and pathological processes, such as cancer, apoptosis, infection and inflammation, pulmonary diseases, and aging [21]. During the past 5 years, many scholars began to focus on gelsolin's possible role in the development of cardiovascular diseases [22]. Activated platelets play a pivotal role in the formation of arterial thrombi, and antiplatelet drugs become the core in the prevention and treatment of CVD. Platelet activation not only causes the changes of membrane protein, but also a series of morphological changes, from inviscid, discotic circulating platelets to a paste-like, protruding platelet jelly, that affects the regulation of platelet cytoskeletal proteins.

Using differential proteomics of platelet, our previous study [9] indicated that platelet gelsolin was the main platelet differential functional proteins between patients with

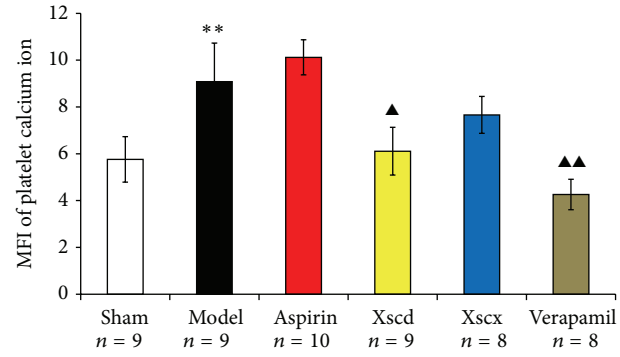

FIGURE 6: Effect of Xiongshao Capsule (XSC) on MFI of platelet calcium ion concentration of AMI rats. $^{**}P < 0.01$ compared to Sham group, and $^{\blacktriangle}P < 0.05$ or $^{\blacktriangle\blacktriangle}P < 0.01$ compared to Model group.

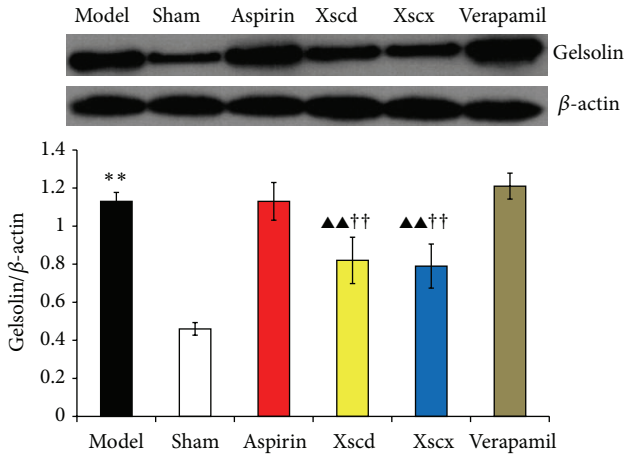

FIGURE 7: Effect of Xiongshao Capsule (XSC) on protein level of gelsolin in ischemic heart tissue of AMI rats. $^{**}P < 0.01$ compared to Sham group, and $^{\blacktriangle\blacktriangle}P < 0.01$ compared to Model group, $^{\dagger\dagger}P < 0.01$ compared to Aspirin group.

coronary heart disease and healthy people. In addition, data from our previous clinical studies demonstrated that [10, 11] platelet gelsolin was highly expressed in patients with acute coronary syndrome (ACS) and the blood stasis syndrome (BSS) of traditional Chinese medicine. Meanwhile, based on the Chinese medicine principle of "prescription to syndrome." Platelet gelsolin may be viewed as a new target for ABC herbs. In this study, we evaluated the biological role of platelet gelsolin in the development of platelet activation in a rat model of AMI and the potential contribution of XSC prophylaxis in this progress *in vivo*.

As we know, P-selectin is a 140 kD glycoprotein that is presented in the granules of platelets and translocates rapidly to the cell surface after platelet activation; it is generally considered as the gold marker of platelet activation [23]. In this study, after ligation of LAD for 3 hours, the concentration of CK-MB and cTnI and the P-selectin level of the Model rats increased significantly compared with Sham rats, which indicated that the model rats had myocardial injury and platelet activation.

The actin cytoskeleton plays a central role in many fundamental cellular processes involving the generation of force and facilitation of movement, which are enabled by the assembly of actin monomers into filaments and cooperation with a wide variety of ABPs [21], including gelsolin. Actin monomers (G-actin) spontaneously associates to form F-actin under physiological conditions and vice versa. This dynamic progress keeps in balance all the time. In the presence of tissue injury or cell death, G-actin is released into the circulation where it can interact with components of the haemostatic and fibrinolytic systems, or polymerize and form F-actin excessively. Studies [24] have suggested that F-actin can lead to platelet aggregation directly *in vitro*, and the presence of excessive F-actin in blood vessels, which can plug smaller vessels and decrease blood flow to promote the formation of blood clots, can be fatal. Infusion of high doses of G-actin in rabbits caused the rapid and fatal formation of massive F-actin-containing thrombi in arterioles and capillaries of pulmonary veins [25]. An extracellular actin-scavenger system (EASS) [12] was therefore likely to exist. Plasma gelsolin, together with vitamin D binding protein (VDBP), another extracellular ABPs, were regarded as potentially important components of this system. They are capable of removing F-actin from the circulation and inhibiting F-actin elongation to alleviate the vascular toxicity of excessive F-actin. In this study, the concentration of gelsolin in PRP of AMI rats increased accompanied by the high platelet activation and increased level of F-actin while gelsolin in PPP decreased which indicates the EASS of AMI rats was suppressed. Correlation analysis showed that platelet gelsolin concentrations were high, positively correlated with plasma P-selectin levels in the Model group.

Xiongshao capsule (XSC) is a patent drug developed from Xue Fu Zhu Yu Decoction. It is the classic formula used for activating blood circulation (ABC) in China for hundreds of years. Clinical studies have shown that XSC can effectively prevent restenosis after percutaneous coronary intervention (PCI) [17]. XSC was shown to enhance the protective effect of ischemic postconditioning on rat with myocardial ischemic reperfusion injury [26]. It was also shown to stabilize atherosclerotic plaque by suppressing inflammation and the expression of FcγRIIIA [27]. But the potential antiplatelet mechanism of XSC prophylaxis is unclear. In this study, we found that high dose of XSC prophylaxis could decrease the concentration of myocardial injury markers, CK-MB and cTnI, and reduce the plasma P-selectin level of AMI rats as well. The antiplatelet mechanism of aspirin involves the inhibition of COX-1 and TXA_2, our study shows that high dosage of XSC can inhibit the activation of TXB_2 and COX-1 *in vivo*, which has similar cardioprotection effect with aspirin *in vivo*. Meanwhile, high dosage of XSC prophylaxis inhibited the expression of platelet gelsolin in AMI rats by inhibiting the platelet calcium influx, but increased the concentration of plasma gelsolin and plasma VDBP simultaneously, so the EASS was activated, and the concentration of F-actin in AMI rats decreased which indicated that the F-actin was being removed from the circulation. Calcium ions not only promote gelsolin secretion but also play a vital role in the development of platelet activation. Studies have shown increased platelet

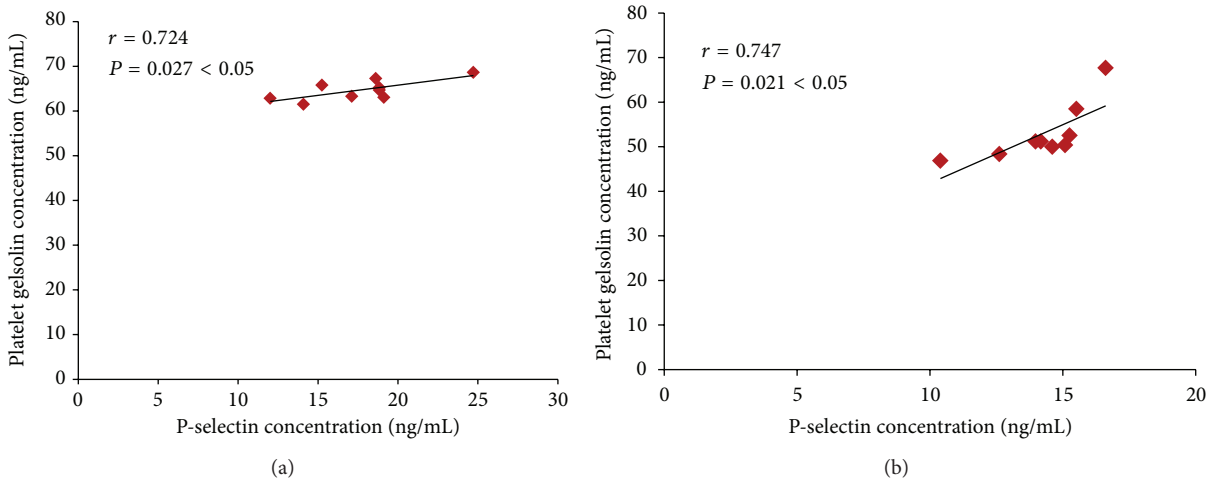

Figure 8: Correlation between gelsolin concentration in PRP and plasma P-selectin level of Model group and Xscd group. (a) Model group and (b) Xscd group.

$[Ca^{2+}]_i$ in patients with CVD [28], and that calcium channel blocker (CCB) could reduce platelet $[Ca^{2+}]_i$ and inhibit platelet aggregation [29]. Verapamil is a classic CCB agent and a previous study *in vivo* [30] shows that verapamil exhibits a dose-dependent inhibitory effect on platelet aggregation and thrombus formation in rats. In this study, our results show that high dosage of XSC can mimic the calcium channel antagonist effect.

We have also investigated the expression of the gelsolin in infarcted myocardium of AMI rats. The results indicate that the gelsolin expression of infarcted myocardium of the Model group increased markedly; while XSC can inhibit gelsolin expression of infarcted myocardium significantly, verapamil or aspirin has no such effect, holding that other pathway existed in the regulation of gelsolin as well. Heart failure (HF) is the end stage of CVD (including after AMI). It is of great importance to know the effects and mechanism of XSC on cardioprotection at earlier stages of CVD. Ventricular remodeling after AMI is the main pathological change of HF. A previous study [31] has showed that gelsolin is an important contributor to heart failure progression through novel mechanisms of HIF-1α and DNase I activation and downregulation of antiapoptotic survival factors. Based on these results and our study, we propose that gelsolin inhibition is a promising target for CVD therapy besides antiplatelet agent.

5. Conclusion

We have provided experimental evidence supporting our conclusion that high correlation between platelet gelsolin and platelet activation level in AMI rats, the aspirin-like cardioprotection, and antiplatelet effects of XSC are related to its inhibition on platelet gelsolin, platelet calcium influx and activated the EASS. Taken together, our results suggest that platelet gelsolin is a potential antiplatelet target and XSC is a promising lead compound for antiplatelet and cardiovascular therapy.

Conflicts of Interests

The authors declare that there is no conflict of interests.

Author Contributions

H. Yin conceived and designed the animal experiments and helped to draft the paper. Y. Liu, Y. Jiang, M. Xue, and C. Guo performed the experiments. Y. Liu participated in its design, analyzed the data, and prepared the paper. K. Chen and D. Shi participated in its design and coordination, and gave final approval for this paper to be published.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (nos. 81073086, 81030063, and 81102845).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 120732, 9 pages
http://dx.doi.org/10.1155/2013/120732

Review Article

Nigella sativa and Its Protective Role in Oxidative Stress and Hypertension

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Received 1 November 2012; Accepted 31 January 2013

Academic Editor: Waris Qidwai

Hypertension increases the risk for a variety of cardiovascular diseases, including stroke, coronary artery disease, heart failure, and peripheral vascular disease. The increase in oxidative stress has been associated with the pathogenesis of hypertension. Increase of blood pressure is due to an imbalance between antioxidants defence mechanisms and free radical productions. Excessive production of reactive oxygen species reduces nitric oxide bioavailability leading to an endothelial dysfunction and a subsequent increase in total peripheral resistance. Hypertension can cause few symptoms until it reaches the advanced stage and poses serious health problems with lifelong consequences. Hypertensive patients are required to take drugs for life to control the hypertension and prevent complications. Some of these drugs are expensive and may have adverse reactions. Hence, it is timely to examine scientifically, complimentary therapies that are more effective and with minimal undesirable effects. *Nigella sativa* (NS) and its active constituents have been documented to exhibit antioxidant, hypotensive, calcium channel blockade and diuretic properties which may contribute to reduce blood pressure. This suggests a potential role of NS in the management of hypertension, and thus more studies should be conducted to evaluate its effectiveness.

1. Introduction

According to The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, hypertension is diagnosed as systolic blood pressure (BP) which is greater than 140 mmHg and/or diastolic BP which is greater than 90 mmHg [1]. The prevalence of hypertension in most developing countries is comparable to the developed countries [2, 3]. Hypertension is a major global health disorder due to prolonged human life span. Familial influence and environmental factors such as obesity, sedentary life style, and unhealthy dietary habit contribute to the high prevalence of hypertension [4–6]. The prevalence of hypertension in Malaysians aged 30 years and above was 42.6% in year 2006, a relative increase of 30% compared to 10 years earlier [7]. In the year 2000, it was estimated about 972 million world's adult population had hypertension. This number will increase to 1.56 billion by the year 2025 [8].

In recent years, there has been a growing interest and demand in using medicinal plants for treating and preventing various diseases including cardiovascular diseases. Traditional medicines of plants origin have received much attention due to several factors such as easy availability, affordable cost, safety, and efficacy as well as cultural acceptability. *Nigella sativa* (NS), or also known as black cumin or its Arabic name *habat-ul sauda*, has been used for centuries in medicinal and culinary purposes throughout the Middle East, India, and Northern Africa. It is an annual flowering plant with pale blue flowers that belongs to the Ranunculaceae family. The plant has a fruit which contains angular black seeds, and the seeds are considered to be the most valuable part contributing beneficial health effects. NS as a natural

remedy has been documented to possess numerous therapeutic values, including diabetes, tumour, hypercholesterolemia, hypertension, inflammation, and gastrointestinal disorders [9–14]. The present paper is, therefore, to examine the current literature on the cardiovascular protective effects of NS and its constituents against oxidative stress and hypertension in addition to the possible mechanisms of actions underlying these beneficial effects.

2. *Nigella sativa* (NS)

The seed oil of NS was found to be rich in polyphenols and tocopherols [25, 26]. The seeds contain 36–38% fixed oils, 0.4–2.5% essential (volatile) oil, proteins, alkaloids, and saponins [27]. The fixed oil is composed mainly of fatty acids, namely, linoleic (C18:2), oleic (C18:1), palmitic (C16:0), and stearic (C18:0) acids [28]. Thymoquinone (TQ) is the most pharmacologically active ingredient found abundantly (30–48%) in the black seeds, together with its derivatives such as dithymoquinone, thymohydroquinone, and thymol [29].

3. Antioxidant Property of NS

The seed oil of NS is well known for its strong antioxidant properties [30]. Previous studies have documented that pretreatment with TQ, the main active constituents in seed oil, protected organs against oxidative damage induced by a variety of free radical generating agents, such as carbon tetrachloride [31] and including the alkylating agents, cisplatin [32], and doxorubicin [33]. The free radical scavenging effects of TQ, dithymoquinone, and thymol were tested against several reactive oxygen species (ROS) [34]. All the tested compounds from NS exerted strong antioxidant effects; thymol acted as singlet oxygen quencher, while TQ and dithymoquinone showed superoxide dismutase (SOD)-like activity [34]. In addition, a study carried out by Mansour et al. [35] revealed that both TQ and dithymoquinone acted not only as superoxide anion scavengers, but also as general free radical scavengers with half maximal inhibitory concentration (IC_{50}) in the nanomolar and micromolar ranges, respectively. These findings suggest the importance of such free radical scavenging compounds in the treatment of hypertension which is closely associated with oxidative stress.

TQ is a potent superoxide radical scavenger which is as effective as SOD against superoxides generated either photochemically, biochemically, or derived from calcium ionophore (A23817) [36]. Furthermore, TQ has an inhibitory effect on lipid peroxidation induced by Fe^{3+}/ascorbate. In rats, TQ is protective against doxorubicin-induced cardiotoxicity by reducing the elevation of serum lactate dehydrogenase and creatine phosphokinase levels [36]. Ismail et al. [37] showed that both TQ-rich fraction and TQ markedly improved plasma antioxidant status by inhibiting formation of hydroxyl radicals. Moreover, liver antioxidant enzymes (SOD and glutathione peroxidase GPx) are significantly increased in rats treated with TQ-rich fraction and TQ. In the same study, both TQ-rich fraction and TQ caused an enhanced expression of antioxidant genes (SOD-1, catalase CAT, and GPx-2) in hypercholesterolemic rats [37].

Erşahin et al. [38] reported that NS oil with its potent free radical scavenging properties, inhibited subarachnoid-haemorrhage-(SAH-) induced lipid peroxidation of the brain tissue in rat against the reactive hydroxyl, peroxyl, and superoxide radicals. In addition, the level of antioxidant glutathione (GSH) was preserved [38], thereby ameliorating oxidative damage. The SAH-induced reduction of Na^+/K^+-ATPase activity indicated the presence of membrane damage. The Na^+/K^+-ATPase is involved in the generation of the membrane potential through the active transport of sodium and potassium ions in cellular membrane. It maintains neuronal excitability and controls cellular volume in the central nervous system. Treatment with NS oil was able to restore Na^+/K^+-ATPase activity back to normal levels [38].

Administration of TQ restored the activities of nonenzymatic (GSH and vitamin C) and enzymatic (SOD, CAT, GPx, and glutathione-S-transferase GST) antioxidants as well as reduced the levels of malondialdehyde (MDA) in the rat brain to normal levels [39]. Besides that, TQ supplementation resulted in a complete reversal of the gentamicin-(GM-) induced increase in blood urea nitrogen, creatinine, thiobarbituric acid reactive substances (TBARS), and nitric oxide (NO) and decrease in GSH, GPx, CAT, and adenosine triphosphate (ATP) to control values [40]. Histopathological examination of kidney tissues was in agreement with the biochemical data, wherein TQ supplementation prevented GM-induced degenerative changes in kidney tissues [40]. The findings from this study demonstrated the strong protective effect of TQ by its ability to decrease oxidative stress and to preserve the activities of the antioxidant enzymes [40].

4. Pathogenesis of Hypertension

4.1. Role of Oxidative Stress in Hypertension. Free radicals possess one or more unpaired electrons in their outer electronic orbits. ROS such as superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are highly reactive. Free radicals and ROS are formed continuously in normal physiological process [41]. However, excessive production leads to oxidative stress with an increase in the formation of nitrogen-oxygen derivative free radicals as well as a decrease in antioxidant capacity [42].

Oxidative stress occurs when there is an imbalance between production of ROS and antioxidant defence system in favour of the former [43, 44]. ROS are highly reactive and unstable by nature; hence, they can damage various cellular components including lipid membranes. Lipid peroxides are derived from polyunsaturated fatty acid (PUFA) oxidation and are capable to initiate lipid peroxidation via free radicals chain reaction. MDA is a major end product of PUFA peroxidation and is often used as an indicator of cell injury.

Increase in the production of MDA may be due to the formation of reactive oxidants. Lipid peroxidation leads to structural changes of the lipid molecules, and the changes are more severe as lipids are the main constituent of biological membranes. Generally, lipid peroxides pose a risk factor

for atherosclerotic complications. Increase in free radical formation is linked to a reduction in NO generation [45]. Elevation in serum MDA level found in hypertensive patients suggests a relationship with the increased oxidative stress [46].

Endothelium-derived relaxing factor (EDRF) or better known as NO plays an important regulatory role in the maintenance of vascular homeostasis. BP is regulated by cardiac output and peripheral resistance of blood vessels. NO causes vasodilatation, subsequently reducing total peripheral resistance. Endothelial dysfunction is associated with abnormal endothelium-dependent relaxation as observed in hypertension [47–49]. Reduced NO bioavailability, that is, a reduction in NO production by free radicals or an increase in deactivation of NO due to imbalance between antioxidant and oxidant levels may be the mechanism underlying endothelium dysfunction.

NO is synthesized predominantly in the vascular endothelium. Endothelial nitric oxide synthase (eNOS) is required for the synthesis of NO from amino acid L-arginine. There are two other isoforms of NOS, namely, neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS). Both eNOS and nNOS are constitutive enzymes that are produced in normal physiological process, while iNOS is mainly induced during inflammation. NO is small in size and lipophilic by nature. This allows NO to diffuse rapidly through cell membranes to the adjacent smooth muscle cells. Eventually, NO activates guanylate cyclase (GC), and stimulates the formation of cyclic guanosine monophosphate (cGMP) that leads to vascular smooth muscle relaxation. Generation of ROS such as superoxide anions may cause cellular injury by oxidizing membrane lipids, proteins, and nucleic acids. Furthermore, superoxide anions may reduce NO bioavailability by binding to the gaseous molecule and forming peroxynitrite which itself is a free radical [50].

Angiotensin-converting enzyme (ACE) plays a vital role in the regulation of BP and electrolytes balance. ACE is mainly located on the surface of endothelium and epithelium. It hydrolyses angiotensin I (Ang I) to angiotensin II (Ang II), a potent vasoconstrictor and aldosterone-stimulating peptide. Ang II is an important factor in cardiovascular homeostasis [51]. The importance of ACE in maintaining the BP can be observed via the beneficial effect of ACE inhibitors in treating hypertension [52, 53]. Ang II induces oxidative stress via activation of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) oxidase and the production of ROS [54]. In addition to that, Ang II increases lipid peroxidation [55] and stimulates the production of prooxidant cytokines [56, 57], in which these subsequently lead to elevation of BP. Ang II decreases NOS expression and stimulates the generation of ROS [54].

Ang II-induced hypertension is associated with increased vascular superoxide production and impaired vasorelaxation to acetylcholine [54]. Ang II exacerbates oxidative stress, and the increase in the superoxide level could result in endothelial dysfunction via scavenging NO and decreasing NO bioavailability [58]. Hence, the NO-Ang II imbalance may be an important component in the vascular pathophysiology of hypertension. Ang II has been suggested to cause an increase in oxidized low-density lipoprotein cholesterol (ox-LDL) uptake, eventually causing endothelial cell injury [59]. On the other hand, it has also been suggested that ox-LDL upregulates Ang II type 1 receptor expression [60]. These observations may indicate the presence of a relationship between ox-LDL and rennin-angiotensin systems in hypertension. Hence, these two systems may be responsible for the development of endothelial dysfunction, leading to an increase in BP.

4.2. Evidences of Oxidative Stress Involved in Hypertension. There has been a growth evidence suggesting that hypertension may be attributable to the increased production of ROS [61, 62]. Oxidative stress may play a vital role in the development of hypertension via the following mechanisms: enhanced sequestration of NO by ROS [63], formation of lipid peroxidation products [64], and depletion of NOS cofactor (tetrahydrobiopterin) [65]. Lastly, it may cause functional and structural changes in vascular wall and blood vessel [66]. These vascular alterations may be mediated by several ways, including direct injury to endothelial and vascular smooth muscle cells, effects on endothelial cell eicosanoid metabolism, altered redox state, increases in intracellular free calcium concentration, stimulation of inflammatory, and growth signalling events [67, 68].

Oxidative stress promotes proliferation of vascular smooth muscle cells as well as collagen deposition which cause thickening of tunica media and narrowing of the vascular lumen [69], which eventually leads to an increase in the total peripheral resistance. Furthermore, increase in oxidative stress may damage the endothelium and impair endothelium-dependent vasodilatation, consequently increasing vascular contractile activity [69]. ROS may also induce endothelial permeability with the recruitment of inflammatory protein and cells, in which can compromise endothelial function and worsen vascular damage [70]. All these observed effects on the vasculature further support the pathological role of oxidative stress in hypertension.

Amanullah et al. [70] reported that higher MDA levels and lower antioxidant activities such as SOD, GSH, and GPx in hypertensive subjects may be due to increased production of free radicals, indicating the presence of oxidative stress in hypertension. There were notable a positive correlation between BP and lipid peroxidation products in the hypertensives and a negative correlations between BP with plasma antioxidant capacity, plasma vitamin C levels, erythrocyte activity of antioxidant enzymes, and erythrocyte reduced/oxidized glutathione (GSH/GSSG) ratio [71]. These findings demonstrated a possible role of oxidative stress in the pathophysiology of hypertension. In another study, hypertensive patients exhibited higher plasma lipid peroxides along with decreased nonenzymatic antioxidant levels, which could be associated with oxidative stress and depleted antioxidant defence potential [72]. Oxidative stress may occur due to a reduction in antioxidant activities or due to an elevation in ROS concentration. This can lead to oxidative damage to the structure of biomolecules which mostly involve the

Table 1: Significant cardiovascular effects of NS and its constituents.

Reference	Study model	Constituents	Laboratory findings
[12]	Renovascular hypertensive rat	NS oil (i.p.) 0.2 mL/kg	↓ SBP, tissue MDA, luminol, and lucigenin CL ↑ tissue Na^+ and K^+-ATPase ↓ plasma CK, LDH, and ADMA ↑ plasma NO
[15]	Rat	(a) NS oil (i.v.) 4–32 μL/kg (b) TQ (i.v.) 0.2–1.6 mg/kg	↓ arterial BP and heart rate (dose dependent)
[16]	Guinea pig	NS oil (i.v.) 4–32 μL/kg	↓ arterial BP and heart rate (dose dependent)
[17]	Rat	(a) De-TQ volatile oil (i.v.) 2–16 μL/kg (b) α-pinene (i.v.) 1–4 μL/kg (c) p-cymene (i.v.) 2–16 μL/kg	↓ arterial BP and heart rate (dose dependent) *De-TQ volatile oil and p-cymene: 4, 8, and 16 μL/kg *α-pinene: 2 and 4 μL/kg
[18]	Rat	Thymol (*in vitro*)	↓ aortic contraction (dose dependent)
[19]	Canine and guinea pig	Thymol (*in vitro*)	Negative inotropic action (dose dependent)
[20]	Spontaneously hypertensive rat	NS seed extract (p.o.) 0.6 mL/kg	↑ diuresis ↓ arterial BP
[21]	Spontaneously hypertensive rat	NS extract (p.o.)	↓ SBP ↑ GFR, urinary and electrolyte output
[22]	L-NAME-induced hypertensive rat	TQ (p.o.) 0.5 mg/kg and 1 mg/kg	↓ SBP and serum creatinine ↑ kidney GSH
[23]	L-NAME-induced hypertensive rat	NS seed extract (p.o.) 400 mg/kg	↓ arterial BP, SBP, DBP, and serum LDH ↑ serum NO
[24]	Patients with mild hypertension	NS seed extract (p.o.) 100 mg/kg and 200 mg/kg	↓ SBP and DBP (dose dependent) ↓ total and LDL cholesterol

NS: *Nigella sativa*; L-NAME: L-NG-nitroarginine methyl ester; i.p.: intraperitoneal; i.v.: intravenous; p.o.: per os; TQ: thymoquinone; De-TQ: de-thymoquinonated; SBP: systolic blood pressure; DBP: diastolic blood pressure; MDA: malondialdehyde; CL: chemiluminescence; CK: creatine kinase; LDH: lactate dehydrogenase; ADMA: asymmetric dimethylarginine; NO: nitric oxide; GFR: glomerular filtration rate; GSH: glutathione; LDL: low-density lipoprotein.

antioxidant enzymes, thus contributing to the oxidative stress in hypertensives instead of normotensive subjects.

A variety of antioxidant treatments ameliorate hypertension in animal and human studies. Veratric acid, a phenolic acid, was found to decrease the BP, significantly restored NO, enzymatic and nonenzymatic antioxidants, and reduced lipid peroxidation products against L-NG-nitroarginine methyl ester-(L-NAME-) induced hypertension in Wistar rats [73]. Park et al. [74] demonstrated that soy isoflavone supplementation elevated serum NO and total radical trapping antioxidant potential (TRAP) with a reduction in systolic BP after 30 days of feeding period to spontaneously hypertensive rats (SHRs). The results suggest that protective effect of isoflavone against hypertension occurs possibly via the mitigation of oxidative stress and augmentation of NO production. Consumption of green tea which possess strong antioxidant polyphenols was able to reduce BP, serum tumour necrosis factor-α, c-reactive protein, and triglycerides, and total and low-density lipoprotein cholesterol while increasing total antioxidant status and high-density lipoprotein cholesterol in patients with obesity-related hypertension [75].

Administration of vitamins C and E for eight weeks in patients with essential hypertension (EH) had significantly lowered systolic BP, diastolic BP, and mean arterial pressure compared to placebo [76]. The BP reduction was associated with higher erythrocyte and serum antioxidant capacity. BP correlated positively with plasma 8-isoprostane levels and negatively with ferric reducing ability of plasma (FRAP) levels in the vitamins C and E and placebo-treated groups [76]. The findings support the view that oxidative stress is involved in the pathogenesis of EH and that enhancement of antioxidant status by supplementation with vitamins C and E in patients with EH is associated with reduced BP. Hence, this suggests that intervention with antioxidants is a potential adjunct therapy for hypertension.

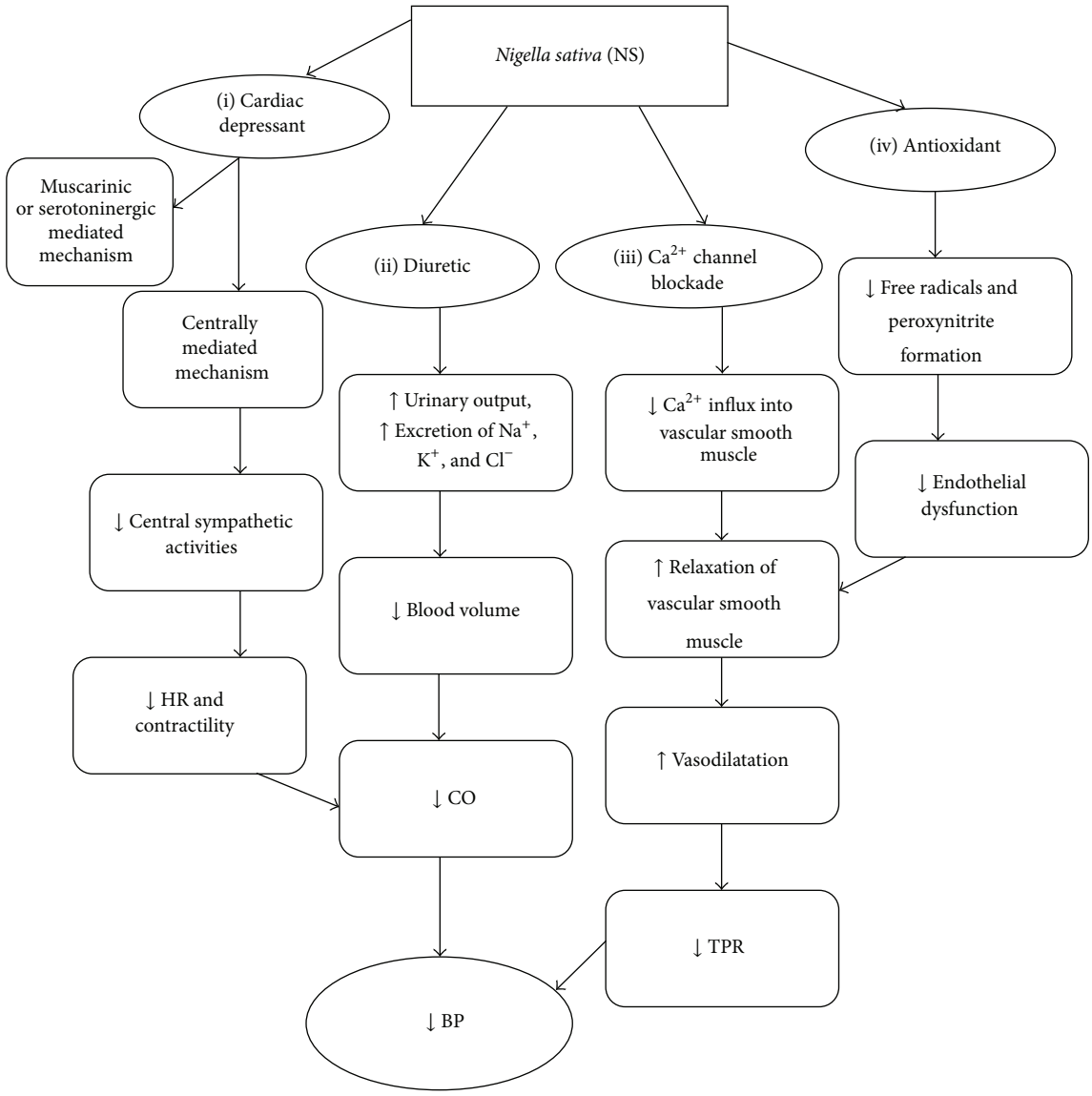

Figure 1: Proposed pathways for *Nigella sativa* (NS) in reducing blood pressure (BP). Ca^{2+}, calcium (II) ion; Na^+, sodium ion; K^+, potassium ion; Cl^-, chloride ion; HR, heart rate; CO, cardiac output; TPR, total peripheral resistance.

5. Possible Pharmacological Actions of NS against Hypertension

The exact mechanism on how NS reduces BP is not exactly known. The antihypertensive effects of NS may be due to the many active compounds, each with distinct mechanisms of actions. There are several possible mechanisms involved in BP reduction, which include cardiac depressant effect, calcium channels blocking property, and diuretic effect. Table 1 summarized the effects of NS and its active compounds on BP.

5.1. Cardiac Depressant. Previous studies reported that the volatile oil and TQ decreased both the arterial BP and heart rate [15, 16]. The effects of NS on BP and heart rate were reversed by cyproheptadine (a nonselective serotonin receptor blocker) and atropine (antimuscarinic M_2 agent) [15, 16]. This finding suggests that the protective effects of NS were mainly mediated centrally either directly or indirectly via mechanisms involving serotoninergic and muscarinic receptors [15, 16]. In contrary, El-Tahir et al. [17] documented that de-thymoquinonated volatile oil, α-pinene, and p-cymene from NS reduced BP and heart rate. However, hypotensive effect of NS was not reversed by atropine or cyproheptadine although these two drugs antagonized the effect of NS on heart rate [17].

The cardiac depressant effects of NS in the rats were significantly reversed by hexamethonium (a ganglionic blocker) suggesting a mechanism involving nicotinic receptors [17]. In addition, destruction of connection between the vasomotor centre in the medulla and preganglionic sympathetic by

spinal pithing prevented NS-induced cardiovascular changes [17]. Therefore, the cardiac depressant and hypotensive effects of NS appeared to be mediated via central mechanism involving vasomotor centre in the medulla and sympathetic outflow to the periphery.

Failure of indomethacin (prostaglandin cyclooxygenase inhibitor), mepacrine and hydrocortisone (phospholipase A_2 inhibitor), mepyramine (histamine H_1 receptor blocker), ranitidine (histamine H_2 receptor blocker), and methylene blue (NO-cGMP formation antagonist) to affect NS-induced cardiovascular depressant effects suggests a lack of involvement of eicosanoid, histaminergic, platelet-activating factor (PAF), and NO-induced mechanisms [17].

5.2. Calcium Channel Blockade. Thymol, another active compound of NS, has been documented to be able to reduce BP via its action on calcium (Ca^{2+}) ion channels. Peixoto-Neves et al. [18] reported that thymol produced dose-dependent relaxation in rat isolated aorta. The NS-induced endothelium-independent relaxation may be mediated via mechanisms involving inhibition of Ca^{2+} release from sarcoplasmic reticulum, reduced Ca^{2+} sensitivity of the contractile system, and/or blockade of Ca^{2+} influx across the membrane [18]. Thymol induced a dose-dependent negative inotropic action on both canine and guinea-pig isolated cardiac preparations [19]. The observed effect may be due to a decrease in Ca^{2+} content in sarcoplasmic reticulum via inhibition of Ca^{2+} channel [19]. Effect of thymol on Ca^{2+} current in human and canine ventricular cardiomyocytes was investigated. Magyar et al. [77] demonstrated that thymol inhibits L-type Ca^{2+} current in a dose-dependent manner. When Ca^{2+} channels are blocked, Ca^{2+} entry into vascular smooth muscles is reduced eventually leading to an increase in vasorelaxation.

5.3. Diuretic. The kidney plays a vital role in the control of BP and in the pathogenesis of hypertension. Zaoui et al. [20] reported that 0.6 mL/kg of NS extract for 15 days caused 16% increase in diuresis in SHRs. The diuretic effect of NS was comparable to 5 mg/kg of furosemide which is a high ceiling diuretic. The diuretic effect was associated with an increase in urinary excretion of Na^+, K^+, Cl^-, and urea [20]. This suggests that NS may decrease BP via its diuretic action. A reduction in electrolytes and water content leading to decrease in blood volume, which subsequently reducing cardiac output, is one of the main determinants for BP regulation. In another study, NS extract also demonstrated similar results with an increase in glomerular filtration rate, urinary, and electrolyte output [21]. Rennin-angiotensin-aldosterone (RAA) systems may contribute in regulating BP by controlling blood volume and peripheral vascular resistance. However, the observed effects of NS extract neither have influence on plasma ACE nor rennin activities of SHRs after 20 days of treatment [21]. Therefore, antihypertensive action of NS seems to be independent of RAA system. Nevertheless, more studies need to be performed to evaluate this hypothesis.

6. Conclusion

The cardiovascular protective effects of NS in hypertension are possibly contributed by its multitude actions including cardiac depressant, diuretic, calcium channel blockade, and antioxidant properties (Figure 1). NS is a promising medicinal plant with many therapeutic properties. Various studies have documented the protective effects of NS on the cardiovascular system against the damaging effects of various ROS, protecting the heart from cardiotoxicity as well as reducing adverse effects due to ROS involved in hypertension. NS has been used as a traditional medicine for the treatment of hypertension for many years with no report of adverse events. Further studies should be carried out on human to confirm its efficacy. It is an important area for further research and development to combine NS with other antihypertensive drugs to investigate their possible synergistic effects and preferable pharmacological properties.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 610694, 11 pages
http://dx.doi.org/10.1155/2013/610694

Research Article

Tanshinone IIA and Cryptotanshinone Prevent Mitochondrial Dysfunction in Hypoxia-Induced H9c2 Cells: Association to Mitochondrial ROS, Intracellular Nitric Oxide, and Calcium Levels

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Received 12 August 2012; Accepted 27 January 2013

Academic Editor: Kashmira Nanji

The protective actions of tanshinones on hypoxia-induced cell damages have been reported, although the mechanisms have not been fully elucidated. Given the importance of nitric oxide (NO) and reactive oxygen species (ROS) in regulation of cell functions, the present study investigated the effects of two major tanshinones, Tanshinone IIA (TIIA) and cryptotanshinone (CT), on hypoxia-induced myocardial cell injury and its relationships with intracellular NO and ROS, calcium, and ATP levels in H9c2 cells. Chronic hypoxia significantly reduced cell viability which accompanied with LDH release, increase in mitochondrial ROS, intracellular NO and calcium levels, decrease in superoxide dismutase (SOD) activity, and cellular ATP contents. TIIA and CT significantly prevented cell injury by increasing cell viability and decreasing LDH release. The protective effects of tanshinones were associated with reduced mitochondrial superoxide production and enhanced mitochondrial SOD activity. Tanshinones significantly reduced intracellular NO and Ca^{2+} levels. ATP levels were also restored by TIIA. These findings suggest that the cytoprotective actions of tanshinones may involve regulation of intracellular NO, Ca^{2+}, ATP productions, mitochondrial superoxide production, and SOD activity, which contribute to their actions against hypoxia injuries.

1. Introduction

It has been established that chronic hypoxia is associated with cardiac dysfunctions in certain pathological conditions such as ischemia reperfusion, myocardial infarction (MI), and hypertrophy [1]. Hypoxia causes changes of various cellular mechanisms related to mitochondrial dysfunction and oxidative stress [2]. Among these, hypoxia-induced changes of ROS and NO productions, intracellular calcium, and ATP levels may have particular importance, given the role of these molecules in regulation of cell functions in general [3]. For example, a recent study shows that hypoxia-increased mitochondrial superoxide anion ($O_2^{\bullet -}$), not cytosolic $O_2^{\bullet -}$, plays an important role in hypoxia-induced cell apoptosis [4, 5]. Studies have also found that excess NO production by hypoxia can result in mitochondrial ROS increase by inhibiting mitochondrial electron transport chain function, which in turn promotes peroxynitrite formation and cell apoptosis [6, 7]. On the other hand, hypoxia may modulate NO production by regulating intracellular calcium which is important for Ca^{2+}/calmodulin-dependent eNOS and nNOS activity, and NO increase in turn may inhibit mitochondrial complex IV [8]. This indicates an interaction among NO, ROS, intracellular calcium, and regulation of ATP synthesis in mitochondria. Understanding the relationship of these factors may help to interpret the mechanisms of cellular injury in hypoxia condition [9, 10].

Tanshinones are a group of bioactive compounds isolated from *Salvia miltiorrhiza* (Danshen), a traditionally medicinal plant used in management of angina pectoris, atherosclerosis,

and MI [11]. Among these, tanshinone IIA (TIIA) and cryptotanshinone (CT) are two major bioactive tanshinones [12]. They have been reported to have actions against oxidative stress, myocardial infarction, and myocardial ischemia reperfusion injury [13]. For example, studies *in vitro* have revealed antioxidant actions of TIIA by attenuating intracellular ROS level and enhancing antioxidant enzymes activity [14, 15]. TIIA and CT have also been shown to influence vasodilation by regulating NO and intracellular Ca^{2+} levels in endothelial cells [16, 17]. However, the actions of TIIA and CT on ROS and NO pathways under hypoxic conditions are still not clear. Thus, the present study was conducted to investigate the effects of TIIA and CT on hypoxia-induced cardiac injury and their regulations of intracellular NO, ROS, calcium levels, and ATP contents in H9c2 cells.

2. Materials and Methods

2.1. Chemicals. Tansinone IIA (TIIA) and cryptotanshinone (CT) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (>99% purity) (Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, and strep-tomycin were purchased from Gibco BRL (Grand Island, NY, USA). GasPak EZ Anaerobe Container System Sachets with Indicator and GasPak EZ Standard Incubation Container were from Becton Dickinson and company (Sydney, NSW, Australia). Trypsin-EDTA solution, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 2′,7′-dichlorodihydrofluorescein diacetate, Superoxide dismutase assay kit, dihydroethidium, diphenyleneiodonium chloride, 4-hydroxy-TEMPO (TEMPOL), rotenone, antimycin A and nitro-L-arginine methyl ester (L-NAME) were from Sigma-Aldrich (St. Louis, MO, USA). Fura-2 AM and MitoSOX were from Molecular Probes (S. San Francisco, CA, USA). Lucigenin and MnTBAP were from Santa Cruz Biotechnology (CA, USA). CytoTox96 NonRadioactive Cytotoxicity assay kit and ENLITEN ATP Assay System Bioluminescence Detection Kit were from Promega (Madison, WI, USA). 4, 5-Diaminofluorescein (DAF-2) was purchased from Sapphire Bioscience Biochemicals (Sydney, NSW, Australia). Mitochondria Isolation Kit for Cultured Cells was purchased from Thermo Scientific (Rockford, USA).

2.2. Cells Culture and Hypoxia. The H9c2 embryonic rat heart-derived the cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal bovine serum and 100 μg/mL penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 (passage 25–35).

To mimic hypoxia condition, cells were placed in a GasPak EZ Gas generating Pouch System (Becton-Dickinson) for 8 hr and incubated with serum-free and glucose-free DMEM as described previously [18]. As normoxia control, serum-free DMEM was added to cells and incubated for 8 hr in normoxia condition (21% O_2). For the treatment groups, TIIA or CT (3 μM) was added 2 hr before and during the hypoxia period. The experimental condition was established from a preliminary study involving different concentrations of tanshinones (0.1–10 μM) at different (2 and 24 hr) pre- and posthypoxia incubation periods. In some experiments, MnTBAP (1 μM), rotenone (10 μM), antimycin A (AA: 10 μM), TEMPOL (10 mM), and L-NAME (1 mM) were treated 1 hr before inducing hypoxia as positive controls.

2.3. MTT Assay. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described previously with a modification [19]. The cells (1×10^4 cells/well) were seed in 96 wells. At the end of hypoxia period, MTT solution was added into plates at a final concentration of 0.5 mg/mL and incubated for 2 hr at 37°C. Then, the culture medium was discarded and 150 μL DMSO was added to each well to dissolve dark blue formazan crystals. The absorbance was read at 570 nm using POLARstar OPTIMA microplate reader (BMG LabTech).

2.4. LDH Release Measurement. LDH release was determined by CytoTox 96 NonRadioactive Cytotoxicity Assay kit according to the manufacturer's instructions (Promega). After 8 hr hypoxia, the supernatant was collected and placed in 96 wells and 50 μL of reconstitute substrate mixture was added in each well. After 30 mins incubation, 50 μL of stop solution was added and absorbance was measured at 490 nm using Flexstation multiplate reader (Molecular Devices).

2.5. Cellular ATP Content Measurement. Cellular ATP content was measured by ENLITEN ATP Assay System Bioluminescence Detection Kit according to the manufacturer's instructions (Promega). After hypoxia, The cells were washed with PBS and lysated, and supernatants were collected. Proteins (10 μg/20 μL) were added in white optiplate and initiated action by adding reconstituted reagent. Then, luminescence was measured in POLARstar OPTIMA microplate reader (BMG LabTech).

2.6. NADPH Oxidase Activity. NADPH oxidase activity was measured by lucigenin chemiluminescence as described previously with minor modification [20]. After 8 hr hypoxia, the cells were collected and centrifuged at 750 g for 10 mins at 4°C. The supernatant was discarded and the pellet was resuspended in lysis buffer (50 mM KH_2PO_4, pH 7.0, 1 mM EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 1 μg/mL pepstatin, and 0.5 mM PMSF). Next, the cells were homogenized by quick freeze and thaw step. 10 μg of proteins were added in optiwhite 96-well plates with the 100 μL assay buffer (50 mM KH_2PO_4 (pH 7.0), 150 mM Sucrose, 100 μM NADPH, and 1 mM EGTA). Then, the reaction was started by 5 μM lucigenin. 5 μM DPI was added as an inhibitor. Chemiluminescence was measured with POLARstar OPTIMA microplate reader (BMG LabTech).

2.7. Intracellular Hydrogen Peroxide/Peroxynitrite Production. Intracellular hydrogen peroxide/peroxynitrite (H_2O_2/$ONOO^-$) production was measured by using 2′,7′-dichlorofluoresceindiacetate (DCFH-DA). The nonfluores-

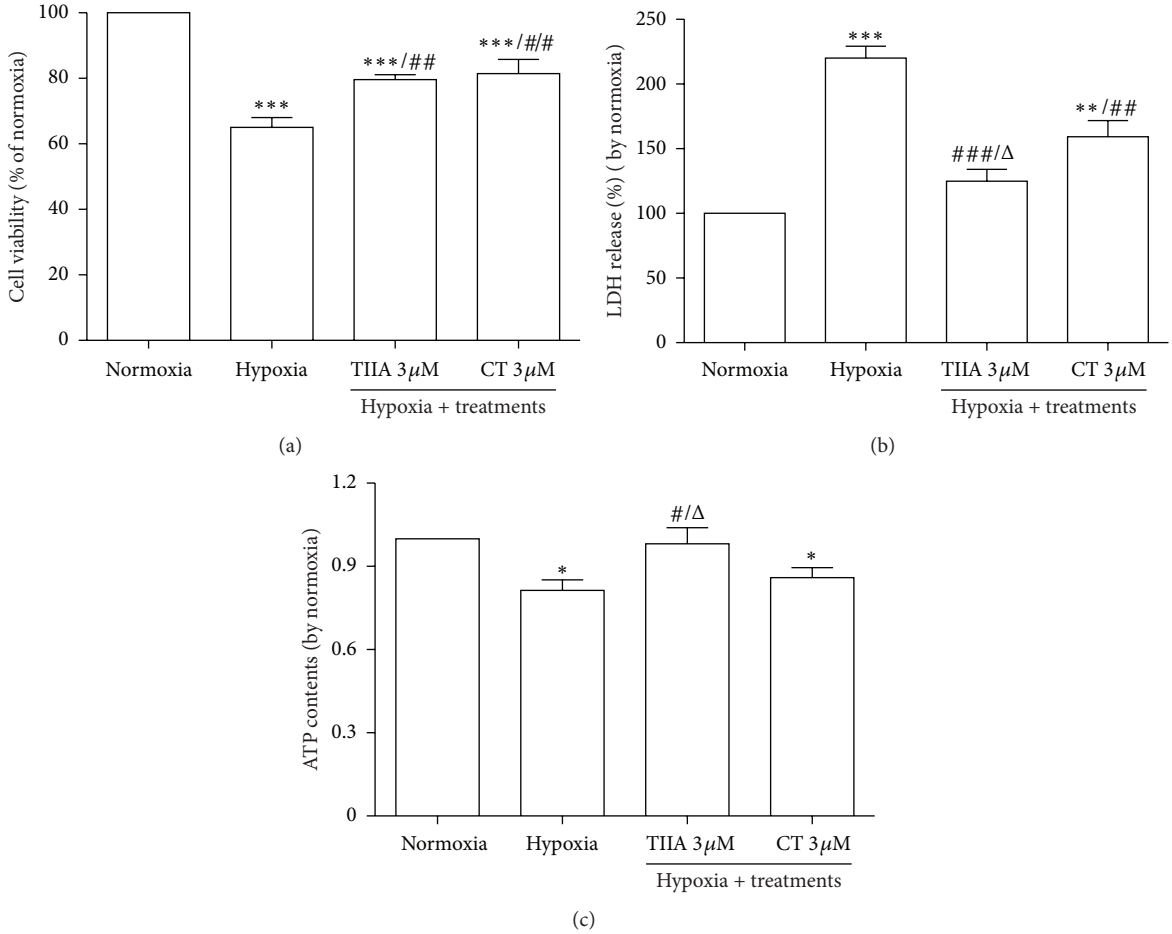


Figure 1: Effects of TIIA and CT on hypoxia-induced H9c2 cell injury. (a) Cell viability by MTT assay. The cell viability of normoxia was adjusted to 100% ($n = 5$), $^{\#\#}P < 0.01$ *versus* hypoxia, $^{***}P < 0.001$ *versus* normoxia. (b) LDH release. The LDH release of normoxia was adjusted to 100% ($n = 3$), $\Delta P < 0.05$ *versus CT*, $^{\#\#}P < 0.01$ *versus* hypoxia, and $^{\#\#\#}P < 0.001$ *versus* hypoxia, $^{***}P < 0.001$ *versus* normoxia. (c) Cellular ATP contents. The cellular ATP contents of normoxia was adjusted to 1 ($n = 6$), $^{*}P < 0.05$ *versus* normoxia, $^{*}P < 0.05$ *versus* normoxia, $^{\#}P < 0.05$ *versus* hypoxia, and $^{\Delta}P < 0.05$ *versus CT*.

cent DCFH-DA readily diffuses into the cells, where it is hydrolysed to the polar derivative DCFH, which is oxidized in the presence of H_2O_2 or $ONOO^-$ to the highly fluorescent DCF. At the End of hypoxia, the cells were incubated with 20 μM DCFH-DA for 20 mins at 37°C. The fluorescence was measured at an excitation wavelength of 488 nm and emission at 530 nm in Flexstation multiplate reader (Molecular Devices). The fluorescence intensity was normalized to total protein contents and expressed as arbitrary units per mg protein.

2.8. Intracellular and Mitochondrial Superoxide Production. Intracellular and mitochondrial superoxide production was measured by loading cells with 20 μM dihydroethidium (DHE) and 2 μM MitoSOX, respectively, by following method described previously with minor modification [21]. End of hypoxia, DHE and MitoSOX were added and incubated for 30 mins at 37°C including 10 mins DAPI (1 μM) staining. After the incubation, the cells were washed with PBS. The cell images were obtained using Image Xpress MICRO system (Molecular Devices) at 20X magnification with binning of 1 and gain of 2 using laser-based focusing. Images were captured using a DAPI filter (350/70 nm Ex, 470/50 nm Em for DAPI) and Cy3 filter (550/35 nm Ex, 570/30 nm Em for DHE and MitoSOX). The cell images were analysed by Meta express software (Molecular Devices).

2.9. Superoxide Dismutase Activity. Superoxide dismutase activity was determined by superoxide dismutase assay kit according to the manufacturer's instructions (Sigma-Aldrich). Briefly, cytosolic and mitochondria fractions were prepared by Mitochondria Isolation kit (Thermofisher). The protein amount was measured by Bradford assay. 20 μg of protein was added in 96-well plates and then the reaction was initiated by adding enzyme solution. Absorbance was measured at 450 nm with POLARstar OPTIMA microplate reader (BMG LabTech).

2.10. Intracellular Nitric Oxide Production. Direct measurement of intracellular nitric oxide production was performed by loading 4,5-diaminofluorescein (DAF-2) [22]. The cells

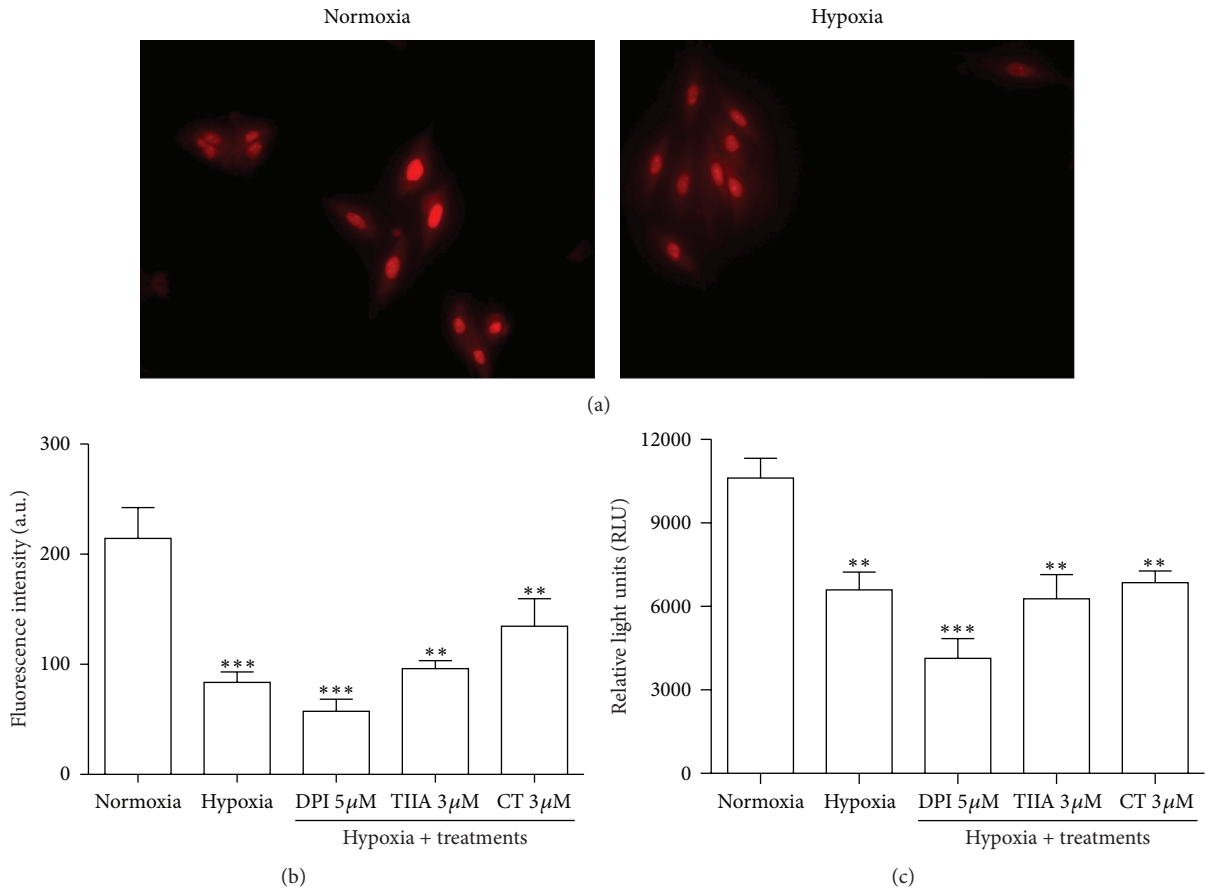


Figure 2: Effects of TIIA and CT on hypoxia-induced decrease in intracellular superoxide level and NADPH oxidase activity. (a) Images of cells labelled with DHE in normoxia and hypoxia groups. (b) The quantified value of DHE fluorescence intensity. Represented data are mean value of 500 each cells with 4 independent experiments. (c) Quantitative value of NADPH oxidase activity. Data shown are representative of 4 independent experiments. $^{**}P < 0.01$ *versus normoxia,* $^{***}P < 0.001$ *versus normoxia.*

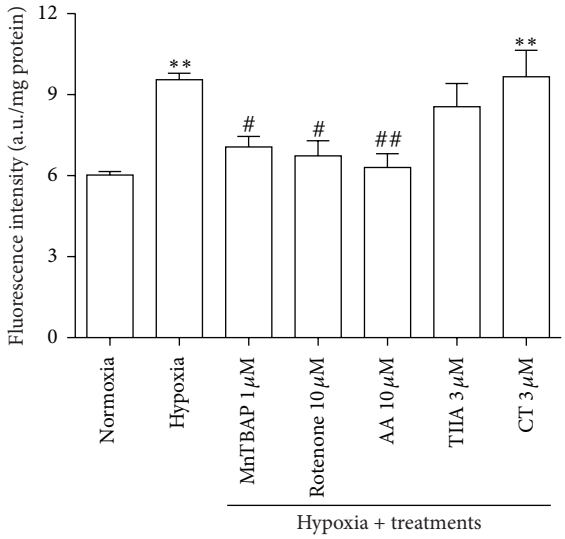
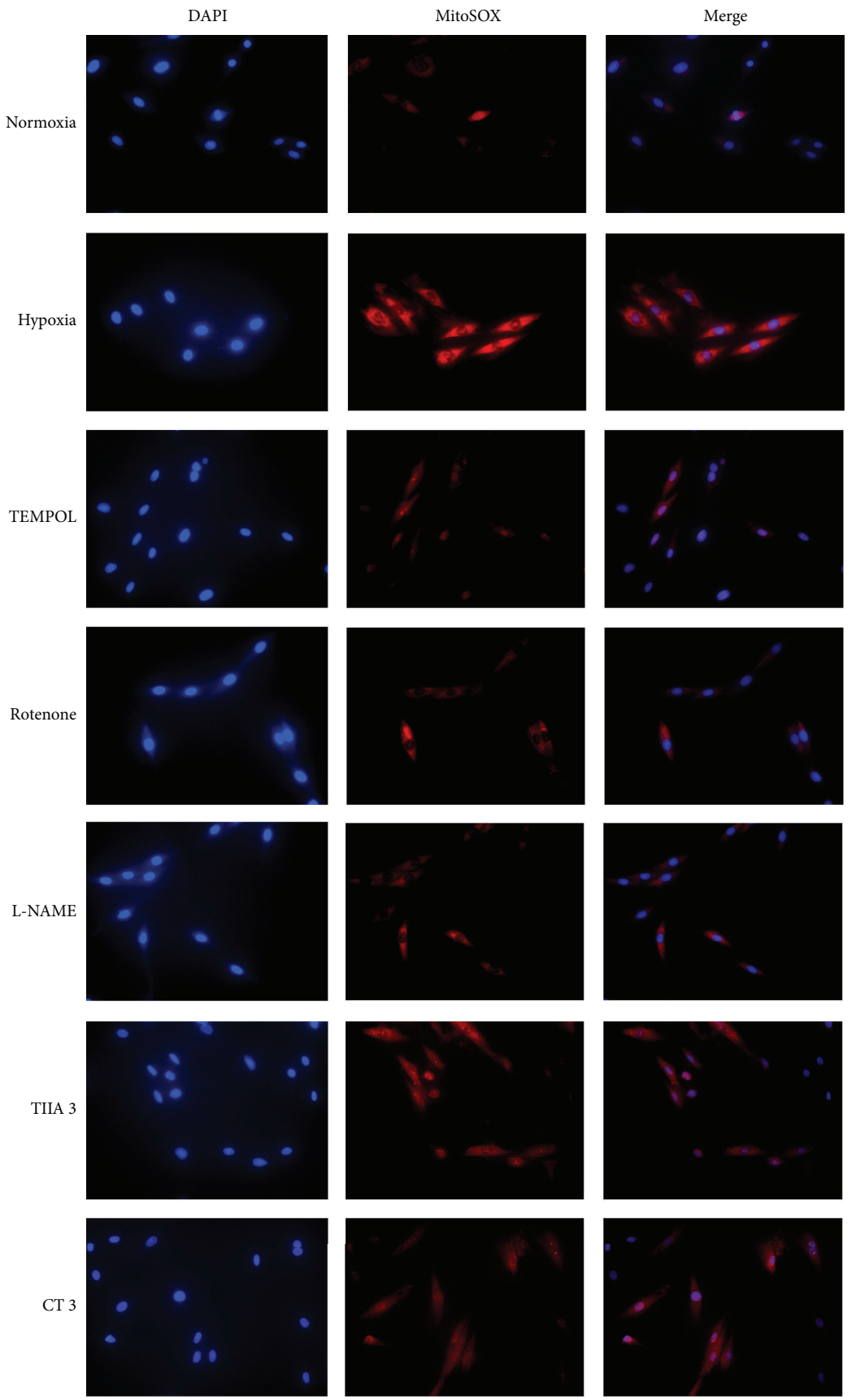


Figure 3: Effects of TIIA and CT on hypoxia-induced increase in $H_2O_2/ONOO^-$ production. Quantitative value of DCFH-DA fluorescence intensity ($n = 4$). $^{\#}P < 0.05$ *versus hypoxia,* $^{\#\#}P < 0.01$ *versus hypoxia,* and $^{**}P < 0.01$ *versus normoxia.*

were incubated with 5 μM DAF-2 for 1 hr after hypoxia. Following the incubation, the cells were washed twice with PBS and visualized in Image Xpress MICRO system (Molecular Devices) at 20X magnification with binning of 1 and gain of 2 using laser-based focusing. Images were captured using a DAPI filter (350/70 nm Ex, 470/50 nm Em for DAPI) and GFP filter (490/40 nm Ex, 510/50 nm Em for DAF-2). The cell images were analysed by Meta Express software (Molecular Devices).

2.11. Intracellular Calcium Level Measurement. Intracellular calcium level was determined by Fura-2AM as described previously with minor modification [23]. After hypoxia, the cells were rinsed twice with PBS and detached by trypsinization. The detached cells were centrifuged and washed with PBS once. Then, the cells were incubated with HBSS with Ca^{2+} buffer (140 mM NaCl, 4.2 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.3 mM $MgCl_2$, 5 mM glucose, and 0.2% bovine serum albumin, pH 7.4) supplemented with 2 μM Fura-2AM for 30 mins at 37°C. After the incubation, the cells were resuspended to HBSS buffer only and incubated for 30 mins at room temperature.



(a)

Figure 4: Continued.

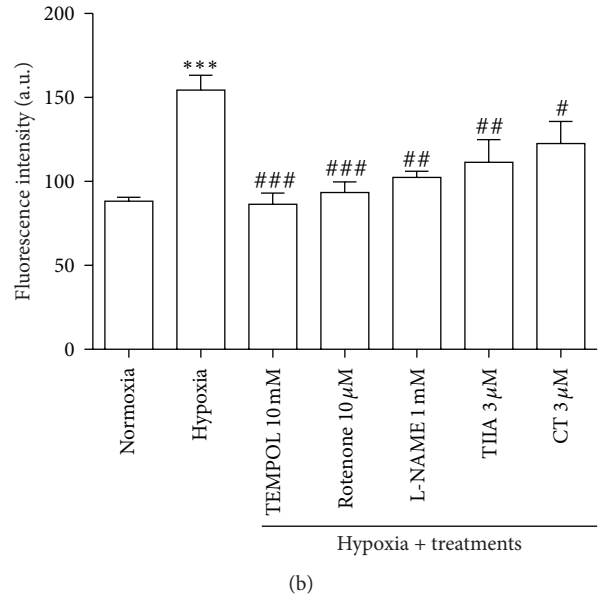


Figure 4: Effects of TIIA and CT on hypoxia-induced increase in mitochondrial superoxide production. (a) Cell images illustrate MitoSOX (red) and DAPI (blue) in each group. (b) The quantified value of MitoSOX fluorescence intensity. Represented data are mean value of 500 each cells with 5 independent experiments. $^{\#}P < 0.05$ *versus* hypoxia, $^{\#\#}P < 0.01$ *versus* hypoxia, $^{\#\#\#}P < 0.001$ *versus* hypoxia, and $^{***}P < 0.001$ versus normoxia.

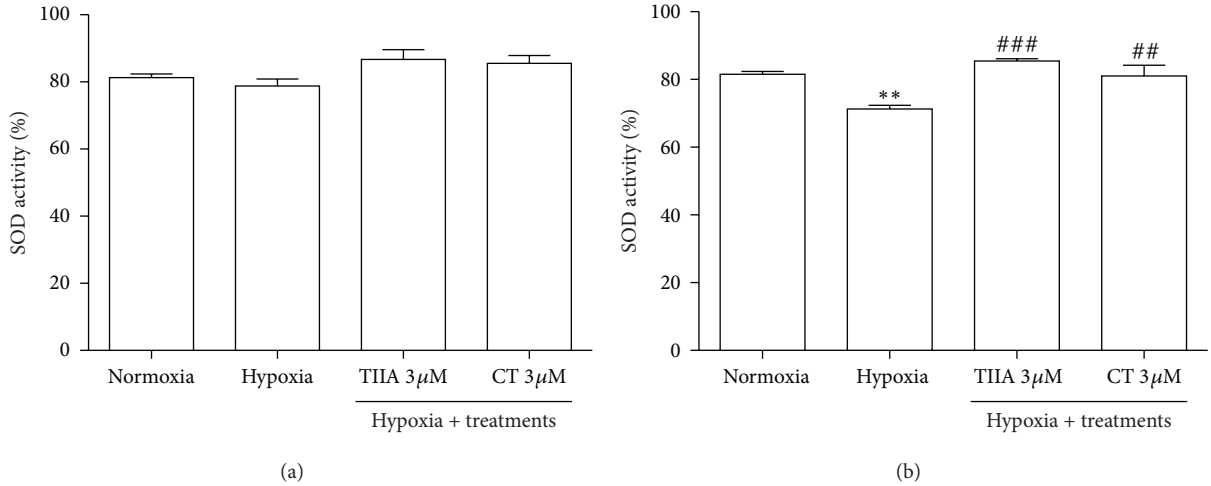


Figure 5: Effects of TIIA and CT on SOD enzyme activity. (a) Cytosolic SOD activity. (b) Mitochondrial SOD activity. Data shown are representative of four independent experiments. $^{**}P < 0.01$ *versus normoxia,* $^{\#\#}P < 0.01$ *versus hypoxia,* and $^{\#\#\#}P < 0.001$ *versus hypoxia.*

1×10^5 cells were transferred to 96-well plates, and then Fura-2AM fluorescence was obtained by alternate excitation at 340 and 380 nm and the emission was detected at 510 nm. The fluorescence maximum was determined by lysing cells with 0.2% Triton X-100 and fluorescence minimum was obtained by recording fluorescence following addition of 40 mM EDTA. The calcium concentration was calculated by equation according to what previously described [24].

2.12. Statistical Analysis. Results were expressed as means ± SEM. Statistical differences among groups were analysed by one-way analysis of variance (ANOVA) using GraphPad Prism Software version 5.0. $P \leq 0.05$ was considered significant.

3. Results

3.1. Effects of TIIA and CT on Hypoxia-Induced Cell Injury. Cells exposed to a 8 hr hypoxia exhibited a significant decrease in cell viability ($P < 0.001$), measured by MTT assay, which was significantly inhibited by pretreatment of TIIA and CT (3 μM) ($P < 0.01$) (Figure 1(a)). 8 hr hypoxia also significantly increased LDH release (to 220.0%, $P < 0.001$), which was significantly inhibited by 3 μM TIIA and CT ($P < 0.001$). The value of LDH release in TIIA-treated group was

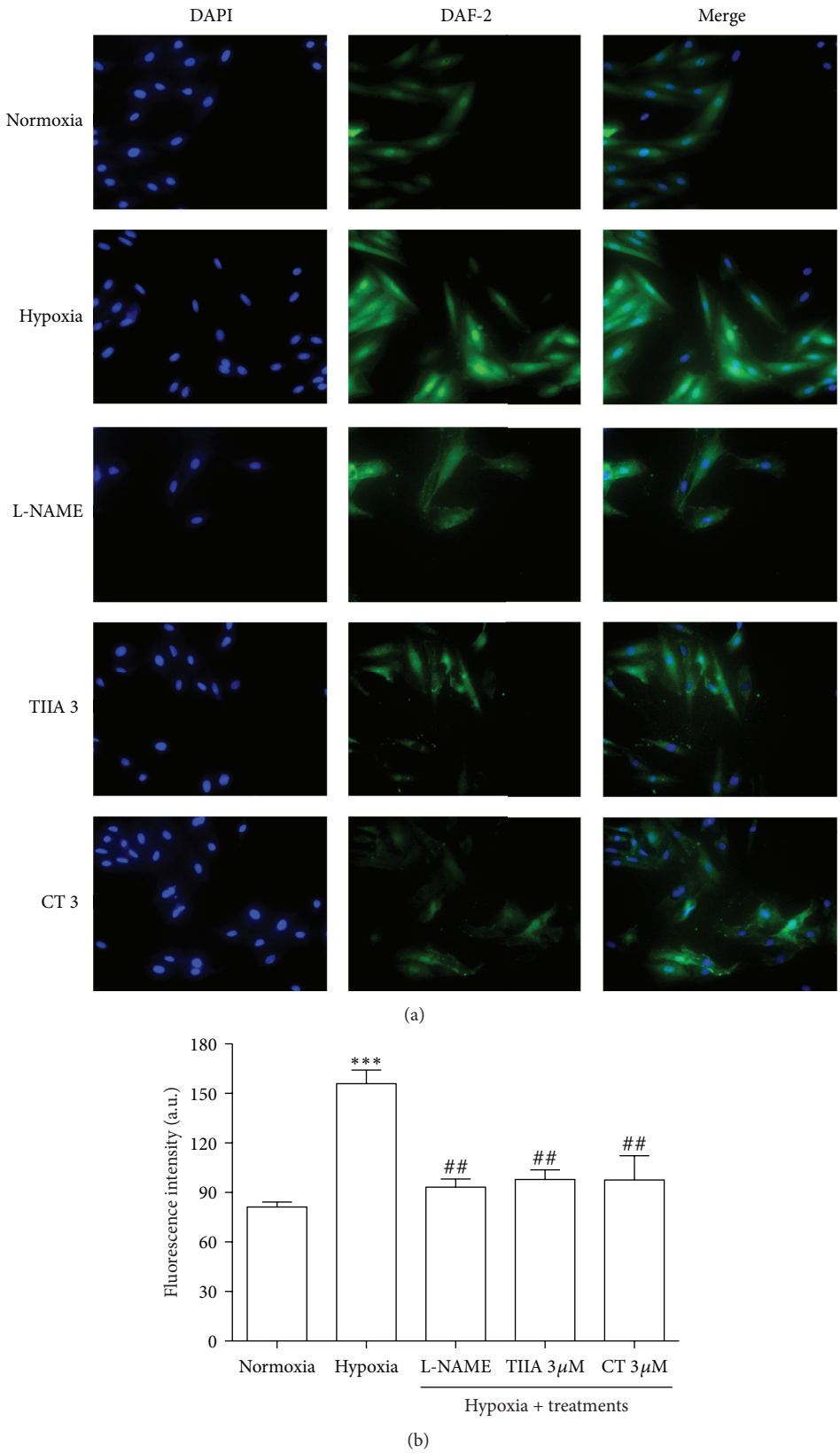


Figure 6: Effects of TIIA and CT on hypoxia-induced increase in intracellular nitric oxide production. (a) Cell images illustrate DAF-2 (green) and DAPI (blue) in each group. (b) Quantitative DAF-2 fluorescence intensity. Represented data are mean value of 500 each cells with 3 independent experiments ($n = 3$). $^{\#\#}P < 0.01$ *versus hypoxia*, $^{***}P < 0.001$ *versus normoxia*.

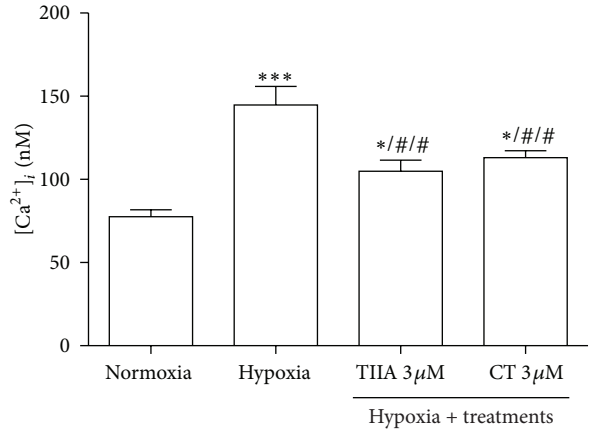


Figure 7: Effects of TIIA and CT on hypoxia-induced increase in intracellular calcium level. Data shown are representative of four independent experiments. $^{*}P < 0.05$ *versus normoxia,* $^{\#\#}P < 0.01$ *versus hypoxia,* and $^{***}P < 0.001$ *versus normoxia.*

significantly less than that of LDH release in CT group ($P < 0.05$) (Figure 1(b)). After 8 hr hypoxia, a significant reduction of the cellular ATP contents (by 20.9%, $P < 0.05$) was observed compared to normoxia control, which was restored by pretreatment with TIIA. The cellular ATP contents in TIIA-treated group were significantly higher ($P < 0.05$) than those in CT group (Figure 1(c)).

3.2. Effects of TIIA and CT on Hypoxia-Induced Decrease in Intracellular Superoxide Generation and NADPH Oxidase Activity. 8 hr hypoxia significantly decreased intracellular superoxide generation ($P < 0.001$) and NADPH oxidase activity ($P < 0.01$) compared to normoxia control. TIIA-, CT- and DPI-treated groups did not significantly affect the intracellular superoxide generation and NADPH oxidase activity compared to hypoxia control (Figures 2(b) and 2(c)).

3.3. Effects of TIIA and CT on Hypoxia-Induced Increase in H_2O_2/$ONOO^-$ Production. After 8 hr hypoxia, the DCF fluorescence intensity was significantly elevated to 9.57 ± 0.50 a.u/mg protein ($P < 0.01$), presenting 59% increase compared to normoxia group. The hypoxia-induced increase in DCF fluorescence was abolished by 1 μM MnTBAP (peroxynitrite inhibitor), 10 μM rotenone (complex I inhibitor), and antimycin A (AA) (complex III inhibitor), presenting 70.1%, 79.2%, and 91.4% inhibition rates, respectively. Also, 3 μM TIIA reduced the increase in DCF fluorescence by 28.9% without statistical significance difference compared to hypoxia control (Figure 3).

3.4. Effects of TIIA and CT on Hypoxia-Induced Increase in Mitochondrial Superoxide Production. Figure 4(a) illustrates the fluorescence images of cells stained with MitoSOX. When compared with normoxia group, distinct intensification in fluorescence was observed in hypoxia group. The cells exposed to hypoxia significantly increased MitoSOX fluorescence intensity to 154.3 ± 19.9 arbitrary units (a.u) while normoxia showed 88.2 ± 5.3 a.u. 1 hr pretreatment with 10 mM TEMPOL (SOD mimic), 10 μM rotenone (complex I inhibitor), and 1 mM L-NAME (NO synthase inhibitor) significantly decreased the mitochondrial superoxide production. In the presence of TIIA and CT (3 μM), the mitochondrial superoxide production was significantly reduced to 111.4 ± 30.2 a.u ($P < 0.01$) and 122.5 ± 29.7 a.u ($P < 0.05$), respectively (Figure 4(b)).

3.5. Effects of TIIA and CT on SOD Activity. Cytosolic SOD activity in hypoxia group (78.8 ± 4.1%) did not significantly ($P > 0.05$) change compared to normoxia control (81.3 ± 2.1%). In contrast, the cells in hypoxia showed a significant decrease in mitochondrial SOD activity by 12.6% compared to normoxia control. In the presence of TIIA and CT (3 μM), the cytosolic SOD activity did not significantly change compared to hypoxia control. The decrease in mitochondrial SOD activity by hypoxia was restored when the cells were pretreated with 3 μM TIIA or 3 μM CT. There was no statistically significant difference in mitochondrial SOD activity between tanshinones treated groups and normoxia control (Figures 5(a) and 5(b)).

3.6. Effects of TIIA and CT on Hypoxia-Induced Increase in Intracellular Nitric Oxide Production. Figure 6(a) illustrates the fluorescence images of cells stained with DAF-2. When compared with normoxia control, distinct intensification in DAF-2 fluorescence was observed in hypoxia group. The quantitative values of the florescence intensity of images were presented in Figure 6(b). Pretreatment with TIIA and CT significantly decreased the intracellular NO production. There was no statistical difference between tanshinones-treated groups and normoxia control. L-NAME (1 mM, NOS inhibitor) significantly ($P < 0.01$) reduced the intracellular NO production compared to hypoxia control.

3.7. Effects of TIIA and CT on Hypoxia-Induced Increase in Intracellular Ca^{2+} Level. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) level was significantly elevated in hypoxia group ($P < 0.001$) compared to normoxia control. Pretreatment with 3 μM of TIIA and CT significantly ($P < 0.01$) prevented the $[Ca^{2+}]_i$ elevation compared to hypoxia control (Figure 7).

4. Discussion

The main finding of the present study is that TIIA and CT protect against chronic hypoxia-induced H9c2 cells injury by restoring cellular ATP contents, decreasing mitochondrial superoxide, intracellular NO, and calcium levels in H9c2 cells. This is consistent with previous observations that hypoxia-induced apoptosis was associated with ROS, NO, and calcium in myocardial cells [5, 25]. Additionally, cellular ATP contents, NO, and calcium are closely associated in mitochondrial ROS production and this suggests that chronic hypoxia-induced cell damages are related to mitochondrial dysfunction.

Myocardial hypoxia is a main cause of cardiac dysfunction due to its triggering cell injury, apoptosis, and/or necrosis [1, 26]. The present study showed that the main cause of cell injury or death under the chronic hypoxia condition was associated with mitochondrial dysfunction with accompanying LDH release and cellular ATP depletion, which is consistent with previous reports [5, 27]. The protective actions of tanshinones against chronic hypoxia-induced cell injury indicate that these compounds may conserve mitochondrial function. Previous studies have reported cardioprotective effects of TIIA on H_2O_2-induced cell injury [28] and doxorubicin-induced cell apoptosis [29] in neonatal cardiomyocytes by protecting DNA integrity mitochondrial proteins and reducing intercellular ROS production. Similarly, the antiapoptotic effect of CT has been shown previously with actions of preventing mitochondrial-dependent apoptosis in nitric oxide induced neuroblastoma cells apoptosis [30]. The increase of ATP level by TIIA may be related to its protection of mitochondrial electron transport chain (ETC) function as ATP is mostly generated by oxidative phosphorylation, a process translocating protons by complex I/III/IV and subsequently uptake of the protons by ATP synthase accompanying the synthesis of ATP, in mitochondria ETC [31]. The finding that CT was less effective than TIIA in restoring cellular ATP contents may be related to a previous observation that CT enhanced AMP-activated protein kinase (AMPK) [32], as it has been known that AMPK is associated with energy homeostasis, mitochondrial function, and cell survival [33]. It will be interesting to investigate further the effects of tanshinones on AMPK activity in chronic hypoxia condition.

Previous studies on hypoxia-induced ROS generation have shown conflicting results. This could be due to a confusion of ROS examined (cytosolic and mitochondria). It has been shown that hypoxia decreased cytosolic superoxide generation but increased mitochondrial superoxide generation [34]. Consistent with this, a significant decrease in cytosolic superoxide generation, but increase in mitochondrial superoxide generation after hypoxia, was observed in the present study. Decreased cytosolic superoxide generation may be associated with lower oxygen level during hypoxia condition and/or decreased NADPH oxidase activity [2, 21]. Interestingly, the activity of cytosolic antioxidant enzyme superoxide dismutase was not significantly changed after hypoxia, indicating that this cytosolic antioxidant enzyme may not play a major role in cell injury and death pathway during chronic hypoxia. On the other hand, there was a significant increase of intracellular hydrogen peroxide/peroxynitrite production, as indicated by DCFH-DA fluorescence probe, suggesting that a mitochondrial-derived ROS component may be involved as shown by the effects of complex I and III inhibitors (rotenone and antimycin A).

The present result is in line with a previous report showing that increased NO and $ONOO^-$ generations resulted in enhanced mitochondrial superoxide generation by blocking mitochondrial electron transport chain [35]. NO can act as a physiological regulator of respiration by reversibly inhibiting cytochrome c-oxidase at the low concentrations (nanomolar). However, at higher concentrations NO can oxidize ubiquinol of ubiquinol-cytochrome c-reductase (Complex III) to increase unstable ubisemiquinone, which produces superoxide by univalent electron transfer to O_2 [36]. Additionally, exposure to higher concentrations of NO can increase peroxynitrite formation which causes an inhibition of mitochondrial respiration at multiple sites (complex I, complex II, cytochrome c oxidase, the ATP synthase, aconitase, MnSOD, and creatine kinase) [37]. This implies that restoring electron transport chain function by reducing NO production, in addition to antioxidant enzyme activity, may help to reduce mitochondrial superoxide production during chronic hypoxia condition.

Since cytosolic ROS may not play major role in hypoxia-induced cell damages, it is not surprising to observe the lack of effect of TIIA and CT in intracellular ROS and NADPH oxidase activity. The important finding in this study is that the mitochondria superoxide generation was increased by hypoxia. This increase was significantly inhibited by TIIA and CT treatments, indicating that mitochondrial ROS plays a major role in cell damage-induced hypoxia. Interestingly, NO synthase inhibitor L-NAME also significantly inhibited mitochondrial superoxide generation, which suggests that endogenous NO may regulate the ROS production in hypoxia condition. It is possible that ROS may be generated from mitochondrial nitric oxide syntheses which may be uncoupled under hypoxic condition [38]. The observation of increase in mitochondrial superoxide dismutase activity and decrease in intracellular NO level by TIIA and CT in the present study is consistent with previous studies showing actions of tanshinones on regulating NO level and SOD activity in H_2O_2-induced cell injury and inflammation-induced cell death in endothelial cells [16, 39].

Interestingly, TIIA and CT significantly decreased intracellular NO and mitochondrial superoxide generations, but not peroxynitrite/hydrogen peroxide levels. Previous studies using the same DCF-DH probe found that TIIA significantly inhibited ROS generation induced by doxorubicin [14, 15]. However, it is not clear if the ROS in those studies is peroxynitrite/hydrogen peroxide specific as no specific inhibitors were used to validate the species of ROS observed. One possible explanation is that ROS labelled with DCF-DH may mainly be peroxynitrite as specific peroxynitrite inhibitor MnTBAP markedly reduced ROS generation (about 70%) in the present study. Thus, TIIA and CT may have a capacity to direct ROS production from peroxynitrite to hydrogen peroxide, as both compounds showed no significant effects on peroxynitrite/hydrogen peroxide production; even they significantly reduced NO and superoxide productions which theoretically should reduce peroxynitrite formation. Partial supporting evidence is that TIIA and CT increased mitochondrial SOD activity, which may result in increase in hydrogen peroxide formation. Further study is required to confirm this hypothesis. The effects of tanshinones on other antioxidant enzymes such as glutathione peroxidase and catalyse were not examined in this study which also requires further investigation.

Changes in intracellular calcium level during hypoxia are important in mitochondrial functions, especially in mitochondrial membrane permeability transition pore opening [40]. Decreased intracellular ATP by hypoxia can decrease cellular pH by glycolysis activation and this elicits imbalancing in intracellular ion exchange and, subsequently, increases in intracellular calcium level [41]. This is consistent with the present finding showing that hypoxia-induced ATP depletion was accompanied with increased intracellular calcium level. The finding of inhibition of intracellular calcium by TIIA and CT is consistent with previous reports in neonatal cardiomyocytes and rat coronary artery [42, 43]. The increased intracellular calcium level is likely due to ATP depletion caused by hypoxia. Thus, it is possible that TIIA may regulate intracellular calcium through affecting ATP level. However, CT reduced intracellular calcium without affecting ATP levels. This suggests that other mechanisms such as endoplasmic reticulum-related stress, which also regulate intracellular calcium production [44], may also be involved. Additionally, studies have shown that increased intracellular calcium may increase mitochondrial ROS production [45, 46]. Therefore, tanshinones may have multiple targets of reducing mitochondrial ROS production.

In summary, the findings from the present study indicate that TIIA and CT protect H9c2 cells via preserving mitochondria function by reducing excess production of mitochondrial superoxide, SOD activity, intracellular NO, and calcium levels and restoring cellular ATP contents. These molecular mechanisms may be involved in the cardioprotective actions of TIIA and CT in hypoxic injuries.

Acknowledgments

This work was supported by grants from The National Institute of Complementary Medicine and RMIT University. H.-J. Jin was supported by a RMIT University International Research Scholarship.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 340267, 10 pages
http://dx.doi.org/10.1155/2013/340267

Research Article

Inflammatory Regulation Effect and Action Mechanism of Anti-Inflammatory Effective Parts of Housefly (*Musca domestica*) Larvae on Atherosclerosis

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Received 18 November 2012; Accepted 27 January 2013

Academic Editor: Kashmira Nanji

The protein-enriched extracts of housefly larvae were segregated by gel-filtration chromatography (GFC) and then anti-inflammatory activity screening in RAW264.7 (induced by LPS) was carried out. After acquire the anti-inflammatory effective parts, its anti-atherosclerotic properties *in vivo* were then evaluated. Results showed that the anti-inflammatory effective parts of housefly larvae were low-molecular-weight parts. After treated with the effective parts oral gavaged for 4 weeks, the atherosclerotic lesions of the mouse were significantly decreased. The inflammatory and lipid parameters were also reduced (except HDL which was increased). Western blot analysis demonstrated that the effective parts exerted potent inhibitory effect on expression of p65 in nucleus and cytoplasm. The results of immunofluorescence microscopy analysis also showed that the expressions of p65 both in cytoplasm and nucleus were significantly reduced. The hypothesis that the anti-inflammatory effective parts of housefly larvae possessed anti-atherosclerosis activity in mouse and the possible mechanism could be associated with the inhibition of expression and nuclear transfer of NF-κB p65 could be derived.

1. Introduction

Insects and insect derivatives have been widely used in folk medicine across the world since ancient times [1]. The use of insects is particularly common in China, and in many other countries including Brazil, Mexico, India, Africa, and South Korea [2]. At present, there are approximately 300 medicinal insects distributed in 70 genera, 63 families, and 14 orders. An estimated 1700 traditional Chinese medicine prescriptions include medicinal insects or insect-derived crude drugs [3]. The housefly (*Musca domestica*) belongs to the order of Diptera. The housefly larvae have been used clinically to cure malnutritional stagnation, decubital necrosis, osteomyelitis, ecthyma, and lip boil which was described by Li Shizhen (1518–1593 AD) in the pharmaceutical text of *Compendium of Materia Medica* [4]. Recently, effects of antioxidant [5], antibacterial, and *in vitro* antitumor properties of the protein extracts of housefly larvae have been reported [6, 7]. Additionally, the oil of housefly larva could be used as a natural ointment to heal burn wound [8]. In our earlier studies, we studied whether the protein-enriched fraction/extracts of housefly larva could potently inhibit multiple proinflammatory responses in atherosclerotic lesions. The results showed that the concentrations of TC, TG, and LDL were lower in the extracts treatment group than in the negative control animal which was treated with cholesterol-enriched diet and LPS (intraperitoneal injection). Also the expressions of TNF-α, IL-1α, and MCP-1 were also decreased after treated with the extracts *in vitro* [9]. However, the composition of the protein-enriched extracts of housefly larva is very complex and also the specific antiatherosclerotic mechanism is not clear. So the objective of this study was to screen

the anti-inflammatory effective parts of housefly larvae and to investigate inflammatory regulation effect and action mechanism of those parts on atherosclerosis. In the present study, the anti-inflammatory effective parts of housefly larvae were obtained by gel-filtration chromatography and (macrophages induced by LPS) anti-inflammatory activity screening *in vitro*. Then the antiatherosclerotic effects in mouse, changes of inflammation-related factors, and expression and nuclear transfer of NF-κB p65 in macrophages were also examined.

2. Materials and Methods

2.1. Preparation of Extracts of Housefly Larvae. After the protein-enriched fraction/extracts of housefly larva were harvested [9], the extracts were redissolved in deionized-distilled water (DDW), and the supernatant was collected by centrifuge at 3000 r/min, 4°C for 10 minutes, and concentrated by solid polyethylene glycol. The gel filtration material used in the experiment was Sephadex G-75. Sephadex G-75 exposed in 0.05 mol/L Tris buffer (pH 7.5) was incubated in boiling water for 30 min to the column (2.5 cm × 100 cm). The column was equilibrated with 0.05 mol/L Tris buffer (pH 7.5) at a constant flow rate of 0.25 mL/min. The concentrated extracts were applied to the column and eluted with the same buffer. 1.5 mL fractions were collected at a flow rate of 0.25 mL/min. Protein profile was monitored by measuring the absorbance at 280 nm. The fractions were combined at the peaks of elution curves and dialyzed overnight against 0.02 mol/L Tris buffer (pH 8.5), and then the vacuum freeze-drying was used to remove the solvents for the next step.

2.2. Screening of Housefly Larvae Extracts for Anti-Inflammatory Activity

2.2.1. Cell Culture. The mouse macrophage cell line, RAW264.7, was purchased from Cell Bank, Center of Experimental Animals, Sun Yat-Sen University. Guangzhou, China. RAW264.7 cells grown in Dulbecco's modified Eagle's medium (DMEM) were supplemented with 10% (v/v) endotoxin-free fetal calf serum, 2 mmol glutamine/L, and 100 U/mL penicillin/streptomycin at 37°C in atmosphere of 10% CO_2 and 95% relative humidity.

2.2.2. Cell Activation and Treatment. 1×10^5 RAW264.7 cells were plated in 24-well plates, incubated for 24 h and pretreated with the indicated concentrations of the extracts of housefly larvae (40 μg/mL), or a vehicle for another 2 h, then challenged with LPS (1 μg/mL) for an additional 18 h.

2.2.3. Measurement of TNF-α and IL-6. The culture supernatants were collected. Supernatant levels of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in cultured RAW cells were measured by ELISA according to the manufacturer's instructions. The requested solutions were provided with the ELISA compact kits and additional toolkits. Within 30 minutes, optical density of each well was determined by a microplate reader setting at 450 nm, and the correction wavelength setting at 570 nm.

2.2.4. Evaluation of Cell Morphology. At the indicated times after treatment of different extracts of housefly larvae, the cell-culture medium were washed with PBS, and changes in cell morphology were examined using a microscope with computer imaging system analysis. Cells were counted in four fields of vision, and the total number was determined. Then the change of cellular morphology was observed, and the number of pseudopodia (foot like projections) of each cell was counted.

2.3. Evaluation of Antiatherosclerosis Properties

2.3.1. Animals. Healthy female C57BL/6 mice at age of 6 weeks old were purchased from Medical Laboratory Animal Center (Guangzhou, Guangdong, China). All animals received humane care and the present study was carried out in accordance with the guidelines for the humane treatment of animals set by the Association of Laboratory Animal center at Guangdong Pharmaceutical University, Guangzhou, China. All mice were housed in individual cages respectively and kept for at least 1 week with commercial solid diet under controlled conditions (25 ± 2°C, 12 h light/dark cycle, 55 ± 5% humidity) with free access to food and water before treatment.

2.3.2. Treatment. After acclimatization, mice were divided into 2 groups: natural control group ($n = 6$) and model group ($n = 12$). Mice in natural control group were treated with standard diet, whereas mice in model group were treated with LPS (2 mg/kg, i.v.) three times a week, and atherogenic solution (Fat-soluble solution was composed of 20% cholesterol and 80% peanut oil, and water-soluble solution was composed of 10% glucose, 10% sodium cholate, and 80% distilled water, gavage) to induce atherosclerosis. All animals had free access to food and water. After the model was established, all mice in model group were divided into anti-inflammatory effective parts of housefly larvae treated group (200 mg/kg, gavage; $n = 6$) and negative control group (distilled water equivalent to the same dose, gavage; $n = 6$) and received the treatment once a day for 4 weeks.

2.3.3. Measurement of Blood Biochemical Variables. After 4 weeks, all mice were killed, and their serum was analyzed for elevation of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), and high density lipoprotein cholesterol (HDL-C). Serum lipids were measured using commercially available kits. Total cholesterol, HDL-C, and triglycerides were measured by standardized automated methods, and LDL-C was calculated by the Friedewald equation: LDL-C = TC − HDL-C − TG/5. The levels of L-6 and TNF-α were also determined (refer to Section 2.2.3).

2.3.4. Histology and Morphometric Analysis. A section of the thoracic aorta and myocardial tissues were fixed in 100 mL/L buffered formalin for 24 hours. The tissue was embedded in paraffin, and a 4 μm section was examined through hematoxylin and eosin (HE) staining. Then the histological

slides were imaged under bright field using a digital color CCD camera.

2.4. Analysis of Expression and Nuclear Transfer of Nuclear Factor κB (NF-κB) p65

2.4.1. Cell Culture and Treatment. The RAW264.7 cells were plated in 24-well plates, incubated for 24 h and pretreated with the extracts of housefly larvae (40 μg/mL), then challenged with LPS (1 mg/mL) for additional 18 h.

2.4.2. Western Blot Analysis of Cytoplasmic and Nuclear Protein Extracts from RAW264.7 Cells. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit. Protein contents were measured taking bovine serum albumin as a criterion. Protein concentration was determined by a commercial protein assay reagent. Proteins of cytoplasmic and nuclear extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All gels were electrically transferred to PVDF membranes before being blocked for 1 h at RT in 0.01% TBS/Tween containing 3% nonfat milk powder (TBS/Tween with nonfat milk). Membranes were incubated with primary antibodies overnight at 4°C in TBS/Tween with nonfat milk and then washed three times for 5 min in TBS/Tween. Visualization of proteins was performed via the addition of a secondary antibody conjugated to horseradish peroxidase (HRP), which was incubated for 1 h at RT in TBS/Tween with nonfat milk. Membranes were washed three times for 10 min in TBS/Tween. Then protein-antibody complexes were visualized using Vectastain ABC and TMB (3,3′,5,5′-tetramethylbenzidine) substrate kits. Protein bands and NF-κB p65 (65 kDa) and β-actin (42 KD) scanning and quantification of signal intensities were performed using a Bio-Rad Gel Doc XR densitometer with Quantity One software. Antibodies were used in the following dilutions: rabbit anti-mouse NF-κB p65 (dilution 1 : 500), anti-mouse β-actin (dilution 1 : 500). All secondary antibodies were used at the following dilutions: goat anti-rabbit IgG, 1 : 1000, goat anti-mouse IgG, 1 : 1000.

2.4.3. Immunofluorescence-Microscopy Analysis of Nuclear Transfer NF-κB p65. Briefly, cells were fixed in 3.7% (wt/vol) formaldehyde in PBS for 10 min and permeated with acetone-methanol (1 : 1, vol/vol) at −20°C for 15 min. Following a 30 min blocking in PBS with 3% (wt/vol) bovine, serum albumin samples were exposed to rabbit polyclonal anti-p65 (PE-conjugated). Antibodies were used at a 1 : 100 (vol/vol) diluted in blocking solution for 1 h at 20°C, washed with PBS, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (5 min) for the identification of nuclei. Coverslips were finally mounted on mounting medium and fluorescent images were taken under the Laser Confocal Microscope.

2.5. Statistical Analysis. All data were expressed as the mean ± standard error of the mean (SEM). Each measurement was performed at least in triplicate. The Student's t-test was applied to compare means of data of two independent samples. For multiple comparisons, one-way analysis of variance (ANOVA) was performed followed by the Dunnett's T 3 (i.e., equal variance was assumed) or Duncan's t tests (i.e., equal variance was not assumed) where appropriate. Differences were considered statistically significant when $P < 0.05$. All statistical analysis was performed with SPSS statistical software (version 13.0 for Windows).

3. Results

3.1. Chromatographic Behavior of Housefly Larvae Extracts. Gel chromatography with Sephadex G-75 (separated molecules with molecular weights from 3,000 to 70,000) was used for segregation and purification of the extracts of housefly larvae. According to the basic principles of chromatography, the small molecules entering into the interior of the gel should be eluted out of the system. Molecular weight distributions and elution curve were shown in Figure 1. The active fractions of major peaks were pooled, concentrated, and loaded according to the molecular weight (MW) which were recovered in three main fractions of the void volume (part I, high MW; part II, middle MW; part III, low MW). The percentage of each part was also shown.

3.2. Anti-Inflammation Activity Screening of Gel-Filtration Chromatography Fractions of Housefly Larvae Extracts. The concentrations of TNF-α and IL-6 in the RAW264.7 cell supernatants were measured by ELISA. RAW264.7 cells treated with LPS alone resulted in significant increases in cytokine production compared to the control group. Compared with the LPS-treated group, part I and part II treated group, the levels of TNF-α and IL-6 were both significantly decreased in part III treated group ($P < 0.01$ for all). Although the cytokines inpart II treated group also decreased compared with that in LPS-treated group, the decrease was not as significant as that ofpart III treated group (Table 1).

The cell morphology of the macrophage RAW264.7 cells treated with different fractions of housefly larvae extracts in the presence or absence of LPS was presented in Figure 2. In the non-LPS-stimulated cells, the cell morphology generally showed a round form, whereas in the LPS-activated RAW264.7 cells, the cell morphology changed into an irregular form with pseudopodia developing rapidly. The cotreatment of LPS with part III inhibited the cell spread and formation of pseudopodia by suppressing cell differentiation. But it was not evident in the other two groups.

The above observations indicate that compared with other fractions (part I and II), part III had more significant anti-inflammatory activity in LPS-stimulated RAW264.7 cells. In general, these results support the hypothesis that part III of housefly larvae extracts was the anti-inflammatory effective parts.

3.3. Antiatherosclerosis Properties of the Anti-Inflammatory Effective Parts of Housefly Larvae in Mouse. After mice were treated with LPS (i.v.) and atherogenic solution (gavage)

Table 1: Effect of different gel-filtration chromatography fractions of housefly larvae extracts on the secretion of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. (pg/mL, $n = 6$).

	LPS−	LPS+	LPS+ part I	LPS+ part II	LPS+ part III
TNF-α	256.5 ± 8.7	1847.9 ± 27.2	1795.5 ± 27.1	1579.0 ± 32.2**	665.1 ± 12.9**##
IL-6	82.8 ± 4.4	632.5 ± 8.2	624.7 ± 4.8	562.3 ± 11.6**	131.7 ± 5.9**##

** $P < 0.01$ versus LPS group, ## $P < 0.01$ versus part II group.

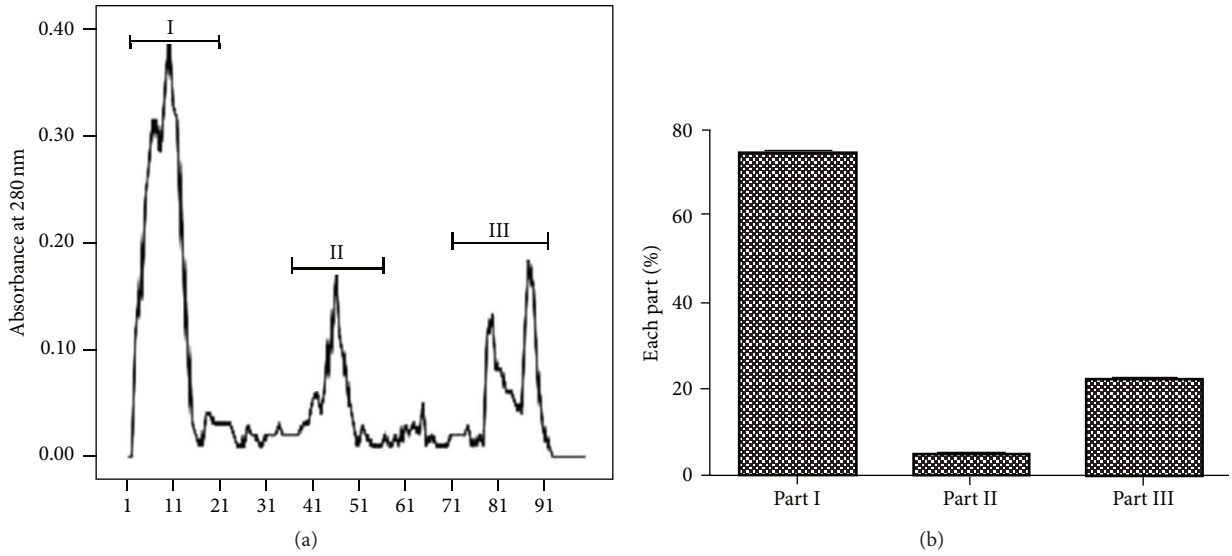

Figure 1: Gel filtration profile (Sephadex G-75) of housefly larvae extracts and percentage of each part.

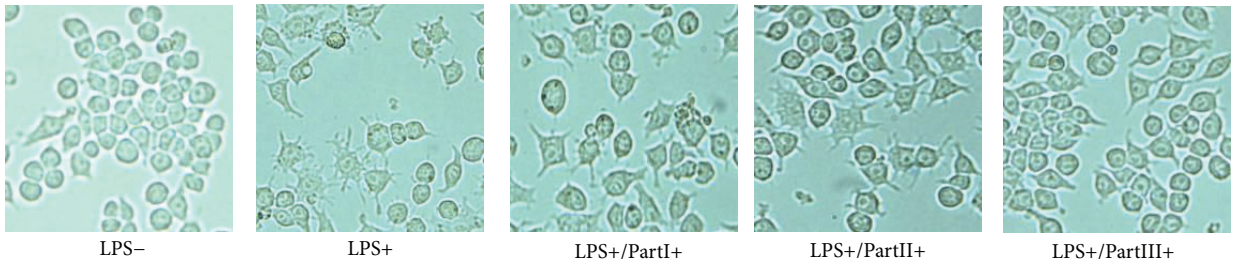

Figure 2: Morphological change in macrophageRAW264.7 cells after treated with/without different parts of extracts of housefly larvae (×400).

for 8 weeks, the histology and morphometric of thoracic aorta and myocardial tissues (HE staining) and the levels of TC, TG, HDL, LDL, TNF-α, and IL-6 in serum were analyzed. Compared with the nature control group, the result of HE staining showed that the aortic walls were thickened (Figure 3), and the myocardial cells became hypertrophic and arranged loosely (Figure 3). Additionally mononuclear infiltrate both in thoracic aorta and myocardial tissues in the model group were observed (Figure 3). The TC and TG serum levels of the model group were significantly higher than those of the normal control group (TC: 3.33 ± 0.54 versus 1.31 ± 0.24, TG: 3.28 ± 0.45 versus 1.16 ± 0.31, $P < 0.01$, for all) (Figure 4). High fat solution reduced HDL-C level (1.13 ± 0.25 versus 0.68 ± 0.13, $P < 0.01$) and increased LDL-C level (1.42 ± 0.34 versus 3.49 ± 0.41, $P < 0.01$). The expression levels of TNF-α and IL-6 were higher in the model group (TNF-α: 46.55 ± 8.35 versus 257.32 ± 18.95, IL-6: 47.47 ± 7.48 versus 360.07 ± 19.47, $P < 0.01$, for all) (Figure 4).

After treated with or without the anti-inflammatory effective parts for 4 weeks, compared with the negative control group, the size of atherosclerotic lesions in aortas was reduced and the inflammatory infiltrates both in thoracic aorta and myocardial tissues were decreased in effective parts treated group (Figure 5). And also except HDL which was increased (0.93 ± 0.04 versus 0.74 ± 0.03, $P < 0.01$), the other inflammatory and lipid parameters associated with atherosclerosis were reduced (TNF-α: 126.32 ± 11.08 versus 275.26 ± 12.35, IL-6: 114.66 ± 7.98 versus 314.33 ± 15.68, TC: 3.52 ± 0.68 versus 6.12 ± 1.04, TG: 1.88 ± 0.42 versus 2.79 ± 0.56, LDL: 2.23 ± 0.25 versus 3.87 ± 0.63, $P < 0.01$, for all) (Figure 6).

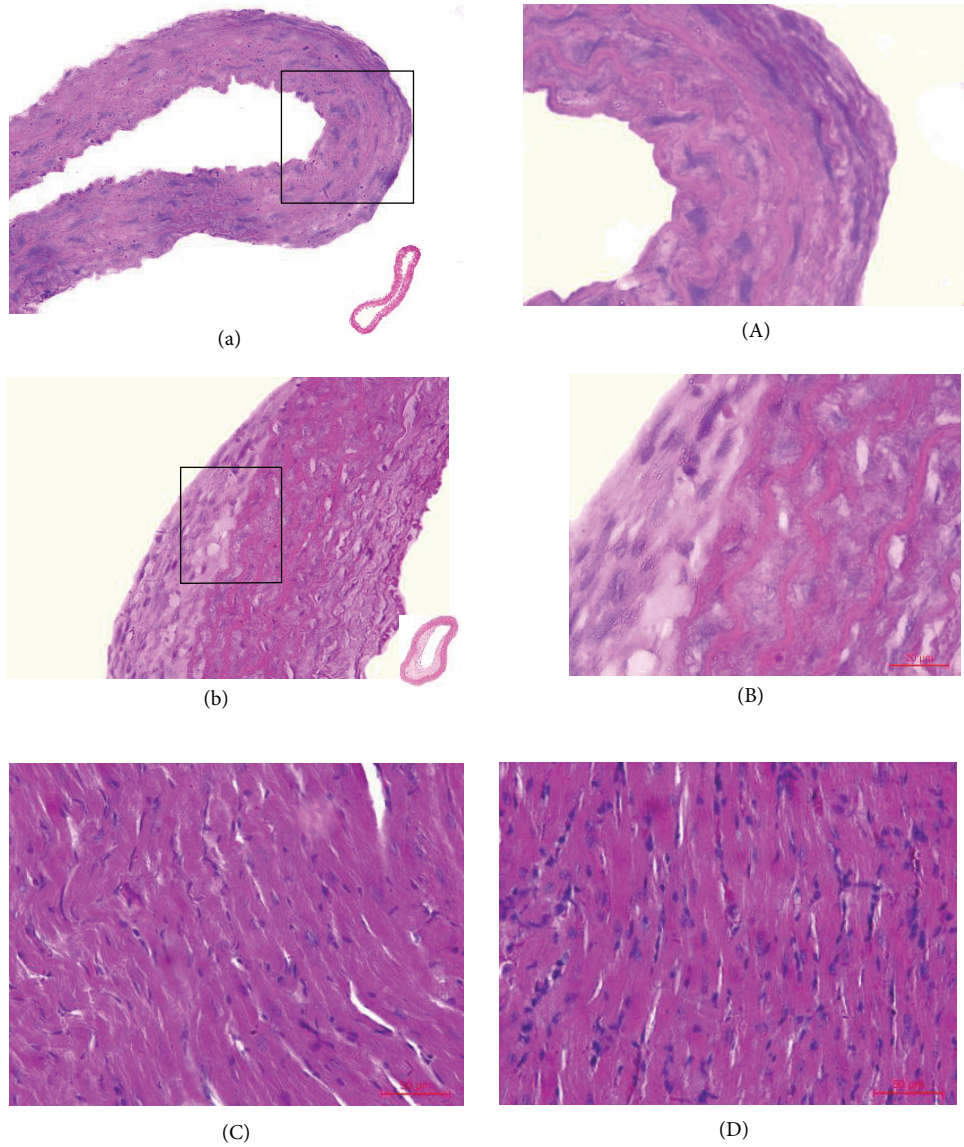

Figure 3: Histology and morphology of thoracic aorta and myocardial tissues treated with LPS (i.v.) and atherogenic solution (gavage) for 8 weeks. (a) Thoracic aorta in normal control group (×200). (b) Thoracic aorta in model group (×200). (A) Thoracic aorta in normal control group (×400). (B) Thoracic aorta in model group (×400). (C) Myocardial tissues in normal control group (×400). (D) Myocardial tissues in model group (×400).

3.4. Affect of Expression and Nuclear Transfer of NF-κB p65 of Effective Parts. RAW264.7 macrophages were treated with or without effective parts and incubated for 18 h with LPS. The protein expressions of NF-κB p65 in nucleus and cytoplasm were measured by western blot assay. It was demonstrated that LPS stimulation resulted in significant increase of p65 in nucleus and cytoplasm compared with that of natural control group ($P < 0.01$, Figure 7). Effective parts exerted potent inhibitory effect on expression of p65 in nucleus and cytoplasm compared with that in LPS-treated group ($P < 0.01$, Figure 7).

To confirm that effective parts inhibit LPS-induced nuclear translocation of NF-κB p65 protein in RAW264.7 macrophage, confocal microscopy was used to visualise the expression of p65 in cytoplasm and nucleus (Figure 8). The results also showed that the expressions of p65 both in cytoplasm and nucleus were reduced significantly (red fluorescence intensity declined, $P < 0.01$, data not show) compared to those in the LPS-treated group.

4. Discussion

Traditional medicines such as traditional Chinese medicine use a great array of insects and their products as drugs. Insects "are a kind of large, unexplored, and unexploited sources containing useful compounds for modern medicine" [10]. In china, housefly (*Musca domestica*) larvae have been used clinically to cure inflammation and inflammation-related

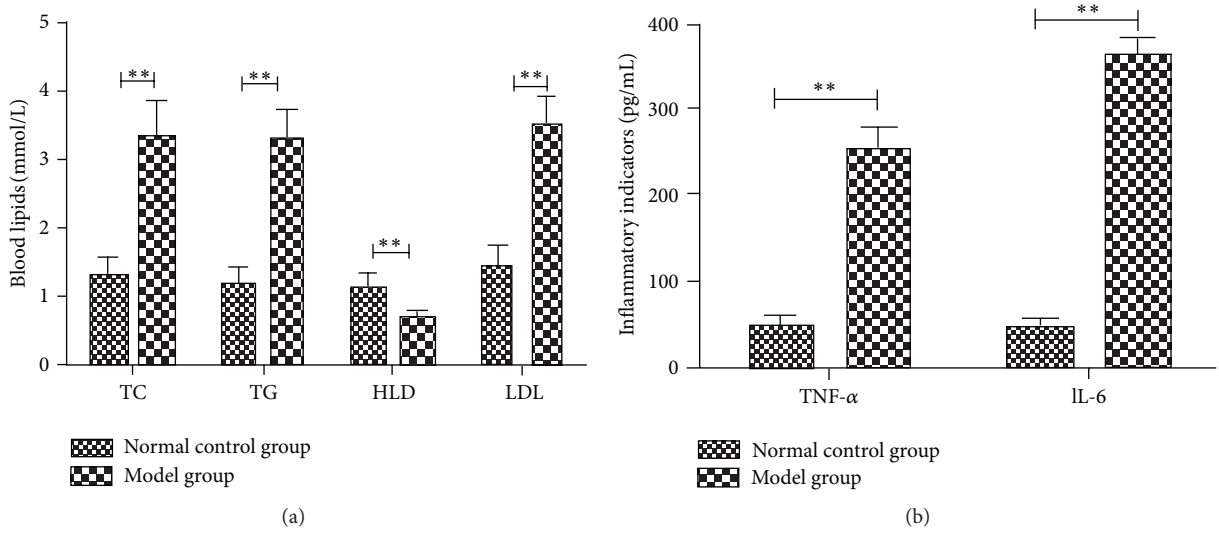

Figure 4: Blood biochemical variables in mice treated with LPS (i.v.) and atherogenic solution (gavage) for 8 weeks. $^{**}P < 0.01$ versus normal control group.

diseases, such as osteomyelitis, decubital necrosis, lip boil, and ecthyma ever since Ming Dynasty (1368 A. D) [4]. During the past decade, due to the antibacterial activity of housefly larvae and its products, they have been used mainly in preservation [11], environmental protection [12], and inhibition of multiresistant bacteria [13]. In recent years, several reports suggested that atherosclerosis is a chronic inflammatory disease [14, 15] that can be accelerated by microbial infection in its early phase [16, 17]. Based on the above information, the antiatherosclerotic effect of the protein-enriched extracts of housefly larvae had been previously studied. On the basis of preliminary studies, the present study tested the effect of anti-inflammatory effective parts on atherosclerosis *in vivo*, and explored the anti-inflammatory mechanism *in vitro*.

In the present work, Gel-filtration chromatography (GFC) was used for proteins segregation in order of molecular size: large to small, which is similar to the method used by purification of a specific β-glucosidase from the digestive fluid of larvae of palm weevil (*Rhynchophorus palmarum*) [18], and small cationic protein from Tobacco Hornworm *(Manduca sexta)* [19]. Compared with other methods such as organic solvent precipitation [20], salting out precipitation [21], and high pressure/performance liquid chromatography (HPLC) [22], gel-filtration was the most promising method with the advantages of high specificity and efficiency. Gel filtration was carried out with fine grade Sephadex G-75 which had optimum range of fractionation (3,000–70,000). The crude protein-enriched extracts of housefly larvae were tested by SDS-page in previous studies which showed that there were three parts of proteins with similar weight of molecule whichwas consistent with the results of the present study. In the present study, three main parts of extracts were obtained based on thedifferent molecular weights. So in the next step, the anti-inflammatory activity screening of all the three main parts of extracts was carried out *in vitro*.

The stimulation of macrophages induced by LPS is a useful protocol *in vitro* screening to identify new anti-inflammatory compounds [23]. The release of TNF-α and IL-6 could be used as indicators for anti-inflammatory activity [24]. In this study, LPS-stimulated RAW264.7 macrophages were used and the levels of TNF-α and IL-6 were investigated. The results of anti-inflammatory activity showed that the part III (small molecular weight) could reduce pseudopodia formation and the levels of TNF-α and IL-6 significantly. So this part was considered as the anti-inflammatory effective parts of housefly larvae.

With these *in vitro* results, the antiatherosclerotic properties in mouse of the anti-inflammatory effective parts of housefly larvae were explored. The atherosclerosis model was established by LPS (i.v.) and atherogenic solution (gavage) for 8 weeks. Although most mouse strains are highly resistant to atherosclerosis, when the atherosclerosis was induced by LPS and atherogenic diet for several weeks, the animal model could be established [25]. In the present study, the atherogenic diet was instead by atherogenic solution to guarantee that every animal could intake the same amount of cholesterol and fat and ensure the atherosclerotic model could be built quickly. Then the atherosclerotic mice were treated with the anti-inflammatory effective parts for 4 weeks by oral gavage. The hypothesis that the anti-inflammatory effective parts of housefly larvae had the effect of antiatherosclerosis *in vivo* was confirmed. The small molecular weight parts were initially postulated to be as antimicrobial peptides [26]. Although the present study was not aimed at optimizing the metabolic stability, approaches were available for the medicinal chemist to increase the metabolic resistance against degradation as described recently [27]. So the effect of anti-atherosclerosis *in vivo* of effective parts could be explained.

Today, atherosclerosis is recognized as a complex disease with serious inflammation. Consistent with its key role

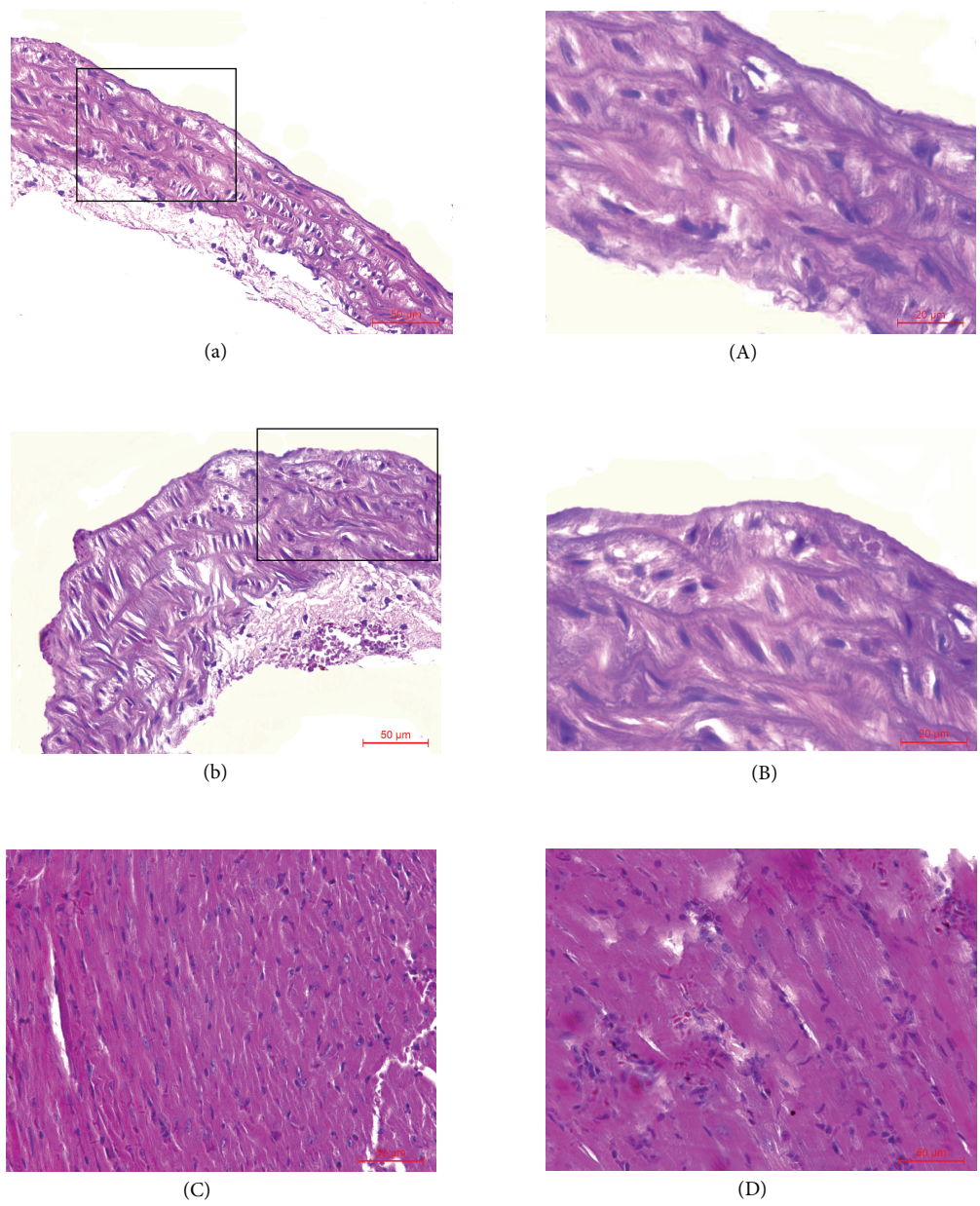

Figure 5: Histological and morphometric observation of thoracic aorta and myocardial tissues from atherosclerotic mice treated with anti-inflammatory effective parts of housefly larvae. (a) Thoracic aorta in anti-inflammatory effective parts of housefly larvae treatment group (×200). (b) Thoracic aorta in negative control group (×200). (A) Thoracic aorta in anti-inflammatory effective parts of housefly larvae treatment group (×400). (B) Thoracic aorta in negative control group (×400). (C) Myocardial tissues in anti-inflammatory effective parts of housefly larvae treatment group (×400). (D) Myocardial tissues in negative control group (×400).

in coordinating inflammatory responses, numerous studies have suggested that the transcription factor nuclear factor κB (NF-κB) is one of the most important proinflammatory pathways in atherogenesis [28, 29]. It was reported that TNF-α expression was mainly regulated by the transcription factor NF-κB [30]. In the present work, the level of TNF-α was decreased *in vitro* and *in vivo*, suggesting that regulation of NF-κB signal pathway might be one of the possible mechanisms underlying anti-inflammatory effect of effective parts. In order to examine this possibility, effects on expression and nuclear transfer of NF-κB p65 of anti-inflammatory effective parts of housefly larvae were detected. The results indicated that the expression and nuclear transfer of NF-κB p65 were inhibited. In fact, entomic NF-κB activation in response to immune challenge was homologous or related to molecules found in mammalian signal pathways activated during innate immune defence. Complex signal pathways regulate the innate immune system of insects, with NF-κB transcription factors playing a key role in the activation of antimicrobial peptides and other immune genes [31].

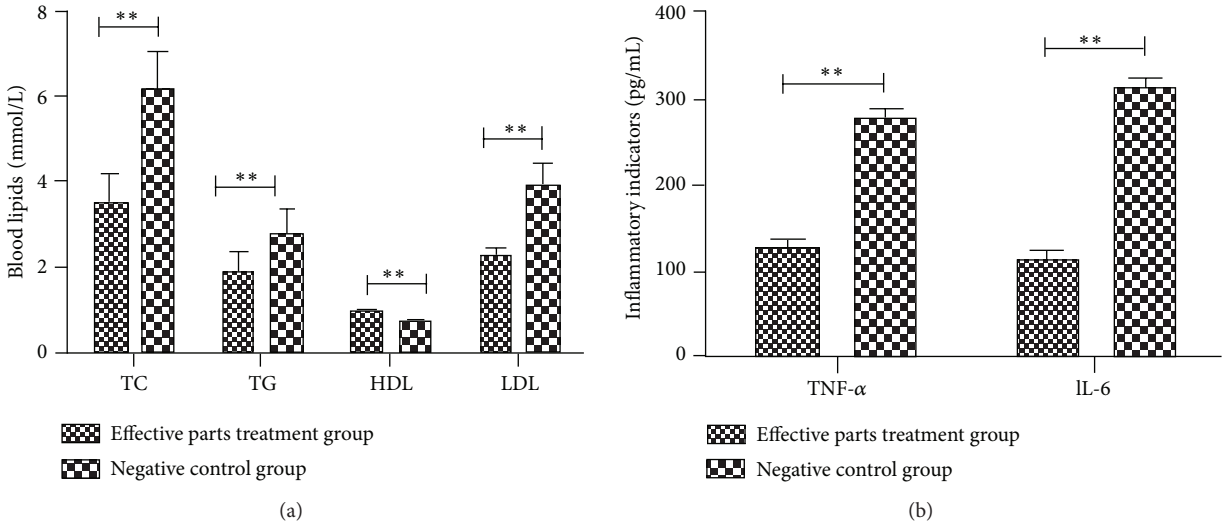

Figure 6: Blood biochemical variables in anti-inflammatory effective parts of housefly larvae treated group and negative control group. $^{**}P < 0.01$ versus negative control group.

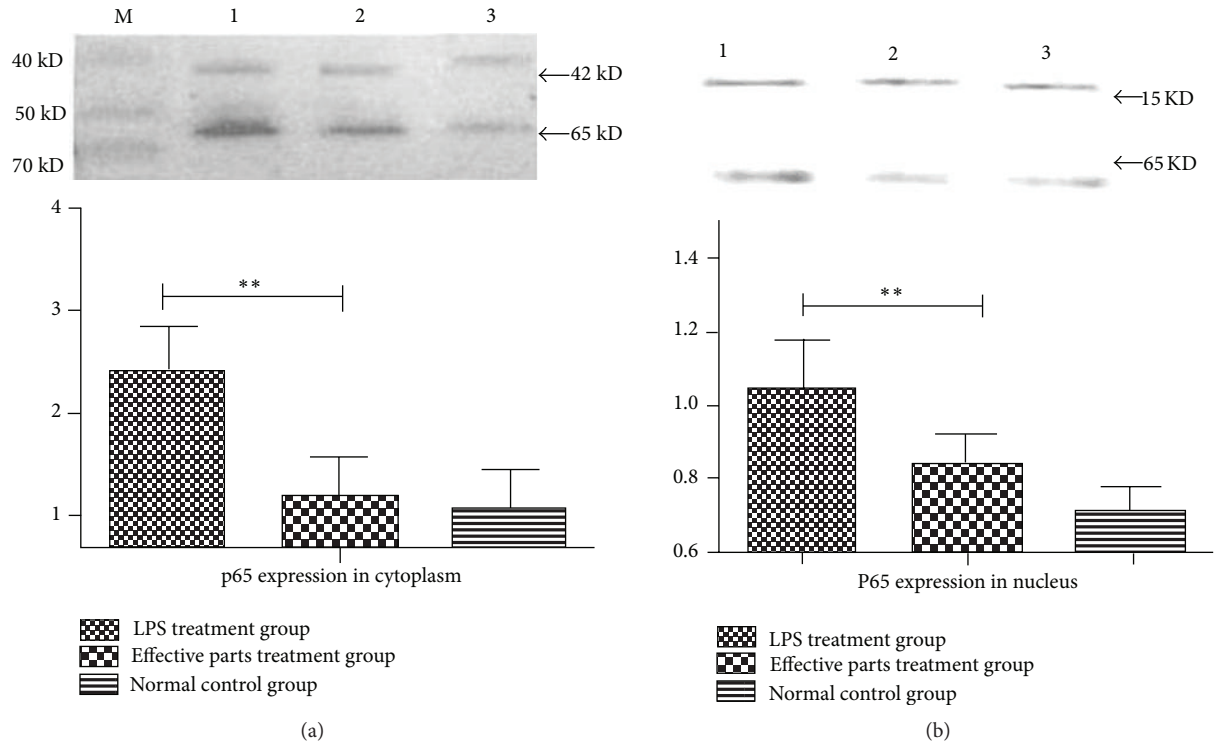

Figure 7: Effects of anti-inflammatory effective parts of housefly larvae on protein expression of NF-κB p65 in LPS-induced RAW264.7 macrophages measured by Western blot. (a) Anti-inflammatory effective parts of housefly larvae decreased LPS-induced p65 expression in cytoplasm. β-actin was set as control normalization. Lane M: protein mark, lane 1: LPS-treated group, lane 2: anti-inflammatory effective parts of housefly larvae treated group, lane 3: natural control group. (b) Anti-inflammatory effective parts of housefly larvae inhibited LPS-induced p65 expression in nucleus. Histon H 3.1 was set as control normalization. Lane 1: LPS-treated group, lane 2: anti-inflammatory effective parts treatedgroup, lane 3: natural control group. $^{**}P < 0.05$ versus LPS-treated group.

In a further study, the composition and structure of the anti-inflammatory effective parts of housefly larvae will be explored by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), sequencing, and bioinformatics analysis.

5. Conclusion

Anti-inflammatory effective parts of housefly larvae have the function of antiatherosclerosis in the mouse. There is a possibility that the mechanism could be associated with

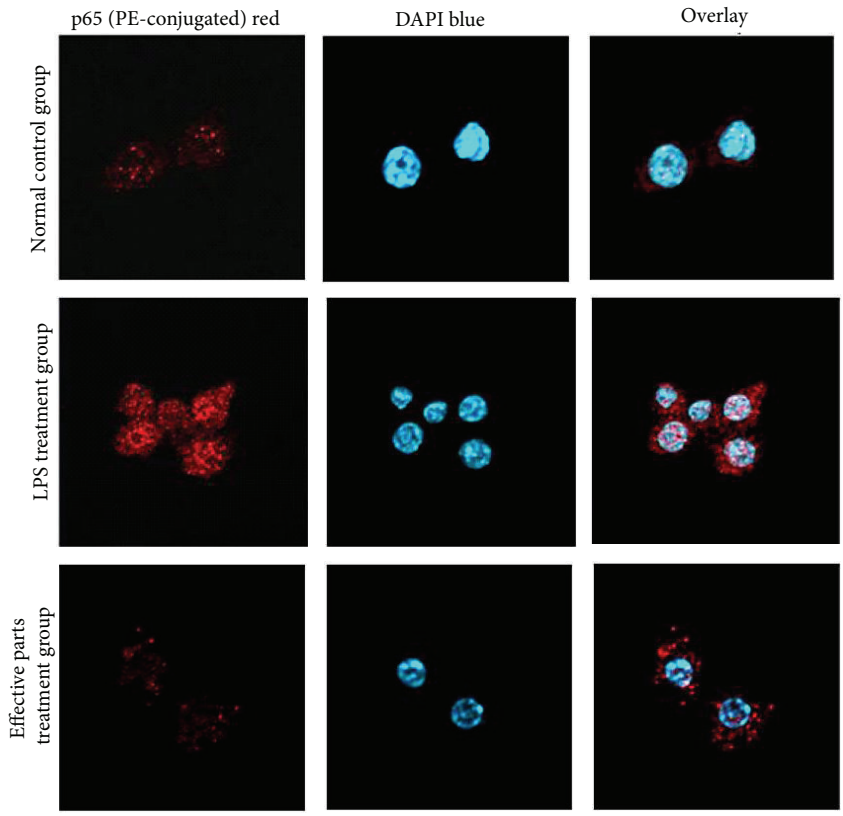

Figure 8: Immunofluorescent images of NF-κB p65 under the laser scanning confocal microscopy in anti-inflammatory effective parts of housefly larvae treated group, negative control group, and normal control group. (1000x). NF-κB p65 was stained with PE (red) and nuclei were stained with DAPI (blue).

the inhibition of expression and nuclear transfer of NF-κB p65.

Authors' Contribution

F. J. Chu, X. B. Jin, and J. Y. Zhu equally contributed to this work.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This study was supported by Core Technology Research Projects of Strategic Emerging Industries, Guangdong Province, China (Grant no. 2012A080800016), Science and Technology Plan Projects, Panyu district, Guangzhou City, Guangdong Province, China (Grant no. 2010-Special-12-3), and National Natural Science Foundation of China (Grant no. 81274061).

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Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 912769, 8 pages
http://dx.doi.org/10.1155/2013/912769

Research Article

The Involvement of a Polyphenol-Rich Extract of Black Chokeberry in Oxidative Stress on Experimental Arterial Hypertension

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Received 1 September 2012; Accepted 29 January 2013

Academic Editor: Peng Nam Yeoh

The aim of this study is to characterize the content of *Aronia melanocarpa* Elliott (black chokeberry) extract and also to estimate the influence of polyphenolic compounds contained in chokeberries on oxidative stress, on an L-NAME-induced experimental model of arterial hypertension. The rat blood pressure values were recorded using a CODA Noninvasive Blood Pressure System. HPLC/DAD coupled with ElectroSpray Ionization-Mass Spectrometry allowed identification of five phenolic compounds in berries ethanolic extract as follows: chlorogenic acid, kuromanin, rutin, hyperoside, and quercetin. The serous activity of glutathione-peroxidase (GSH-Px) has significantly lower values in the hypertensive (AHT) group as compared to the group protected by polyphenols (AHT + P). The total antioxidant capacity (TAC) values are lower in the AHT group and they are significantly higher in the AHT + P group. All the measured blood pressure components revealed a biostatistically significant blood pressure drop between the AHT group and the AHT + P group. The results reveal the normalization of the reduced glutathion (GSH) concentration as well as a considerable reduction in the malondialdehyde (MDA) serum concentration in the AHT + P group. Ethanolic extract of black chokeberry fruits not only has a potential value as a prophylactic agent but also may function as a nutritional supplement in the management of arterial hypertension.

1. Introduction

Hypertension is a significant cardiovascular risk factor, associated to endothelial dysfunction and oxidative stress. The oxidative process participates in increasing systemic arterial pressure, reducing NO availability and vasodilation [1]. Oxidative stress is involved in remodelling the myocardial architecture and as a consequence, in the development of left ventricular hypertrophy [2, 3].

Assessment of antioxidant activities and lipid peroxidation byproducts in hypertensive subjects indicates an excessive amount of ROS and a reduction of antioxidant mechanism activity in both blood as well as in several other cellular systems, including not only vascular wall cells but also those found in circulating blood [4].

Dietary polyphenols are mostly derivatives and/or isomers of flavones, isoflavones, flavonols, catechins, and phenolic acids. *Aronia melanocarpa* Elliot (*Rosaceae*, black chokeberry) is a shrub native to North America. Its berries, which are rich in polyphenols, have been used by native Indians both as a remedy and as a food [5]. Anthocyanins (cyanidin glycosides), flavonoids (quercetin glycosides), chlorogenic acids, and proanthocyanidins are the main polyphenols identified in *Aronia* berries [6]. Several reports indicated that extracts from *Aronia* berries exhibited different biological effects both *in vitro* and *in vivo* (antioxidant, gastroprotective, hepatoprotective, and antiproliferative activities not only via antioxidant pathways, but also via impacting signal transduction/intracellular signalling cascades, impacting apoptosis, etc.) [7, 8].

The aim of this study is to characterize the content of *Aronia melanocarpa* extract and also to estimate the influence of polyphenolic compounds contained in chokeberries on oxidative stress, on an L-NAME induced experimental model of arterial hypertension.

2. Materials and Methods

The experimental study fulfils all the requirements of the guide regarding the use of laboratory animals and biological preparations issued by the International Society of Pain Study (IASP) and the European Council Committee (86/609/EEC). Also, the study was evaluated and accepted by the professional ethics committee of Grigore T. Popa University of Medicine and Pharmacy of Iasi (approval no. 9803/12.09.2006).

2.1. Preparation of Extract and Chemical Determinations

2.1.1. Chemicals. Folin-Ciocalteu's phenol reagent, chlorogenic acid, and rutin trihydrate were from Merck (Darmstadt, Germany). Quercetin dihydrate, hyperoside, and kuromanin chloride were from Carl Roth (Karlsruhe, Germany). Gallic acid, caffeic acid, and (+)-catechin hydrate were purchased from Sigma-Aldrich (Steinheim, Germany). Except for HPLC grade solvents, all other solvents and reagents were of analytical grade. Ultrapure water was obtained using a SG Water Ultra Clear TWF water purification system (Barsbüttel, Germany).

2.1.2. Plant Material. Ripe berries of *Aronia melanocarpa* Elliott (*Rosaceae*, black chokeberry) were sampled in Botanical Garden, Iasi, Romania. A herbarium voucher sample (AMF.09) is deposited in the Department of Pharmacognosy, School of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa," Iasi, Romania. The berries were shade-dried at room temperature for one week.

2.1.3. Extraction. Dried berries (100 g) were chopped into small pieces and extracted with 3 × 700 mL ethanol using a magnetic stirrer (FALC F30ST), each time for 3 h. The combined extracts were taken to dryness by evaporation under reduced pressure (BÜCHI R-210 Rotavapor, BÜCHI V-850 vacuum controller, and BÜCHI V-700 vacuum pump).

2.1.4. Total Phenolic Content. Total phenolics quantification was performed by Folin-Ciocalteu method [9]. Briefly, berries extract was mixed with 3.16 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent. After 5 min., 0.6 mL of 20% sodium carbonate were added. The absorbance was measured at 765 nm after 2 h of incubation at room temperature. A calibration curve was plotted using gallic acid as standard. The total phenolic content was expressed as mg gallic acid equivalents/g extract. Sample was assayed in triplicate and the results were given as the mean ± SD.

2.1.5. Total Anthocyanin Content. Anthocyanins quantification was performed according to a described procedure [10, 11]. Berries extract was mixed with methanol-hydrochloric acid (99 : 1, v : v) and kept at room temperature, in dark, for 2 h followed by centrifugation (1000 ×g, 15 min). Anthocyanin content in supernatant was measured both at 530 nm and 657 nm. Absorbance values were converted into anthocyanin concentration using an extinction coefficient of 31.6 M^{-1} cm^{-1}. The results were expressed as μmole anthocyanin/g extract. Sample was assayed in triplicate and the results were given as the mean ± SD.

2.1.6. HPLC/DAD/ESI-MS Analysis. (High-Performance Liquid Chromatography coupled with Diode Array Detection and ElectroSpray Ionization-Mass Spectrometry) was conducted on an Agilent 1200 Series HPLC system with a diode array detector coupled to an Agilent 6520 Accurate-Mass Q-TOF LC/MS system (Quadrupole Time-of Flight Liquid Chromatography/Mass Spectrometry) equipped with an ESI source. Separations were done on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, i.d. 5 μm). The mobile phase consisted of (A) water and acetic acid (99 : 1, v/v) and (B) acetonitrile and acetic acid (99 : 1, v/v). The elution profile was as follows: 0% B (0–3 min, isocratic); 0–7% B in A (3–5 min, linear gradient); 7% B in A (5–20 min, isocratic); 7–10% B in A (20–30 min, linear gradient); 10–15% B in A (30–35 min, linear gradient); 15% B in A (35–50 min, isocratic); 15–20% B in A (50–65 min, linear gradient); 20–30% B in A (65–80 min, linear gradient); 30–40% B in A (80–85 min, linear gradient); 40–100% B (85–100 min, linear gradient); 100% B (100–103 min, isocratic); 100–0% B in A (103–120 min, linear gradient). The flow rate was 0.4 mL/min. Volumes of 20 μL were injected. The compounds were monitored at 254, 280 and 320 nm; anthocyanins were monitored at 515 nm. Mass spectrometric detection was performed in the negative ion mode for nonanthocyanin polyphenols and in the positive mode for anthocyanins. The mass spectrometric conditions for negative and positive ion mode were as follows: drying gas (N_2) flow rate 7.0 L/min; drying gas temperature 220°C; nebuliser pressure 15 psig. In negative ion mode the capillary voltage was set to −4.2 kV and the skimmer voltage to −60 V. In positive ion mode the capillary voltage was set to 4.2 kV and the skimmer voltage to 60 V. A fragmentor voltage of 200 V was used in both modes. The full-scan mass spectra of the investigated compounds were acquired in the range 50–2000 m/z [6]. Data were collected and processed using a MassHunter Workstation software.

2.2. Animal Treatments and Biochemical Determinations. The dry polyphenol extract was diluted in DMSO, 100 mL polyphenolic solution containing 840 mg natural polyphenols, 95 mL distilled water, and 5 mL DMSO. After repeated testing, it was found that the dose of polyphenols extracted from the fruits of *Aronia melanocarpa* to be administered as enteral solution (by tube feeding) is 0.050 g/kg body every two days. The experiment used active therapeutic doses, well-determined fractions of DL50 on an experimental model of arterial hypertension. Fractions of DL50 are doses representing 1/5, 1/10, 1/20, and 1/40 of DL50. The dose representing 1/20 of DL50 was chosen, as it is the smallest dose that determined the pharmacodynamic effect that is being researched, without producing significant toxic effects.

The research was performed on Wistar white rats, with an average weight of 250–280 g, which were divided into 4 groups of 12, namely: (i) Group W—control, normal animals, that did not receive natural polyphenols; (ii) Group AHT—animals that were administered L-NAME 40 mg/kg body/day, i.p., at every 2 days, for 8 weeks; (iii) Group P—animals that were administered polyphenols under the form of solution, from the extract obtained from the *Aronia melanocarpa* fruit, at every 2 days, for 8 weeks; (iv) Group AHT + P—animals that were administered polyphenols in the dosage mentioned p.o. at every 2 days, concomitantly with L-NAME, for 8 weeks.

The blood samples necessary to the biochemical determinations were drawn from the retrorbital venous sinus. *The malondialdehyde (MDA) concentration*—the index of lipid peroxidation—was determined by the Ohkawa method using the thiobarbituric acid [12]. The MDA concentration was expressed in nmol/mL. *Glutathione peroxidase (GSH-Px)* (H_2O_2*: GSH oxidoreductase*) was determined by the Gross and Beutler method [13]; the GSH-Px activity was expressed in μM oxidized GSH per minute/g Hb or mg protein. *Reduced glutathione (GSH)* was also determined by the Beutler method, through the use of 5,5′ dithio-bisnitrobenzoic acid (DTNB), and was expressed in μg GSH/mg protein or g Hb in erythrocyte. For the extracellular response the *total antioxidant capacity (TAC)* was determined by using a RANDOX kit for manual use by Randox Laboratories Ltd. The major advantage of this test is to measure the antioxidant capacity of all antioxidants in a biological sample and not just the antioxidant capacity of a single compound. The method is based on formation of the ABTS•+ cation (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) and its scavenging by antioxidant sample constituents (e.g., serum or food) measured by spectrophotometry (decay of green/blue chromophore absorbance is inversely associated with antioxidant sample content and the control antioxidant is Trolox, a hydrophilic vitamin E analog).

The rat *blood pressure* values were recorded using a CODA Non-invasive Blood Pressure System, purchased from Kent Scientific Corporation, which uses a noninvasive blood pressure measuring method. It records the blood volume-pressure by a band attached to the tail, homologated by Bland-Altman testing [14], designed to reveal conformity with an invasive method (radiotelemetry), which enjoys proven accuracy yet is difficult to use in our study. The method is also recommended by the American Heart Association in its blood pressure measuring guide for laboratory animals [15]. The experiment consists in performing at least 6 blood pressure measurements in each laboratory animal, and data collection is by means of the CODA.

2.3. Statistical Data Interpretation. All the data are shown as mean value ± standard error of the mean (SEM). In order to assess the normal distribution of the groups, Shapiro-Wilk test was performed. Additionally, Levene test was performed to confirm the homoscedasticity of the groups, followed by ANOVA and paired or unpaired t-test to reveal the pairs of groups that differ biostatistically significantly in term of means. Statistical data interpretation considered the corresponding differences for a given significance threshold: $P > 0.05$: statistically insignificant; $P < 0.05$: statistically significant; $P < 0.01$: strong statistical significance; $P < 0.001$ very strong statistical significance.

3. Results

3.1. Extraction. Ethanol extraction of black chokeberry fruits yielded 47.17 g extract. The extract was stored at −20°C until used.

3.2. Phenolic Contents. Berries ethanolic extract contained 24.87±0.54 mg total phenolics/g and 4.46±0.06 μmole anthocyanin/g.

3.3. HPLC/DAD/ESI-MS Analysis. In the present study, the phenolic profile of berries ethanolic extract was characterized by HPLC/DAD/ESI-MS (Figure 1).

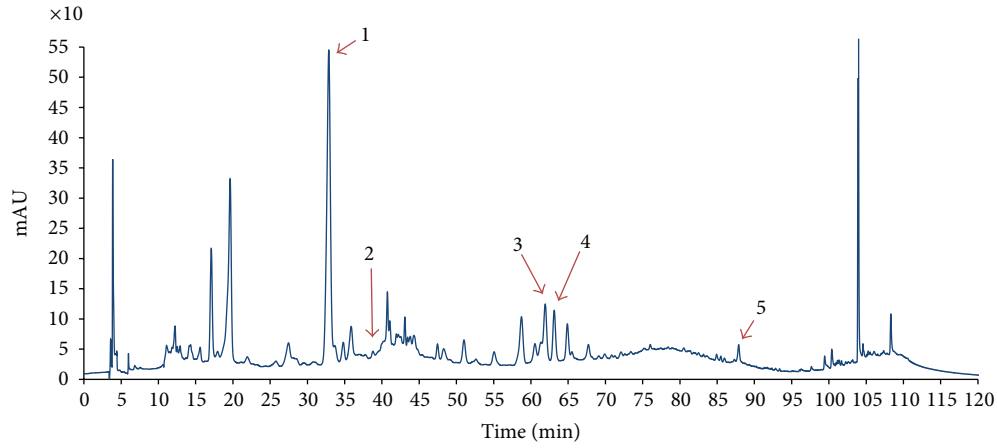
HPLC/DAD/ESI-MS allowed identification of chlorogenic acids, quercetin and cyanidin glycosides, and proanthocyanidins as major polyphenols in black chokeberry fruits [16]. (+)-Catechin hydrate, chlorogenic acid, caffeic acid, rutin trihydrate, hyperoside, quercetin dehydrate, and kuromanin chloride were used as standards. Main phenolic constituents were identified by comparison of their retention times and mass spectral data to those of authentic standards. Five phenolic compounds have been detected in berries ethanolic extract as follows: chlorogenic acid, kuromanin, rutin, hyperoside and quercetin; their retention times and mass spectral data are given in Table 1.

3.4. GSH-Px Serum Activity in Hypertensive Rats (AHT). The GSH-Px serum activity in hypertensive rats (AHT) has significantly low values ($P < 0.001$) when compared to the rats in groups W and AHT + P, which is a consequence of oxidative stress increase (Table 2).

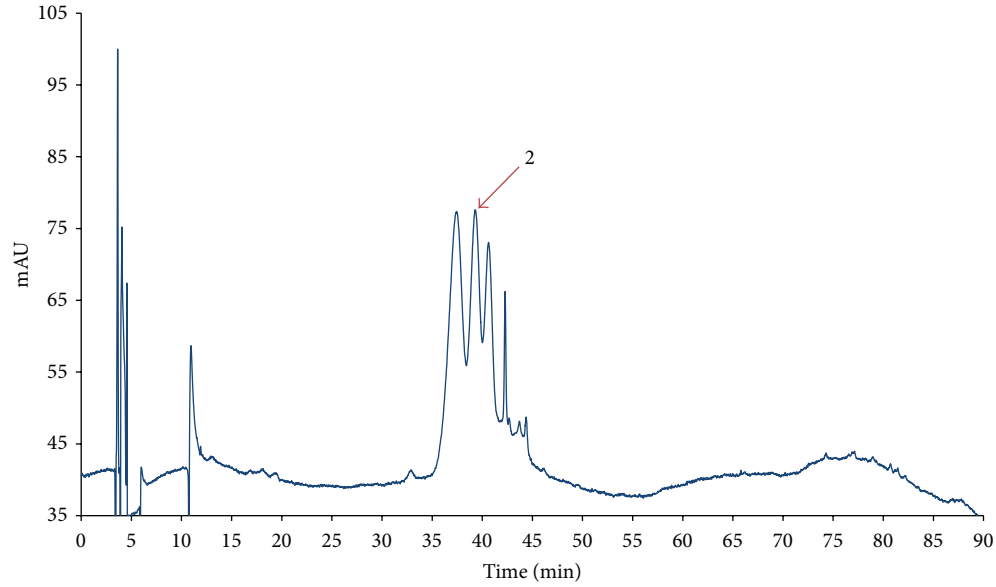
As a consequence of oxidative stress increase in hypertensive rats (AHT), the reduced glutathione (GSH) values are significantly low ($P < 0.001$) as compared to the rats in the W and AHT + P groups. The group of hypertensive rats protected by the administration of polyphenolic extract showed significantly higher ($P < 0.01$) values of GSH serum concentration compared to the hypertensive group.

The results achieved reveal a significant serum antioxidant capacity improvement ($P < 0.001$) in the AHT + P rats, the normalization of the GSH concentration, and a considerable reduction in the MDA serum concentration, causing a significant lipid peroxide diminution in the serum. Depending on the significance threshold values (P), the statistical analysis of the MDA values reveals significant differences ($P < 0.01$) between the AHT + P and the AHT groups, respectively, and highly significant differences ($P < 0.001$) between the AHT and the W groups.

The TAC levels were significantly decreased in AHT group ($P < 0.001$) as compared to the rats in the W and AHT + P groups. As expected, severe oxidative stress disturbs the antioxidant balance by generating reactive species and decreasing the total antioxidant capacity in the extracellular



(a) Detection at 280 nm



(b) Detection at 515 nm

Figure 1: HPLC-DAD chromatograms of ethanolic extract of black chokeberry fruits (1—chlorogenic acid, 2—kuromanin, 3—rutin + unknown compound, 4—hyperoside, and 5—quercetin).

space. There are similar TAC values in group W and group P and the differences between the AHT and AHT + P groups are statistically significant ($P < 0.01$).

The systolic and diastolic blood pressures, as well as their calculated mean, were measured. *The Shapiro-Wilk test* was positive, which supports sample normality, and the descriptive statistics and box-and-whisker plots are shown in Figure 2.

The Levene test confirmed group homoscedasticity, whereas the *ANOVA* test revealed a significant difference between the means of the 4 groups, as concerns systolic and diastolic blood pressure (Table 3). All the measured blood pressure components revealed a biostatistically significant ($P < 0.05$) blood pressure drop between the AHT and the AHT + P groups.

4. Discussion

Polyphenols act as free radicals scavengers by donating hydrogen atoms or electrons from phenolic hydroxyls. This is the main mechanism by which polyphenols scavenge many ROS (superoxide anion radical, hydroxyl radical).

During arterial hypertension, due to the high oxygen consumption, the reactive oxygen species act chiefly on unsaturated lipids, belonging to the membrane, with the formation of certain peroxidation products, which generate MDA. Since the most reactive radicals are short-lived they might be expected to react close to the site where they are formed. Polyphenols vary strongly in their absorption and distribution. They show high affinity for different structures and may therefore be able to decrease oxidative damage

Table 1: Retention time and mass spectral data of polyphenolic compounds detected in ethanolic extract of black chokeberry fruits.

Nonanthocyanin polyphenols				
Peak no.	Rt (min)	Mass spectral data		Peak assignment
		Deprotonated molecule $[M–H]^-$ (m/z)	Fragment ions (m/z)	
1	32.9	352.90	190.93 [M-H-Caffeoyl]	Chlorogenic acid (5-O-caffeoyl quinic acid)
3	61.9	608.87	—	Rutin* (quercetin-3-O-glucorhamnoside)
4	63.2	462.86	—	Hyperoside (quercetin-3-O-galactoside)
5	87.9	300.86	—	Quercetin
Anthocyanins				
Peak no.	Rt (min)	Mass spectral data		Peak assignment
		Molecular ion $[M]^+$ (m/z)	Fragment ions (m/z)	
2	39.3	449.21	287.13 [M-Glucose]	Kuromanin (cyanidin-3-O-glucoside)

*Rutin coeluted with a compound (462.86 m/z), possibly another quercetin glycoside.
Rt: retention time.

Table 2: GSH-Px, GSH, and TAC modifications in the studied groups.

	W	P	AHT	AHT + P
MDA (nmol/mL)	0	0	$8.76 \times 10^{-2***}$	$6.43 \times 10^{-2\#\#}$
GSH-Px (μmol/mL)	2.53 ± 0.19	2.37 ± 0.49*	1.17 ± 0.20***	1.56 ± 0.21##
GSH (μmol/mL)	7.29 ± 0.21	7.53 ± 0.40*	5.10 ± 0.49***	6.71 ± 0.35##
TAC (mmol/L)	1.55 ± 0.29	1.58 ± 0.31	1.31 ± 0.16**	1.53 ± 0.27##

Values are mean ± SEM ($n = 12$ animals). Statistical analyses:
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, versus W group.
#$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$, versus AHT group.

Table 3: ANOVA test.

Blood pressure	F value	P^* value
Systolic	22.901	0.001
Diastolic	13.199	0.001
Mean	16.970	0.001

*$P < 0.05$ indicates biostatistically significance.

mainly at such particular sites. A clear idea about the antioxidant potentials and the dependence of antioxidant activities on the quality and the quantity of phenolic substances can be obtained by comparison of the antioxidant activities of different phenolic extracts at equal total phenol concentrations.

Recent studies have revealed a total phenolic content of 20–90 mg/kg fresh wt (or mg/L) in strawberry, of 20–40 mg/kg fresh wt (or mg/L) in apple, and of 15–40 in black grape [17]. The results of this study show that the total phenolic content in the black chokeberry extract is similar to that found in fresh strawberries, apples, and black grapes.

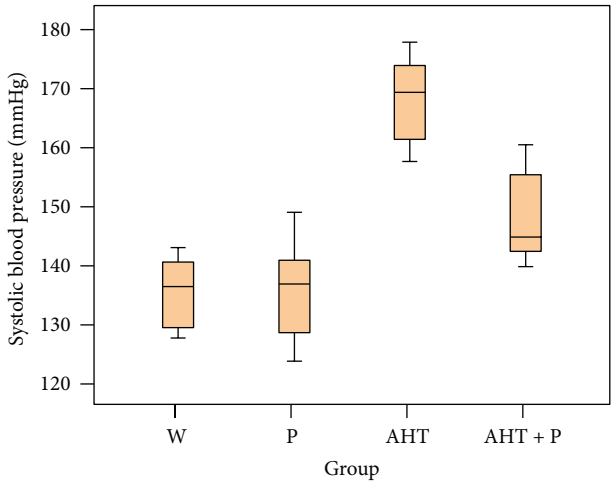
In normal conditions, the intrinsic antioxidant systems counteract the effects of oxidative stress. Therefore, the polyphenolic extract used contributes to actively maintain the effects of these systems (this can be construed as an explanation to why the MDA values in the animals from P group are lowered less than the ones in AHT + P group).

Free radicals scavenging activity and metal chelation partially explain polyphenols inhibitory effects on lipid peroxidation and LDL oxidation [18]. In addition, some polyphenols increase the activity of several endogenous antioxidant defence systems (GSH-Px, superoxide dismutase (SOD), and catalase (CAT)) and induce a significant increase in GSH level [19].

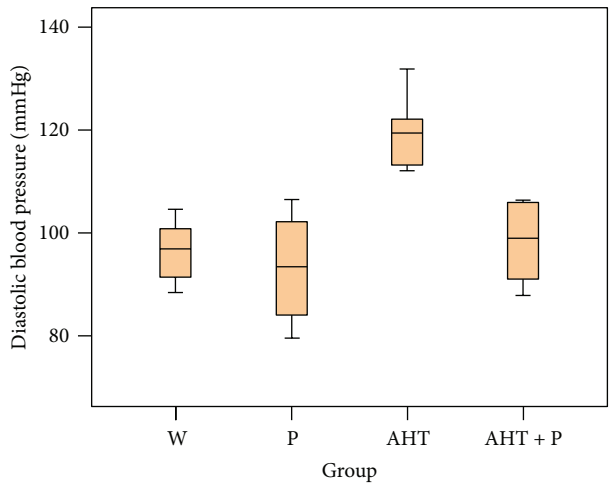
Phenolic compounds detected in ethanolic extract of black chokeberry fruits have shown antioxidant effects. Chlorogenic acid, quercetin, and cyanidin are effective radical scavengers and iron chelators; glycosylated derivatives of quercetin and cyanidin have a lower scavenging activity in comparison to quercetin and cyanidin, respectively [20].

However, it may still be true that specific antioxidants are preventive, and since polyphenols are both potent antioxidants and abundant in plant foods they are likely candidates for fulfilment of the antioxidant hypothesis.

The current study revealed an important reduction in the antioxidant mechanisms, both in GSH levels and antioxidant enzymatic activities in the hypertensive group. Reduced levels of GSH have been related to an extensive number of metabolic and gene expression disturbances, since the tripeptide is not only an efficient antioxidant but also an important regulatory substance in biological systems. The low activity of GSH is a consequence and not a cause of the increase in the oxidative status. Reactive oxygen species oxidized GSH to



(a) The *box-and-whisker* plot of systolic blood pressure



(b) The *box-and-whisker* plot of diastolic blood pressure

Figure 2: Systolic and diastolic blood pressure at studied groups.

GSSG, leading to a decrease in GSH and an increase in GSSG concentrations. Long-time oxidative stress can consume antioxidants and reduce SOD, CAT, and GSH-Px levels in cardiovascular diseases in general, and, especially, in arterial hypertension.

A wide range of evidence suggests that the Keap1 (Kelch ECH associating protein)-Nrf2 (nuclear-factor-erythroid 2-related factor) complex constitutes a sensor of oxidative stress involved in triggering antioxidant-response-element- (ARE-) mediated gene expression to restore the cellular redox status [21]. Under basal conditions, Nrf2 interacts with a cytosolic repressor protein Keap1 limiting Nrf2 mediated gene expression [22]. In cells exposed to oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it activates ARE dependent transcription of phase II and antioxidant defense enzymes, such as NADPH:quinone oxidoreductase, glutathione-S transferase, glutathione peroxidase, and heme oxygenase-1 [23]. Polyphenols may modify the capability of Keap1 to seclude Nrf2 and/or activate MAPK proteins (ERK, JNK and p38). Polyphenols could also be involved in Nrf2 stabilization [24].

The role of antioxidant nutrients in fighting against oxidative stress is well established in several diseases including cardiovascular and neurological pathologies [25]. In this sense, researchers had materially proved that following consumption of diets rich in fruits and vegetables there was an increase in serum TAC [26]. The decline of MDA levels may be caused by increased antioxidant status.

The blood pressure decrease effect of polyphenols could be due to particular actions, nonmediated by estrogenic receptors, of nitric oxide or superoxide anion bio-availability modulation by polyphenols [27, 28]. The significant blood pressure value drop in the hypertension group protected by polyphenols could be related to their ability to decrease *in vivo* reactive oxygen species production. In our study, polyphenols intake is associated with blood pressure decrease, not through the lowering of heart rate, but through the antioxidant mechanism especially.

The TAC levels were significantly decreased in the hypertensive group. In this experimental model, all groups were put on the same diet. The reduction in antioxidant mechanisms can be neutralized through natural polyphenol compounds of *Aronia melanocarpa* which will maintain TAC to levels capable of neutralizing ROS effects. The decay in TAC, as significant as it was, was not as severe as the changes observed in intracellular enzyme activity.

The TAC evaluation, used with other oxidative stress and antioxidant defense biomarkers, constitutes the first step in search for a healthy body status. In order to form strategies for the intervention and prevention of cardiovascular diseases, an understanding of the basic molecular mechanisms by prophylactic agents is required.

The study demonstrated that an ethanolic extract from fruits of *Aronia melanocarpa* Elliott is able to reduce endothelial dysfunction and improve total antioxidant capacity in early arterial hypertension. Evidence shows that polyphenols can increase the antioxidative capacity of plasma and that this effect is directly related to the plasma concentration and the intrinsic antioxidant capacity of the compounds. Evidence that the intake of catechins and related procyanidins are linked to the increased antioxidant capacity of plasma is necessary in order to assess more accurately whether there is a relationship between polyphenol intakes and health risks mediated by antioxidation.

A good way to raise the intake of antioxidants from *Aronia melanocarpa* fruits is to increase the proportion of consumption, and another effective way is to substitute the fruit and vegetables that have low antioxidant capacity with antioxidant-rich extract from *Aronia* fruits. In addition, a colorful variety of all fruit and vegetables, healthfully prepared, makes a significant contribution to a diet that promotes good health.

There are potential clinical benefits in using the polyphenolic extract coupled with the antihypertensive drugs with therapeutic purposes. This would lead to using a smaller dose of antihypertensive drugs and thus diminishing the secondary effects they produce.

5. Conclusions

Ethanolic extract of black chokeberry fruits has a potential value as prophylactic agent, but also may function as a nutritional supplement in the therapy of arterial hypertension. The role of the polyphenolic extract of *Aronia melanocarpa* is to prevent the total antioxidant capacity decrease and also to reduce the oxidative stress. Knowing the cellular and molecular mechanisms through which each compound of the *Aronia melanocarpa* extract acts in arterial hypertension requires further studies.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The work was supported by the Romanian Ministry of Education and Research, CNCSIS, plan PN2, program IDEI 2008, section PCE, Research Grant ID: 2519/2008–2011.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 480597, 9 pages
http://dx.doi.org/10.1155/2013/480597

Research Article

QiShenYiQi Pills, a Compound Chinese Medicine, Ameliorates Doxorubicin-Induced Myocardial Structure Damage and Cardiac Dysfunction in Rats

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Received 1 November 2012; Accepted 22 December 2012

Academic Editor: Kashmira Nanji

QiShenYiQi Pills (QSYQ) is a compound Chinese medicine used for treatment of cardiovascular diseases. The present study investigated the effects of QSYQ on the Doxorubicin- (DOX-) induced disorders in rat cardiac structure and function and the possible mechanism underlying. A total of 24 male Sprague-Dawley rats were administrated by intraperitoneal injections with DOX at a dose of 2.5 mg/kg, once every day for a total of 6 times. After the 6th injection, the rats were evaluated by echocardiographic analysis, and the animals with injured heart ($n = 14$) were divided into 2 groups and further treated with ($n = 7$) or without ($n = 7$) QSYQ by gavage at a dose of 0.2 g/day, once a day, over the next 2 weeks. Two weeks after QSYQ treatment, the following variables were assessed: myocardial blood flow (MBF) by Laser-Doppler Perfusion Imager, the ratio of heart weight to body weight (HW/BW), myocardial histology, myocardial content of ATP, AMP, free fatty acids (FFAs) and AMP/ATP by ELISA, and expression of PPARα, PGC-1α, and ATP 5D by Western blot. Statistical analysis was performed using one-way ANOVA followed by Turkey test for multiple comparisons. DOX challenge significantly increased left ventricular internal diameter and HW/BW and decreased the thickness of the left ventricular posterior wall, the left ventricle ejection fraction, and the left ventricle fractional shortening. DOX also increased AMP, FFA, and AMP/ATP, decreased ATP, and downregulated the protein content of ATP 5D, PPAR α, and PGC-1 α. All these DOX-induced cardiac insults were attenuated significantly by QSYQ treatment. These results show the potential of QSYQ to ameliorate DOX-induced disorders in cardiac structure and function; this effect may be related to the increase in myocardial ATP content via the upregulation of ATP 5D, PPAR α, and PGC-1 α and the oxidation of FFA.

1. Introduction

Cancer and cardiovascular diseases (CVDs) have become the main cause of death for the adults in China [1]. More importantly, the therapy of cancer may evoke or aggravate CVD due to the toxicity of antineoplastic [2]. Therefore, the development of management to limit the adverse effect of antineoplastic on cardiovascular system is of clinical significance for the therapy of cancer.

Doxorubicin (DOX), an anthracycline antibiotic [3], is one of the most effective antineoplastic medicines at present. However, continuous use of DOX may lead to chronic congestive heart failure in a dose dependent manner, which limits its clinic application [4]. Increasing study has been

published with respect to the mechanism responsible for the cardiac toxicity of DOX. The myocardial damage caused by DOX is reported to be relevant to free radicals [5], iron ion unbalanced metabolism [6], calcium overload [7], mitochondria damage [8], and cell apoptosis [9]. Recent researches revealed that DOX could also reduce myocardial ATP content [10], cause the downregulation of peroxisome proliferator-activated receptor α (PPAR α) in kidney [11]. These results suggest that metabolism disorder may be implicated in DOX-induced cardiac injury.

The activation of PPAR α is known to mediate the expression of cardiac fatty acid oxidation (FAO) enzyme gene, while peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC-1 α) is the essential regulator for cardiac metabolism and function. Coactivation of PGC-1 α and PPAR α can regulate FAO [12], which plays an important role in cardiac energy metabolism and injury. Shortage of ATP is known to play a dual role in the pathogenesis of ischemia-reperfusion (I/R) injury; in addition to trigger reactive oxygen species (ROS) production, it leads to the degradation of F-actin located on thin filament and thus the abnormality of cardiac structure and function. ATP synthase δ (encoded by ATP 5D), as one of the subunits of ATP synthase, is critical for ATP synthesis [13]. However, the role of ATP 5D in DOX-induced cardiac injury is yet unclear. Furthermore, to date no Chinese medicine has been proven to improve cardiac structure and function via myocardial energy metabolism.

QSYQ is a compound Chinese medicine composing of Radix Astragali (RA), Salvia miltiorrhiza (SM), Panax notoginseng(PN) and rosewood. In 2003, QSYQ was approved for treating coronary heart disease and angina by the Chinese State Food and Drug Administration. Our laboratory has proved that QSYQ ameliorates pressure overload-induced cardiac hypertrophy, myocardial fibrosis, myocardial blood flow, and cardiac function [14]. Zhang et al. confirmed that QSYQ can protect against cardiac injury and fibrosis in ischemia-reperfusion rat and increase the expression of vascular endothelial growth factor (VEGF) [15]. However, it is not clear whether QSYQ can reduce DOX-induced cardiac disorder in structure and function, and, if yes, what is the underlying mechanism? The present study was conducted to address the effect of QSYQ on DOX-induced disorder by testing ATP, ATP 5D, F-actin, PPAR α, and PGC-1 α in rat heart.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley (SD) rats weighing 189 ± 12 g were purchased from the Animal Center of Peking University Health Science Center (Beijing, certificate no. SCXK 2006-0008). The animals were housed in cages at 22°C ± 2°C and humidity of 40% ± 5% under a 12-hour light/dark cycle, received standard diet and water ad libitum. The animals were fasted for 12 h before the experiment, while allowing free access to water. The experimental procedures were carried out in accordance with the European commission guidelines (2010/63/EU). All animals were handled according to the guidelines of the Peking University Animal Research Committee. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University (LA2011-67).

2.2. Reagents. QiShenYiQi Pills (QSYQ, lot number: 110708) was obtained from Tasly Pharmaceutical Co., Ltd. (Tasly, Tianjin, China), 0.5 g per pouch. Doxorubicin Hydrochloride for Injection (DOX, lot number: 9QL0161) was purchased from Pfizer Pharmaceuticals Ltd. (Pfizer, Latina, Italy), one bottle containing 10 mg of Doxorubicin Hydrochloride, 50 mg of lactose, and 1 mg of methylparaben. Isoflurane was purchased from Jiupai Pharmaceutical Co. Ltd. of HeBei (Jiupai, Shijiazhuang, China). Tetramethylethylenediamine and ammonium persulfate were purchased from Sigma-Aldrich Co. (Sigma, St. Louis, MO, USA). Supper ECL Plus was purchased from Pierce Biotechnology, Inc. (Pierce, Rockford, IL, USA). RIPA tissue lysate was purchased from Cell Signaling Technology, Inc. (CST, Danvers, MA, USA). Protease inhibitor was obtained from Merck Drugs & Biotechnology Co. (Merck, Darmstadt, Germany). Protein marker was purchased from MBI Fermentas (MBI, Burlington, ON, Canada). Whole protein extracting kit was purchased from Bio-Rad Laboratories, Inc. (Bio-Rad, Hercules, CA, USA). BCA protein assay kit was purchased from Bio-Rad Laboratories, Inc. (Bio-Rad, Hercules, CA, USA). ELISA kit for ATP, AMP, and free fatty acid (FFA) of rat was purchased from R & D Systems, Inc. (R & D, Minneapolis, USA). The antibodies against PPAR α and PGC-1 α were purchased from Abcam, Inc. (Abcam, Cambridge, USA). The antibody against ATP 5D was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were of reagent grade.

2.3. Animal Model and Drug Administration. Rats were randomly divided into 5 groups: Control 2W ($n = 6$), Control 4W ($n = 6$), DOX 2W ($n = 14$), DOX 4W + NS ($n = 7$), and DOX 4W + QSYQ group ($n = 7$). For the last three groups, twenty-four rats were administrated with DOX (2.5 mg/kg in saline) by intraperitoneal injection once every other day for a total of 6 times, as described previously by others [16]. After the sixth injection, left ventricular ejection fraction (LVEF) was measured by echocardiographic analysis. Of 24 rats tested, fourteen had LVEF reduced by 10%, which were scored as DOX-injured animals (DOX 2W) and used for subsequent experiments. The DOX-injured rats were further randomly divided into DOX 4W + NS group and DOX 4W + QSYQ group. The animals in the DOX 4W + NS group were administrated with 1 mL of saline daily by gavage for the subsequent 2 weeks. Over the same period of time, animals in the DOX 4W + QSYQ group received 1 mL of QSYQ saline solution (concentration of 0.2 g/mL), as described [14], instead of saline alone, daily by gavage. The rats in the Control 2W group were administrated with saline by intraperitoneal injection at the dose of 1 mL every other day for 14 days, while the animals in Control 4W group were treated in the same way as those in Control 2W group, and followed by administration of saline once a day by gavage for subsequent 2 weeks.

2.4. Echocardiographic Analysis. The left ventricular wall thickness and cardiac function were evaluated at week 2 and

4, respectively, using a Vevo 770 High-Resolution Imaging System (Vevo 770, Visual Sonics Inc, Toronto, ON, Canada) with a 17.5 MHz linear array transducer (model 716). The following parameters were measured as indicators of cardiac function and remodeling: left ventricular internal diameter at end-diastole (LVIDd), left ventricular internal diameters at end-systole (LVIDs), left ventricular posterior wall at diastole (LVPWd), left ventricular posterior wall at systole (LVPWs), left ventricle ejection fraction (%EF), and left ventricle fractional shortening (%FS) [14].

2.5. Measurement of Myocardial Blood Flow (MBF). Images of MBF in the territory supplied by left anterior descending coronary artery were acquired by Laser-Doppler Perfusion Imager (PeriScan PIM3, Perimed, Stockholm, Sweden) equipped with a computer and evaluated on an area of $3 \times 4\,mm^2$ with the software LDPI win 3.1 (LDPIwin 3.1, Perimed, Stockholm, Sweden) [17].

2.6. Measurement of HW/BW. Rats were killed at the end of experiment, and the hearts were removed and washed with normal saline. Both body weight (BW) and heart weight (HW) were determined by an electronic balance (CPA64-0CE, Sartorius AG, Goettingen, Germany), and the ratio of HW to BW (HW/BW) was calculated [14].

2.7. Histological Investigation of Myocardial Tissues. Hearts were removed at the end of the experiment, fixed in 4% formaldehyde, and further prepared for paraffin sectioning. The paraffin sections (5 μm) were rehydrated and stained with hematoxylin and eosin (HE). The images were captured by a digital camera connected to a stereo microscope (SZ-40, Olympus, Tokyo, Japan) and an optical microscope (Digital Sight DS-5 M-U1, Nikon, Tokyo, Japan) and processed with the software Image-Pro Plus 5.0 (Image-Pro Plus 5.0, Media Cybemetrics, Rockville, MO, USA).

2.8. F-Actin Staining. Paraffin sections were treated with 0.01 M sodium citrate for antigen retrieval. After washing, sections were then incubated with rhodamine phalloidin (1 : 40, R415, Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C and washed with PBS. To label nucleus, the sections were incubated with Hoechst 33342 (1 : 100, Molecular Probes, New York, NY, USA) [18]. Sections were observed with a laser scanning confocal microscope (TCS SP5, Leica, Mannheim, Germany).

2.9. Assessment of ATP, AMP, FFA, and AMP/ATP. At the end of the experiment, the rat was perfused with NS, and a tissue block about $2\,mm^3$ was removed from the heart for the assessment of the concentrations of ATP, AMP, and FFA in myocardial tissues by ELISA kits according to the manufacturer's protocol. OD values were determined by enzyme microplate reader (Thermo multiskan Mk3, Thermo Fisher Scientific Inc., Barrington, IL, USA), with a detection wave length of 450 nm. The concentrations of ATP, AMP, FFA, and AMP/ATP were calculated based on the standard curves.

2.10. Western Blotting Assay. A piece of about 200 mg of tissue was cut from the heart of each animal and preserved at −80°C ($n = 3$). The whole protein was extracted. The concentration of whole protein was detected in duplicate with BCA protein assay kit according to instruction, and the mean values were computed. All the concentrations of whole proteins were adjusted to the lowest concentration detected and the samples were preserved at −80°C.

The whole protein was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk or 3% BSA and, after washing, incubated overnight at 4°C with primary antibody against PPARα (1 : 1000), PGC-1 α (1 : 500), and ATP5D (1 : 200). Following rinsing, the membranes were incubated for 1 h at room temperature with respective HRP-conjugated secondary antibody. The membranes were developed with ECL, exposed in dark box, and the protein signal was quantified by scanning densitometry in the X-film by bioimage analysis system (Image-Pro plus 6.0, Media Cybernetics, Bethesda, MD USA). The result of each group was expressed as relative optical density compared with that from Control group.

2.11. Statistical Analysis. All parameters were expressed as mean ± S.E. Statistical analysis was performed using one-way ANOVA followed by Turkey test for multiple comparisons. A probability of less than 0.05 was considered to be statistically significant.

3. Results

3.1. QSYQ Ameliorates DOX-Induced Disorder in Left Ventricular Wall Thickness and Heart Function in Rats. The results of echocardiography analysis in various groups are displayed in Table 1. Compared with Control 2W group, the DOX 2W group had a significant increase in LVIDs and a decrease in LVPWd, LVPWs, EF%, and FS%, all of which were attenuated by QSYQ treatment for 2 weeks. The representative echocardiograms in different groups are presented in Figure 1.

3.2. Effect of QSYQ on DOX-Induced Reduction in MBF in Rats. Figure 2(a) shows the MBF images acquired by Laser-Doppler Perfusion Imager in different groups. Of notice, as compared to the Control 4W group, MBF apparently decreased in DOX 4W + NS group. In contrast, the image of DOX 4W + QSYQ group shows that QSYQ treatment for two weeks obviously attenuated DOX-induced decrease in MBF. This result was verified by the quantitative evaluation of myocardial coronary blood flow (Figure 2(b)).

3.3. Effect of QSYQ on DOX-Induced Reduction in HW/BW in Rats. Figure 3 shows the statistical results of the ratio of the HW/BW in different groups. As noticed, DOX 4W + NS group had a 30% increase in HW/BW compared to that

Table 1: Echocardiography parameters of rat hearts in different conditions.

GROUP	n	LVIDd (mm)	LVIDs (mm)	LVPWd (mm)	LVPWs (mm)	EF (%)	FS (%)
Control 2W	6	7.468 ± 0.18	4.338 ± 0.14	1.985 ± 0.12	3.046 ± 0.06	71.03 ± 1.27	41.88 ± 1.07
DOX 2W	14	6.901 ± 0.023	4.985 ± 0.13*	1.372 ± 0.11*	1.780 ± 0.14*	51.68 ± 3.51*	27.49 ± 2.28*
Control 4W	4	7.654 ± 0.34	4.521 ± 0.21	1.873 ± 0.09	2.782 ± 0.07	69.73 ± 2.62	40.86 ± 2.27
DOX 4W + NS	6	7.624 ± 0.29	5.439 ± 0.29	1.395 ± 0.09$^{\blacktriangle}$	2.111 ± 0.32$^{\blacktriangle}$	53.52 ± 2.57$^{\blacktriangle}$	28.78 ± 1.64$^{\blacktriangle}$
DOX 4W + QSYQ	7	7.279 ± 0.10	4.106 ± 0.17$^{\#}$	1.565 ± 0.06$^{\#}$	2.428 ± 0.08$^{\#}$	72.93 ± 2.37$^{\#}$	43.60 ± 2.12$^{\#}$

$^{*}P < 0.05$ versus Control 2W group; $^{\#}P < 0.05$ versus DOX 4W + NS group; $^{\blacktriangle}P < 0.05$ versus Control 4W group. LVIDd: left ventricular internal diameter at end-diastole; LVIDs: left ventricular internal diameters at end systole; LVPWd: left ventricular posterior wall at diastole; LVPWs: left ventricular posterior wall at systole; EF: left ventricle ejection fraction; FS: left ventricle fractional shortening. The data are presented as mean ± S.E. The treatments for each group are detailed in Section 2.

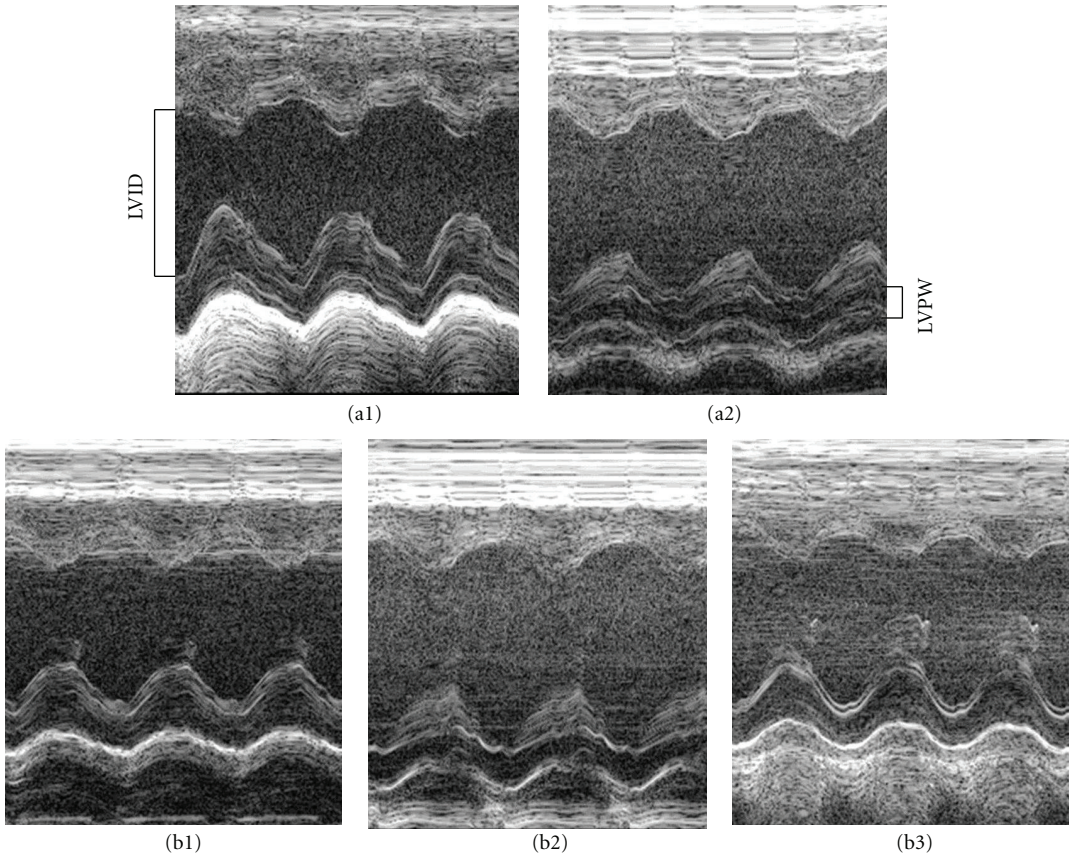

Figure 1: Representative rat echocardiograms in various conditions. (a1) Control 2W group, (a2) DOX 2W group, (b1) Control 4W group, (b2) DOX 4W + NS group, and (b3) DOX 4W + QYSQ group. LVID: left ventricular internal diameter at end diastole; LVPW: left ventricular posterior wall at diastole.

of Control 4W group, indicating a significant myocardial hypertrophy after DOX challenge, which was significantly attenuated by 2 weeks of QSYQ treatment.

3.4. Effect of QSYQ on DOX-Induced Myocardial Injury in Rats. Figures 4(a) and 4(b) illustrate the results of histological examination of myocardial tissues in different groups. Compared with the Control 4W group (a1), distinct alterations occurred in myocardial tissues from the DOX 4W + NS group, including myocardial edema and fiber breakage (a2), all of which were noticeably ameliorated by 2-week QSYQ treatment (a3). Figure 4(b) shows the images of rhodamine phalloidin-labeled F-actin, wherein decreased F-actin and myocardium rupture were observed in the DOX 4W + NS group (b2) in comparison to Control 4W group (b1). Administration of QSYQ for 2 weeks significantly attenuated F-actin reduction and myocardium rupture (b3).

3.5. Effect of QSYQ on DOX-Induced Changes in the Energy Metabolism. The ATP, AMP, FFA content, and the ratio of AMP/ATP were determined by ELISA at the end of the experiment in different groups (Figure 5). In comparison with Control 4W group rats, the ATP content of rat myocardial tissue in DOX 4W + NS group significantly decreased (a),

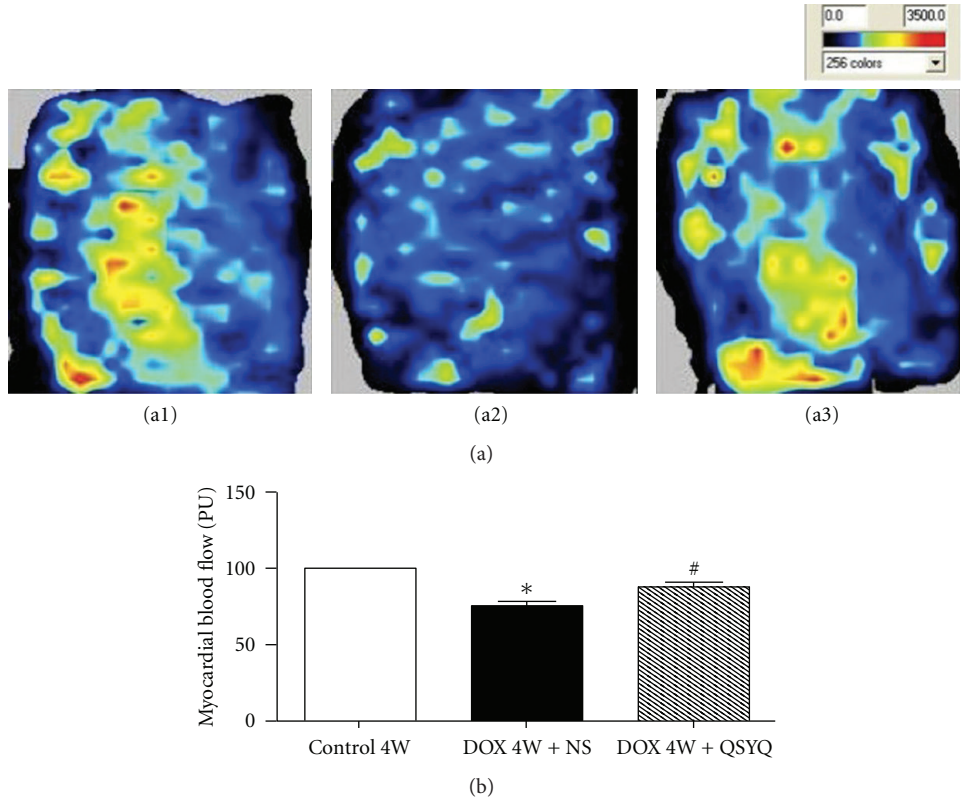

Figure 2: The effect of QSYQ on rat MBF. (a) the MBF images acquired by Laser-Doppler Perfusion Imager in Control 4W group ((a1), $n = 6$), DOX 4W + NS group ((a2), $n = 7$), and DOX 4W + QSYQ group ((a3), $n = 7$). Red color represents high MBF and blue color represents low MBF. (b) The statistical results of MBF in different groups. The data are presented as mean ± S.E. $^{*}P < 0.05$ versus Control 4W group; $^{\#}P < 0.05$ versus DOX 4W + NS group.

while the content of AMP (b), the ratio of AMP/ATP (c), and FFA (f) significantly increased. In DOX 4W + QSYQ group the ATP content were remarkably recovered, and so did the content of AMP, FFA, and the ratio of AMP/ATP.

To explore the cause of the observed change in ATP content, the expression of ATP 5D was detected by Western blots. As shown in Figure 5(d), the expression of ATP 5D was reduced significantly by DOX, as compared to Control 4W group. Administration of QSYQ for 2 weeks relieved the decline of ATP 5D expression evoked by DOX.

3.6. Effect of QSYQ on DOX-Induced Alteration in the Expression Level of PPAR α and PGC-1 α. Western Blotting was undertaken to assess the expression levels of PPAR α and PGC-1 α in myocardial tissues from different groups at the end of the experiment (Figure 6). As noticed, both qualitative survey and quantitative evaluation indicate that the expression levels of PPAR α and PGC-1 α in DOX 4W + NS group were apparently decreased compared with Control 4W, while these decreases restored significantly in the DOX 4W + QSYQ group.

4. Discussion

The present study revealed that the intraperitoneal injection of DOX for 2 weeks in rats leads to an increase in LVID and HW/BW, a reduction of LVPW and LVPW, and a decrease in EF and FS. In addition, DOX caused reduction of ATP synthase subunit ATP 5D, the degradation of myocardial F-actin, and myocardium rupture. All the DOX-induced alterations can be evidently ameliorated by the administration of QSYQ for 2 weeks, suggesting the potential of QSYQ to relieve the DOX-induced cardiac insufficiency. In addition, we also found that QSYQ promotes the expression of PPAR α and PGC-1 α in the DOX-injured myocardium cells, facilitates the oxidation of fatty acid, degrades myocardial free fatty acid, and finally increases the content of ATP.

Previous studies reported that intraperitoneal injection of DOX in rats provokes cardiomyopathy, exhibiting as a larger LVIDS and a thinner LVPW [19]. In the present study, using the same animal model with decreased cardiac function induced by DOX, we proved that continuous post-intervention by QSYQ for 2 weeks obviously rescues the cardiac function. Parallel with functional assessment, the beneficial role of QSYQ in DOX-induced myocardial injury was demonstrated by morphological study as well. QSYQ is a compound traditional Chinese medicine which is mainly used to promote blood circulation. Previous studies showed that QSYQ could attenuate the myocardial injury and fibrosis induced by overload pressure [14] and ischemia reperfusion [17], and improve myocardial blood flow and cardiac function in rats [14]. The present study provided evidence

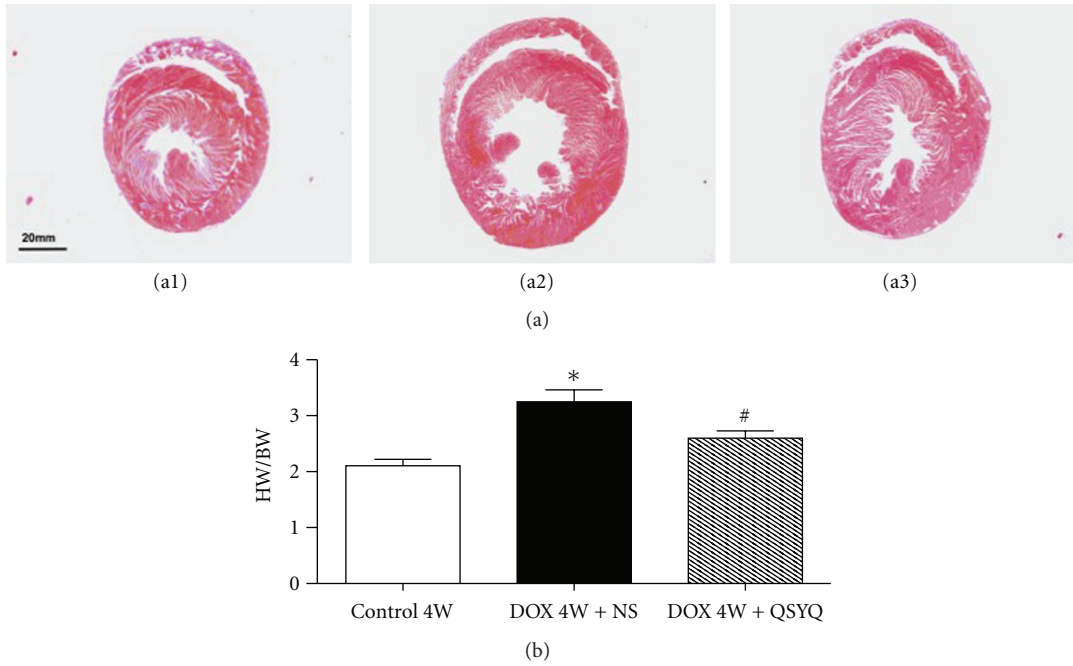

Figure 3: The effect of QSYQ on rat HW/BW. (a) Representative transverse heart slice from Control 4W group (a1), DOX 4W + NS group (a2), and DOX 4W + QSYQ group (a3). Myocardial tissues of rat were sampled in cross-sections 5 mm in thickness throughout the left and right ventricles. Sections were fixed in formalin and embedded in paraffin. Transverse sections were cut at 5 μm thickness. (b) The statistical results of HW/BW in Control 4W group ($n = 6$), DOX 4W + NS group ($n = 7$), and DOX 4W + QSYQ group ($n = 7$). The data are presented as mean ± S.E. $^{*}P < 0.05$ versus Control 4W group; $^{\#}P < 0.05$ versus DOX 4W + NS group.

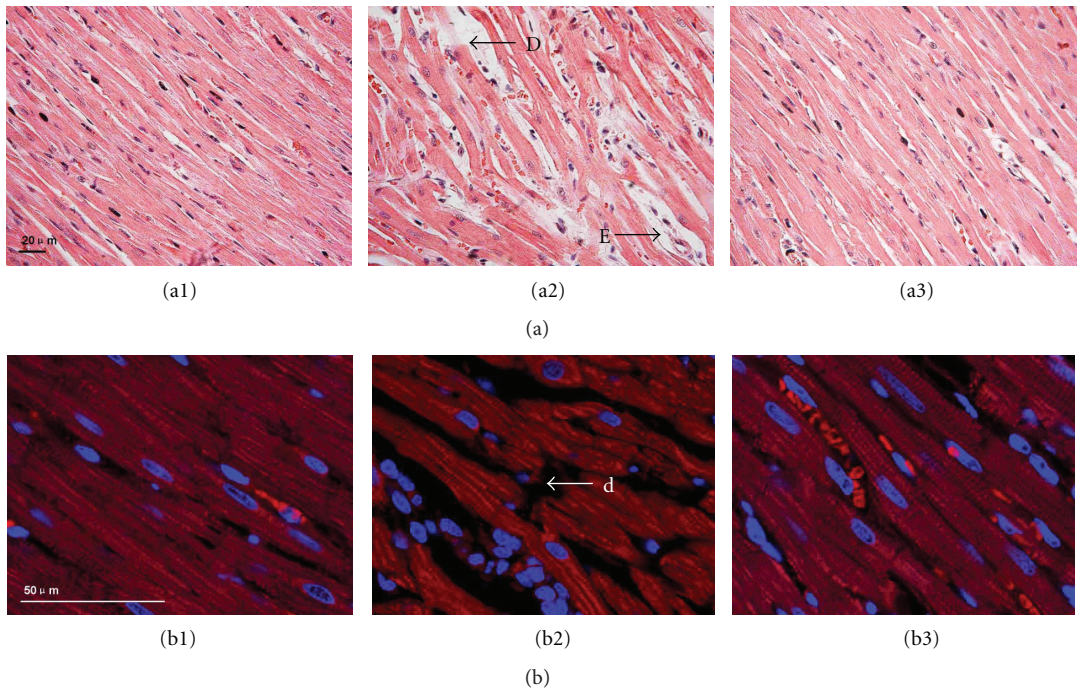

Figure 4: Effect of QSYQ on the structure of rat myocardial tissue. (a) Representative histological images by H & E staining in Control 4W group (a1), DOX 4W + NS group (a2), and DOX 4W + QSYQ group (a3). (b) Representative photographs of F-actin stained by rhodamine phalloidin in Control 4W group (b1), DOX 4W + NS group (b2), and DOX 4W + QSYQ group (b3). E: edema; D: disrupted myocardial fiber; d: disrupted myocardial fiber.

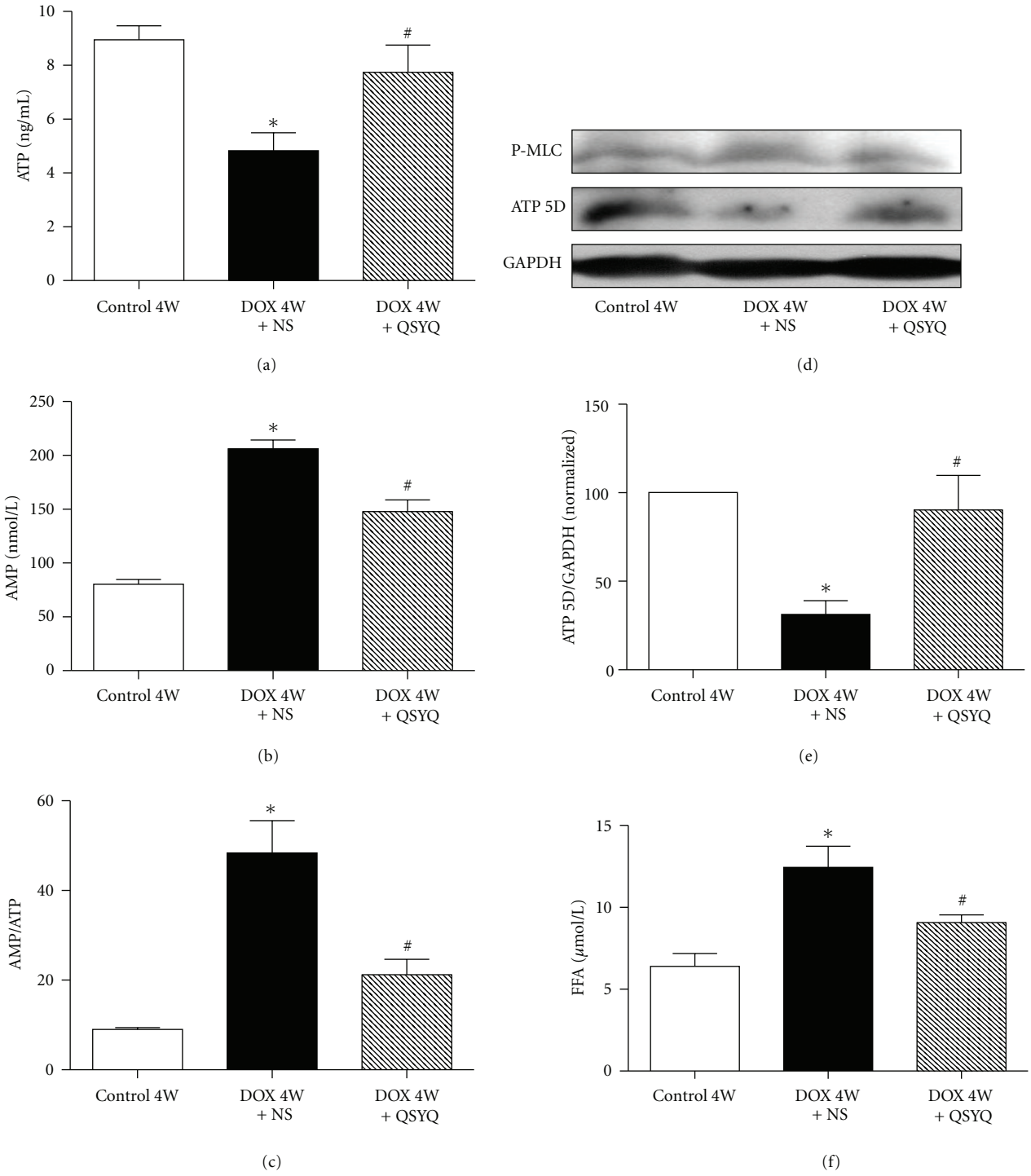

Figure 5: Effect of QSYQ on the content of ATP, AMP, AMP/ATP, and FFA in rat myocardial tissue. (a), (b), (c), and (f) show the statistical results from ELISA for ATP, AMP, AMP/ATP, and FFA, respectively, in Control 4W group ($n = 6$), DOX 4W + NS group ($n = 7$), and DOX 4W + QSYQ group ($n = 7$). (d) Representative Western blotting for ATP 5D in rat myocardial tissue in various groups. (e) Quantitative results of the Western blotting for ATP 5D. The data are presented as mean ± S.E. $^{*}P < 0.05$ versus Control 4W group; $^{\#}P < 0.05$ versus DOX 4W + NS group.

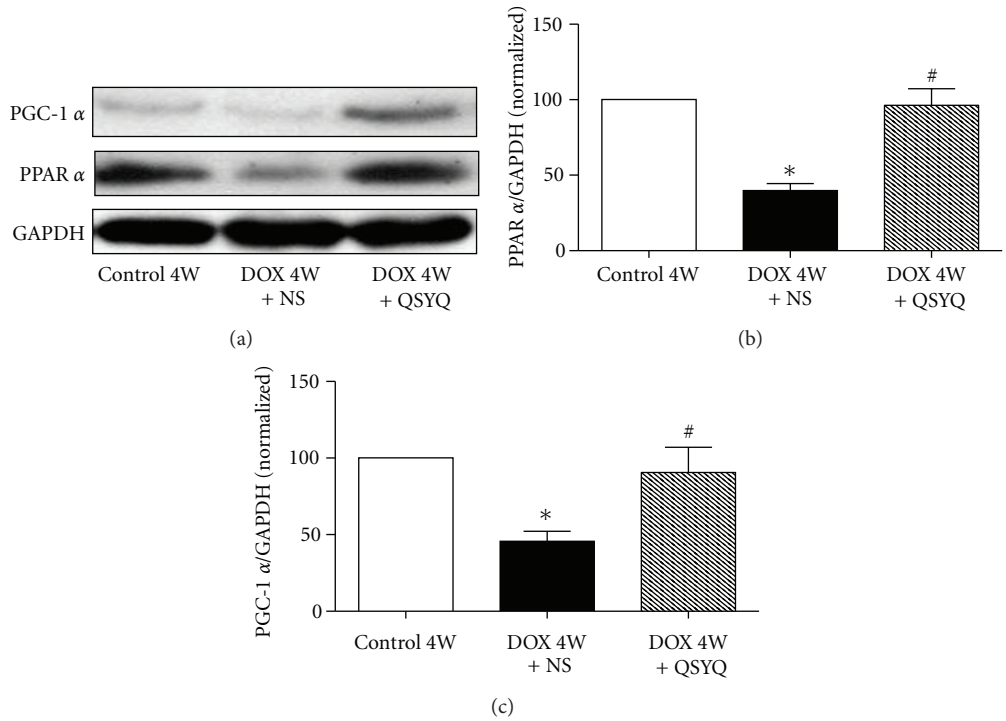

Figure 6: Assessment of PGC-1 α and PPAR α protein in rat myocardial structure. (a) Representative Western blotting for PGC-1 α and PPAR α in Control 4W group ($n = 6$), DOX 4W + NS group ($n = 7$), and DOX 4W + QSYQ ($n = 7$). (b) and (c) show the quantitative results of the Western blotting of PGC-1 α and PPAR α, respectively. The data are presented as mean ± S.E. $^{*}P < 0.05$ versus Control 4W group; $^{\#}P < 0.05$ versus DOX 4W + NS group.

supporting for the effectiveness of QSYQ in ameliorating cardiac disorders in an unexplored condition, the DOX-induced myocardial injury and cardiac dysfunction. Cardiac toxicity is the main problem limiting the clinical use of DOX; the finding of the present study may open a potential avenue to surmount the adverse effect of DOX for its clinical application.

Fatty acid beta-oxidation takes place in mitochondrial matrix, a process that constitutes the major source of energy for myocardial cell activity [20]. Numerous genes are involved in fatty acid beta-oxidation, which are primarily regulated by PPAR α/PGC-1 α complex [12], with PGC-1 playing an important role in regulating cardiac mitochondrial number and function [21]. In line with others, the present study revealed an upregulating cardiac FFA level and downregulating myocardial ATP content in rats after DOX challenge [22]. Importantly, we demonstrated for the first time that administration of QSYQ to the DOX-challenged rats for 2 weeks remarkably suppressed myocardial FFA level and elevated cardiac ATP content, meanwhile, increased PPAR α/PGC-1 α expression. These results suggest that QSYQ increases myocardial fatty acid beta-oxidation and ATP content and improves myocardial energy metabolism and cardiac function most likely through interference in PPAR α/PGC-1 α pathway.

ATP 5D is the gene encoding ATP synthase subunit δ which contributes to the synthesis of ATP [13]. However, no study has been reported about the changes of ATP synthase subunits after DOX. The present study revealed that ATP 5D protein decreased significantly after DOX, probably accounting for the reduction of ATP content. Interestingly, treatment with QSYQ restrained the decrease of ATP 5D and the increase of AMP/ATP.

F-actins constitute myocardial thin filaments, which, alone with thick filament, are responsible for the actin-based myofilament motility [23]. We demonstrated in the present study the degradation of F-actin followed by the rupture of myocardial fibers and the hypofunction of cardiac contractility after DOX, an insult most likely due to the reduction of ATP content [24]. Furthermore, the beneficial role of QSYQ in maintaining the myocardial structure and cardiac function after DOX is presumably achieved through increasing the expression of ATP 5D and the synthesis of ATP leading to the preservation of F-actin.

Heart ejection function depends on cardiac energy supply and normal myocardial structure. We demonstrated that DOX caused cardiac energy depletion and myocardial structure damage, which contribute to the reduced heart ejection and perfusion function. QYSQ could restore the cardiac energy, myocardial structure and improve myocardial blood flow and cardiac function implying QYSQ as a promising remedy for reducing the adverse effects of DOX in clinic.

In summary, QSYQ is able to ameliorate DOX-induced myocardial structure injury and cardiac dysfunction. This beneficial role of QSYQ is correlated with its potential to modulate energy metabolism, involving upregulating PPAR

α/PGC-1 α and fatty acid oxidation, reducing myocardial FFA and increasing ATP level. This result suggests QSYQ as a potential management to cope with the obstacle that DOX confronts in clinical use, and provides insight for better understanding the mechanism behind the QSYQ effect. Nevertheless, the detailed mechanisms thereby QSYQ protects heart from injury by DOX need further clarification, and mor studies, particularly using lager animals, are required to verify the feasibility for QSYQ application in clinic.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This study was supported financially by The Key Program Foundation of Beijing Administration of Traditional Chinese Medicines (2004-IV15), The Joint Fund of Guizhou Provincial Department of Science and Technology-Guiyang College of Traditional Chinese Medicine (2011-LKZ7041) and The Production of New Medicine Program of Ministry of Science and Technology of the People's Republic of China (2008ZX09401), and Tianjin Tasly Group, Tianjin, China (20050230).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 540613, 8 pages
http://dx.doi.org/10.1155/2013/540613

Review Article

Is Yangxue Qingnao Granule Combined with Antihypertensive Drugs, a New Integrative Medicine Therapy, More Effective Than Antihypertensive Therapy Alone in Treating Essential Hypertension?

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Received 19 November 2012; Accepted 15 January 2013

Academic Editor: Tabinda Ashfaq

Background. Yangxue Qingnao granule (YQG) combined with antihypertensive drugs, a new integrative medicine therapy, has been widely used for essential hypertension (EH) in China. This study aims to assess the current clinical evidence of YQG combined with antihypertensive drugs for EH. *Methods*. Randomized controlled trials (RCTs) published between 1996 and 2012 on YQG combined with antihypertensive drugs versus antihypertensive drugs in treating EH were retrieved from six major electronic databases, including The Cochrane Library, PubMed, Chinese National Knowledge Infrastructure, Chinese Scientific Journal Database, Chinese Biomedical Literature Database, and Wanfang Data. Meta-analysis was performed on the overall effects on blood pressure. *Results*. Twelve randomized trials were included. Methodological quality of the trials was evaluated as generally low. Meta-analysis showed that YQG combined with antihypertensive drugs demonstrated potential effect for lowing either SBP (MD: −7.31 [−11.75, −2.87]; $P = 0.001$) or DBP (MD: −5.21 [−8.19, −2.24]; $P = 0.0006$) compared to antihypertensive drugs alone. *Conclusions*. It indicated that YQG combined with antihypertensive drugs is more effective than antihypertensive drugs alone in treating EH. However, more RCTs of larger scale, multicentre/country, longer follow-up periods, and higher quality are required to verify the efficacy of integrative medicine therapy over all antihypertensive therapies.

1. Introduction

Hypertension is one of the most important modifiable cardiovascular risk factors, contributing to half of the coronary heart disease and almost two-thirds of the cerebrovascular disease burdens [1]. Among them, approximately 90% to 95% of hypertension, affecting more than 1 billion adults worldwide, is the essential hypertension subtype [2, 3]. There is a robust evidence from randomized trials showing that the treatment of hypertension is remarkably effective, and a small reduction in blood pressure (BP) may result in a large reduction in the risk of stroke and myocardial infarction [4]. Although great prevention efforts have been made, the absolute numbers of hypertensive patients are increasing, given the large population and the increasing prevalence of hypertension in developing countries [5]. Chung et al. [6] estimated that the prevalence of hypertension and related cardiovascular morbidity and mortality has increased

dramatically in the past 30 years throughout Asia, placing a considerable and growing economic and social burden on countries throughout the region. The hypertensive patients continue to progress to hypertension-related cardiovascular mortality. Thus, more than two-thirds of elderly patients with hypertension worldwide resort to various kinds of complementary or alternative medicine [7].

In China, the prevalence of hypertension increased from 7.8% in 1980 to 27.2% in 2001. Hypertension-related cardiovascular mortality in China is predicted to cost $6–9 million per year until 2030. And the economic impact will be particularly pronounced as a high proportion of deaths will occur in people of working age [6]. Fortunately, there is one important characteristic of China's national medical system, that is, traditional Chinese medicine (TCM) and western medicine (WM) complement and cooperate with each other (also known as integrative medicine), being responsible for the health care of Chinese people together [8, 9]. Integrative medicine (IM), therefore, combines the latest scientific advances with the most profound insights of traditional healing systems to regain and preserve health and to assist the patient's own capacities to recover from illness [10]. In the last decades, an increasing popularity of IM has been gained for the treatment of chronic and acute states of illnesses in in-patient treatment [11]. Until now, a variety of high-quality clinical trials in IM have been conducted and published. There are more and more evidence of safety and effectiveness in IM [12]. As we know that TCM has played an important role in the medical care of patients with hypertension-related signs and symptoms for thousands of years in China [13–15]. Modern researches found out that, compared to antihypertensive drugs alone, IM has better efficacy both in blood pressure (BP) and clinical symptoms such as headache, neck and shoulder pain, dizziness, and fatigue [16, 17]. There is no doubt that, in modern time, IM therapy will be widely used for hypertension treatment both in China and other countries [16–20].

Yangxue Qingnao granule (YQG) is a popular Chinese traditional patent medicine (CTPM) for the treatment of essential hypertension (EH), which has been authoritatively recommended by Newly Edited National Chinese Traditional Patent Medicines [21] and reasonable application manual of traditional Chinese patent medicine in internal medicine [22]. YQG contains eleven commonly used herbs, including angelica sinensis, *Ligusticum chuanxiong* Hort, white peony root, prepared radix rehmanniae, uncaria, *Spatholobus* suberectus *Dunn*, *Prunella vulgaris*, cassia seed, pearl shell, rhizoma corydalis, and asarum herb. The mechanism of the prescription maybe related to nourish blood, calm liver, and activate blood circulation according to the theory of TCM [21, 22]. It has been widely used to treat hypertension-related symptoms in clinical practice in China. The most common symptoms include headache, dizziness, giddiness, irritability, and insomnia, which belong to the liver yang hyperactivity and blood deficiency syndrome [21, 22]. Recently, more and more researches demonstrated that YQG could contribute to lowing BP with few side effects both in vitro and in vivo when tested alone [23–28]. Biochemically, BBTD also showed good effect in improving plasma levels of endothelin (ET), calcitonin gene-related peptide (CGRP), and nitric oxide (NO), regulating rennin-angiotensin system (RAS) and decreasing serum levels of urea nitrogen, uric acid, and creatinine [26–28].

Currently, YQG combined with antihypertensive drugs, a new integrative medicine therapy, has been widely used as an alternative and effective method for EH in China. And, until now, a large number of randomized controlled trials (RCTs) and case series have been published in Chinese language but have not been evaluated according to the PRISMA systematic review standard. This study aims to assess the current clinical evidence of YQG combined with antihypertensive drugs for EH.

2. Methods

2.1. Database and Search Strategies. The literature searches were conducted in The Cochrane Library (October, 2012), PubMed, Chinese National Knowledge Infrastructure (CNKI), Chinese Scientific Journal Database (VIP), Chinese Biomedical Literature Database (CBM), and Wanfang data. We also searched the reference list of retrieved papers. Four main databases in Chinese were searched to retrieve the maximum possible number of trials of YQG for EH because YQG is mainly used and researched in China. All of the searches ended on October 20, 2012. Ongoing registered clinical trials were searched in the website of Chinese clinical trial registry (http://www.chictr.org/) and international clinical trial registry by U.S. national institutes of health (http://clinicaltrials.gov/). The following search terms were used individually or combined: "hypertension", "essential hypertension", "Yangxue Qingnao granule", "nourishing the blood and clearing brain granule", "clinical trial", and "randomized controlled trial." The bibliographies of included studies were searched for additional references.

2.2. Inclusion Criteria. All the randomized controlled trials (RCTs) based on YQG combined with antihypertensive drugs compared with antihypertensive drugs in patients with essential hypertension were included. There were no restrictions on population characteristics, language, and publication type. The main outcome measure was blood pressure. Duplicated publications reporting the same groups of participants were excluded.

2.3. Data Extraction and Quality Assessment. Two authors conducted the literature searching (Xiong, Chu), study selection (Xiong, Yang), and data extraction (Xiong, Qian) independently. The extracted data included authors, title of study, year of publication, study size, age and sex of the participants, diagnosis standard, details of methodological information, treatment process, courses, details of the control interventions, outcomes, and adverse effects for each study. Disagreements were resolved by discussion and reached consensus through a third party (Wang). The methodological quality of trials was assessed independently using criteria from the *Cochrane Handbook for Systematic Review of Interventions*, Version 5.1.0 (Xiong, Yang) [29]. The seven items included random sequence generation (selection bias),

allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias. The quality of all the included trials was categorized to low/unclear/high risk of bias ("yes" for a low of bias, "no" for a high risk of bias, and "unclear" otherwise). Then, trials were categorized into three levels: low risk of bias (all the items were in low risk of bias), high risk of bias (at least one item was in high risk of bias), unclear risk of bias (at least one item was unclear).

2.4. Data Synthesis. RevMan 5.1 software provided by the Cochrane Collaboration was used for data analyses. Continuous outcome will be presented as mean difference (MD) and its 95% CI. The statistical heterogeneity was presented as significant when I square (I^2) is over 50% or $P < 0.1$. Fixed effects model was used if there is no significant heterogeneity of the data; random effects model was used if significant heterogeneity existed ($I^2 > 50\%$). Publication bias would be explored by funnel plot analysis if sufficient studies were found.

3. Result

3.1. Description of Included Trials. We identified 220 potentially relevant articles from electronic and manual searches in the six databases. Twelve RCTs [30–41] were included. All the RCTs were conducted in China and published in Chinese. The screening process is summarized in a flow diagram (Figure 1). The characteristics of included trials were summarized in Table 1.

A total of 1985 patients with essential hypertension were included. Twelve trials specified two diagnostic criteria of essential hypertension, three trials [32–34] used Chinese Guidelines for the Management of Hypertension-2005 (CGMH-2005), three trials [35, 38, 40] used 1999 WHO-ISH guidelines for the management of hypertension (1999 WHO-ISH GMH), and the other six trials [30, 31, 36, 37, 39, 41] only demonstrated patients with essential hypertension without detailed information. Only two trials [35, 38] specified the diagnostic criteria of liver-kidney yin deficiency syndrome and blood stasis syndrome by Guidelines of Clinical Research of New Drugs of Traditional Chinese Medicine (GCRNDTCM) and Diagnostic Criteria of Blood Stasis Syndrome (DCBSY). And the rest ten trials [30–34, 36, 37, 39–41] did not report any TCM diagnostic criteria.

The interventions of all the trials [30–41] included YQG combined with antihypertensive drugs as shown in Table 1. The controls included antihypertensive drugs alone. The total treatment course duration ranged from 2 to 12 weeks. All of the sixteen trials used the BP as the outcome measure. Adverse effect was described in details.

3.2. Methodological Quality of Included Trials. According to the criteria introduced above, no trial was evaluated as having a low risk of bias. The majority of the included trials were assessed to be of general poor methodological quality. Only one trial of the twelve trials reported the method to generate the allocation sequence (random number table) in the paper [37]. We have tried to contact the authors for further information; however, no detailed information could be get. Therefore, it could not judge whether or not it was conducted properly because of the insufficient information provided. Allocation concealment, blinding of participants and personnel, and blinding of outcome assessment were not mentioned in all trials. No trials reported dropout or withdraw. None of trials had a pretrial estimation of sample size. Only two trials [34, 36] mentioned followup. The results of the assessment of risk of bias are presented in Table 2.

3.3. Effect of the Interventions. All the included trials [30–41] compared YQG combined with antihypertensive drugs with antihypertensive drugs alone. A change in blood pressure was reported in all the RCTs. Among them, six trials [33, 34, 38–41] used three classes to evaluate treatment effects, including significantly effective, effective, and ineffective, according to the changes of BP data in GCRNDTCM. Thus, only the rest six trials could be considered for further meta-analysis [30–32, 35–37].

3.3.1. Systolic Blood Pressure (SBP). The six independent trials [30–32, 35–37] did not show homogeneity in the trial results, chi-square = 130.49, ($P < 0.00001$); $I^2 = 96\%$. Thus, random-effects model should be used for statistical analysis. The meta-analysis showed there are significant beneficial effect on the combination group compare to the antihypertensive drugs using alone (MD: −7.31 [−11.75, −2.87]; $P = 0.001$) (Table 3).

3.3.2. Diastolic Blood Pressure (DBP). The six independent trials [30–32, 35–37] did not show homogeneity in the trial results, chi-square = 141.51, ($P < 0.00001$); $I^2 = 96\%$. Thus, random-effects model should be used for statistical analysis. The meta-analysis showed there are significant beneficial effect on the combination group compare to the antihypertensive drugs using alone (MD: −5.21 [−8.19, −2.24]; $P = 0.0006$) (Table 4).

3.4. Publication Bias. The number of trials was too small to conduct any sufficient additional analysis of publication bias.

3.5. Adverse Effect. Eight out of twelve trials mentioned the adverse effect [30–33, 35, 36, 38, 41]. Among them, no adverse events were found in three trials [30, 36, 38]. Four trials reported five specific symptoms including dry cough, nausea, epigastric fullness, sore throat, and constipation [31, 32, 35, 41]. One trial reported adverse effect in valsartan group including paroxysmal headache and dizziness [33]. One trial mentioned hypokalemia in indapamide group which may be the adverse effect of indapamide [35]. One trial mentioned dizziness and fatigue in enalapril group [41]. All of the adverse events were not serious.

4. Discussion

This systematic review included twelve randomized trials and a total of 1985 participants. The main findings of this systematic review were that YQG combined with antihypertensive drugs demonstrated potential effect for lowing either SBP

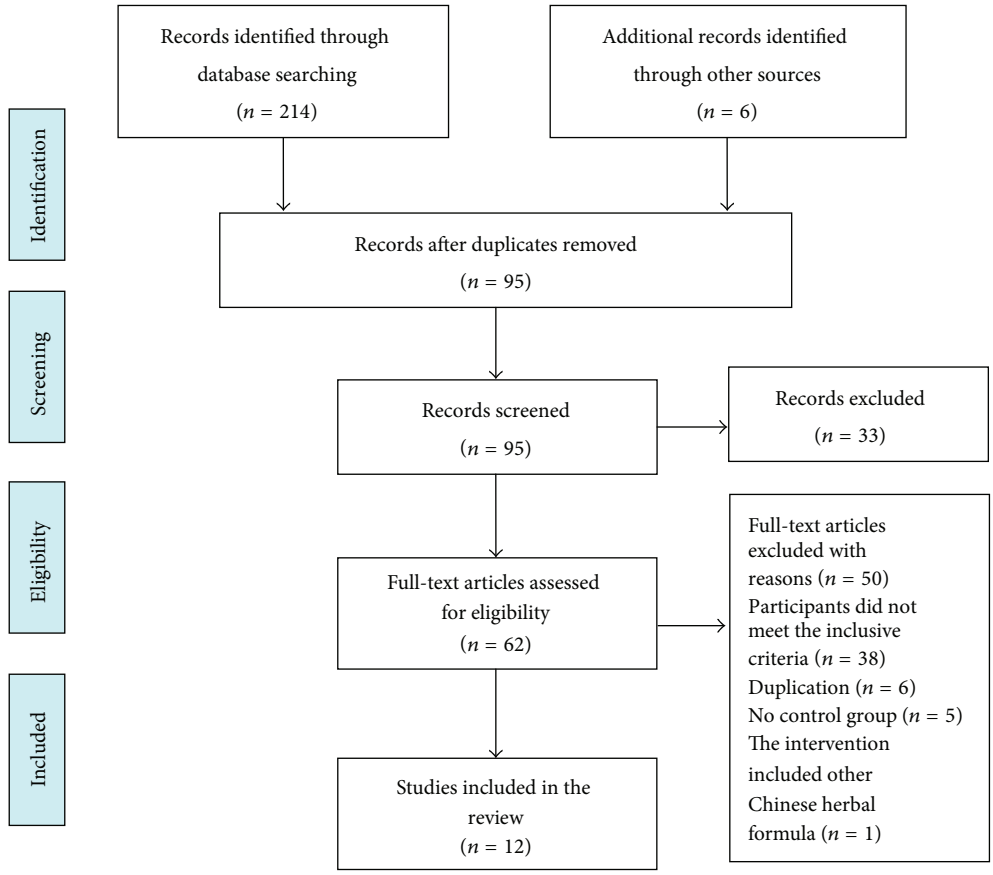

Figure 1: PRISMA 2009 flow diagram.

(MD: −7.31 [−11.75, −2.87]; $P = 0.001$) or DBP (MD: −5.21 [−8.19, −2.24]; $P = 0.0006$) compared to antihypertensive drugs alone. YQG is an effective adjunctive treatment to antihypertensive drugs in patients with hypertension. However, the evidence remains weak due to poor methodological quality of including studies. Thus, available data are not adequate to draw a definite conclusion of combination therapy for essential hypertension. And the positive findings should be interpreted conservatively due to the following facts.

Firstly, the methodology of this systematic review is generally low. (1) Randomization: all the included trials claimed randomization; however, only one trial demonstrated on the generation of random allocation [37]. The other trials did not provide sufficient information on randomization and only mentioned that "patients were randomized into two groups." So, we could not rule out the possibility that the claimed randomization may not be real actually. It could lead to selection bias. (2) Blinding: all of the studies were lack of any blinding method, either blinding of participants and personnel or blinding of outcome assessment. There were six trials [33, 34, 36, 37, 39, 40] conducted by only one author, and three trials [32, 35, 38] conducted by only two authors. It is difficult to accomplish an RCT such as randomization, allocation concealment, blinding and analysis only by one or two doctors. It could lead to performance bias. (3) Analysis of data: only two trials have reported the dropouts, but without the intention-to-treat analysis [31, 36]. Therefore, the positive findings should be interpreted conservatively. It could lead to attrition bias. (4) Placebo controlled: none of the included trials have placebo control. All of them used "A + B versus B" design where patients were randomized to receive YQG plus antihypertensive drugs treatment versus antihypertensive drugs control treatment without a rigorous control for placebo effect. Thus, positive conclusions would be made due to nonspecific placebo effects [42]. (5) Sample size: none of trials had a pretrial estimation of sample size, which indicated the lack of statistical power to ensure appropriate estimation of the therapeutic effect. And all of the included trials were of single center. Sample size calculation should be conducted before enrollment. It is well known that, if methodologically poorly designed, all the trials would show larger differences between experimental and control groups than those conducted rigorously [43–45].

Secondly, another major limitation was the publication bias. All of the trials were conducted in China and published in Chinese. Almost all the RCTs claimed that the positive effect of YQG combined with antihypertensive drugs is better than antihypertensive drugs alone. Negative findings almost have not been reported. We tried to conduct extensive

Table 1: Characteristics and methodological quality of included studies.

Study ID	Sample	Diagnosis standard	Intervention	Control	Course (week)	Outcome measure
Liu et al., 2009 [30]	118	Hypertension diagnostic criteria (unclear)	YQG 4 g tid + control	Valsartan 80 mg qd	4	BP; adverse effect
Chen et al., 2006 [31]	130	Hypertension diagnostic criteria (unclear)	YQG 4 g tid + control	Antihypertensive drugs (no detailed information)	8	BP; adverse effect
Ji and Han, 2011 [32]	160	1999 WHO-ISH GMH	YQG 4 g tid + control	Antihypertensive drugs (no detailed information)	12	BP; adverse effect
Yuan, 2006 [33]	103	1999 WHO-ISH GMH	YQG 4 g tid + control	Valsartan 80 mg qd	2	BP; adverse effect
Shi, 2011 [34]	80	1999 WHO-ISH GMH	YQG 4 g tid + control	Felodipine 5 mg qd	4	BP
Li and Wan, 2006 [35]	80	CGMH-2005; GCRNDTCM; DCBSY	YQG 4 g tid + control	Indapamide 1.25 mg qd	3	BP; adverse effect
Liang, 2011 [36]	100	Hypertension diagnostic criteria (unclear)	YQG 4 g tid + control	Antihypertensive drugs (no detailed information)	8	BP; adverse effect
Fu and Xiao, 2011 [37]	992	Hypertension diagnostic criteria (unclear)	YQG 4 g tid + control	Antihypertensive drugs (no detailed information)	4	BP
Yang et al., 2008 [38]	122	CGMH-2005; GCRNDTCM	YQG 4 g tid + control	Antihypertensive drugs (no detailed information)	4	BP; adverse effect
Lin and Zhou, 2004 [39]	100	Hypertension diagnostic criteria (unclear)	YQG 4 g tid + control	Antihypertensive drugs (no detailed information)	2	BP
Ai, 2012 [40]	102	CGMH-2005	YQG 4 g tid + control	Antihypertensive drugs (no detailed information)	6	BP
Qin, 2008 [41]	68	Hypertension diagnostic criteria (unclear)	YQG 4 g tid + control	Enalapril 10 mg qd	4	BP; adverse effect

searches for unpublished material, but no unpublished "negative" studies were found.

Thirdly, syndrome (also known as "pattern" or "zheng") is the basic unit and key concept in TCM theory, which has been used in China for over 3,000 years. The Chinese herbs and formulas should match the type of syndrome differentiation, which is the basic rule in TCM clinical practice. In this paper, only two trials reported TCM diagnostic criteria with liver-kidney yin deficiency syndrome and blood stasis syndrome [35, 38]. Thus, the trial assessed the clinical effect of combination therapy with positive findings. The rest ten trials have not mentioned any TCM diagnostic criteria at all [30–34, 36, 37, 39–41]. Six trials reported good effect on improving symptoms such as headache, dizziness, and insomnia, which were common symptoms in hypertensive patients. Chinese medicine practitioners believed that treating patients without syndrome differentiation will impair the advantages of Chinese herbs [46–49]. Therefore, the process of syndrome differentiation should be explained clearly and assessed rigorously.

Fourthly, with the increasing awareness of self-care, natural plants as raw materials are favored by people all over the world for their advantages in preventing and curing diseases. However, the safety problem of Chinese herbal medicines is generally concerned [50]. What is more is, as that integrative medicine therapy with both conventional western medicine and tradition medicine becomes the new trend in current medical care, the combined applications of herbs and drugs are increasing. And the potential of interactions between them causes more and more attention worldwide [51–55]. Herb-drug interaction has hence become an important focus of this systematic review. As most of the trials did not reported adverse events of combination therapy strictly, the safety of YQG combined with antihypertensive drugs needs to be monitored rigorously and reported appropriately in the future clinical trials.

In conclusion, there is some encouraging evidence of YQG combined with antihypertensive drugs for lowering BP, but the evidence remains weak due to the poor methodological quality of including studies. More randomized trials

Table 2: Quality assessment of included randomized controlled trials.

Included trials	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other sources of bias	Risk of bias
Liu et al., 2009 [30]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Chen et al., 2006 [31]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Ji and Han, 2011 [32]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Yuan, 2006 [33]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Shi, 2011 [34]	Unclear	Unclear	Unclear	Unclear	Yes	Yes	Unclear	High
Li and Wan, 2006 [35]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Liang, 2011 [36]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Fu and Xiao, 2011 [37]	Table of random number	Unclear	Unclear	Unclear	Yes	Yes	Unclear	Unclear
Yang et al., 2008 [38]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Lin and Zhou, 2004 [39]	Unclear	Unclear	Unclear	Unclear	Yes	Yes	Unclear	High
Ai, 2012 [40]	Unclear	Unclear	Unclear	Unclear	Yes	Yes	Unclear	High
Qin, 2008 [41]	Unclear	Unclear	Unclear	Unclear	Yes	No	Unclear	High

Table 3: Analyses of systolic blood pressure.

Trials		MD [95% CI]	*P* Value
YQG plus valsartan versus valsartan	1	−7.30 [−10.25, −4.35]	<0.00001
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−9.00 [−10.45, −7.55]	<0.00001
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−4.90 [−7.10, −2.70]	<0.0001
YQG plus indapamide versus indapamide	1	−4.38 [−8.28, −0.48]	0.03
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−16.00 [−17.66, −14.34]	<0.00001
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−1.85 [−4.00, 0.30]	0.09
Meta-analysis	6	−7.31 [−11.75, −2.87]	0.001

Table 4: Analyses of diastolic blood pressure.

Trials		MD [95% CI]	*P* Value
YQG plus valsartan versus valsartan	1	−11.70 [−13.70, −9.70]	<0.00001
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−8.00 [−9.03, −6.97]	<0.00001
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−2.11 [−3.60, −0.62]	0.006
YQG plus indapamide versus indapamide	1	0.57 [−1.39, 2.53]	0.57
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−7.00 [−8.27, −5.73]	<0.00001
YQG plus antihypertensive drugs versus antihypertensive drugs	1	−3.04 [−4.12, −1.96]	<0.00001
Meta-analysis	6	−5.21 [−8.19, −2.24]	0.0006

with well design and adequate sample size are warranted to support or refute the positive findings in future [56]. In addition, all clinical trials must be carried out and reported according to the CONSORT Statement [42, 57].

Conflict of Interests

All authors declare that they have no conflict of interests.

Authors' Contribution

J. Wang, X. Yang, B. Feng, W. Qian, Z. Fang, W. Liu, H. Li, X. Li, and F. Chu contributed equally to this paper.

Acknowledgments

The current work was partially supported by the National Basic Research Program of China (973 Program, no. 2003CB517103) and the National Natural Science Foundation Project of China (no. 90209011). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the paper.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2012, Article ID 697237, 7 pages
doi:10.1155/2012/697237

Review Article

Outcome Measures of Chinese Herbal Medicine for Hypertension: An Overview of Systematic Reviews

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Received 11 November 2012; Accepted 10 December 2012

Academic Editor: Tabinda Ashfaq

Objective. The aim of this overview was to summarize the outcome measures of Chinese herbal medicine (CHM) for the treatment of hypertension based on available systematic reviews (SRs), so as to evaluate the potential benefits and advantages of CHM on hypertension. *Methods.* Literature searches were conducted in the Cochrane Database of Systematic Reviews, MEDLINE, and 4 databases in Chinese. SRs of CHM for hypertension were included. Two independent reviewers (J. Wang and X. J. Xiong) extracted the data. *Results.* 10 SRs were included. 2 SRs had primary endpoints, while others focused on secondary endpoints to evaluate CHM for hypertension such as blood pressure (BP) and Traditional Chinese Medicine (TCM) syndrome. 6 SRs have reported the adverse effects, whereas the other 4 SRs have not mentioned it at all. Many CHM appeared to have significant effect on improving BP, TCM syndrome, and so on. However, most SRs failed to make a definite conclusion for the effectiveness of CHM for hypertension due to poor evidence. *Conclusion.* Primary endpoints have not been widely used currently. The benefits of CHM for hypertension need to be confirmed in the future with randomized controlled trials (RCTs) of more persuasive primary endpoints and high-quality SRs.

1. Introduction

Hypertension is one of the most common and important health problems affecting millions of people throughout the world and about 20% of the adult population in many countries [1]. It could lead to severe complications, such as hypertensive cardiovascular disease, hypertensive renal disease, and atherosclerotic complications including stroke, coronary heart disease, renal insufficiency, and heart failure [2]. However, hypertension in most individuals remains untreated or uncontrolled [3]. Effective treatment of hypertension is limited by availability, cost, and adverse effects of antihypertensive medications. Some hypertension-related symptoms could not be completely relieved by conventional medicine. Hypertension is the major cause of morbidity and mortality and is the third highest risk factor for lifetime burden worldwide [4]. Therefore, some patients have turned to complementary and alternative therapies (or traditional medicine), especially Chinese medicine (CM) [5–9], hoping that such treatments might improve their symptoms. Chinese medicine (CM) has a history for more than 2500 years with unique theory of diagnosis and treatment [10–15]. In recent years, with the popularity and prevalence of Chinese medicine (CM), there has been a growing interest in Chinese herbal medicine (CHM) for patients with hypertension both in China and the West [16–20]. Until now a number of clinical studies of CHM reported the clinical effectiveness in hypertensive patients ranging from case reports and case series to controlled observational studies and randomized clinical trials. However, the evidence needs to be reviewed systematically [21].

As the evidence gathering tools, systematic reviews (SRs) of randomized controlled trials (RCTs) are considered to provide the best evidence about the effectiveness of interventions [22, 23]. Physicians and policy makers need evidence from SRs for decision making and policy making. Patients and researchers also need such information to support shared decisions and to set priorities for research. Recently, an increasing number of SRs about CHM for hypertension have been reported. However, few of them have shown that CHM was definitely effective for hypertension due to the weak evidence. There is a need for combining multiple

reviews into overviews to provide users with easily available information. In addition, when people making decisions about health care look for guidance from research, the outcomes reported are key, which also plays an important role in drawing a more persuasive conclusion [24]. However, there is a general lack of consensus regarding the choice of outcomes in particular clinical settings, which affect trial design, conduct, analysis, and reporting [25]. The aim of this overview was to summarize the outcome measures of CHM for treatment of hypertension based on available SRs both in English and Chinese, so as to display the current situation and evaluate the potential benefits and advantages of CHM on hypertension.

2. Methods

Literature searches were conducted in the Cochrane Database of Systematic Review (October, 2012), MEDLINE (2002–2012), Chinese National Knowledge Infrastructure (CNKI, 2002–2012), Chinese Biomedical Literature Database (CBM, 2002–2012), Chinese Scientific Journal Database (VIP, 2002–2012), and Wanfang Databases (2002–2012). All of those searches ended on October 10, 2012. CNKI, CBM, VIP, and Wanfang were four main databases in China. All of the databases in Chinese were searched to retrieve the maximum possible number of systematic reviews or meta-analyses of CHM for hypertension because CHMs are mainly used and researched in China. We searched papers from 2002 to 2012 for high-quality RCTs and SRs mainly focusing on the recent ten years.

The strategy below was used to search The Cochrane Library and adapted appropriately for use in different electronic databases: #1 herb*; #2 medic*; #3 (#1 and #2); #4 Chinese; #5 (#3 or #4); #6 blood pressure; #7 hypertension; #8 high blood pressure; #9 (#6 or #7 or #8); #10 (#5 and #9). Two reviewers (J. Wang and X. J. Xiong) independently scanned the relevance of all references based on title and abstract of each record. If the information included a systematic review or a meta-analysis of CHM for hypertension, the full paper was obtained for further assessment. Papers were excluded when problems occurred with: repeat publication, methodological studies, quality assessment report, research on acupuncture, qigong, massage, or other treatments (Figure 1).

Outcome measures included primary endpoints and secondary endpoints. Primary endpoints include mortality, stroke, coronary heart disease, and hypertensive renal damage. Secondary endpoints mainly indicate blood pressure, the level of blood lipids, pulse pressure (PP), quality of life (QOL), and Traditional Chinese Medicine (TCM) syndrome. In addition, PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) was used as an assessment tool to evaluate the quality of the included SRs [26]. As shown in the article written by Moher et al. [26], the checklist consists of 27 items in 7 key areas and a four-phase flow diagram in order to help authors improve the reporting of systematic reviews and meta-analyses. It describes the preferred way to present the title, abstract, introduction, methods, results, discussion, and funding sections in detail of systematic reviews and meta-analyses. It requires each reviewer to follow the research process and include a flow diagram providing information about the number of studies identified, included, and excluded through database searching and other sources, and reasons for excluding them such as duplicates. Information of each included SRs was imported into PRISMA statement for analysis. One author (J. Wang or X. J. Xiong) independently extracted data from each included review using predefined criteria and discussed the data with the other author to reach a consensus when there is a disagreement.

3. Results

After primary search of 6 databases, 182 articles were screened out from electronic and manual searches (as shown in Figure 1), and the majority were excluded due to obvious ineligibility which including irrelevant titles and abstracts (some papers were found from more than one database). After reading the titles and abstracts, a majority of them were excluded. 170 articles were excluded because of duplicates, nonclinical studies, case reports, and research on acupuncture, moxibustion, cupping, qigong, Tai Chi, and other treatments. Then 12 full articles were retrieved for more detailed evaluation. Due to methodological study and quality assessment report, 2 out of them were excluded respectively based on the assessment tool. In the end, 10 SRs were reviewed [27–36]. All the SRs were conducted in China with 1 in English and 9 in Chinese. 9 SRs from the Chinese electronic databases were published between 2006 and 2012. Since 2011, the number of SRs increased markedly. Only 1 SR from the Cochrane Database of Systematic Review was published in 2012 [31].

8 SRs were concerned with essential hypertension, and the other 2 were related to elderly hypertension. We also retrieved the related clinical trials for further analysis. These clinical trials in SRs were mainly conducted in China. The methodological quality of clinical trials was assessed independently with criteria from the Cochrane Handbook for Systematic Review of Interventions, Version 5.1.0 (J. Wang and X. J. Xiong) [37]. The items included random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias. It was found out that although the original trials included all claimed "RCTs" or "quasi-RCTs", only few of them were typical RCTs. Almost all the trials mentioned that "patients were randomized into two groups" without detailed information about randomization. So, it is hard to judge whether randomization was conducted properly and really. Most of them have not mentioned allocation concealment and double-blind. That is to say, the claimed RCTs may not be true RCTs actually. Therefore, most of the trials in the SRs were of low quality. However, only 10 RCTs were of high quality: three were concerned with replenishing spleen and kidney therapy, one was related to promoting blood circulation and removing blood stasis therapy, two were associated with clearing heat therapy such as *Bidens bipinnata L.* and *Qinre jiangya*

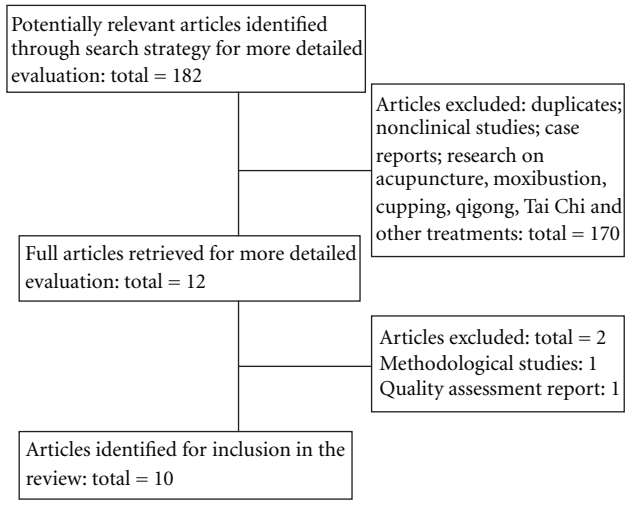

Figure 1: Flow-chart of SRs selection.

mixture, and four were about calming the liver therapy such as *Pingjiangyin* capsule, *Pinggan jiangya* capsule, *Niuhuang jiangya* tablet, and *Tiaopingkang* tablet. Among these 10 SRs, 3 kinds of CHM were reviewed, including capsules, pellets, and herbal decoction as follows: *Niuhuang Jiangya* preparation ($n = 1$) [27]; *Tianma Gouteng* Yin ($n = 2$) [30, 31]; herbal decoction ($n = 7$) [28, 29, 32–36]. The characteristics of 10 SRs were summarized in Table 1.

As shown Table 1, 2 SRs analyzed primary endpoints and the remaining nine SRs all focused on secondary endpoints to evaluate CHM for hypertension. This is mainly due to whether there was detailed information in the original research or not. 4 primary endpoints were analyzed in 2 SRs including essential hypertension and elderly hypertension. 1 SR about *Niuhuang Jiangya* preparation showed no effect on the mortality, stroke, coronary heart disease, and hypertensive renal damage [27]. The other 1 SR about herbal products appeared to be effective on improving hypertensive renal damage [36]. Blood pressure was the most common secondary endpoint in the SRs. All the included SRs reported blood pressure changes. Among them, 8 SRs showed improvement in blood pressure, but the other 2 SRs showed insufficient evidence [27, 31]. 5 SRs analyzed TCM syndrome changes [32–36]. There are 3 SRs that reported Triglycerides (TG) [28], pulse pressure (PP) [35], and quality of life (QOL) [36], respectively. Many CHMs appear to be effective on improving signs and symptoms, level of blood lipids, and so forth. Some SRs also reflected that CHM may be effective to prevent progression to severe complications of hypertension. However, due to poor methodological quality in the majority of included trials, most SRs could not draw confirmative conclusions on the beneficial effect of CHM for hypertension.

Adverse effects, providing a guideline to both doctors and patients for reasonable medication, should also be regarded as an essential outcome measure in clinical trials [38, 39]. However, there is a widespread misunderstanding of CHM. Most people, especially in East Asia, think that the application of TCM has a long history, natural origination, good health care effects, efficacy of treating symptoms and root causes, and no toxic and side effects [40–42]. Even it is widely accepted that it is safe to use herbal medicines for various diseases in China. However, along with the development of pharmacology study, there are more and more reports of liver toxicity and other adverse events associated with CHM [43–45], so this paper makes the analysis on the adverse effects of CHM for hypertension. In this paper, adverse effects are ignored. 6 SRs [27, 30, 32–34, 36] have reported the adverse effects, whereas the other 4 SRs [28, 29, 31, 35] have not mentioned it at all. Only 2 trials in the 1 SR [34] had long-term data on adverse effects. Most of adverse effects of CHM were mentioned as "none obvious," "low adverse effect" or even "no adverse effect." The reported adverse reactions in control groups were more severe than in treatment groups. Adverse events reported in 4 SRs [27, 30, 32, 34], including headache, dizziness, cough, dry stool, and diarrhea. Thus, adverse reactions of CHM should be highlighted in systematic reviews, and the safety of CHMs needs to be monitored rigorously and reported appropriately in the future clinical trials.

The Cochrane Collaboration is an international organization which aims to prepare and maintain rigorous systematic reviews in order to help people make well-informed decisions about health care [46]. As we know that Cochrane reviews are regarded as the highest standard of evidence with a greater methodological quality [47]. Outcome measures of the included SRs in Cochrane Database of Systematic Reviews are more credible than non-Cochrane reviews [48, 49]. They adopt primary endpoints, secondary endpoints, and safety as outcome measures. Unfortunately, in our paper, only one SR about *Tianma Gouteng* Yin for essential hypertension was retrieved from Cochrane Library [31]. The authors of the SR identified no study which met the inclusion criteria for review. As no trials could be identified for the review, no conclusions can be made about the role of *Tianma Gouteng* Yin in the treatment of essential hypertension. When referring to non-Cochrane reviews, primary endpoints and adverse effects are seldom taken as outcome measures in most SRs.

In addition, it was found out that most of the included SRs were generally of low quality according to PRISMA statement. Review methods were not fully reported in most SRs. The characteristics of included clinical trials were not described with detailed information in 5 SRs [29, 30, 32–34]. No flow-chart of information through the different phases of a systematic review was provided. Sensitivity analysis, subgroup analysis, and potential publication bias were not analyzed sufficiently in the reviews. Convincing outcome measures were lacked in most SRs.

4. Discussion

In our overview, the primary endpoints and secondary endpoints are all used to evaluate the efficacy of CHM for hypertension. It is widely known that the primary goal of essential hypertension treatment is to reduce mortality, or prevent progression to severe complications such as stroke, coronary heart disease, heart failure, and hypertensive renal damage. However, there is a lack of data on the final indicator

Table 1: Outcome measures of CHM for hypertension in systematic reviews.

Outcome measures (number of SR)	Condition (number of SR)	CHM	First author	Number of RCTs/total	Conclusion	Risk of publication bias
Primary endpoints						
Mortality, stroke, coronary heart disease, and hypertensive renal damage (1)	Essential hypertension (1)	*Niuhuang Jiangya* preparation	Wang et al. (2008) [27]	3/3	B	NA
hypertensive renal damage (1)	Elderly hypertension (1)	Herbal products	Han (2012) [36]	4/45	A	NA
Secondary endpoints						
Blood pressure (10)	Essential hypertension (8)	*Niuhuang Jiangya* preparation	Wang (2008) [27]	3/3	B	NA
		Herbal products	Hu (2009) [28]	24/24	A	L
		Herbal products	Ren (2006) [29]	11/11	A	H
		Tianma Gouteng Yin	Dong (2011) [30]	6/6	A	L
		Tianma Gouteng Yin	Zhang (2012) [31]	0/0	B	NA
		Pinggan qianyang	Xu (2012) [32]	8/8	A	H
		Buyi shenqi	Shi (2012) [33]	5/5	A	H
		Buyi pishen	Liu (2011) [34]	13/15	A	L
	Elderly isolated systolic hypertension (1)	Herbal products	Li (2012) [35]	17/17	A	L
	Elderly hypertension (1)	Herbal products	Han (2012) [36]	45/45	A	NA
Triglycerides (1)	Essential hypertension (1)	Herbal products	Hu (2009) [28]	4/24	A	L
Pulse pressure (1)	Elderly isolated systolic hypertension (1)	Herbal products	Li (2012) [35]	4/17	A	L
Quality of life (1)	Elderly hypertension (1)	Herbal products	Han (2012) [36]	4/45	A	NA
TCM syndrome (5)	Essential hypertension (3)	Pinggan qianyang	Xu (2012) [32]	3/8	A	H
		Buyi shenqi	Shi (2012) [33]	4/5	A	H
		Buyi pishen	Liu (2011) [34]	9/15	A	L
	Elderly isolated systolic hypertension (1)	Herbal products	Li (2012) [35]	6/17	A	L
	Elderly hypertension (1)	Herbal products	Han (2012) [36]	4/45	A	NA

Notes: Pinggan qianyang: calming the liver and suppressing liver-yang to patients with hyperactivity of liver yang syndrome; Buyi shenqi: replenishing kidney qi to patients with kidney qi deficiency syndrome; Buyi pishen: replenishing spleen and kidney to patients with spleen and kidney deficiency syndrome; A: CHM may be or appear to be effective; B: the evidence is insufficient and inclusive; H: high; L: low; NA: not mentioned.

at endpoint. Most of the included SRs have not reported the mortality rate or the incidence of complications. The primary endpoints are seldom used due to the difficulty of clinical implementation, limitations of the research funding and other reasons. On the contrary, secondary endpoints are most commonly adopted in clinical trials. The outcome measures from all the included SRs are mainly blood pressure and TCM symptom. It is probably related to the feasibility and operability either in inpatients or outpatients in small sample size and short-term clinical trials. Although it is helpful to reduce future cardiovascular risk to some extent by decreasing blood pressure and improving TCM symptoms, primary endpoints are widely recognized as more persuasive outcome measures when evaluating the efficacy of CHM for hypertension. Moreover, adverse effect, which is also very important in evaluating the safety of CHM, should be taken as outcome measures too. All of these problems affect the generation of high-level evidence of CHM for hypertension.

Ever since 1999 when the first Cochrane review of CHM was published [50], there is an increasing number of similar systematic reviews/meta-analysis. Thus, it is necessary to systematically identify and assess the quality of these reviews. The methodology and reporting quality of systematic reviews/meta-analyses of CHM have attracted great attention [51–54]. According to PRISMA statement, the quality of the current included SRs is judged as generally poor, especially those published in Chinese journals. Reviews had methodological and reporting flaws that could have influenced the reviews validity. The deficiencies mainly lies in searching literature, reporting of characteristics of included and excluded studies, extracting relevant data, evaluating primary trials' quality, and merging data. Also, the report of less persuasive outcome measures in most of the SRs has reduced the validity of the conclusions. So, in future, reviewers should attach more importance to the method of performing SR and receive relevant training of skills in reporting to reduce the amount of bias in their reviews. Researchers of clinical trials in TCM should also pay more attention to experimental design and methodological quality and improve the reporting quality according to the Consolidated Standards of Reporting Trials (CONSORT) statement [55], so as to improve the quality of TCM clinical research and ensure truth and reliability of conclusions. Although CHM appeared to be effective for hypertension in clinical use, most SRs were inconclusive that CHM had a definite effect for hypertension due to the poor evidence.

More specifically, the following deficiencies in this overview should be taken into consideration before recommending the conclusion. Firstly, both the majority of included SRs and the original clinical trials are of low quality due to poorly designed and low-quality methodology. Secondly, as CHM is mainly used in China, SRs published in Chinese and English are retrieved. However, electronic databases in other languages have been omitted. Thirdly, unpublished studies and many negative randomized, double-blind, and controlled trials have not been taken into account for further analysis.

In summary, although both primary and secondary endpoints were all used to evaluate the effectiveness of CHM for hypertension, primary endpoints have not widely been used currently. Although this overview may show potential effectiveness of CHM for hypertension in terms of some outcome measures, most SRs failed to draw a confirmative conclusion for recommendation on the beneficial effect of CHM in hypertensive patients due to poor evidence. The benefits of CHM for hypertension still need to be confirmed in the future with more rigorous RCTs of more persuasive primary endpoints and high-quality SRs.

Conflict of Interests

All authors declare that they have no conflict of interests.

Acknowledgments

This work was supported in part by the National Basic Research Program of China (973 Program, 2003CB517103) and the National Natural Science Foundation Project of China (90209011).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2012, Article ID 828170, 6 pages
doi:10.1155/2012/828170

Research Article

Reheated Palm Oil Consumption and Risk of Atherosclerosis: Evidence at Ultrastructural Level

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Received 19 October 2012; Accepted 29 November 2012

Academic Editor: Kashmira Nanji

Background. Palm oil is commonly consumed in Asia. Repeatedly heating the oil is very common during food processing. *Aim*. This study is aimed to report on the risk of atherosclerosis due to the reheated oil consumption. *Material and Methods*. Twenty four male Sprague Dawley rats were divided into control, fresh-oil, 5 times heated-oil and 10 times heated-oil feeding groups. Heated palm oil was prepared by frying sweet potato at 180°C for 10 minutes. The ground standard rat chows were fortified with the heated oils and fed it to the rats for six months. *Results*. Tunica intima thickness in aorta was significantly increased in 10 times heated-oil feeding group ($P < 0.05$), revealing a huge atherosclerotic plaque with central necrosis projecting into the vessel lumen. Repeatedly heated oil feeding groups also revealed atherosclerotic changes including mononuclear cells infiltration, thickened subendothelial layer, disrupted internal elastic lamina and smooth muscle cells fragmentation in tunica media of the aorta. *Conclusion*. The usage of repeated heated oil is the predisposing factor of atherosclerosis leading to cardiovascular diseases. It is advisable to avoid the consumption of repeatedly heated palm oil.

1. Introduction

Palm oil is one of the commonly consumed oils in Malaysia. It contains 50% unsaturated fat and 50% saturated fat [1]. Consumption of saturated fat is believed to predispose cardiovascular disease. However, fresh palm oil contains tocopherols and tocotrienols which are antioxidants and the antioxidant effect of tocotrienols is 40–60 times higher than that of tocopherols [2]. Frying is one of the common methods to prepare Asian food. Therefore, the consumed fats in our diet are exposed to extreme temperature during cooking. Furthermore, the practice of reusing oils for repeated frying is also prevalent in an attempt to save cost. Repeated heating increases lipid peroxidation and reduces antioxidant properties of the oils, leading to produce free radicals [3]. Moreover, free radical induced oxidative stress is associated with the atherosclerosis development [4]. Therefore, the ingestion of repeatedly heated oil might produce harmful effect, attributing to the development of atherosclerosis.

Atherosclerosis is a chronic progressive disease which commonly affects arteries, resulting in reduced blood flow that eventually predisposes to various ailments such as coronary artery disease and cerebrovascular disease. Atherosclerosis is prevalent in all over the world. It has been proven that nutrition and cholesterol intake in the diet are ranked as the highest risk factors in atherosclerosis [5]. The incidence of the disease is usually associated with the circulating low-density lipoprotein (LDL). Polyunsaturated fatty acid residues in lipoprotein are vulnerable to free radical oxidation and these modified oxidative LDLs are scavenged by the macrophage, forming the cholesterol laden "foam-cells" in the atherosclerotic plaques [4]. Furthermore, these

LDLs are chemoattractants for macrophage and smooth muscle cells [6]. The atheromatous plaques formation might be related to the growth factor action and angiogenesis property of vasoactive small molecules produced by the mast cells [7]. Therefore, cholesterol laden macrophage, mononuclear cells migration, and migratory smooth muscle cells in tunica media are expected to reveal in atherosclerotic plague at ultrastructural level. The present study aimed to highlight the impact on the cardiovascular health by consumption of reheated palm oil and review the literature on the mechanism of development of atherosclerosis.

2. Materials and Methods

The aortic samples of this study were obtained from the previous research conducted by the postgraduate student of the Department of Pharmacology, Faculty of Medicine, UKM [8]. The protocol of the study was as follows.

2.1. Experimental Animals. Twenty-four healthy adult male Sprague-Dawley rats (200–280 g) were obtained from the institutional animal resource unit. The rats were reared in stainless steel cages with a room temperature of 27 ± 2°C with 12 hours light and dark cycle. All rats were allowed to access food and tap water *ad libitum*. All the animals handling procedures were in accordance with the institutional animal ethical guideline with the ethical approval number UKMAEC: FP/FAR/2008/Kamsiah/9-Apr/220-Apr-2008-Feb-2011.

2.2. Source and Preparation of Diets. Commercial palm oil (Lam Soon Edible Oil, Malaysia) was used as fresh, five times heated and ten times heated as described by Owu et al. [9]. Briefly, the 2.5 L of the oil was used to fry 1 kg of sweet potatoes in a stainless-steel wok at 180°C for 10 minutes. To prepare five times and ten times heated oil, the hot oil was allowed five hour cooling interval, and the entire frying process is then repeated four and nine more times, respectively, with a fresh batch of sweet potatoes. No fresh oil was added between batches to replace any loss due to evaporation and absorption of oil. The test diets were formulated by mixing 15% weight/weight of the respective prepared oils with ground standard rat chow (Gold Coin Sdn Bhd, Malaysia), reformed into pellets, and then dried in an oven overnight at 70°C. The preparation of test diet was in accordance with the experimental protocol of Adam et al. [10]. However, cholesterol was not added in the present study.

2.3. Study Design. This study was a randomized control study. The rats were acclimatised for one week prior to feed the test diets. They were randomly divided into 4 groups of six based on the diet, namely, basal diet feeding group (C), basal diet fortified with 15% weight/weight fresh palm oil (FPO), 5 times heated palm oil (5HPO), and 10 times heated palm oil (10HPO) feeding groups. After 6 months of feeding with the respective diets, all the rats were sacrificed using diethyl ether. The proximal portion of the ascending aorta and the arch of aorta were taken for light and electron microscopic studies.

2.4. Sample Preparation. Each aortic sample was sectioned into 3 segments of less than 1.0 mm thickness. They were initially immersed for 12–16 hours at 4°C in glutaraldehyde fixative. The samples were then washed 3 times in 0.1 M phosphate buffer, bulk stained with 1% buffered osmium tetroxide for 1-2 hours, and washed in distilled water for 3 times. They were then treated with uranyl acetate for 30 minutes, dehydrated in an ascending series of ethanol solution, infiltrated in propylene oxide, and finally embedded in resin at 60°C for 24 hours. After the resin had polymerized, the samples were sectioned with glass knives.

2.5. Histomorphometric Study. Semithin sections of 1 μm thickness with 1% toluidine blue staining were viewed using a computerized image analyzer of 100 times magnification with the software Image-Pro Plus (Version 5.0.2.9, Media Cybernetics, Inc., Bethesda, USA) together with light microscope (Eclipse 80i, Nikon Corporation, Tokyo, Japan). The aortic section was nominally divided into 4 quarters, and the tunica intima and tunica media thickness were measured at 5 different random areas for each quarter. The mean of the 20 readings was then taken as a representative of a particular treatment group and used for statistical analysis. Thickened tunica intima in the sample was selected for qualitative electron microscopic study.

2.6. Qualitative Electron Microscopic Study. The resin blocks were further trimmed at the areas of interest (thickened tunica intima) to identify the ultrastructural changes. Ultrathin sections of 80 nm thickness were then collected and stained with 3% uranyl acetate and Reynold's lead citrate. These specimens were examined with a transmission electron microscope (Philips HMG 400, Philips, Eindhoven, The Netherlands) for the presence of vacuoles and mononuclear cells in the tunica intima. Micrographs were then taken for qualitative description.

2.7. Statistical Analysis. The data was presented as the mean ± standard error of mean (SEM). Normally distributed data were analysed using parametric tests analysis of variance (ANOVA) test. Data that were not normally distributed were analysed using nonparametric tests, Mann-Whitney U test. Results were considered significant if the P value is <0.05. All mentioned statistical analyses were conducted using Statistical Product and Service Solutions (SPSS) software, version 13.

3. Results

3.1. Quantitative Analysis. The quantitative data were shown in Table 1. The analysis of all the measurements was done by comparing between the frequencies of heating.

3.1.1. Tunica Intimal (TI) Thickness. In general, there was a significant difference in the TI thickness among all groups ($P = 0.013$). However, in terms of types of diets used, there was no significant difference of TI thickness among control and the fresh oil groups ($P = 0.065$).

Table 1: Quantitative analysis of changes in the aortic wall of rats fed with different frequencies of heated palm oil.

Groups	Tunica intima (TI) thickness (μm) ± SEM	Tunica media (TM) thickness (μm) ± SEM	TI : TM ± SEM
Control (C)	23.9200 ± 1.6405	442.5800 ± 18.7733	0.0546 ± 0.0040
Fresh (FPO)	21.6250 ± 1.4244	380.2100 ± 11.9707	0.0570 ± 0.0029[b]
5 times heated (5HPO)	26.7817 ± 1.7856[a]	450.7383 ± 43.6100	0.0659 ± 0.0048[b]
10 times heated (10HPO)	30.1883 ± 2.1573[a]	423.2150 ± 14.8780	0.0673 ± 0.0059[b]

[a]Significant difference between heated oil and fresh oil ($P < 0.05$).
[b]Significant difference between control and palm oil feeding groups ($P < 0.05$).
Results shown as mean ± standard error of mean (SEM).

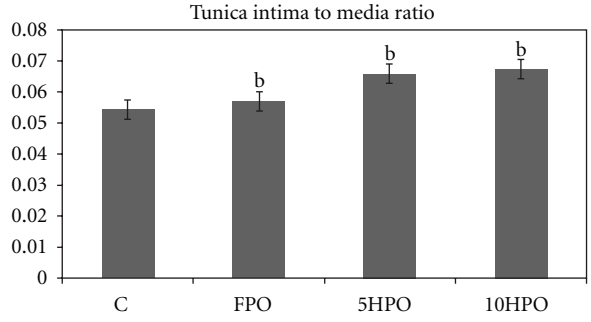

Figure 1: Tunica intima to tunica media ratio (IMR) in the aortic wall of the rats fed with different frequencies of heated palm oil. [b]Significant difference between control and palm oil fed groups ($P < 0.05$).

Comparison among groups of rats fed with palm oil with different frequency of heating (fresh (FPO), 5-times (5HPO) and 10-times heated (10HPO)) was done. Results show that there was a significant difference of TI thickness in the palm oil group ($P = 0.012$), whereby the 10-times heated group has the thickest TI, followed by 5-times heated and lastly, fresh palm oil.

3.1.2. Tunica Media (TM) Thickness. There was no significant difference of TM thickness between control and fresh oil feeding groups as well as among the frequencies of heating and fresh oil feeding groups ($P > 0.05$).

3.1.3. Tunica Intima to Tunica Media Ratio (IMR). The analysis of IMR was done by comparing between the frequencies of heating and fresh oil. In general, there was a significant difference in the IMR among all groups ($P = 0.009$). In terms of types of diets, there was also a significant difference in IMR between the control and palm oil groups ($P = 0.019$).

Comparison among groups of rats fed with fresh (FPO) and repeatedly heated palm oil (5HPO and 10HPO) showed no significant difference of IMR ($P = 0.281$). However, the IMR among the palm oil groups showed an increasing trend (Figure 1).

3.2. Qualitative Analysis. For qualitative descriptive analysis, the changes in the ultrastructure of aortae of the rats ($n = 7$) were examined using transmission electron microscope. Only one sample from each treatment group which showed the greatest IMR was chosen. The electron micrographs (EM) of the respective groups, as well as their descriptions, are as in Figure 2. The ultrastructure of the aorta of control rat was shown in EM 1 (Figure 2(a)).

The endothelium of the majority of the test diet feeding groups consists of an intact layer of endothelial cells, maintaining their squamous characteristic. In FPO group as shown in EM 1 (Figure 2(b)), the denuded endothelial cells (dEC), with no mononuclear cells (MNC), were found in the TI. The internal elastic lamina (IEL) appeared intact, continuous, and regular and appeared to be normal thickness. The TM consisted mainly of smooth muscle cells (SMC), some of which appeared to be fragmented. No intracellular vacuoles (V) were seen. In 5HPO group as shown in EM 1 (Figure 2(c)), the intact endothelial cell (EC) layer was found as in the FPO. However, there was a thickening with MNC and granular material (GM) in the subendothelial layer. The lipid accumulation and collagenous connective tissue might be the content of GM. There was a gap found in the IEL with migratory features of SMC cytoplasm into the TI was also revealed. The fragmented SMC (fSMC) was found in the TM. However, no intracellular vacuoles were seen. In 10HPO group, the TI appeared to be prominently thickened secondary to the plaque formation. The plaque was characterized by a central necrotic core (NC) with surrounding MNC, myointimal cells (MIC), vacuoles (V), and foam cells (FC) embedded in the granular material and collagenous connective tissue. The lesion projected prominently into the vessel lumen and it was covered by an intact EC layer. The plaque was rested on the IEL which appeared to be intact, continuous, and regular (EM 1 (Figure 2(d)). However, the discontinuous and irregular IEL was found in the other area of the specimen. The fragmented SMC was noted in the TM. The massive lipid accumulation (LP) was also found in the subendothelial layer (EM 1 (Figure 2(e)).

4. Discussion

Consumption of saturated fat generally attributes to cardiovascular ailments. Palm oil which is rich in monounsaturated fatty acids is derived from the tropical plant *Elaeis guineensis* [1]. Although it is generally regarded as saturated oil, we must also take into account its antioxidant properties. It contains vitamin E, tocopherols, and tocotrienols which act as potent antioxidants [2]. It helps to protect against lipid peroxidation by trapping free radicals [11]. It has also been

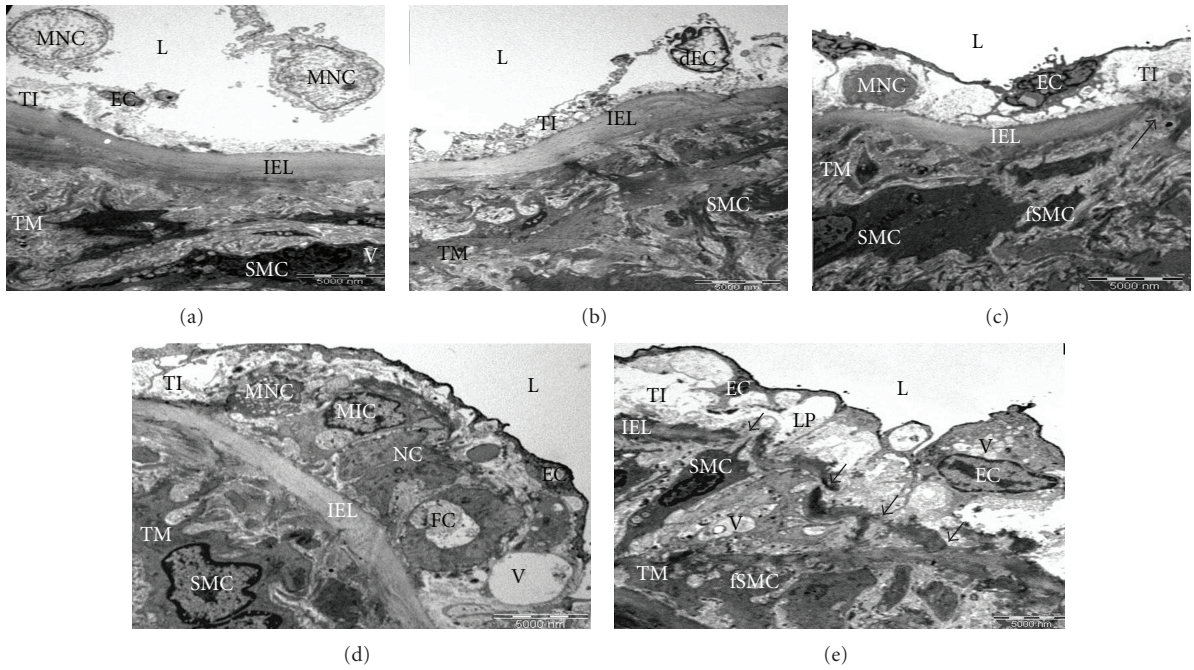

Figure 2: EM 1: Ultrastructure of the aortic wall of rats fed with different frequencies of heated oil (3200x magnification). (a) Control, (b) fresh palm oil (FPO), (c) 5 times heated palm oil (5HPO), and ((d) and (e)) 10 times heated palm oil (10HPO). Tunica intima (TI); tunica medica (TM); vessel lumen (L); denuded endothelial cells (dEC); mononuclear cells (MNC); internal elastic lamina (IEL); smooth muscle cells (SMC); endothelial cell (EC); granular material (GM); fragmented SMC (fSMC); central necrotic core (NC); myointimal cells (MIC); vacuoles (V); foam cells (FC); disrupted IEL (arrows); lipid accumulation (LP).

proved that the tocotrienol-rich fraction (TRF) in palm oil is known to exhibit cardioprotective effects [12]. Furthermore, the palm oil derived vitamin E possesses the serum lipid lowering properties mainly on cholesterol and low density lipoprotein (LDL) [13].

However, as the antioxidants (vitamin E) are extremely sensitive to heat, the repeated heating reduces the antioxidant properties of oil [3]. Repeated thermal exposure may generate more free radicals in the oil due to the underlying oxidative process [14]. These free radicals are highly reactive to bind with the lipids, proteins, carbohydrates, and DNA in the body system, enhancing oxidative stress [5]. Therefore, consumption of repeatedly heated oil might aggravate the lipid peroxidation, leading to damage the arterial wall and increase uptake of lipid, and, subsequently, develop atherosclerosis [10]. Therefore, the heated oil feeding rats in the present study showed obvious changes in the aortic wall as the oil lost its protective antioxidant properties. The gradually increased TI thickness in the heated oil treated groups indicated that the antioxidant effect of palm oil was gradually lost when reheating frequency was increased. The histomorphometric and morphological findings in our study complimented the fact that heating destroyed the tocotrienols and other heat labile vitamins in the oil, resulting in reduction of antioxidant properties of the oil [3].

As polyunsaturated fatty acid residues in lipoprotein are vulnerable to free radical oxidation, the oxidized LDLs are atherogenic via its cytotoxic effect towards arterial endothelial cells [15]. The oxidatively modified LDLs were found in the human and rabbit atherosclerosis lesion [16]. The modified LDLs induced the transmigration of monocyte into the subendothelial space and it was prevented by pretreatment with antioxidant vitamin E [17]. Moreover, atherosclerotic changes develop when the LDLs infiltrate into the tunica intima and accumulate in macrophages [18]. This event initiates the proliferation of intimal fibroblasts and myointimal cells together with collagen deposition, producing a plaque which causes the intima thickening that is identified as the earliest indicator of atherosclerotic process [19]. The tunica intima thickening in the control group of the present study could be explained by the effect of aging because the duration of study was the total of 6 months. It has been proved that aging is a responsible factor for the differential changes in the TI and TM thickness [20]. However, the intimal thickening in the heated oil treated groups might be the adverse consequence of oil consumption, eventually leading to atherosclerosis.

Furthermore, the increase in IMR indicated the increase in subendothelial ground material accumulation. The slight elevation of IMR in the FPO group was probably due to the fatty acids in nature of the oil although fresh oil possesses antioxidant properties. However, the qualitative results showed no obvious changes in FPO group. The palm oil decreases the serum triglyceride (TG) and cholesterol level [21]. It also increases the level of high density lipoprotein (HDL) [22, 23]. It is well documented that high HDL level may lower the risk of cardiovascular problem [24]. The ultrastructural finding in FPO group of our study implicated

the protective role of FPO due to its antioxidant vitamin compositions.

Several studies have been done on consumption of repeatedly heated oil and the impact on cardiovascular diseases. Although one study found the temporary increase in serum TG and LDL level in 5HPO feeding ovariectomised rats, the morphological results showed no obvious changes under light microscopic study [25]. In another study, the ovariectomised rats were fed with cholesterol diet fortified with heated palm oil and they found that the atherosclerotic changes were revealing at the ultrastructural level without significant alteration in the plasma lipid profile [26]. The present study was conducted on the male rats feeding with basal diet without being fortified with cholesterol, yet the ultrastructural level study also revealed the similar finding as in the study done by Adam et al. [26]. This can be concluded as the male rats are more liable to develop cardiovascular diseases.

Several other studies were conducted on the consequence of atherosclerosis which attributes to the aetiology of hypertension and cardiac problem. The study found that the consumption of repeatedly heated oil resulted in high blood pressure [27]. The authors also pointed out that the free radicals generated by repeated heating might impair the nitric oxide bioavailability on the blood vessel wall leading to hypertension [27]. The evidence of atherosclerotic plaque formation in the present study might also contribute the development of hypertension. The necrosis in cardiac tissue was found in repeatedly heated palm oil fed rats [8]. It might be due to atherosclerosis plague formation in the coronary artery, leading to narrowing of the lumen which diameter is much narrower than that of the aorta. The above findings indicated the harmful effect of consumption of reheated oil.

In conclusion, the antioxidant property of oil is reduced by repeated heating that increases the lipid peroxidation which aggravates the development of atherosclerosis. Therefore, it is important that we should utilize protective nutritional value of palm oil in full and discourage the usage of repeated heating oil in our daily diet to reduce the risk of atherosclerosis.

4.1. Limitations and Recommendations. Firstly, the development of atherosclerosis in animal models may be different from human, despite the obvious histological similarities of atherosclerosis between both species. Therefore, it is recommended to develop the relevant model to conduct the extended study.

Secondly, as the selected area of the aortic sample was examined under electron microscope, the certain parts of the aortic sample which would have more relevance to our study might have been missed. In our humble opinion, further detailed and quantitative studies are recommended to explore the exact nature of disease development.

In addition, it would be interesting to investigate the relationship between biochemical parameters and the histomorphometric as well as electron microscopic study in one single research. The future research should be aimed to determine the safe threshold of heating frequency by using the lower frequencies of heating such as one or two times and so on.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

High appreciation goes to the Universiti Kebangsaan Malaysia Research committee, the staff from the electron microscopy unit of the Pathology Department and Institute of Medical Research, Malaysia, and the staff of Anatomy Department, UKM for the technical support. The authors are also grateful to the statistician and the postgraduate student [8] from the Pharmacology Department, UKM, for kindly providing the samples.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2012, Article ID 485262, 10 pages
doi:10.1155/2012/485262

Research Article

Dual Roles of Quercetin in Platelets: Phosphoinositide-3-Kinase and MAP Kinases Inhibition, and cAMP-Dependent Vasodilator-Stimulated Phosphoprotein Stimulation

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Received 25 August 2012; Revised 29 October 2012; Accepted 30 October 2012

Academic Editor: Kashmira Nanji

Background. Progressive diseases including cancer, metabolic, and cardiovascular disorders are marked by platelet activation and chronic inflammation. Studies suggest that dietary flavonoids such as quercetin possess antioxidant, anti-inflammatory, and antiplatelet properties, which could prevent various chronic diseases including atherosclerosis and thrombosis. However, the mechanism and the signaling pathway that links quercetin's antiplatelet activity with its anti-inflammatory property is limited and thus further exploration is required. The aim of this paper was to examine the link between antiplatelet and anti-inflammatory roles of quercetin in agonist-induced platelet activation. *Methods*. Quercetin effects on agonist-activated platelet-aggregation, granule-secretion, $[Ca^{2+}]_i$, and glycoprotein-IIb/IIIa activation were examined. Its effects on PI3K/Akt, VASP, and MAPK phosphorylations were also studied on collaged-activated platelets. *Results*. Quercetin dose dependently suppressed collagen, thrombin, or ADP-induced platelet aggregation. It significantly inhibited collagen-induced ATP release, P-selectin expression, $[Ca^{2+}]_i$ mobilization, integrin-$\alpha_{IIb}\beta_3$ activation, and augmented cAMP and VASP levels. Moreover, quercetin attenuated PI3K, Akt, ERK2, JNK1, and p38 MAPK activations, which were supported by platelet-aggregation inhibition with the respective kinase inhibitors. *Conclusion*. Quercetin-mediated antiplatelet activity involves PI3K/Akt inactivation, cAMP elevation, and VASP stimulation that, in turn, suppresses MAPK phosphorylations. This result suggests quercetin may have a potential to treat cardiovascular diseases involving aberrant platelet activation and inflammation.

1. Introduction

Platelets play a major role in hemostasis and thrombosis [1], in which the latter causes a serious problem leading to myocardial infarction, atherosclerosis, ischemia, and stroke [2]. At the sites of vascular damage, platelet activation by agonists such as collagen, adenosine diphosphate (ADP), and thrombin resulted in an increase in $[Ca^{2+}]_i$ concentration, platelet shape change, secretion, and aggregation [3]. Activated platelets also release various aggregation mediators including ADP, adenosine triphosphate (ATP), thromboxane A2 (TXA2), serotonin, and various proteins [4]. These released mediators stimulate G-protein coupled receptors (GPCRs) that are necessary for phospholipase C (PLC), protein kinase C (PKC), phosphoinositide-3 kinase (PI3K) [4], and MAP kinases activations [5]. Furthermore, activated platelets secret P-selectin, which stabilizes the initial $\alpha_{IIb}\beta_3$ integrin-fibrinogen binding to more stable platelet aggregate formation [6] and links platelet activation with inflammation [7].

Platelet activation and chronic inflammation are sequels of a wide range of progressive diseases, including cancer, metabolic and cardiovascular disorders [8], suggesting that prevention of inflammation and aberrant platelet activation by dietary flavonoids, such as quercetin, is one of the ways to prevent various chronic diseases including atherosclerosis

and thrombosis [8–11]. Several animal and clinical studies have suggested that flavonoids such as quercetin are rich in fruits, vegetables, red wine, and tea where consuming them may protect the development of cardiovascular disease risks through their antioxidant and anti-inflammatory properties [10–14]. The results of population studies [11, 12], and animal and clinical intervention studies [10, 13, 14] using quercetin-rich diets, have suggested antiatherosclerosis and antithrombosis effects of quercetin through their antioxidant, antiplatelet, and anti-inflammatory properties. Studies have reported an inverse association between dietary quercetin intake and mortality from coronary heart disease [10, 11]. Quercetin may also be a promising dual antiplatelet and anti-inflammatory/antiatherosclerosis agent that warrants comprehensive evaluation of its potential as a new lead class of drug development. However, its mechanism of action and signaling pathways that links quercetin's antiplatelet property with its anti-inflammatory activity is limited in platelets and further exploration is required to add data on the existing reports and expand the knowledge base about the relationship of the antiplatelet and anti-inflammatory properties of the compound in association with cardiovascular disorders.

Previous reports indicated that quercetin possesses a potent antioxidant, immunomodulatory, anti-inflammatory, and antiatherosclerotic and antiplatelet properties [15, 16]. It has also been reported to inhibit MAPKs, Akt, Src, JAK-1, and Tyk2 activations [17]. However, the modulatory effects of quercetin in agonist-induced platelet activation, protein and/or lipid kinases phosphorylations, and cyclic nucleotide activities are only partially explored. In addition, information on the effect of quercetin in aberrant platelet activation and inflammation is limited. In this study, therefore, we determined that quercetin inhibits agonist-induced platelet activation through inhibition of PI3K/Akt activation with subsequent cAMP elevation and VASP stimulation that, in turn, suppresses ERK2, JNK1, and p38 MAPK phosphorylations.

2. Materials and Methods

2.1. Materials. Collagen and ADP were purchased from Chronolog (Havertown, PA, USA). Thrombin, Fura-2/AM, quercetin and forskolin and 3-isobutyl-1-methyl xanthine (IBMX), SB203580, SP600125, and PD98059, LY-294002, and wortmannin were procured from Sigma (St. Louis, MO, USA). ATP assay kit was obtained from Biomedical Research Service Center University (Buffalo, NY, USA). Mouse monoclonal to CD62P antibody and goat polyclonal to mouse IgG antibody (FITC) were obtained from Abcam (Cambridge, UK). Antibodies to phospho-p44/42, total-p44/42, phospho-p38, total-p38, phospho-JNK, total JNK, phospho-Akt, total Akt, phospho-PI3K p85/p55, PI3 kinase p85, phospho-VASP (Ser 157), and β-actin were purchased from Cell Signaling (Beverly, MA, USA). Monoclonal antibody to VASP (phosphorylated) (pSer239) (16C2) was obtained from Enzo Life Sciences (PA, USA). Alexa Fluor 488 fibrinogen conjugate was obtained from Molecular Probes (Eugene, OR, USA). Cyclic AMP Kit was procured from Ann Arbor (MI, USA). All other chemicals were of reagent grade.

2.2. Platelet Preparation. Blood was collected from the abdominal artery of 8∼10 weeks old rats with citrate phosphate dextrose solution (CPD; 90 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, 14 mM $C_6H_8O_7 \cdot H_2O$, 128.7 mM $NaH_2PO_4 \cdot H_2O$, 2.55 g/100 mL dextrose). Platelet-rich plasma (PRP) was prepared by centrifugation of the blood samples at 1000 rpm for 7 min twice, and platelets were washed with washing buffer. Washed platelets were then gently resuspended in Tyrode buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $NaHPO_4$, pH 7.4) to a final concentration of 5×10^8 platelets/mL.

2.3. Platelet Aggregation. Washed 5×10^8 platelets/mL were preincubated for 3 min at 37°C in the presence of 1 mM exogenous $CaCl_2$ with or without various concentrations (12.5–100 μM) of quercetin, and then platelet aggregation was stimulated by collagen (2.5 μg/mL), ADP (10 μM), or thrombin (0.1 U/mL). The aggregation was monitored by using an aggregometer (Chronolog, Havertown, PA, USA) at a constant stirring of 1200 rpm and aggregation rates were measured as the light transmission changes were recorded for 8 min.

2.4. P-Selectin Secretion. P-selectin (CD62) expression on platelets was measured using FITC-labeled anti-CD62P antibody. Quercetin-pretreated platelets were activated by collagen and incubated for 5 min at 37°C with stirring condition. Washed platelets were then centrifuged followed by resuspension in ice-cold PBS containing 10% FBS, and 1% sodium azide. Samples were blocked with ice-cold PBS containing 3% BSA and labeled with CD62P primary antibody for 30 min at 4°C in dark condition. The sample was washed repeatedly in ice-cold PBS and labeled with FITC-conjugated secondary antibody in 3% BSA/PBS for 30 min at 4°C in the dark. After repeated washing with ice-cold PBS, the sample was resuspended in ice-cold PBS, 3% BSA, and 1% sodium azide. Flow cytometry was performed using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), and data was analyzed using CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA, USA).

2.5. Measurement of ATP Release. ATP secretion from the dense granules of platelet was determined in a luminometer (GloMax 20/20, Promega, Madison, WI, USA) using the ATP assay kit (Biomedical Research Service Center, Buffalo, NY, USA) according to the manufacturer's protocol. Briefly, platelets were incubated for 3 min at 37°C with or without various concentrations of quercetin and then stimulated with collagen for 5 min. The reaction was stopped, platelets were centrifuged, and supernatants were used for the assay.

2.6. Determination of Cytosolic-Free Ca^{2+} Concentration. Platelets were prepared as described above and incubated with 5 μM fura-2/AM at 37°C for 60 min. Fura 2-loaded

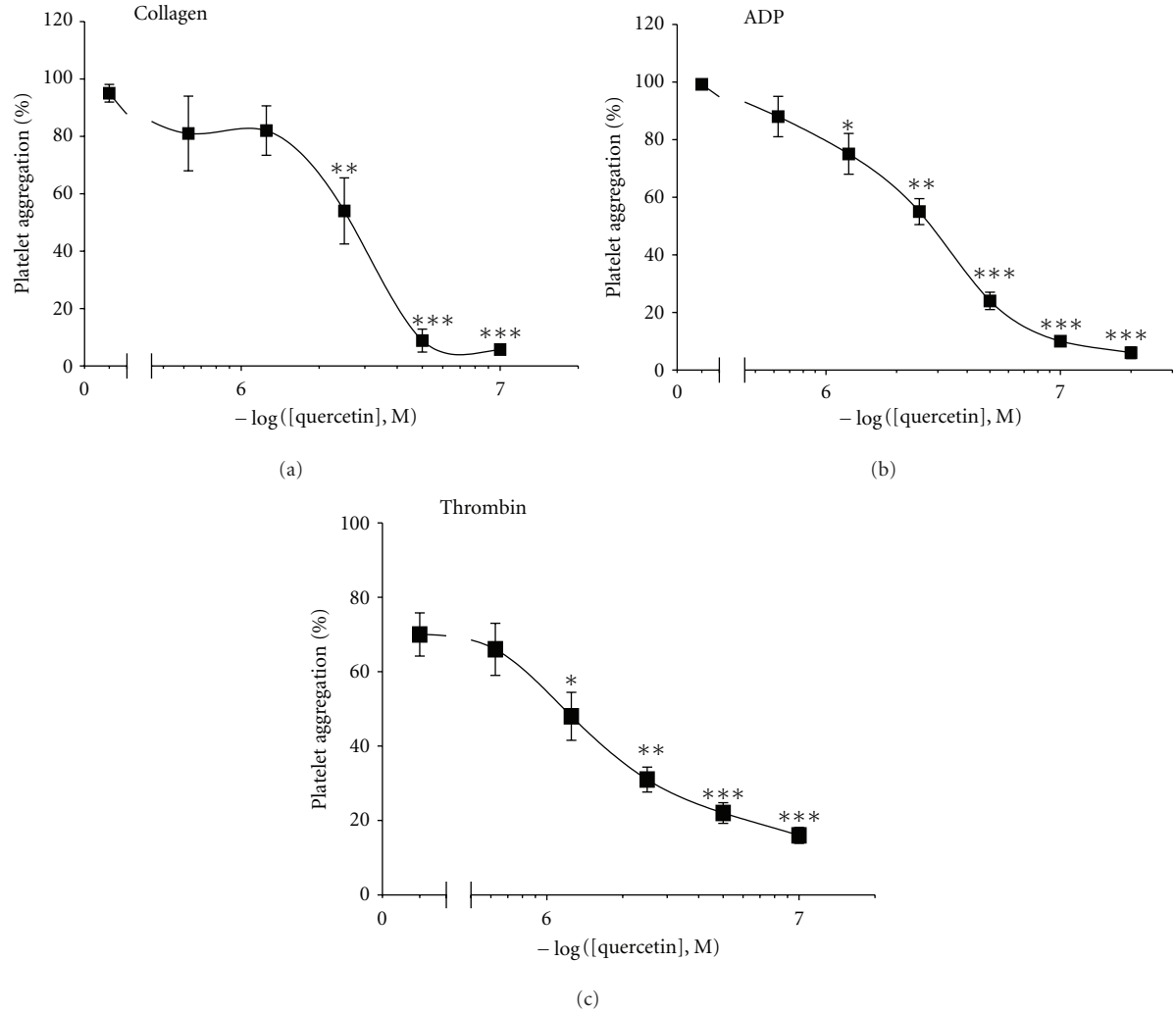

FIGURE 1: Quercetin inhibits agonist-induced platelet aggregation. Platelets were preincubated with quercetin in the presence of 1 mM $CaCl_2$ for 2 min at 37°C and stimulated with collagen (a), ADP (b), and thrombin (c). Aggregation was terminated at 5 min and percent aggregation was determined. Tracings ((a), (b), and (c)) are summary of 8 to 10 experiments with mean ± SEM of at least 8 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ versus agonist activated control.

platelets (5×10^8 platelets/mL) were preincubated for 1 min at 37°C with various concentrations of quercetin in the presence of 1 mM $CaCl_2$ and then stimulated with thrombin for 200 seconds. Fura-2 fluorescence was measured with a spectrofluorimeter (F-2500, Hitachi, Japan) in an excitation wavelength altering every 0.5 sec from 340 nm to 380 nm; the emission wavelength was at 510 nm. Then, the $[Ca^{2+}]_i$ was estimated using the method of Blaustein [18].

2.7. Determination of Fibrinogen Binding. Washed platelets were initially treated with quercetin or vehicle and incubated for 5 min at room temperature. Two hundred μg/mL Alexa Fluor 488-human fibrinogen were added before collagen (2.5 μg/mL) stimulation and then the sample was incubated at 37°C for 15 min. Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry using FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), and data were analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Fibrinogen nonspecific binding was estimated by measuring its binding in the presence of a specific integrin inhibitor, RGDS peptide (1 mM).

2.8. Immunoblotting. Platelets (5×10^8/mL) were activated with collagen for 5 min in the presence of 1 mM $CaCl_2$ with or without quercetin (12.5, 25, 50, and 100 μM) and immediately dissolved in sample buffer (0.125 M Tris-HCL at pH 6.8, 2% FBS, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue in the presence of 1 mM phenylmethylsulfonylfluoride (PMSF), 2 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin). Protein concentration was determined using BCA assay (PRO MEASURE, iNtRON biotechnology, Korea) on ice. After boiling for

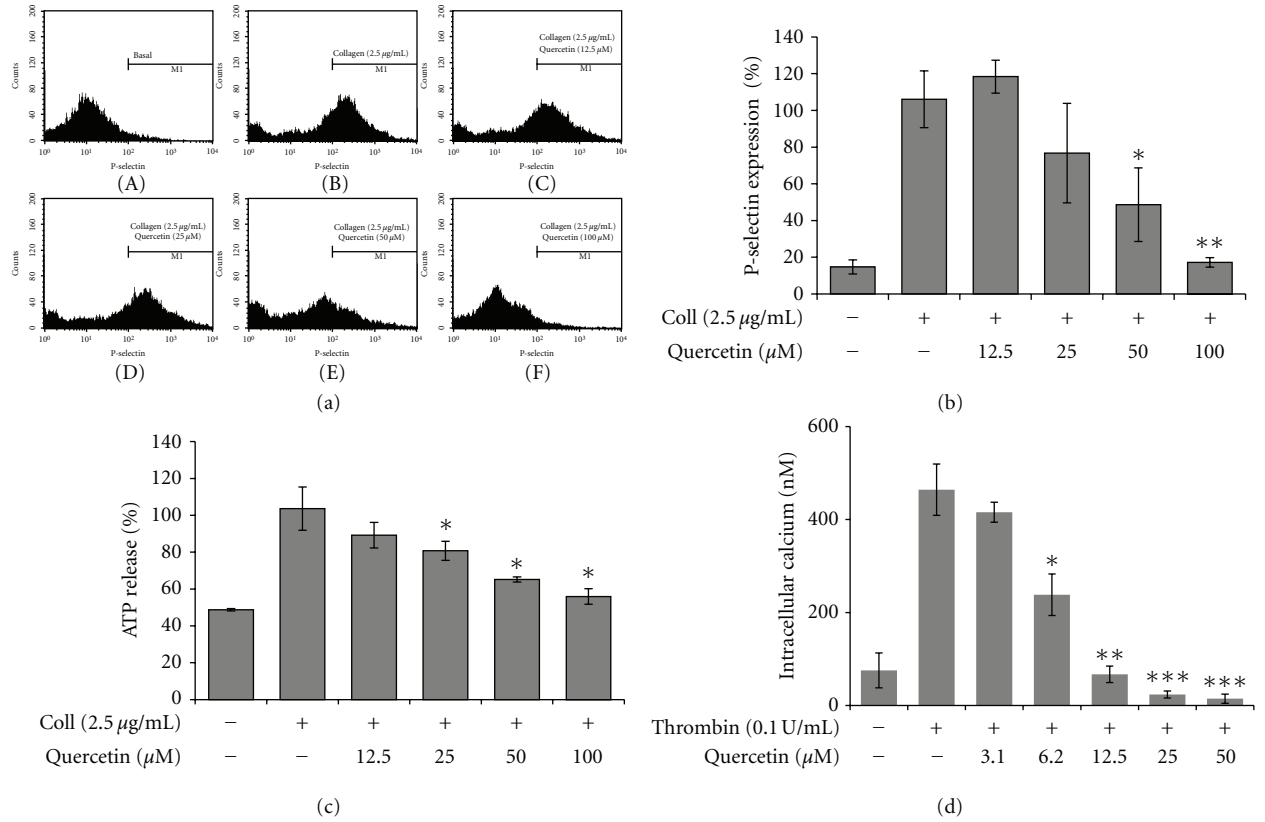

Figure 2: Quercetin influences collagen-activated granule secretions. Platelets were preincubated with quercetin and stirred in an aggregometer for 2 min before collagen or thrombin stimulation for 5 min and the reaction was terminated followed by granule secretion assay. ((a) and (b)) Effect of quercetin on collagen-induced P-selectin expression. (a) Panels ((A) and (B)) represent untreated and collagen stimulated, and ((C)–(F)) represent quercetin dose-dependent effects. (b) The bar graph shows summary of 4 independent experiments. (c) ATP release in response to agonist stimulation was performed as described in the "Materials and Methods." (d) Platelets (3×10^8/mL) were loaded with Fura-2/AM and preincubated with or without quercetin in the presence of 1 mM $CaCl_2$ for 2 min followed by thrombin (0.1 U/mL) stimulation for 5 min at 37°C and $[Ca^{2+}]_i$ levels were determined. Bar graphs show mean ± SEM of at least 4 independent experiments performed. $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ versus agonist-activated control.

5 min, the proteins were resolved by electrophoresis in 10% SDS-PAGE and then transferred to PVDF membranes in a transfer buffer (25 mM Tris (pH 8.5) and 20% methanol). Membrane was blocked with 5% skim milk, washed, and subjected to immunoblotting with antiphospho ERK1/2, anti-ERK1/2, antiphospho p38, anti-p38, antiphospho JNK, anti-JNK, antiphospho AKT, anti-AKT, antiphospho-p55, antiphospho-p85 PI3K, anti-PI3K, antiphospho-VASP^{Ser157}, and antiphospho VASP^{Ser239} antibodies. The immunoblots were again incubated with HRP secondary antibody and the membranes were visualized using enhanced chemiluminescence, ECL (iNtRON Biotechnology, Korea).

2.9. Measurement of cAMP. Platelets were preincubated at 37°C for 1 min and treated with quercetin (25, 50, and 100 μM) or FSK (1 μM) and incubated for 5 min at stirring condition. The mixture was boiled for 5 min and cooled at 4°C. Then, the precipitate was centrifuged and supernatant used to determine the cyclic AMP content using EIA kits (Ann Arbor, MI, USA) following acetylation as described by the manufacturer.

2.10. Statistical Analysis. Data were analyzed by one-way ANOVA, using Statistical Analysis Software, version 9.1 (SAS Institute Inc., Cary, NC, USA) tool, followed by a *post hoc* Dunnett's test in order to determine the statistical significance of the differences between treatment groups. All data are presented as means ± SEM, and $P \leq 0.05$ were considered to be statistically significant.

3. Results

3.1. Quercetin Inhibits Agonist-Induced Platelet Aggregation. Quercetin inhibited platelet aggregation induced by collagen (2.5 μg/mL), ADP (10 μM), and thrombin (0.1 U/mL), respectively (Figure 1). The fifty percent inhibitory concentrations (IC_{50}) of quercetin to the above indicated agonists-activated platelet aggregations were estimated to be 25.0 ± 4.4, 25.0 ± 3.1, and 12.5 ± 3.1 μM (Figures 1(a), 1(b), and 1(c)), respectively.

3.2. Quercetin Reduces Agonist-Induced ATP Release, P-Selectin Expression, and $[Ca^{2+}]_i$ Mobilization. Since granule

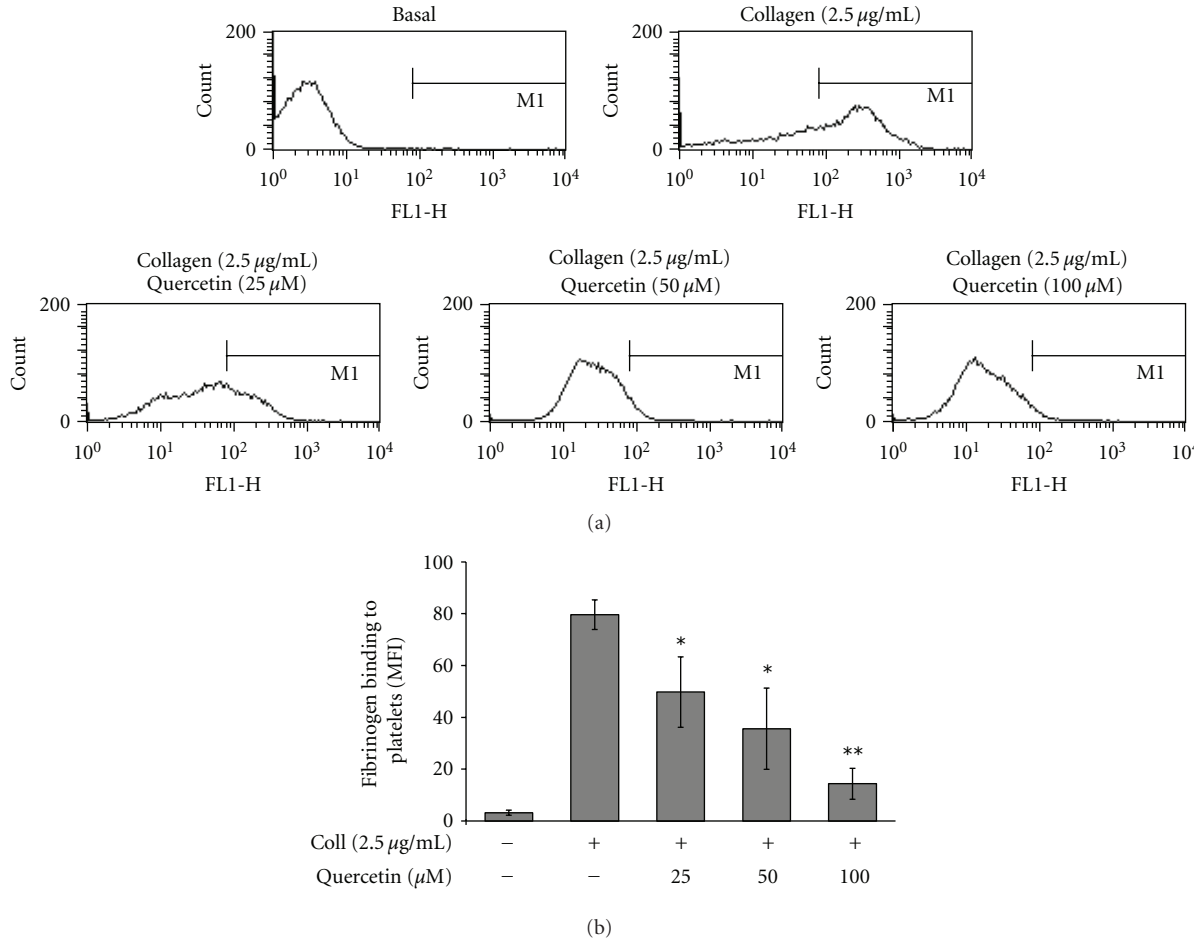

Figure 3: Quercetin attenuates fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. Fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in platelets pre-treated with quercetin and stimulated by collagen (2.5 μg/mL) together with Alexa Fluor 488-human fibrinogen (200 μg mL^{-1}) followed by incubation at 37°C for 15 min. (a) Tracings are representatives of 4 independent experiments. (b) Bar graph represents summary of quercetin effects on fibrinogen binding. $^*P < 0.05$, $^{**}P < 0.01$.

secretions are crucial early events of platelet activation, we examined the influence of quercetin treatment on collagen-induced dense and α-granule secretions. As shown in Figure 2, quercetin reduced collagen-induced P-selectin secretion (Figures 2(a) and 2(b)) and ATP release (Figure 2(c)) in a dose-dependent manner, respectively. In addition, it significantly attenuated thrombin evoked $[Ca^{2+}]_i$ mobilization in the concentrations indicated in Figure 2(d).

3.3. Quercetin Increases Platelet cAMP Levels and Enhances Vasodilator-Stimulated-Phosphoprotein (VASP) Phosphorylation. Cyclic AMP generation and cyclic nucleotide-dependent protein kinase activity are known to be inhibited by platelet activation [3], and agents that can enhance cAMP reverse platelet activation. We, therefore, investigated whether quercetin influences platelet cAMP levels. Quercetin markedly increased the level of cAMP in collagen-stimulated platelets (Figure 4(a)). Besides, we further assessed effect of quercetin with adenylyl cyclase activator and phosphodiesterase inhibitor on platelet aggregation. As such, coincubation of low-dose quercetin with forskolin (2.5 μM), adenylyl cyclase activator or IBMX (50 μM), broad spectrum cyclic phosphodiesterase inhibitor, highly potentiated quercetin-mediated platelet aggregation inhibition and augmented individual effects upon combination (Figures 4(c) and 4(d)).

Since VASP, a substrate of cyclic nucleotide- (cAMP/cGMP-) dependent protein kinases (PKA/PKG), inhibits agonist-induced platelet aggregation [19], we examined the effect of quercetin in platelet VASP expression. Though no basal VASP expression was detected (Figure 4(b)), quercetin treatment dose dependently increased $VASP^{Ser157}$ and $VASP^{Ser239}$ phosphorylations with increased translocation of $VASP^{157}$ from 46 to 50 kDa protein. This suggests that quercetin has a role in stimulating cyclic nucleotide-dependent protein kinase mediated VASP phosphorylation.

3.4. Quercetin Reduces Fibrinogen Binding to Activated Integrin $\alpha_{IIb}\beta_3$. The ligand-binding functional change of integrin $\alpha_{IIb}\beta_3$ is the main outcome of adhesion and activation in platelets [20] followed by aggregation as a result of the adhesive substrates bound to the membranes of activated platelets [21]. Thus, we examined the role of

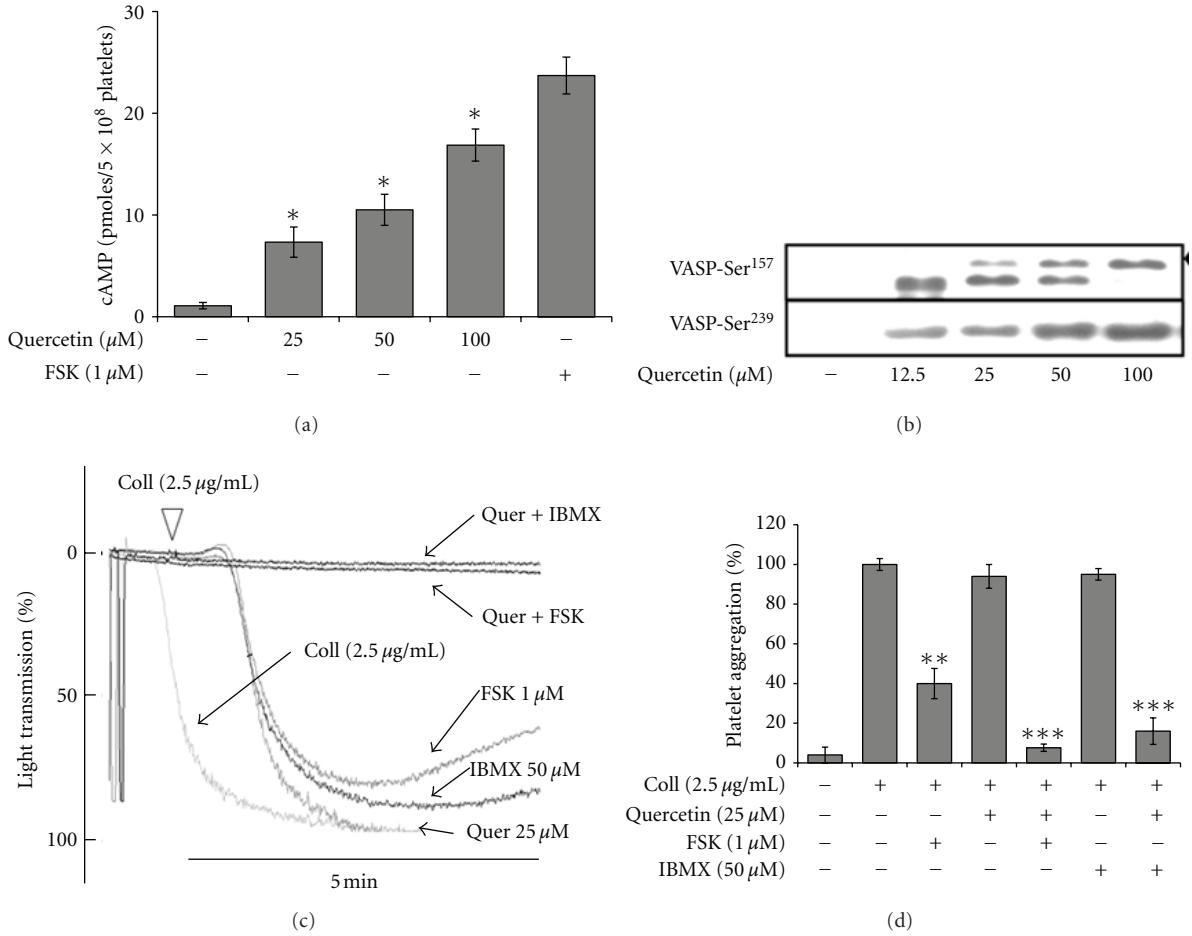

Figure 4: Quercetin enhances basal cyclic AMP levels and VASP phosphorylation. Rat platelets were stirred with either the presence of vehicle or quercetin in an aggregometer, the reaction was terminated, and cAMP enzyme immunoassays and western blot for VASP activation were performed. (a) Dose-dependent quercetin effects in resting platelets cAMP levels. (b) Dose-dependent effect of quercetin on VASP activation. ((c) and (d)) Forskolin and IBMX before treatment strongly potentiated quercetin-induced platelet aggregation. Results are summary of at least 3 independent experiments performed and bar graphs presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ versus control.

quercetin on functional response of integrin $\alpha_{IIb}\beta_3$ activation. Collagen-induced fibrinogen binding to its receptor was dose dependently reduced in quercetin-treated platelets (Figures 3(a) and 3(b)). This finding suggests that quercetin may impair integrin $\alpha_{IIb}\beta_3$ conformational changes for high affinity fibrinogen binding site exposure (inside-out signaling) that occurs as a result of prior platelet agonist interactions.

3.5. Quercetin Suppresses Collagen-Stimulated Platelet MAP Kinase Phosphorylations. Quercetin is known to inhibit MAPKs, and the presence of p38 MAPK, ERK, and JNK has been demonstrated in blood platelets and reported to be phosphorylated by various platelet agonists [22]. As a result, we thought to determine whether collagen-induced MAPK phosphorylations are affected by quercetin. Our findings show that quercetin markedly inhibited collagen-stimulated ERK, JNK, and p38 MAP kinases in a dose-dependent manner (Figure 5(a)). The involvement of the above indicated MAP kinases in the antiplatelet activity of quercetin was further confirmed by using the respective inhibitors (PD98059 (30 μM), SB203580, and SP600125 (10 μM)) in collagen-induced platelet aggregation, respectively (data not shown).

3.6. Quercetin Arrests PI3K/Akt Signaling. Quercetin is a known inhibitor of PI3K and is a parent compound from which LY294002 (PI3K inhibitor) was derived, and PI3K plays a crucial role in platelet function such as activation, adhesion, spreading, and aggregation [23], with Akt, the main target of PI3K signaling [4]. Thus, the effect of quercetin on collagen-induced platelet PI3K/Akt activation was examined. Interestingly, quercetin significantly and dose dependently suppressed collagen-induced platelet Akt and PI3K phosphorylations (Figure 5(b)). Further, wortmannin or LY294002 (PI3K inhibitors, 20 μM) suppressed platelet adhesion and activation via reducing $[Ca^{2+}]_i$ mobilization and $\alpha_{IIb}\beta_3$ activation (data not shown).

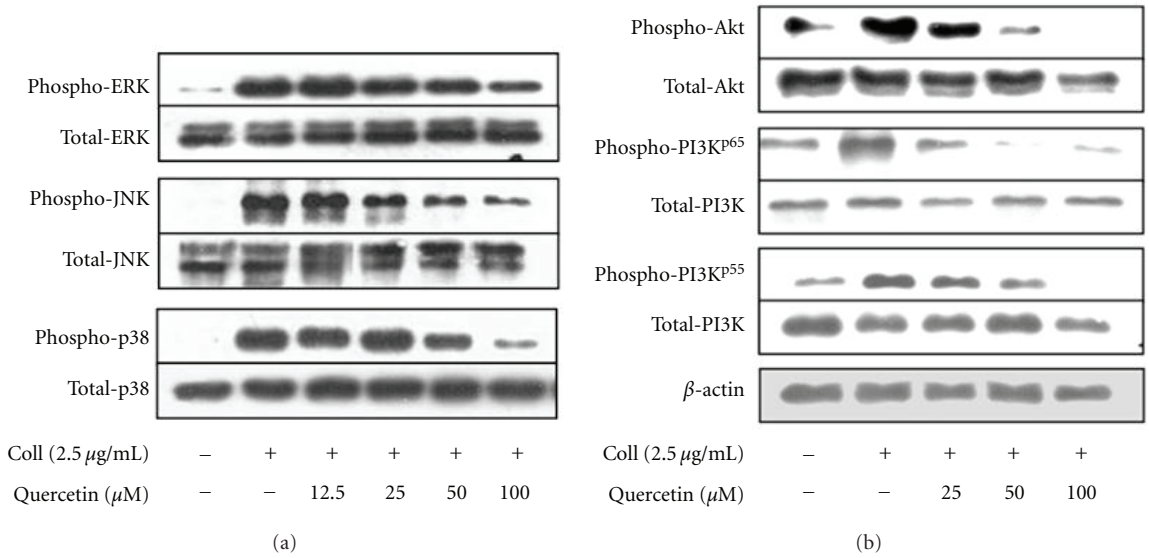

Figure 5: Quercetin suppresses collagen-activated PI3K/Akt and MAP kinase phosphorylations. ((a) and (b)) Washed platelets were stirred in an aggregometer with quercetin or vehicle at the concentrations indicated for 3 min and stimulation with collagen for 5 min before termination of the reactions. Proteins were extracted, separated by SDS-PAGE transferred to PVDF membranes, and immunoblotted with antibodies. Blots were visualized by ECL. (a) Quercetin dose-dependent effect on p38, JNK1, and ERK2 phosphorylations. (b) Effects of quercetin on platelet PI3K and Akt phosphorylation. Immunoblots are representatives of 3 to 4 experiments.

4. Discussion

Quercetin is known to be a negative regulator of cardiovascular disease risks as consumption of this compound is related to reduced incidences of stroke [24] and myocardial infarction [25]. Apart from its antioxidant activity [26], a multitude of anticipated mechanisms for quercetin mediated reduction of such risks have been reported. These mechanisms include inhibition of platelet activation [27], thrombus formation [28, 29], 5-HT secretion, and TXA2 release or binding to its receptor [30, 31]. In addition, inhibition of tyrosine [27], lipid [32], and serine/threonine kinases [33] have been reported as quercetin effects. In this regard, the ability of quercetin to bind competitively with ATP at the nucleotide binding site makes the compound an inhibitor of several protein kinases [32]. As a result, it was used as a lead compound to develop LY294002 and other inhibitors of PI3K [34]. A critical consideration for quercetin-mediated inhibition of platelet function may lie on the link between its anti-inflammatory property and antiplatelet activity. However, data on the relationship of quercetin-mediated antiplatelet effects with cAMP and/or PDE activity as well as MAPK and PI3K/Akt phosphorylation is scarce.

We in the present study showed that quercetin suppressed the main pathways involved in platelet aggregation through inhibition of agonist-induced platelet activation, $[Ca^{2+}]_i$ mobilization, granule secretion, and fibrinogen binding. Our results also showed that quercetin inhibited collagen-induced PI3K and Akt phosphorylations downstream of collagen receptor. This effect was supported by suppressive effect of PI3K-inhibitors (wortmannin or LY294002) to platelet activation via inhibition of $[Ca^{2+}]_i$ mobilization and $\alpha_{IIb}\beta_3$ activation. An enhanced integrin $\alpha_{IIb}\beta_3$ receptor binding to fibrinogen is particularly considered to be the final common pathway for platelet aggregation [35]. In accordance to the present study, quercetin is reported to inhibit collagen-induced PI3K, Akt, and PLCγ activations and $[Ca^{2+}]_i$ mobilization in platelets [27, 36]. Akt is the key downstream molecule of PI3K signal that can be phosphorylated by collagen-induced platelet activation [37] and thrombus formation [23] where its inhibition by quercetin may have a negative role in platelet function.

In the present study, quercetin significantly elevated cAMP-mediated VASP phosphorylation in resting platelets and addition of IBMX increased this effect further. Such an effect may provide a sound rationale for considering quercetin as a potential antiplatelet therapy in combination with cAMP elevating agents or alone. An increase in intracellular cAMP concentration either through enhancing adenylyl cyclase (AC) or suppressing phosphodiesterase (PDE) has been reported to inhibit platelet responses activated by various agonists such as collagen, thrombin, ADP, and TXA2 [38] or attenuate the $[Ca^{2+}]_i$ mobilization, which is an essential factor for platelet aggregation [3]. VASP phosphorylation has also been reported to inhibit integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation [39]. The proposed mechanism of quercetin action in this study may include inhibition of PI3K/Akt pathway with a subsequent increase in cAMP-mediated VASP phosphorylation, and a reduction in $[Ca^{2+}]$ mobilization. Recent reports indicated that Akt activation decreased cAMP levels through increment of PDE activity [40, 41]. On the other hand, cAMP-elevating agents such as cilostamide and cilostazol (PDE3 inhibitors) or forskolin

(AC activator) are reported to show inhibitory effects to the PI3K-Akt signaling pathway in collagen-stimulated platelets [42]. This study, however, did not rule out whether PI3K/Akt or cyclic nucleotide pathway is upstream signaling and if the latter involves negative feedback mechanism. Thus, exploring the exact mechanism of interaction between the two signaling pathways in the presence of quercetin requires further investigation.

Our findings in this report show that quercetin attenuated p38, JNK1, and ERK2 phosphorylations in collagen-activated platelets. The involvement of ERK2 p38 and JNK1 signalings on the antiplatelet activity of quercetin was further confirmed by using the respective MAPK inhibitors in collagen-induced platelet aggregation. This result suggests that the antiplatelet effect of quercetin may be linked to its anti-inflammatory effect as its pretreatment involves inhibition of MAPK activation in collagen-induced platelets. We have thus established in this paper that the inhibitory effect of quercetin on platelet activation by collagen might be through inhibition of PI3K/Akt stimulation, induction of cAMP-mediated VASP phosphorylation, and inhibition of MAPKs activation. This is in line with a previous study indicating an inhibition of adenylyl cyclase-mediated MAP kinase phosphorylation in collagen-stimulated platelets [43]. In addition, PDE-inhibitor induced reduction of platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation is reported to be mediated by inhibition of MAPK and Akt activation [44]. Interestingly, quercetin-mediated attenuation of P-selectin expression and MAP kinase activation in this study suggests that the antiplatelet activity of the compound could be linked to its regulation of hemostatic- and inflammatory responses. Since platelets are involved in inflammation, P-selectin expression on the membrane of activated platelets is the main link between platelets and inflammatory cells [7, 45] and quercetin-mediated suppression of P-selectin expression and MAPKs activation in this paper may be attributed to its anti-inflammatory property.

Extensive studies, using various experimental setups, have indicated the role of ERK2, JNK1, and p38 in platelet granule secretion and aggregation [22, 46]. Using collagen [47] and thrombin [48] as agonists, previous reports indicated the involvement of ERK2 activation in platelet secretion and aggregation as well as JNK1 phosphorylation in thrombus formation [49]. Besides, P38 activation has been shown in collagen- [50] or thrombin-induced [51] platelet activation and secretion, which was restored by p38 inhibitors [52]. Platelet aggregation and thrombus formation are also known to involve in MAP kinase activation [53] and platelet-platelet cross-linking of fibrinogen bound to activated-$\alpha_{IIb}\beta_3$ [54]. Thus, the dual antiplatelet and anti-inflammatory properties of quercetin in the present study may have a role in treating aberrant platelet activation as an antiatherothrombotic and anti-inflammatory agent. Therefore, The inhibitory property of quercetin on agonist-induced granule secretion, $[Ca^{2+}]_i$ mobilization, $\alpha_{IIb}\beta_3$, PI3K/Akt and MAP kinases activations, and an enhanced cAMP-dependent VASP phosphorylation in platelet aggregation reflects the potential use of the compound as a candidate dual antiplatelet, anti-inflammatory agent.

In conclusion, this study suggests that the inhibitory property of quercetin in platelet aggregation may involve (i) inhibition of PI3K/Akt signaling, (ii) induction of cAMP-mediated VASP phosphorylation, and (iii) inhibition of the ERK2, p38, and JNK1 MAP kinase phosphorylations in activated platelets. Thus, the ability of quercetin to inhibit $[Ca^{2+}]_i$ mobilization, integrin activation, ATP release, and P-selectin expression during platelet aggregation, in combination with its anti-inflammatory effects, suggests that quercetin could be considered as an antiatherothrombosis and anti-inflammatory agent. Given the observed effects of quercetin on platelet signaling and functional responses, it will be important to identify the specific active metabolite that is responsible for the observed effects that link PI3K/Akt and MAPK inhibition and cAMP-dependent VASP activation. This will enable a more detailed mode of action at the molecular level to be determined, and the therapeutic potential of quercetin supplementation to be assessed.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by Kyungpook National University Research Fund (2012) and Basic Science Research Program from the National Research Foundation of Korea (NRF), Ministry of Education, Science and Technology (2010-0022223).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2012, Article ID 519031, 15 pages
doi:10.1155/2012/519031

Research Article

A Systems Biology Approach to Uncovering Pharmacological Synergy in Herbal Medicines with Applications to Cardiovascular Disease

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Received 19 August 2012; Accepted 10 October 2012

Academic Editor: Kashmira Nanji

Background. Clinical trials reveal that multiherb prescriptions of herbal medicine often exhibit pharmacological and therapeutic superiority in comparison to isolated single constituents. However, the synergistic mechanisms underlying this remain elusive. To address this question, a novel systems biology model integrating oral bioavailability and drug-likeness screening, target identification, and network pharmacology method has been constructed and applied to four clinically widely used herbs Radix Astragali Mongolici, Radix Puerariae Lobatae, Radix Ophiopogonis Japonici, and Radix Salviae Miltiorrhiza which exert synergistic effects of combined treatment of cardiovascular disease (CVD). *Results*. The results show that the structural properties of molecules in four herbs have substantial differences, and each herb can interact with significant target proteins related to CVD. Moreover, the bioactive ingredients from different herbs potentially act on the same molecular target (multiple-drug-one-target) and/or the functionally diverse targets but with potentially clinically relevant associations (multiple-drug-multiple-target-one-disease). From a molecular/systematic level, this explains why the herbs within a concoction could mutually enhance pharmacological synergy on a disease. *Conclusions*. The present work provides a new strategy not only for the understanding of pharmacological synergy in herbal medicine, but also for the rational discovery of potent drug/herb combinations that are individually subtherapeutic.

1. Introduction

Herbal medicine, especially traditional Chinese medicine (TCM) with the longest history in Asia, is a cost-effective system of medical practice that differs in substance, methodology, and philosophy to modern medicine, and plays an important role in health maintenance for the peoples of the world [1]. Because of their extensive use and the therapeutic effects [2, 3], there is an increasing interest and need to evaluate the mechanisms of action of herbal products rigorously.

Herbal medicines are characterized by the use of mixtures of several herbs (multiherbs) in a single formula, in which the pharmacological activities of one single herb is either potentiated or prolonged, and/or its adverse effects reduced by addition of other herbs [4]. This thus will lead to a more favorable response for some herbal combinations than for the constituent herb used alone [2], which suggests that therapeutic effects of these herbal products may arise from synergistic actions of herbal ingredients [3, 4].

Up to now, herbal synergism has been frequently reported [5], which may result from: (1) the potentiation of pharmacokinetics, such that one ingredient enhances the therapeutic effect of another component by regulating the drug absorption and metabolism. For examples, saponins increase absorption of corticosteroids [6] and procyanidin B2 or hyperoside in St. John's wort increases the solubility of hypericin [7, 8]; and (2) reinforcement of pharmacodynamics, thus all ingredients involved in an herbal combination direct at a similar receptor target or physiological system

[4]. For instance, in the case of St. John's wort, individually subtherapeutic effects (e.g., MAO and COMT inhibition) may combine to augment the primary pharmacological mechanism (monoamine reuptake inhibition). However, the molecular mechanism underlying such multicomponent synergy associated with the interacting targets, pathways, and even diseases remains largely uncovered. Clearly, deep understanding of the herb synergism will be not only helpful to optimize the drug combinations in multicomponent therapeutics but also critical for developing novel drug combinations that are individually subtherapeutic but efficacious in combination.

Evaluation of synergy in multicomponent therapeutics is usually performed experimentally in a case-by-case approach [9]. For examples, Wiesner et al. demonstrated that a novel antimalarial drug fosmidomycin had both *in vitro* and *in vivo* synergic effect with clindamycin [10]; Nguyen et al. reported that triple combination of amantadine, ribavirin, and oseltamivir was highly active and synergistic against drug resistant influenza virus strains *in vitro* [11]. However, as we know for any medicinal herb, they might contain hundreds of ingredients, thus it is unfeasible to screen all possible drug combinations for all possible indications, although high-throughput screening was possible to determine drug combinations [12], it is also much expensive. Another drawback of the existing methods is that these "blind" approaches including molecular biology are costly and time consuming. In addition, little is known about the system properties of a full drug interaction network, which hinders the understanding of mechanisms of drug combinations.

Alternatively, computational methods, especially systems biology, that enable to investigate the complex mechanism of action of drugs and circumvent the challenges associated with experiment have recently been developed. Systems biology investigates the biological processes within the complex, physiological milieu systematically through a systems approach integrating experimental, mathematical, and computational sciences. It has the potential to further facilitate the identification and validation of the therapeutic modulation of regulatory and metabolic networks and hence help identify targets and biomarkers, as well as "off-target" and side effects of drug candidates (reviewed by [13]). For example, network-target based techniques were used for virtual screening synergistic drug combinations [14, 15], thereby try to explain how and why the drugs work. But they only work on small drug sets due to the computational and experimental cost. Moreover, drugs are generally combined based on their mechanisms of action, which is characterized by the properties of drugs, such as their targets and pharmacology [16]. Thus the incompleteness of molecular networks and the scarceness of the drug properties limit the application of such approaches to TCM considerably.

In this work, we present a novel concept based on a systems biology framework for the investigation of synergy of four herbs, that is, Radix Salviae Miltiorrhiza (RSM), Radix Astragali Mongolici (RAM), Radix Puerariae lobatae (RPL), and Radix Ophiopogonis Japonici (ROJ) [17]. Among these four herbs, RSM shows diverse biological activities, such as inhibition of angiotensin converting enzyme (ACE), lowering blood pressure, dilate arteries, and decreasing blood clotting [18–20], thus is widely prescribed in different TCM formulae; RAM also shows protective effects against ultraviolet A-induced photoaging in human fibroblasts [21] and on proliferation and Akt phosphorylation of breast cancer cell lines [22]; isoflavones from RPL and their metabolites can inhibit growth and induce apoptosis in breast cancer cells [23]; ROJ plays a role in enhancing immunity, antimyocardial ischemia, lowering blood glucose, and antiviral activity [24]. Impressively, RSM has shown synergisms with each of the other three herbs in clinical trials for cardiovascular disease (CVD) [17], the leading cause of morbidity and mortality all over the world [25].

2. Materials and Methods

We have identified the potential targets of the four herbs on a proteome-wide scale and disclosed the synergistic mechanisms of action of the active ingredients by integrating both molecular and pharmacological features associated with drugs [26, 27]. Our methodology effectively and systematically extends the scope of the previously network-target concept, and is more likely to be successful in achieving the ultimate goal of providing pharmacological synergy in psychoactive herbal medicines. Our systems biology approach proceeds as follows:

(1) all 3D structures of available molecules in the four herbs are collected;

(2) the drug-likeness (DL) and oral bioavailability (OB) of the molecules are calculated to prescreen for the bioactive molecules;

(3) the physicochemical properties and architecture of molecules in four herbs are revisited;

(4) the potential targets of these four herbs are identified on a proteome-wide scale;

(5) the tools of network biology and systematic information about drugs and their targets are combined to uncover the synergistic therapeutic actions of herbal ingredients.

Our approach essentially explores some feature patterns enriched in known combinatorial therapies that can be predictive of new drug combinations and provide insights into the mechanisms underlying combinatorial therapy. Then these compounds and proteins are mapped to functional ontologies such as compound-target associations, compound-pathway connections, and disease-target; we assessed retrospectively and prospectively network-based relationships between drugs and their targets and interrelationships between drug targets and disease-gene products. In this way, our approach has the potential to increase the rate of successful drug discovery and development.

2.1. Database Construction and Molecular Modeling. In order to extract the current state of the art on known chemical ingredients in these four herbs, their information was extracted from the Traditional Chinese Medicine Systems

Pharmacology database and Analysis Platform (TcmSP; http://tcmspnw.com/) that was recently developed in our group. The newest version of TcmSP comprises 510 effective herbal entries registered in Chinese pharmacopoeia with more than 31000 ingredients, which spread over 18 different drug classes. This is currently the most comprehensive small molecular database for systems pharmacology analysis of TCM. In this work, the 3D structures of all molecules in the database were minimized in Sybyl by using the standard Tripos force field (Tripos, Inc.). After removing the duplicated compounds, a total of 532 molecules with 209 in RSM, 95 in RAM, 113 in RPL, and 135 in ROJ were collected in this study. Glycosides in medicinal herbs are usually hydrolyzed to liberate aglycone which is then absorbed at the intestinal mucos [28], thus the corresponding aglycone chemicals were also added into the database.

2.2. Drug-Likeness Calculation. DL is a qualitative concept used in drug design for how "druglike" a substance is with respect to factors affecting pharmacodynamics and pharmacokinetics of molecules which ultimately influences their absorption, distribution, metabolism, and excretion (ADME) in human body [29]. DL between the compound structure x and the drug molecular structure x' obtained from Drugbank was evaluated by Tanimoto coefficient defined as:

$$f(A,B) = \frac{A * B}{|A|^2 + |B|^2 - A * B}, \quad (1)$$

in which A represents the new compound, and B represents the average drug-likeness index of all 6511 molecules in DrugBank database (access time: June 1st, 2011, http://www.drugbank.ca/). In this work, compounds with DL $\geq$ 0.2 were selected as the candidate bioactive molecules, because the mean value of DL for all 6511 molecules in DrugBank is 0.18.

2.3. Oral Bioavailability Prediction. Herbal medicine is administrated mainly by oral route. In the development of herbal drugs intended for oral use, good drug absorption and appropriate drug delivery are very important. OB, the percentage of an oral dose able to produce a pharmacological activity, is one of the most desirable attributes of a new drug. To calculate the OB, we have developed a robust in-house system OBioavail 1.1 [27] integrating the metabolism (cytochrome P450 3A4) and transport (P-glycoprotein) information. This program helped us screen out the compounds with good OB, thus significantly reducing the number of original components to a smaller set for herbal medicine.

In this work, compounds with OB $\geq$ 40% were selected as the candidate bioactive molecules. The threshold determination is based upon careful consideration of the following: (1) information from the studied herbs is extracted as much as possible using the least number of chemical ingredients; (2) the obtained model can be reasonably explained by the reported pharmacological data.

2.4. Comparisons of Four Herbs Based on Chemicals. To analyze differences in molecular properties and structural features between RSM and the other three herbs, eight representative drug-related physicochemical properties including molecular weight (MW), number of rings per molecule (nCIC), octanol-water partition coefficient (MlogP), hydrogen bond donors/acceptors (nHDon and nHAcc), number of rotatable bonds (RBN), hydrophilic factor (Hy), and topological polar surface area (TPSA) were calculated with the dragon software [30]. To obtain a visual representation of the property space, property distribution analyses of both total compounds and bioactive chemicals were carried out considering all eight of the above mentioned physicochemical properties.

2.5. Target Identification. In silico prediction of drug-target interactions from heterogeneous biological data can accelerate the system-level search for drug molecules and therapeutic targets. Recently, we have developed a robust model [26] that efficiently integrates chemical, genomic, and pharmacological information for drug targeting and discovery on a large scale, based on random forest (RF) and support vector machine (SVM) methods. The optimal models show impressive reliability of prediction for drug-target interactions, with the concordance of 85.83%, the sensitivity of 79.62%, and the specificity of 92.76% [26]. In this work, the predicted targets for each bioactive molecule were selected based on the following principles [26, 31]: firstly, the targets should be both presented in the RF and SVM positive prediction list (value > 0.5); secondly, the targets with value of greater than 0.7 for RF and 0.8 for SVM were chosen as the final predicted targets. At last, a total of 87 targets were reserved for further analysis.

2.6. Network Construction. The predicted targets were used to build the compound-target networks by linking with the bioactive compounds. The relationship between CVD and targets was retrieved from the PharmGkb database [32] (http://www.pharmgkb.org/) and therapeutic target database [33] (http://bidd.nus.edu.sg/group/ttd/ttd.asp). In the interactive network diagram, nodes represent either compounds or proteins, and edges indicate compound-target interactions. All networks were generated in Cytoscape 2.8.1, a popular bioinformatics package for biological network visualization and data integration [34]. The quantitative properties of these networks were analyzed by two plugins including NetworkAnalyzer and CentiScaPe 1.2.

3. Results and Discussion

For thousands of years, herbal medicine holds a great promise for medical diagnosis and treatments in Asia and now is considered a complementary or alternative medical system in most Western countries. Different from conventional medicine in which drugs are studied in isolation, herbal medicine typically incorporates several medicinal herbs which contain multiple ingredients that probably produce a more favorable response than an isolated single constituent [4]. Such multicomponent therapeutics thereby is considered as a rational and efficient form of therapy designed to control

complex diseases such as CVD [35]. However, despite the fact that many positive outcomes have been observed in *in vivo* studies [36] and clinical trials [3], the underlying mechanisms of action, especially the synergism, remain to be elucidated for many psychoactive herbal medications. In this study, we applied our method to three representative herb pairs, that is, RSM and RAM, RSM and RPL, RSM and ROJ, with applications to CVD.

3.1. Extracting Active Components by OB and DL Prescreening. The oral route of drug administration is the most convenient way of choice for the formulators and continues to dominate the area of TCM therapy. However, though popular, this route is limited by absorption and bioavailability in the milieu of gastrointestinal tract. Hence OB is undoubtedly one of the most important pharmacokinetic parameters since it is the indicator of the efficiency of the drug delivery to the systemic circulation. Furthermore, a drug-like compound often has sufficiently acceptable ADME properties and can exert the pharmacodynamic effect on target site in human body. In short, the OB and DL prescreening is favorable to determine pharmaceutically active compounds in medicinal herbs.

3.1.1. RSM. A total of 40 compounds with good OB (≥40%) and DL value (≥0.2) are obtained (Table 1), most of which have been reported as bioactive ingredients. For examples, miltirone II (OB = 45%, DL = 0.24) presents sedative activity and is a benzodiazepine receptor agonist [37]; cryptotanshinone (OB = 52%, DL = 0.46) and tanshinone VI (OB = 53%, DL = 0.36) can protect the myocardium against ischemia-induced derangements by eliciting a significant enhanced recovery of the contractile force upon reoxygenation [38]. This further validates the reasonability of our prescreening model. According to the prescreening rule, we also find some other potential bioactive compounds including tanshinaldehyde and tanshinone VI that have not yet been validated. Notably, although salvianic acid A and Tanshinone II B have low DL or OB (0.06 and 22%, resp.), both of them exhibit significant biological activities. For instances, salvianic acid A exerts protective effect on vascular endothelial cells induced by lipopolysaccharide via an antioxidative mechanism, thus inhibiting apoptotic morphological changes of cells [39]; Tanshinone II B (DL = 0.45) exerts neuroprotective effect via inhibition of neuronal apoptosis *in vitro* [40]. Therefore, these two compounds are also involved as the bioactive ingredients of RSM. In summary, 42 (20.6% of all 209) bioactive compounds from RSM are reserved for further target prediction.

3.1.2. RAM. In this herb, total 44 (46.3% of all 95) compounds (Table 1) are obtained after prescreening of OB and DL and reserved for further target prediction. Most of them belong to astragalus flavonoids which have shown well protective effects on CVD [41]. For examples, calycosin (OB = 89%, DL = 0.24) and kaempferol (OB = 66%, DL = 0.24) are demonstrated to have antioxidant activities that benefit for CVD [42]; aglycon of formononetin-7-glucoside (formononetin, OB = 62%, DL = 0.21) reduces circulating concentrations of VCAM-1 which is an adhesion molecule associated with atherosclerosis [43]; quercetin (OB = 13%, DL = 0.27) may exert multiple actions on the NO-guanylyl cyclase pathway, endothelium-derived hyperpolarizing factor(s) and endothelin-1 and protect endothelial cells against apoptosis [41], thus epidemiologically associated with protection from coronary artery disease and cancer [44]. Besides, some molecules belong to triterpenoid saponins which are responsible for the bioactivities and efficacies of RAM on the treatment of CVDs. For instance, after deglycosylation, astragalosides I–IV with high OBs (range from 41% to 81%) can prevent changes of sarcoplasmic reticulum Ca^{2+}-uptake ability and Ca^{2+}-ATPase and Ser16-phosphorylated phospholamban protein expression, thus may prevent the depression in sarcoplasmic reticulum Ca^{2+} transport and improve cardiac function [45]. In addition, gamma-aminobutyric acid (GABA) with a high OB of 62% but low DL value (0.01) is also analyzed since it is reported to be the main hypotensive principle of right anterior measurement [46].

3.1.3. RPL. A total of 12 (10.6% of all 113) potential bioactive compounds (Table 1) are obtained for further target prediction. Most of them come from pueraria isoflavones, which are the main active constituents of RPL [47]. Notably, although daidzin has been demonstrated as a potent, selective inhibitor of human mitochondrial aldehyde dehydrogenase [48], its OB is extremely low (10%). Interestingly, its aglycon (daidzein) possesses relative good OB (38%) and affords protection against CVD [49]. Thus we propose that daidzin serves as a prodrug that is metabolized to the active form by intestinal bacterial deglycosylation. Similarly, after deglycosylation, the compound ononin (formononetin 7-O-β-D-glucoside) has good OB and presents certain pharmacological effects. Although puerarin in RPL has low OB (13%), it is the most abundant isoflavone glucoside and found to act as a β-adrenoreceptor antagonist in isolated arteries and veins [50]. Thus this molecule is also involved as active ingredients.

3.1.4. ROJ. In this herb, 14 compounds with satisfied drug property (OB > 40%; DL > 0.2) are obtained. Most of them belong to homoisoflavonoids which have one more carbon atom than normal isoflavone skeleton. For example, ophiopogonanone B, G, ophiopogonone A, 6-aldehydoisoophiopogonone A, methylophiopogonanone A and B are responsible for bioactivities including antioxidant activity, inhibition of platelet aggregation, cough relief, and hyperglycemia [51–53]. In addition, DL prediction of ophiopogonin D gives a relatively low value of 0.08, but it is also included for further analysis due to its various biological activities, such as inhibition of venous thrombosis, anti-inflammation, and antitussive activity [54]. Similarly, ophiopogonone A, 6-aldehydoisoophiopogonone A, methylophiopogonanone A, and methylophiopogonanone B displaying significant biological activities are also included though have low OBs (from 5% to 19%). To sum up, 19 (14.1% of all 135)

Table 1: 118 bioactive compounds from four herbs RSM, RAM, RPL, and ROJ and corresponding predicted oral bioavailability (OB), and drug-likeness (DL).

NO.	Compound	DL	OB	Herbs*
M161	ophiogenin	0.76	100.00	ROJ
M306	przewalskin b	0.44	100.00	RSM
M476	dimethyl-4,4′-dimethoxy-5,6,5′,6′-mdimethylene-dioxybiphenyl-2,2′-dicarboxylate	0.67	100.00	RAM
M465	7,2′-dihydroxy-3′,4′-dimethoxyisoflavone-7-O-β-D-glucoside	0.86	99.41	RAM
M6	tuberosin	0.76	95.05	RPL
M196	5-hydroxy-7,8-dimethoxy-6-methyl-3-(3′,4′-dihydroxybenzyl)chroman-4-one	0.41	93.42	ROJ
M521	Calycosin	0.24	88.9	RAM
M267	4-hydroxy-1-vinylcarboxy-7-(3,4-dihydroxyphenyl)benzo-β-furan	0.31	88.57	RSM
M462	4′-O-beta-Glucopyranosyl-5-O-Methylvisamminol	0.81	83.95	RAM
M204	N-[β-hydroxy-β-(4-hydroxy)phenyl]ethyl-4-hydroxy cinnamide	0.23	82.91	ROJ
M468	9,10-dimethoxypterocarpan-3-O-β-D-glucoside	0.92	81.91	RAM
M411	przewalskinone b	0.27	81.61	RSM
M182	ophiopogonanone F	0.45	81.49	ROJ
M505	astragaloside IV	0.15	81.32	RAM
M493	riboflavin	0.5	81	RAM
M480	betulinic acid	0.78	78.63	RAM
M510	aglycon of astragaloside I	0.2	77.02	RAM
M433	tanshindiol a	0.46	75.49	RSM
M183	ophiopogonanone G	0.46	75.41	ROJ
M533	aglycon of rhamnocitrin-3-O-glucoside	0.27	75.39	RAM
M461	5,4′-dihydroxy-3,7-dimethoxyflavone kumatakenin	0.29	75.36	RAM
M550	isoquercitrin	0.77	75.03	RAM
M509	astragaloside I	0.11	73.23	RAM
M55	8-prenylgenistein	0.37	72.6	RPL
M286	formyltanshinone	0.42	72.34	RSM
M390	miltionone II	0.44	71.03	RSM
M467	7-O-methylisomucronulatol	0.3	70.95	RAM
M464	aglycon of 5′-hydroxyiso-muronulatol-2′,5′-di-O-glucoside	0.8	70.73	RAM
M365	epiRSMspiroketallactone	0.31	68.27	RSM
M187	ophiopogonone B	0.31	67.52	ROJ
M559	red sandalwood ene	0.48	66.61	RAM
M492	behenic acid	0.26	65.99	RAM
M530	kaempferol	0.24	65.98	RAM
M507	mucronulatol-7-O-glucoside	0.86	65.21	RAM
M328	tanshinol I	0.52	64.81	RSM
M200	6-aldehydoisoophiopogonone B	0.38	64.39	ROJ
M406	prolithospermic acid	0.31	64.3	RSM
M523	aglycon of calycosin-7-O-glucoside	0.24	64.29	RAM
M501	astragaloside III	0.1	63.07	RAM
M485	aglycon of formononetin-7-glucoside	0.21	62.54	RAM
M478	γ-aminobutyric acid	0.01	62.12	RAM
M439	tanshinone VI	0.3	61.7	RSM
M213	ophiopogonanone B	0.3	59.58	ROJ
M421	salvianolic acid g	0.61	59.36	RSM
M522	calycosin-7-O-glucoside	0.81	58.36	RAM
M327	tanshinol II	0.56	58.29	RSM
M198	6-aldehydoisoophiopogonanone B	0.38	58.26	ROJ
M459	3,9-di-O-methylnissolin	0.48	57.75	RAM
M512	aglycon of astragaloside II	0.25	56.75	RAM
M516	rutin	0.68	56.65	RAM

Table 1: Continued.

NO.	Compound	DL	OB	Herbs*
M376	isotanshinone IIb	0.45	56.64	RSM
M551	aglycon of isoquercitrin	0.28	56.54	RAM
M54	8-prenyldaidzein	0.33	56	RPL
M351	danshenspiroketallactone	0.31	55.99	RSM
M311	przewaquinone c	0.4	55.83	RSM
M373	isocryptotanshinone	0.39	55.08	RSM
M197	6-aldehydo-7-methoxyl-isoophiopogonanone B	0.41	54.45	ROJ
M326	tanshinol a	0.41	54.27	RSM
M546	folic acid	0.71	53.33	RAM
M325	tanshinaldehyde	0.45	52.54	RSM
M395	neocryptotanshinone	0.32	52.54	RSM
M343	cryptotanshinone	0.4	52.44	RSM
M495	aglycon of alexandrin	0.75	52.12	RAM
M556	biochain B	0.21	51.72	RAM
M482	aglycon of β-sitosterol-3-O-β-D-glucopyranoside daucosterol	0.75	50.29	RAM
M377	isotanshinone IIa	0.4	50.02	RSM
M389	miltionone I	0.32	49.68	RSM
M506	aglycon of astragaloside IV	0.32	49.67	RAM
M356	deoxyneocryptotanshinone	0.29	49.51	RSM
M554	isomucronulatol-7,2′-di-O-glucosiole	0.62	49.32	RAM
M324	aglycon of tannin	0.26	49.23	RSM
M1	(Z,Z,Z)-8,11,14-eicosatrienoic acid	0.2	48.76	RPL
M469	aglycon of 9,10-dimethoxypterocarpan-3-O-β-D- glucoside	0.42	47.86	RAM
M96	daidzein-4′,7-diglucoside	0.67	47.27	RPL
M348	danshexinkum b	0.26	46.79	RSM
M270	6-O-syringyl-8-O-acetyl shanzhiside methyl ester	0.71	46.69	RSM
M532	rhamnocitrin-3-O-glucoside	0.76	45.82	RAM
M36	3′-methoxydaidzin	0.81	45.13	RPL
M264	3α-hydroxytanshinone IIa	0.44	45.1	RSM
M384	manool	0.2	45.06	RSM
M359	dihydrotanshinone I	0.36	45.04	RSM
M393	miltirone II	0.24	44.95	RSM
M477	sitosterol	0.78	44.72	RAM
M430	stigmasterol	0.76	43.83	RSM
M319	sclareol	0.21	43.67	RSM
M445	Δ1-dehydrotanshinone	0.4	43.67	RSM
M518	chlorogenic acid	0.33	43.43	RAM
M357	dihydroisotanshinone I	0.36	43.39	RSM
M435	tanshindiol c	0.45	42.87	RSM
M434	tanshindiol b	0.45	42.68	RSM
M558	lupenone	0.78	42.39	RAM
M265	3β-hydroxytanshinone IIa	0.45	42.17	RSM
M312	przewaquinone d	0.45	41.31	RSM
M168	5,7-dihydroxy-6,8-dimethyl-3-(2′-hydroxy-3′,4′-methylenedioxybenzyl)chromone	0.53	41.14	ROJ
M258	2-isopropyl-8-methylphenanthrene-3,4-dione	0.23	41.06	RSM
M511	astragaloside II	0.13	40.87	RAM
M489	syringaresinol	0.72	40.79	RAM
M91	soyasapogenol C	0.77	40.74	RPL
M167	5,7,2′-trihydroxy-8-methyl-3-(3′,4′-methylenedioxyb-enzyl)chromone	0.49	40.63	ROJ
M171	methylophiopogonone B	0.34	40.52	ROJ
M250	1,2,5,6-tetrahydrotanshinone	0.36	40.5	RSM

Table 1: Continued.

NO.	Compound	DL	OB	Herbs*
M517	lariciresinol	0.38	40.27	RAM
M170	methylophiopogonone A	0.48	40.24	ROJ
M520	aglycon of ononin	0.21	38.22	RAM
M23	daidzein	0.19	38.19	RPL
M414	salvianic acid a	0.06	35.95	RSM
M24	formononetin	0.21	32.3	RPL
M438	tanshinone IIb	0.45	21.7	RSM
M119	ophiopogonin D	0.06	20.86	ROJ
M203	methylophiopogonanone A	0.48	19.1	ROJ
M186	ophiopogonone A	0.44	14.24	ROJ
M496	quercetin	0.28	13.18	RAM
M64	puerarin	0.69	12.92	RPL
M199	6-aldehydoisoophiopogonone A	0.53	12.6	ROJ
M67	daidzin	0.73	9.83	RPL
M68	formononetin-7-O-b-D-glycoside-ononin	0.78	8.62	RPL
M323	tannin	0.03	7.3	RSM
M235	methylophiopogonanone B	0.34	5.26	ROJ

* RSM (Radix Salviae Miltiorrhiza), RAM (Radix Astragali Mongolici), RPL (Radix Puerariae lobatae), and ROJ (Radix Ophiopogonis Japonici).

candidate compounds are used for further target prediction (Table 1).

3.2. Potential Target Identification. To develop effective and safe therapies is the ultimate goal of medicine. For TCM comprising many molecules, a key challenge remains the identification of the molecular targets underlying the beneficial or detrimental effects of drugs. Thus a systematic, widely applicable, and robust approach is badly needed. Previously, we have developed a simple, universally applicable target identification approach on the basis of the RF and SVM techniques. In this section, with application of this method, we have analyzed the binding of Chinese herbs RSM, RAM, RPL, and ROJ to targets of interest in the CVD.

3.2.1. RSM. There are 68 potential targets identified for 43 bioactive components in RSM (supporting Table S1). This means that one compound hits 1.6 target proteins on average, which elaborates the polypharmacology characteristic of the multicomponent TCM. Among 68 targets, 21 are proven to be related with CVD. For examples, 5-hydroxytryptamine 2A receptor, alpha-1D adrenergic receptor, and beta-2 adrenergic receptor are reported to play an important role in the pathogenesis of hypertension [55–57]; potassium voltage-gated channel and sodium channel protein are demonstrated to be related with cardiac arrhythmias [58, 59]; prothrombin is proved to have a relationship with myocardial infarction, stroke, and venous thrombosis in a large cohort of US men [60].

3.2.2. RAM. A total of 77 target proteins (supporting Table S1 in Supplementary Materials available online at doi:10.1155/2012/519031) are obtained for 44 bioactive compounds in this herb, of which 19 are CVD related. For examples, peroxisome proliferator-activated receptor gamma (PPARγ) is expressed by macro-phages, endothelial cells, and vascular smooth muscle cells. It regulates gene expression of key proteins involved in lipid metabolism, vascular inflammation, and proliferation contributing to atherogenesis and postangioplasty restenosis, thus having beneficial effects on CVD [61]; vascular endothelial growth factor receptor 2 can regulate angiogenesis which is a critical reparative process that occurs subsequent to ischemic injury [62].

3.2.3. RPL. In this herb, 12 candidate drugs are predicted to bind with 34 target proteins (supporting Table S1), of which 13 link with CVD. For example, the major active constituent puerarin (M64) hits 3 potential targets associated with CVD such as estrogen receptor, prostaglandin G/H synthase 2, and prothrombin. Recently, puerarin has been reported to compete with 17β-estradiol binding to estrogen receptors, thereby suppressing invasion and vascularization of endometriosis tissue stimulated by 17β-estradiol [63]. Besides, puerarin modulating the proteins prostaglandin G/H synthase 2 and prothrombin gives insights into the puerarin-induced protection against myocardial infarction and improvement of the blood flow [64]. These findings may explain why puerarin is responsible for the pharmacological effects of RPL on the cardiovascular systems in several animal models with cardiovascular disorders [65].

3.2.4. ROJ. Altogether 77 target proteins (supporting Table S1) identified for 19 candidate drugs in this herb. Among 77 targets, 19 are relevant to CVD such as protein beta-2 adrenergic receptor related with disease myocardial ischemia; potassium voltage-gated channel and sodium channel protein associated with arrhythmia; 5-hydroxytryptamine 2A

Table 2: Comparison of molecular properties between RSM, RAM, RPL, and ROJ.

Index	RSM (mean ± SD)		RAM (mean ± SD)		ROJ (mean ± SD)		RPL (mean ± SD)	
	Total compounds	Active compounds	Total compounds	Active compounds	Total compounds	Active compounds	Total compounds	Active compounds
nHDon	2.09 (2.69)	1.95 (3.88)	4.05 (2.86)	3.89 (2.77)	3.22 (3.23)	2.68 (1.49)	5.00 (4.65)	3.42 (2.19)
nHAcc	4.23 (4.47)	5.19 (6.78)	7.51 (4.70)	7.91 (4.32)	6.72 (6.22)	6.58 (2.46)	8.50 (7.62)	6.42 (3.70)
MLogP	2.45 (2.61)	2.10 (2.28)	0.75 (2.59)	1.18 (2.60)	2.12 (2.48)	1.61 (0.91)	1.28 (3.38)	1.44 (2.57)
MW	310.37 (156.93)	343.28 (219.76)	411.00 (232.39)	438.31 (169.88)	428.72 (262.11)	373.42 (120.83)	484.18 (286.37)	379.77 (92.05)
RBN	3.66 (5.17)	2.09 (4.99)	5.17 (3.68)	5.02 (3.42)	4.99 (4.81)	3.26 (1.45)	6.58 (6.02)	4.08 (3.96)
nCIC	2.74 (1.73)	3.72 (1.35)	3.59 (2.66)	4.14 (1.84)	4.05 (3.01)	3.74 (1.52)	3.89 (2.6971)	3.58 (1.38)
Hy	0.56 (2.00)	0.38 (2.79)	1.92 (2.06)	1.68 (1.95)	1.13 (2.13)	0.74 (0.97)	2.46 (3.28)	1.28 (1.61)
TPSA (Tot)	73.61 (76.27)	86.86 (114.01)	120.61 (72.73)	123.18 (69.35)	102.76 (92.12)	100.13 (35.49)	142.01 (125.68)	106.28 (59.74)

* RSM (Radix Salviae Miltiorrhiza), RAM (Radix Astragali Mongolici), RPL (Radix Puerariae lobatae), and ROJ (Radix Ophiopogonis Japonici).

receptor related with thrombosis, which clarifies the herbal cardiovascular activities such as anti-ischemia, anti-arrhythmic, and antithrombotic. Impressively, multiple targets of this herb such as sodium channel protein, beta-2 adrenergic receptor, and 5-hydroxytryptamine 2A receptor, are shared with RSM.

3.3. Differences in Chemical Space of the Four Medicinal Herbs. In TCM, pharmacological activities of one herb are usually potentiated by addition of other herbs in a formula. The nature of such pharmacological synergy in psychoactive herbal medicine is probably due to the bioactive compounds targeting a similar receptor or physiological system [4]. Since the chemical composition of herbs will provide the building blocks of the pharmacology activities, a question arises whether herbs of similar pharmacology activity (treatment of CVD) have similar chemical composition. To answer this question, we have analyzed the molecular diversity of compounds from four herbs by considering 8 common descriptors including MW, nCIC, RBN, nHDon, nHAcc, Hy, TPSA, and MlogP for these four compound classes (Table 2, Figure 1 and supporting Figure S1 available online at doi: 10.1155/2012/519031), since these parameters can reflect the basic characteristics of a molecule especially its pharmacodynamic properties.

Generally, systematic investigations of chemical space are used as a way of measuring the diversity of a compound library. The main focus of this study is on comparing scaffolds of bioactive natural products inherent to different medicinal herbs, thus for further understanding of scaffold architectures in different herbs that might be suitable for combinatorial library design. We get a first overview of molecular distribution of all molecules in each herb. The distribution curves of different properties are displayed in supporting Figure S1 and the mean values are in Table 2. The distribution characteristics of each descriptor for all herbs are similar to those of natural products observed by Feher and Schmidt [66]. Although the distribution of each descriptor for compounds in RSM peaks at a similar position as those in the other three herbs, they are skewed toward much lower values except MlogP. Besides, RSM has an obviously narrower distribution of each descriptor than that of the other herbs, suggesting that compounds in RSM are substantially less diverse than those in the other three herbs. These results demonstrate that chemical compositions of RSM and the other three herbs have substantially different properties. The following section will be devoted to comparison of molecular distribution of bioactive molecules in each herb.

As shown in Table 2, the average calculated MW is similar for ROJ (373.42 ± 120.83) and RPL (379.77 ± 92.05), which is much lower than that of molecules in RAM (438.31 ± 169.88) while significantly higher than those of RSM (343.28 ± 219.76); the peaks of MW distribution of four (bioactive) natural products' datasets are located at around 300~500. As shown in Figure 1, the MW distribution of RSM is highly overlapped with those of the other three herbs and slightly skewed toward lower molecular weights.

The average lipophilicity MlogP value is the highest for the RSM (2.10 ± 2.28), followed by ROJ (1.61 ± 0.91), RPL (1.44 ± 2.57), and RAM (1.18 ± 2.60) (Table 2 and Figure 1), indicating that the molecules in RSM are more soluble in neutral solvents than those from the other herbs. From Figure 1, interestingly, we find that RSM has a wider

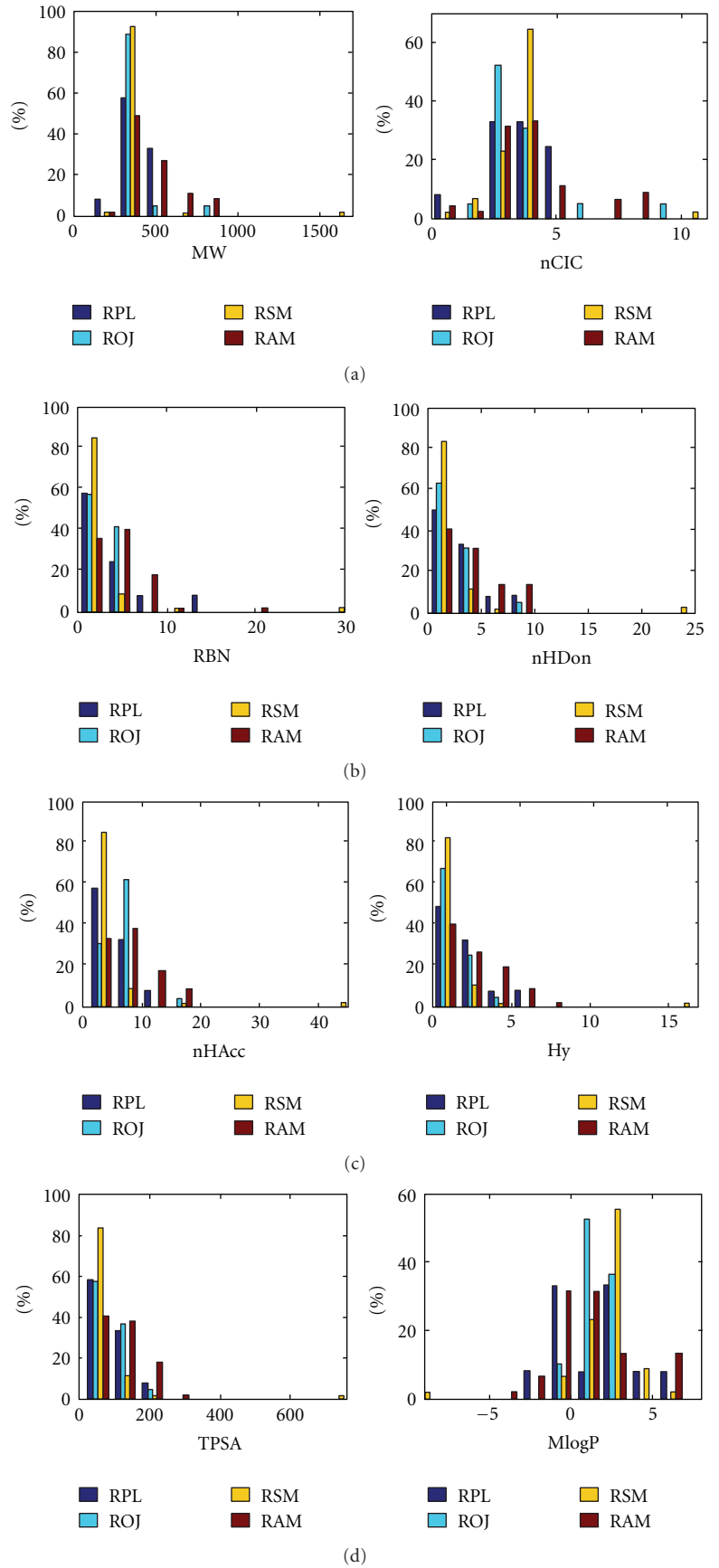


Figure 1: The profile distributions of eight important molecular properties for bioactive molecules from Radix Salviae Miltiorrhiza (RSM), Radix Astragali Mongolici (RAM), Radix Puerariae lobatae (RPL), and Radix Ophiopogonis Japonici (ROJ).

distribution of MLogP than ROJ but less than RAM and RPL. Similarly, the mean value of hydrophilic factor for each molecule is the lowest for RSM (0.38 ± 2.79), followed by ROJ (0.74 ± 0.97), RPL (1.28 ± 1.61), and RAM (1.68 ± 1.95). This result indicates that molecules in RSM are much more hydrophobic than molecules in the other herbs.

The average number of rotatable bonds (RBN) per molecule is the lowest for RSM (2.09 ± 4.99), followed by ROJ (3.26 ± 1.45), RPL (4.08 ± 3.96), and RAM (5.02 ± 3.42). Considering the mean MW of ROJ that is almost equal to that of the RPL, chemicals in RPL are probably more flexible than those in ROJ. Notably, when two molecules with different flexibility follow the same interaction with a target, the rigid molecule has a relative lower entropy loss compared with the flexible one, thus usually leading to the stronger binding affinity of rigid molecule [66]. This finding suggests that the flexibility of a molecule plays a key role in determining its binding to the active site of a target. Therefore, molecules in RPL probably have more thermodynamic advantages to achieve favorable binding properties than those in the ROJ. The prevalence of rings is another measurement for the rigidity of molecules, similar to RBN, the average number of rings per molecule (nCIC) in RAM (4.14 ± 1.84) is the lowest among four classes.

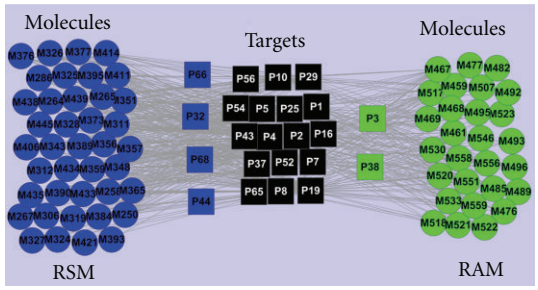
For nHDon and nHAcc, RSM has the least donor/acceptor atoms for H-bonds (HDon = 1.95; nHAcc = 5.19), while the RAM (HDon = 3.89; nHAcc = 7.91) has the largest number, followed by RPL (HDon = 3.42; nHAcc = 6.42) and ROJ (HDon = 2.68; nHAcc = 6.58) has mediate ones. An examination of the frequency of occurrence of elemental composition reveals that molecules in all herbs have many oxygen atoms but few nitrogen atoms. On average, the total number of oxygen and nitrogen atoms for RSM, RAM, ROJ, and RPL are 5.19, 7.64, 6.53, and 6.42, respectively. Therefore, among four herbs, compounds in RAM have the most polar functional groups which can act as H-bond acceptors.

TPSA is a key factor for OB and typically compounds are considered to be orally bioavailable with a TPSA value between about 80 and 150 Å^2 [67]. The peaks of TPSA distribution of four herbs locating around this range further validate our prescreening model. RAM (123.18 ± 69.35 Å^2) has the highest average TPSA values, followed by and RPL (106.28 ± 59.74 Å^2), ROJ (100.13 ± 35.49 Å^2), and RSM (86.86 ± 114.01 Å^2). This is similar to the trends of nHDon and nHAcc since TPSA is relevant to the number of hydrogen-bond donors and acceptors.

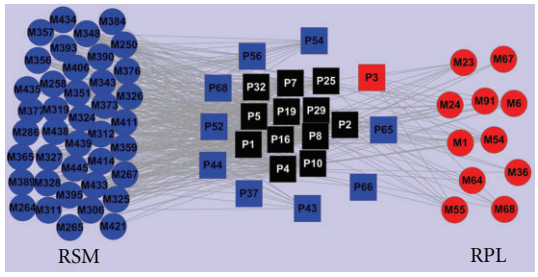
In summary, the distribution profiles of these basic, physicochemical properties for RSM are obviously different from those of the other three herbs, suggesting four herbs with different chemical compositions. However, their pharmacological roles are very similar in treatment of CVD. It is reasonable to speculate that their real interaction with target proteins may be different. Therefore, the mutual enhancement of these herb pairs could be achieved through the different mode of actions to exert a complementary synergistic effect (details in the next section).

3.4. Uncovering the Synergy from Network Pharmacology Level. Herbal medicines are multicomponent therapeutics, in which two or more herbs interact with multiple targets simultaneously, thus are considered as a rational and efficient form of therapy designed to control complex diseases [35]. The fundamental advantage of this therapeutics is the generation of synergism between ingredients (i.e., herbs and/or phytochemicals) within herbal formulas when two or more ingredients within a concoction mutually enhance the effect of the formulation in a certain activity or clinical outcome [68]. A representative herb RSM was reported to have synergic effect with multiple different herbs including RAM, RPL, and ROJ, and clinical trials have demonstrated that multiherb RSM formula enhanced stroke survival and recovery in comparison to RSM alone [2]. However, the molecular mechanism underlying the herbal synergism remains unclear. To solve this problem, the network pharmacology has been employed to understand the multicomponent synergy by its latent network topology properties.

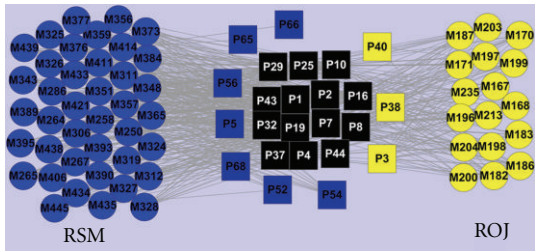
As mentioned above, a total of 68, 77, 34, and 40 targets were identified for RSM, RAM, RPL, and ROJ, respectively. To elucidate the synergic mechanism of different herbs on treating CVD, all target proteins associated with CVD are used to construct the drug-target network for three herb pairs, that is, RSM and RAM, RSM and RPL, RSM and ROJ, by linking with the cognate compounds (Figures 2(a), 2(b), and 2(c)). For RSM and RAM, Figure 2(a) shows a global view of the bipartite graph with color-coded nodes which correspond to either drugs (circle) or target proteins (square): compounds in RAM (green), compounds in RSM (blue), the same compounds in both herbs (black), targets specific to RAM (green), targets specific to RSM (blue). The network consists of 91 nodes and 529 edges. For compounds, M467 (7-O-methylisomucronulatol) exhibits the highest number of target connections (DD = 17), followed by M258 (2-isopropyl-8-methylphenanthrene-3,4-dione, DD = 15), M348 (dan-shexinkum b, DD = 14), M357 (dihydroisotanshinone, DD = 14), M365 (epidanshenspiroketallactone, DD = 14), M459 (3,9-di-O-methylnissolin, DD = 14), while the molecules M384 (manool), M393 (miltirone), M477 (sitosterol), M482 (aglycon of β-sitosterol-3-O-β-D-glucopyranoside daucosterol), M495 (aglycon of alexandrin), M558 (lupenone) have the least targets (DD = 1), The average number of targets per drug is 7.78, indicating the polypharmacology of drugs. For the proteins, the average number of drugs per target is 23. P8 (estrogen receptor) possesses the largest number of connected ingredients (DD = 61), followed by P4 (prostaglandin G/H synthase 2, DD = 60), P7 (nitric oxide synthase, inducible, DD = 52), and P19 (cell division protein kinase 2, DD = 51). Interestingly, 17 targets (73.91%) are found to be shared by both herbs. For examples, the common target P29 (β2-adrenergic receptor), which is related to heart failure, hypertension, and ischemic heart disease [69], can be modulated by 21 compounds in RSM and 8 in RAM. Similarly, another important target P2 (prothrombin) that plays an important role in CVD [60] is impacted by 36 chemicals in RSM and 13 in RAM. This suggests that individual drugs in RSM and RAM can act on the same targets in a single formula, thus exert synergistic therapeutic effect on CVD.



(a)



(b)



(c)

Figure 2: Drug-target interaction networks bioactive molecules from *Radix* Salviae Miltiorrhiza (RSM), Radix Astragali Mongolici (RAM), Radix Puerariae lobatae (RPL), and Radix Ophiopogonis Japonici (ROJ). (a) 40 bioactive compounds (blue circles) from RSM and 28 ones from RAM (green circles) predicted to have 23 potential protein targets (squares). The black squares (17) are the common targets of both herbs. The blue (4) and green (2) squares are the specific targets of RSM and RAM, respectively. (b) 40 bioactive compounds (blue circles) from RSM and 11 ones from RPL (red circles) predicted to have 22 potential protein targets (squares). The black squares (12) are the common targets of both herbs. The blue (9) and red (1) squares are the specific targets of RSM and RAM, respectively. (c) 40 bioactive compounds (blue circles) from RSM and 17 ones from ROJ (yellow circles) predicted to have 24 potential protein targets (squares). The black squares (14) are the common targets of both herbs. The blue (7) and yellow (3) squares are the specific targets of RSM and RAM, respectively.

CVD is a complex disease in which multiple mediators contribute to overall disease pathogenesis by distinct or redundant mechanisms; drugs designed to act against individual molecular targets probably yield less therapeutic efficacy than simultaneous blockade of multiple targets [35]. Thus another way called multicomponent therapy is also conceivable. Among all 23 targets, we find that RSM possess 4 specific potential targets and RAM has 2 specific potential targets (Figure 2(a)), that is, P66 (alpha-2A adrenergic receptor), P68 (alpha-2B adrenergic receptor), P44 (leukotriene A-4 hydrolase), and P32 (coagulation factor VII), are the specific targets of RAM, while P3 (PPARγ) and P38 (vascular endothelial growth factor receptor 2) are only targeted by RSM. To take target-disease-pathway information into consideration, intriguingly, we find that several targets are involved in the same or related pathway. For example, prostaglandin G/H synthase 1 and prothrombin belong to platelet aggregation inhibitor pathway, which is known to be related to myocardial infarction [70], thus individual drugs in RSM and RAM could act on different targets in the same pathways, thereby having synergistic effect on the treatment of CVD. Furthermore, salvianic acid A in RSM can also target multiple proteins including beta-1/2 adrenergic receptor, nitric-oxide synthase (endothelial), and PPARγ which are responsible for dilating coronary arteries and protecting the myocardium from reperfusion injury of the ischemic heart. This indicates that drugs in both herbs can also act on different targets in related pathways. Combined, these results suggest that both herbs hitting more functionally diverse targets with clinically relevant associations would improve polypharmacology in treating CVD.

For RSM and RPL (Figure 2(b)), a total of 22 protein targets (20 for RSM and 12 for RPL) and 51 molecules (40 for RSM and 11 for RPL) were included in the constructed network, to which 457 reactions (edges) were assigned, that is, 17 drugs, 2 targets, and 72 interactions less than the network constructed from both RSM and RAM. This translates into a significant increase in the number of interactions per drug, increasing the value from 7.78 to 8.96, while for the proteins, the average number of drugs per target decreases from 23.00 to 20.78. This difference may result from the different properties of the chemical space in RAM and RPL which link implicitly to some degree of target promiscuity [71]. Similar to RSM and RAM, 12 targets (54.54%) can be commonly modulated by RSM and RPL. For example, P4 (prostaglandin G/H synthase 2, DD = 48) can be modulated by 39 compounds in RSM and 9 in RPL. Besides, multiple drugs in RSM and RPL can also act on different targets in related pathways in treating CVD. For examples, NO is an important protective molecule in the vasculature, and endothelial nitric-oxide synthase (P5) is responsible for most of the vascular NO [72]. Simultaneously, NO can downregulate cell division protein kinase 2 (P19) activity [73] that has been implicated in prevention of coronary arteriosclerosis [74].

For RSM and ROJ, the drug-target network (Figure 2(c)) was based on the 534 interactions connecting 57 drugs (40 for RSM and 17 for ROJ) to 24 targets (21 for RSM and 17 for ROJ), resulting in an average number of interactions per drug of 9.37. In this network, 14 out of 24 target proteins are connected to both herbs. For example, mitogen-activated protein kinase 14 (P25) targeted by 17 chemicals in RSM and 15 in ROJ plays a protective role against cardiac myocyte apoptosis and myocardial remodeling [75]. These data suggest that both herbs used together to treat CVD can enhance the pharmacological effects by acting at the same molecular target, which is probably more effective than RSM alone.

Taken together, the above results provide insights into that the synergic effect between RSM and other three herbs could result from two strategies: (1) multiple drugs act on the same target; (2) multiple drugs act on different targets in related (or even the same) pathways. Although RSM and other herbs have shown progress as CVD treatments, they probably have distinct mechanistic differences from their different chemical compositions which are the foundation of pharmacology. This suggests that the combination of RSM and the other herbs will show great promise to have synergistic combined effect and overcome drug resistances in CVD therapies. Therefore, different medicinal herbs or drugs of lower potencies need to be appropriately combined in accordance with these profiles and probably in a personalized manner to achieve sufficient levels of efficacy.

4. Limitations

In this work, although such approaches have produced a significant amount of knowledge and understanding, only three widely used herb pairs were discussed. As more and more resources have been devoted to uncovering molecular mechanisms of herbal medicine under different conditions, there is a strong need of analysis for more other different herb pairs in a biological meaningful way. Moreover, other factors also have to be considered to evaluate whether two or more herbs can be merged (as a formula): do the herbs act in the same cell type or at the same developmental stage of the cell?

5. Conclusion

Herbal medicine has been widely used for disease treatment and is fast becoming a very popular form of alternative medicine worldwide. In TCM, multiple herbs have been frequently used together (multiherb recipe) to execute therapeutic actions. To formulate these TCM recipes, special herb pairs claimed to be unique combinations have been frequently used for achieving synergism that can modulate the efficacy and toxicity of the chemicals of the constituent herbs. However, the mechanisms of synergistic actions of herbal ingredients are still an unresolved issue. Here, we investigate a three representative herb pairs, that is, RSM and RAM, RSM and RPL, RSM and ROJ, by using a novel modeling system that integrates OB and DL screening, targets identification, and network pharmacology. Our results show the following.

(1) 43, 44, 12, 19 bioactive ingredients have been identified for the herbs RSM, RAM, RPL, and ROJ, respectively, suggesting that chemical compositions of RSM and the other three herbs have substantially different properties.

(2) 21, 19, 13, 17 potential targets associated with CVD have been identified for RSM, RAM, RPL, and ROJ, respectively, which are critical for better understanding of the mechanisms of action of herbs for the treatment of CVD and for the development of novel drugs and TCM modernization.

(3) Despite the pharmacological roles of the four herbs being very similar in treatment of CVD, the interaction of individual drugs in each herb with target proteins may be different.

(4) In TCM, the synergistic effect between RSM and the other three herbs could result from both strategies including (1) multiple drugs act on the same target; (2) multiple drugs act on different targets in related (or even the same) pathways. Therefore, the mutual enhancement of different herb pairs could be achieved through the different mode of actions to exert a complementary synergistic effect. The discovered mechanisms of synergistic actions of herbal ingredients will offer insights into designing new multi-target drugs and drug combinations and into discovering potent drug combinations that are individually subtherapeutic but efficacious in combination.

Based on the approach developed in this study, we can re-evaluate herbal formulae comprising many species of herbs, thus providing professional advice to support development of recipe optimization of TCM, which will promote drug discovery.

Authors' Contribution

X. Wang and X. Xu contributed equally to this work.

Acknowledgment

This work is financially supported by the Fundamental Research Funds of China Academy of Chinese Medical Sciences (ZZ0608).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2012, Article ID 408187, 12 pages
doi:10.1155/2012/408187

Research Article

Oroxylin A, but Not Vasopressin, Ameliorates Cardiac Dysfunction of Endotoxemic Rats

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Received 9 August 2012; Accepted 13 September 2012

Academic Editor: Waris Qidwai

The mortality in septic patients with myocardial dysfunction is higher than those without it. Beneficial effects of flavonoid oroxylin A (Oro-A) on endotoxemic hearts were evaluated and compared with that of arginine vasopressin (AVP) which is used to reverse hypotension in septic patients. Endotoxemia in rats was induced by one-injection of lipopolysaccharides (LPS, 10 mg/kg, i.p.), and hearts were isolated 5-hrs or 16-hrs later. Isolated hearts with constant-pressure or constant-flow mode were examined by Langendorff technique. Rate and force of contractions of isolated atrial and ventricular strips were examined by tissue myography. Isolated endotoxemic hearts were characterized by decreased or increased coronary flow (CF) in LPS-treated-for-5hr and LPS-treated-for-16-hr groups, respectively, with decreased inotropy in both groups. Oro-A-perfusion ameliorated while AVP-perfusion worsened the decreased CF and inotropy in both preparations. Oro-A and AVP, however, did not affect diminished force or rate of contraction of atrial and ventricular strips of endotoxemic hearts. Oro-A-induced CF increase was not affected following coronary endothelium-denudation with saponin. These results suggest that Oro-A ameliorates LPS-depressed cardiac functions by increasing CF, leading to positive inotropy. In contrast, AVP aggravates cardiac dysfunction by decreasing CF. Oro-A is a potentially useful candidate for treating endotoxemia complicated with myocardial dysfunction.

1. Introduction

Sepsis is a systemic response to infection [1], and may lead to septic shock which is one of the primary causes of death in the intensive care unit in many countries [2]. The most common cause of sepsis is an exposure to lipopolysaccharides (LPS), the structural component of a Gram-negative bacterial membrane, and key symptoms may include hypotension [3] and multiple organ failure [4]. During septic shock, myocardial depression also may be present and is characterized by impaired myocardial contractility and reduced ejection fraction [2]. The mortality in septic patients with myocardial dysfunction is higher by 50–70% than those without myocardial dysfunction [5]. Although many factors have been proposed to cause myocardial depression in septic shock [6–8], the complicated pathogenesis of cardiac dysfunction in septic shock remains unclear.

Oroxylin A (Oro-A, 5,7-dihydroxy-6-methoxyflavone), one of the main bioactive component in the root of *Scutellaria baicalensis*, is a conventional herbal medicine widely prescribed as an analgesic, antipyretic, anti-inflammation, anticancer, antiviral and antibacterial infections remedy

[9]. It also is an antioxidant that depresses generation of superoxide and nitric oxide (NO) [10]. In our previous report, Oro-A, via inhibition of nuclear factor-kappa B (NF-κB) activation, blocks LPS-induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in macrophages [11]. In addition, our preliminary in vivo experimentation demonstrated that Oro-A administered (i.v.) after establishment of LPS-induced septic shock (posttreatment) in anesthetized rats reversed the depressed heart rate (HR) and hypotension to normal ranges in 10 min with significantly improved survival rate. The exact mechanisms of action of Oro-A in ameliorating the myocardial dysfunction and hypotension in endotoxemia, however, are not clarified.

In the present study, we tested the hypothesis that Oro-A posttreatment (administered after establishment of endotoxemia or endotoxemic shock) improved coronary flow (CF) and cardiac function of the endotoxemic rat. Clinically, arginine vasopressin (AVP) is increasingly used to raise the systemic arterial blood pressure in septic patients who are refractory to catecholamine or conventional treatments [12]. AVP, however, has been shown to cause constriction of coronary arteries [13], leading to impairment of cardiac index and systemic oxygen delivery [14, 15]. Accordingly, chronic administration of AVP in patients with cardiac dysfunction should be cautious [16, 17]. We, therefore, evaluated and compared the cardiac effects of AVP and Oro-A. Our results indicated that Oro-A reversed while AVP further decreased the diminished CF and cardiac contractile force in isolated hearts from endotoxemic rats.

2. Materials and Methods

2.1. Drugs and Chemicals. LPS (derived from *Escherichia coli*, serotype 0127:B8), dimethyl sulfoxide (DMSO), arginine vasopressin (AVP), 5-hydroxytryptamine (5-HT), sodium nitroprusside (SNP), and saponin were obtained from Sigma Chemical (St. Louis, MO, USA). Acetone, chloroform, methanol, and hexane were purchased from Mallinckrodt (St. Louis, MO, USA).

2.2. Extraction and Purification of Oro-A from S. baicalensis. Oro-A was extracted from dried *S. baicalensis* [18]. In brief, dried *S. baicalensis* roots were cut into small pieces, which were then immersed and extracted with 10-fold (v/w) acetone at room temperature once every 2 weeks for 2 times. The acetone extracts were subjected to column chromatography on silica gel eluted with chloroform and chloroform-methanol and rechromatographed on silica gel eluted with hexane-acetone to yield Oro-A. The compound was identified by direct comparison of its electrospray ionization (ESI-) mass, ^{1}H-, and ^{13}C-nuclear magnetic resonance (NMR) spectroscopic data with authentic samples. The purity exceeded 99.5% as determined by high-performance liquid chromatography (HPLC). Because of the low water solubility, Oro-A was dissolved in 100% DMSO as a 100 mM stock solution and then added to the protein-free Krebs-Henseleit (K-H) buffer to make 10 or 20 μM Oro-A solution before use. The K-H buffer contains (in mmol/L) NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 11, which was filtered through a 0.22 μm filter disk (Millipore, Eschborn, Germany) before use.

2.3. Animal Models. All animal protocols were approved by the Animal Care and Use Committee of Tzu-Chi University. Adult male Sprague-Dawley rats (280–350 g), purchased from the BioLASCO Co., Ltd. Taipei, Taiwan, were housed in the Tzu-Chi University's animal quarters under a 12 hrs light/dark cycle. All rats were fed with a standard ration and tap water ad libitum. Endotoxemia was induced in conscious rats by injection of LPS (10 mg/kg in 1 mL of saline, i.p.) [19], while control group by injection of saline (1 mL/kg, i.p.).

Sixty-five rats were divided into three groups. (I) The control group: rats were treated with saline (1 mL/kg, i.p.) and sacrificed 5 hrs later. (II) The LPS-treated-for-5 hr endotoxemic group: rats were treated with LPS (10 mg/kg, i.p.) and sacrificed 5 hrs later. (III) The LPS-treated-for-16 hr endotoxemic shock group: rats were treated with LPS (10 mg/kg, i.p.) and sacrificed 16 hrs later. Five hrs or 16 hrs after saline or LPS treatment, rats were anesthetized by sodium pentobarbital (50 mg/kg, i.p.) and the mean arterial pressure (MAP, mmHg), HR, and body temperature (BT, °C) were measured. Animals were then heparinized (1000 IU/kg, i.p.) for 15 min before sacrifice (Figure 1(a)). Hearts were removed and prepared for Langendorff studies, and atrial and ventricular strips were dissected from some hearts for tissue bath studies (see below).

The rectal temperature, as an index of steady core body temperature, was recorded with a thermocouple probe coupled with a 7000 H microcomputer thermometer (Jenco Electronics Ltd., Taipei, Taiwan). The probe lubricated with Vaseline was inserted 5 cm into the rectum to ensure a reliable measurement. The femoral artery was cannulated with a polyethylene-50 (PE-50) catheter connected to a pressure transducer (P231D, Statham, Oxnard, CA, USA) for measuring the MAP and HR [20], which were displayed on a MP35 polygraph recorder (Biopac System, Inc., Santa Barbara, CA, USA).

2.4. Choice of Experimental Dosage of Oro-A and AVP. The choice of 10 and 20 μM of Oro-A in the present study was based on our previous biochemical studies using cell lines [11] that 17 μM Oro-A transcriptionally inhibited the expression of iNOS and COX-2 induced by LPS. Concentrations of AVP of 0.2 and 0.4 IU/L (equivalent to 0.003 and 0.006 IU/min calculated as 1 international unit = 4 nM AVP and based on 14 mL/min of perfusion flow rate) used in the present studies are comparable to those used by other investigators [21, 22]. The clinical doses used in the human adults are infused at a rate of 0.01 to 0.04 IU/min [23].

2.5. Langendorff Preparations. The cardiac functions were determined by a modified isovolumetric Langendorff technique [24]. After thoracotomy under anesthesia as described

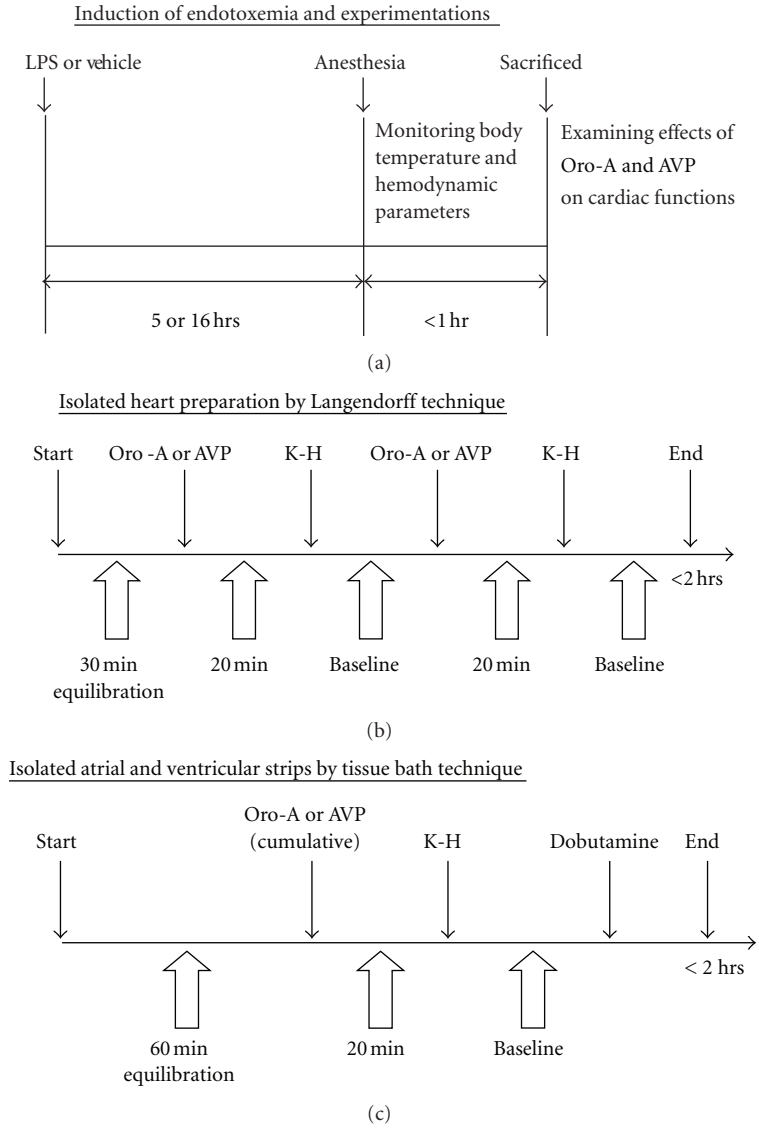

Figure 1: Experimental designs. Time schedules for LPS induction of endotoxemia in rats are shown in panel (a). Time schedules for studying effects of Oro-A and AVP on isolated endotoxemic heart are shown in panel (b), and on isolated atrial and ventricular strips in panel (c). LPS (lipopolysaccharide); K-H (Krebs-Henseleit solution); Oro-A (oroxylin A); AVP (arginine vasopressin).

above, hearts were quickly excised and placed in 4°C K-H buffer. A fluid-filled latex balloon, inserted into the left ventricle (LV) cavity via the mitral valve, was linked to a transducer connected with MP35 polygraph recorder for measuring left ventricular systolic pressure (LVSP) and HR. The balloon was inflated after insertion to reach a left ventricular end-diastolic pressure (LVEDP) of 5 to 10 mmHg, which remained unchanged throughout the experiment. Cardiac functions were evaluated upon left ventricular developed pressure (LVDP) calculated as the difference between LVSP and LVEDP, and the rate-pressure product (RPP, indicative of cardiac work) [25] was calculated as the product of LVDP and HR [26].

The constant pressure heart preparation was that the coronary perfusion pressure (CPP) was maintained constant at 96 cm H_2O monitored by a pressure transducer with MP35 polygraph recorder. This allowed to measure the CF by collecting the effluent dripping from the heart [24]. Thus, an increase or decrease of the CF represents dilatation or constriction, respectively, of the coronary artery. On the other hand, in the constant flow heart preparation, the CF was maintained constant at 14 mL/min. This allowed to measure the CPP (indicative of coronary resistance). An increase or decrease of the CPP indicates constriction or dilatation of the coronary, respectively [27].

The isolated heart was perfused through an aortic cannula with oxygenated K-H buffer (95% O_2 + 5% CO_2, pH 7.4) containing 0.02% (v/v) DMSO (the maximum final concentration of used as Oro-A in K-H buffer). Perfusion fluid and bath temperature were maintained at 37°C by a thermostatically controlled water circulator. After 30 min of baseline equilibration, the heart received Oro-A

(10 and 20 μM) or AVP (0.2 and 0.4 IU/L) in randomized order, each for 15 min to achieve a steady state. After 20 min washout, Oro-A or AVP was administered (Figure 1(b)). All measurements were taken during the last 5 min of each experimental period.

2.6. Coronary Endothelium Denudation. In order to assess possible role of endothelium in coronary vasomotor effect of Oro-A, vasomotor function of the coronary with or without the endothelium was evaluated. After 30 min of baseline equilibration in the Langendorff technique with constant flow heart preparation, 1 μM 5-HT (submaximum concentration) was perfused to induce endothelium-dependent coronary dilatation [28] as indicated by a decrease in the CPP. The hearts were then perfused with saponin (50 μg/mL in the K-H buffer) 3 times (5 min of saponin followed by 5 min K-H buffer each time) to ablate endothelium [28], then the heart received Oro-A (10 μM) and 5-HT (1 μM). A complete denudation was indicated by lack of 5-HT-induced vasodilation or decreased CPP. At the end of the experiment, 100 μM SNP was perfused to induce maximum percent of relaxation of coronary arteries, and drug-induced relaxation was estimated as percent of that induced by SNP. All measurements were taken during the last 5 min of each experimental period.

2.7. Force and Rate of Contraction of Isolated Atria and Ventricular Strips. In order to further confirm whether Oro-A and AVP exhibit direct myocardial effect, beat rate (right atrium) and isometric forces of isolated heart strips were evaluated. The right atrial and ventricular strips were dissected from the isolated heart and were suspended vertically in a 10 mL tissue bath filled with oxygenated K-H buffer (with 0.02% v/v DMSO) at 35°C [29]. The right atrial strips were allowed to beat spontaneously, and the ventricular strips were contracted by electrical stimulation with a Grass SD-9 stimulator (Grass-Telefactor, RI, USA) at the frequency of 2 Hz, 5 ms of duration, twin pulses and supramaximal (threshold + 25%) voltage [30]. The resting force of both atrial and ventricular strips was adjusted at 0.5 g. Changes in beat rate and isometric force were recorded via a transducer bridge amplifier connected to a MP35 polygraph recorder and stored in a public computer.

After 60 min of baseline equilibration, each preparation was treated with different concentrations of Oro-A (10, 20 μM) or AVP (0.2, 0.4 IU/L) for 10 min each. At the end of each experiment, a drug-free K-H buffer was replaced for 20 min, allowing a recovery to the baseline condition. Dobutamine (1 μM, a β_1-adrenoceptor agonist) then was applied and results served as positive control for atrial rate (AR) and isometric force (Figure 1(c)). The force was calculated as follows Force mg/mg = isometric force mg/preparation wet weight mg [31]. All measurements were taken during the last 5 min of each experimental period.

2.8. Statistical Analysis. Data are expressed as means ± SEM. One-way analysis of variance (ANOVA) and Student's *t*-test were employed for comparison within groups the effects of Oro-A, AVP, or 5-HT on their cardiac functions, or coronary vasomotor activities. The differences among groups were compared changes in hemodynamics, cardiac functions or coronary vasomotor activities by two-way ANOVA. Post-hoc analysis was done with SPSS version 13.0 (SPSS Inc., Chicago, IL). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Changes in Hemodynamics in LPS-Treated Rats. Five hrs and 16 hrs after LPS treatment, rats developed endotoxemia and endotoxemic shock, respectively, characterized by the presence of lassitude, pilo-erection, fever, and tachycardia (Table 1). The MAP remained unchanged 5 hr after LPS treatment (endotoxemia without shock, or early-stage endotoxemia), but decreased by 35% 16 hr after LPS treatment (endotoxemia with shock, or late-stage endotoxemia). None of the rats died in 5 hr after LPS treatment (or LPS-treated-for- 5 hr group), while 2 of 20 (10%) rats died between 8 and 12 hrs after LPS in LPS-treated-for-16 hr group.

3.2. Cardiac Dysfunction Induced by LPS. Compared with that of the normal control (Table 1), the CF in constant pressure heart preparation of the LPS-treated-for-5 hr rats was decreased significantly by 45%, while the CPP in constant flow heart preparation was increased significantly by 30%. The CF in the hearts of the LPS-treated-for-16 hr rats, however, was increased significantly by 20%, while the CPP was decreased significantly by 43% (Table 1). From the rats treated with LPS for 5 hrs or 16 hrs, the LVDP and RPP of the isolated hearts were significantly decreased in heart preparations of the constant pressure by 46% and 48%, respectively, and of the constant flow by 52% and 50%, respectively (Table 1). The HR was not significantly changed except a slight but significant (16%) increase in the constant pressure heart preparation from LPS-treated-for-16 hr rats.

Furthermore, contractile force of the isolated right atrial strips (atrial force/AF) from both 5 hr and 16 hr after LPS-treated rats and that of the ventricular strips (ventricular force/VF) from 5 hr after LPS-treated rats were significantly decreased (Table 1). On the other hand, the spontaneous contraction rate of atrial strips (atrial rate/AR) from both 5 hr and 16 hr post-LPS rats were slightly but significantly increased (Table 1), while that of the ventricular rate (VR) was not altered 5 hrs after LPS treatment (LPS-treated-for-5 hr rats).

3.3. Effects of Oro-A and AVP on LPS-Induced Endotoxemic Hearts. In constant pressure heart preparation from LPS-treated-for-5 hr rats (Figure 2(a), solid bar, $n = 6$ each), Oro-A (10 and 20 μM) concentration-dependently increased CF. Similar concentrations of Oro-A, however, did not affect the already-increased CF in the hearts from LPS-treated-for-16 hr rats. In contrast, AVP (0.2 and 0.4 IU/L) concentration dependently decreased the CF in the hearts of both LPS-treated for 5 hr and 16 hr rats (Figure 2(a), open bar).

Table 1: Changes of body temperature and hemodynamic parameters in 5 hr and 16 hr post-LPS (10 mg/kg, i.p.) rats.

Preparations	Parameters	Control	5 hrs	16 hrs
Whole animals	*n*	22	23	18
	BT (°C)	36.9 ± 0.1	39.0 ± 0.1*	38.2 ± 0.1*#
	MAP (mmHg)	111 ± 2.1	112 ± 2.2	72 ± 2.3*#
	HR (bpm)	336 ± 9.9	435 ± 7.5*	454 ± 5.2*
Isolated heart (constant pressure)	*n*	6	6	6
	CF (mL/min)	13 ± 0.1	7.1 ± 0.5*	15.6 ± 0.7*#
	LVDP (mmHg)	80.8 ± 4.0	42.9 ± 4.8*	45.2 ± 4.1*
	HR (bpm)	292.1 ± 6.2	277.5 ± 2.0	338.4 ± 8.6*#
	RPP (mmHg·bpm)	23321 ± 755	10836 ± 912*	13528 ± 560*
Isolated heart (constant flow)	*n*	6	6	6
	CPP (mL/min)	94.1 ± 0.6	122.2 ± 3.6*	53.8 ± 3.0*#
	LVDP (mmHg)	89.9 ± 3.1	45.0 ± 2.2*	41.8 ± 1.9*
	HR (bpm)	299.4 ± 7.1	317.5 ± 7.9	312.5 ± 8.9
	RPP (mmHg·bpm)	26837 ± 841	14181 ± 976*	12763 ± 596*
Isolated right atrium	*n*	5	6	6
	Force (mg/mg)	12.5 ± 1.0	8.0 ± 1.0*	6.2 ± 0.6*
	Rate (bpm)[1]	321.1 ± 6.8	348.1 ± 7.9*	355.4 ± 13.2*
Isolated right ventricle	*n*	5	5	N/P
	Force (mg/mg)	13.9 ± 2.4	6.3 ± 0.3*	N/P
	Rate (bpm)[2]	120.0 ± 0.0	120.0 ± 0.0	N/P

Values are mean ± SEM. $^*P < 0.05$ versus control group, $^{\#}P < 0.05$ versus 5 hr LPS group. [1]The spontaneous beating rate of the atrium was considered as sinoatrial electrical activity. [2]The ventricular rate was triggered by electric stimulation with a frequency of 2 Hz. BT (body temperature); MAP (mean arterial pressure); HR (heart rate); CF (coronary flow); CPP (coronary perfusion pressure); LVDP (left ventricular developed pressure); RPP (rate-pressure product); bpm (beats per min); N/P (not performed).

In constant flow heart preparation from LPS-treated-for-5 hr rats, Oro-A (10 and 20 μM) in concentration-dependent manner decreased the CPP (Figure 2(b), solid bar). Oro-A at the same concentrations, however, did not affect the already decreased CPP in the hearts of LPS-treated-for-16 hr rats. In contrast, AVP (0.2 and 0.4 IU/L) concentration dependently increased the CPP in the constant flow heart preparation from both LPS-treated-for-5 hr and 16 hr rats (Figure 2(b), open bar).

The diminished LVDP of the hearts from LPS-treated for 5 hr and 16 hr rats was reversed by Oro-A, but was further decreased by AVP in constant pressure heart preparation (Figure 3(a)). In constant flow heart preparations from LPS-treated-for-5 hr and 16 hr rats, Oro-A also slightly but significantly increased the LVDP, while AVP was without any effect (Figure 3(b)).

Furthermore, Oro-A perfusion did not affect the HR in either constant pressure (Figure 4(a), solid bar) or constant flow (Figure 4(b), open bar) heart preparations of LPS-treated-for-5 hr and 16 hr rats. In contrast, AVP (0.2 and 0.4 IU/L) perfusion decreased HR in constant pressure (Figure 4(a), solid bar) heart preparation of LPS-treated-for-5 hr and 16 hr rats, but did not affect that in constant flow heart preparations (Figure 4(b), open bar).

In isolated hearts of constant-pressure and constant-flow heart preparations from LPS-treated-for-5 hr and 16 hr rats, Oro-A perfusion increased RPP (Figures 5(a) and 5(b), solid bar, $n = 6$ each). In contrast, AVP significantly reduced RPP in the constant pressure heart preparation but did not significantly affect those in the constant flow heart preparations (Figures 5(a) and 5(b), open bar, $n = 6$ each).

3.4. Oro-A-Induced Decrease of Coronary Perfusion Pressure (CPP) is Independent of the Endothelium. In constant flow heart preparation from normal rats, 5-HT (1 μM) and Oro-A (10 μM) decreased the CPP indicative of decreased coronary resistance. After endothelium denudation by saponin (50 μg/mL), the decrease of CCP induced by Oro-A was not significantly affected. The decrease induced by 5-HT, however, was converted to increase (Figure 6, $n = 4$).

3.5. Failure of Oro-A and AVP to Affect Force or Rate of Isolated Atrial and Ventricular Strips from Endotoxemic Hearts. The spontaneous contractile force of atrial strips from both LPS-treated-for-5 hr and 16 hr rats and the electrically-paced force of the ventricular strips from LPS-treated-for-5 hr rats were significantly decreased comparing to those of the respective controls (Figures 7(a) and 7(b)). On the other hand, the atrial beat rate of both LPS-treated-for-5 hr and 16 hr rats was slightly but significantly enhanced, while the ventricular beat rate of LPS-treated-for-5 hr rats remained unaltered. Oro-A (10 and 20 μM) and AVP (0.2 and 0.4 IU/L) did not affect the atrial force (Figure 7(a), $n = 6$ each) or atrial rate (Figure 7(a), $n = 6$ each) of the isolated atrial strips from both LPS-treated rats. Similarly, Oro-A and AVP

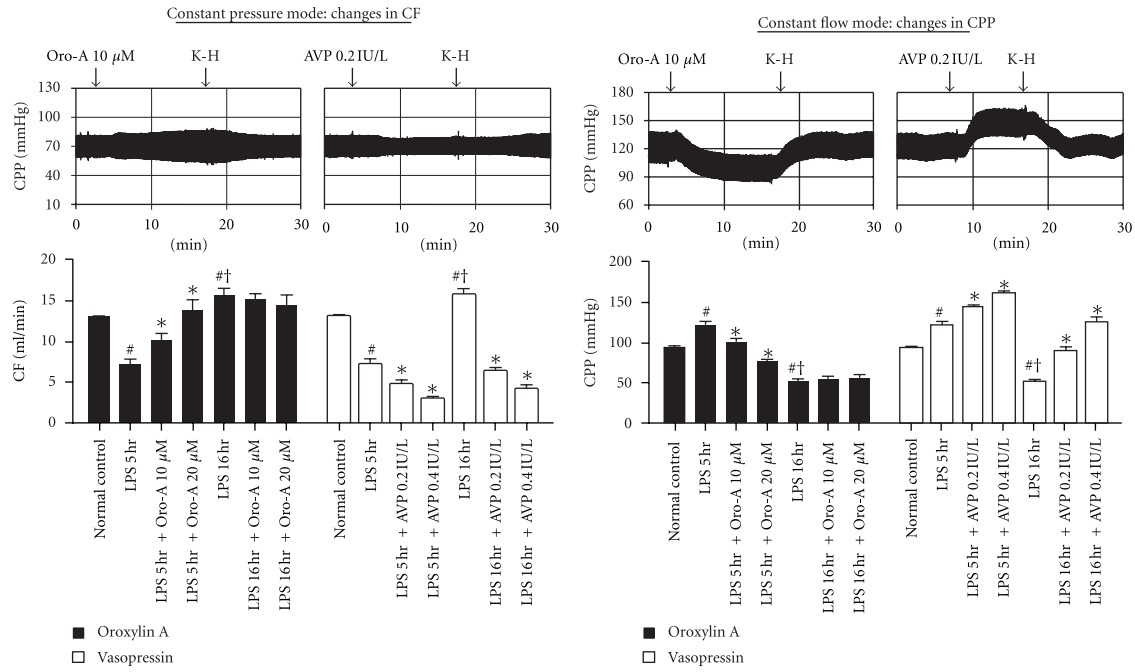

Figure 2: Effects of Oro-A and AVP on the CPP, CF, and coronary arterial function of isolated hearts from endotoxemic rats. The representative tracings in (a) indicate that Oro-A (10 μM) or AVP (0.2 IU/L) perfusion does not affect the mean of CPP; however, Oro-A (10 μM) perfusion increases while AVP (0.2 IU/L) perfusion decreases the pulse of CPP in constant pressure heart preparation from 5 hr post-LPS rats. In constant flow heart preparation (b), Oro-A (10 μM) decreases while AVP (0.2 IU/L) increases the CPP in the constant flow (14 mL/min) heart preparation from 5 hr post-LPS rats. The bar chart in (a) summarize effects of Oro-A (10 and 20 μM, solid bar) and AVP (0.2 and 0.4 IU/L, open bar) on CF in constant pressure heart preparation from 5 hr or 16 hr post-LPS rats. The bar chart in (b) summarize effects of Oro-A (10 and 20 μM, solid bar) and AVP (0.2 and 0.4 IU/L, open bar) on CPP in constant flow heart preparation from 5 hr or 16 hr post-LPS rats. Values of normal control and 5 hr and 16 hr post-LPS rats are from Table 1. Values are mean ± SEM ($n = 6$ each group). $^{*}P < 0.05$ versus respective LPS groups; $^{\#}P < 0.05$ versus normal control; $^{\dagger}P < 0.05$ versus 5 hr post-LPS group. CF (coronary flow); CPP (coronary perfusion pressure).

at similar concentrations did not affect the electrically-paced force or beat rate of the ventricular strips from LPS-treated-for-5 hr rats either (Figure 7(b), $n = 5$ each).

4. Discussion and Conclusion

In the present study, we demonstrated that Oro-A ameliorated while AVP aggravated the LPS-depressed cardiac function in the early-stage (5 hrs after LPS treatment) and late-stage (16 hrs after LPS treatment) endotoxemia by monitoring CF and cardiac inotropy. We reported here also for the first time that Oro-A induced endothelium-independent coronary vasodilation of the isolated hearts. Furthermore, the decreased CF in isolated hearts from the early-stage endotoxemic rats was reversed significantly by Oro-A which, however, did not affect the already increased CF in isolated hearts of the late-stage endotoxemia. In addition, diminished LVDP (indicative of cardiac contractility) and RPP (indicative of cardiac work) of the isolated hearts from both early- and late-stage endotoxemic rats were significantly reversed toward normal ranges by Oro-A without significant effect on the altered HR. The increased CF induced by Oro-A is expected to improve myocardial blood flow and oxygen supplies. No change of the HR by Oro-A suggests additional benefit of no further increase in myocardial oxygen demands. In contrast, AVP further decreased CF, LVDP, and RPP in isolated hearts of both early- and late-stage endotoxemic rats, suggesting its potential detrimental effects on the endotoxemic heart.

The findings of decreased LVDP, right atrial, and right ventricular forces in LPS-induced endotoxemic rats in the present study are in line with the results found in septic patients and other different animal models of sepsis [2, 32]. LPS treatment caused bimodal effects on the CF. In the early-stage endotoxemia, the CF was decreased with increased CPP and coronary microvascular resistance. Similar results have been reported by others [24, 33–35]. This coronary vasoconstriction has been suggested to involve release of vasoconstrictors including endothelin-1 (ET-1, a potent vasoconstrictor and proinflammatory peptide) [36] from the coronary and endocardial endothelial cells and/or impaired vasodilator responses of NO [33]. Therefore, the increased coronary resistance in early-stage endotoxemic hearts may lead to loss of local regulatory mechanism and an increased propensity for coronary vasospasm, myocardial ischemia

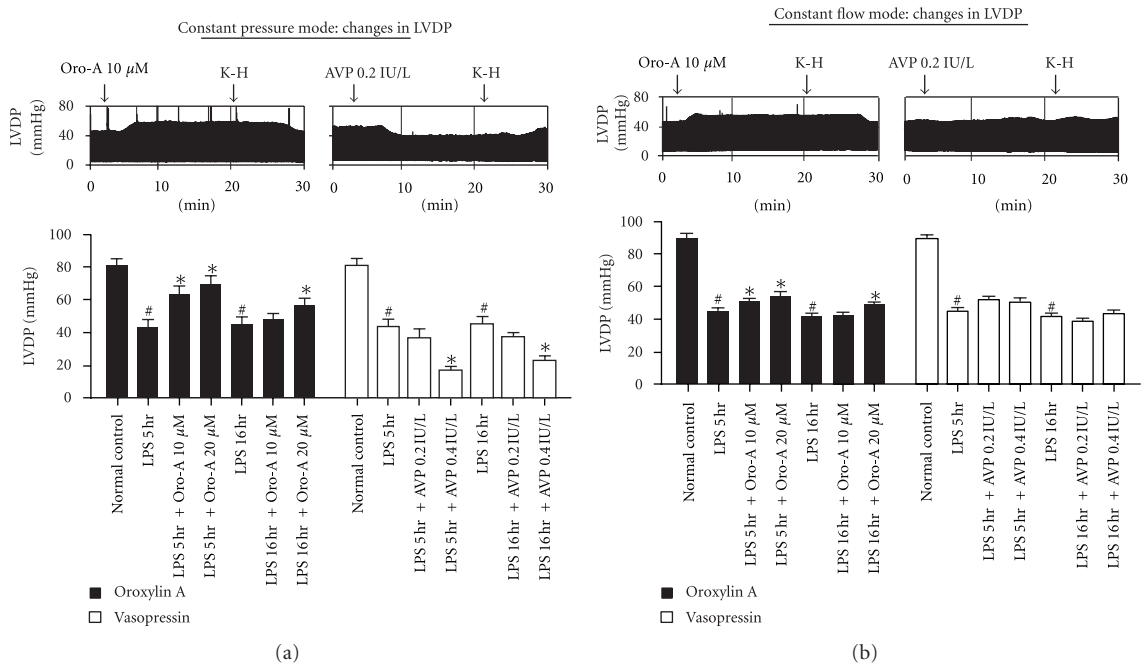

Figure 3: Effects of Oro-A and AVP on the LVDP of isolated hearts from endotoxemic rats. The representative tracings in (a) indicate that Oro-A (10 μM) perfusion increases while AVP (0.2 IU/L) perfusion decreases the LVDP in constant pressure heart preparation from 5 hr post-LPS rats. In constant flow heart preparation (b), Oro-A (10 μM) slightly but significantly increases LVDP, while AVP (0.2 IU/L) is without effect. The bar chart in (a) summarizes effects of Oro-A (10 and 20 μM, solid bar) and AVP (0.2 and 0.4 IU/L, open bar) on LVDP in constant pressure heart preparation from 5 hr or 16 hr post-LPS rats. The bar chart in (b) summarize effects of Oro-A (10 and 20 μM, solid bar) and AVP (0.2 and 0.4 IU/L, open bar) on LVDP in constant flow heart preparation from 5 hr or 16 hr post-LPS rats. Values of normal control, and 5 hr and 16 hr post-LPS rats are from Table 1. Values are mean ± SEM ($n = 6$ each group). $^{*}P < 0.05$ versus respective LPS groups; $^{\#}P < 0.05$ versus normal control. LVDP (left ventricular developed pressure).

and coronary dysfunction [37]. On the other hand, in the late-stage endotoxemia, the CF was increased with decreased CPP and coronary microvascular resistance. The coronary vessel already relaxed considerably and, therefore, Oro-A did not cause any further relaxation beyond the capacity of the vessels. The increased coronary vasodilation in the late-stage endotoxemia is likely due to increased expression of iNOS, which produces high levels of NO, in the myocardium [19]. Thus, coronary vascular tone increases in the early-stage and decreases in the late-stage endotoxemia. This difference in hemodynamic changes in endotoxemia may be determined by the balance between available vasoconstrictors such as ET-1 and dilators such as NO at different stages after endotoxin contamination.

The present results indicated that Oro-A had greater improvement on LVDP and RPP at early-stage than that at late-stage endotoxemia. One logical explanation for this difference is that both CF-related and CF-unrelated mechanisms are involved in positive inotropic effects of Oro-A, and that a smaller CF-related effect than CF-unrelated effect of Oro-A in found at late-stage endotoxemia. This is expected since the coronary vasodilation may already be almost maximal at the late-stage endotoxemia, and therefore may not dilate significantly further or affect the already increased CF in the hearts. These results favor the idea that Oro-A improvement of LVDP and RPP is CF-related in the early-endotoxemic period. It is possible that the CF-unrelated positive inotropic effect may become more determinant in the late-endotoxemic period. The exact mechanisms underlying the CF-unrelated positive inotropic effect of Oro-A remain unknown. It may be due to Oro-A reduction of oxidative stress or removal of free radicals [10, 38] in endotoxemia.

The decreased LVDP and HR induced by AVP administration was found only in the constant-pressure heart preparations, suggesting that altered inotropic and chronotropic effects by AVP also depend on the CF-related mechanisms. Our findings are consistent with those reported by others [15, 21] that AVP-induced decrease of myocardial contractility is most likely due to a decreased CF. However, there was a lack of correlation between CF and corresponding decrease in LVDP and RPP induced by 0.2, U/L AVP in the early-stage endotoxemia, although positive correlation between CF and RPP in the late-stage was found (Figure 5(a)). The exact reason for the difference in findings in different stages is not known. It may be due to that in the late-stage endotoxemia, the coronary vessel relaxed considerably more and, therefore, AVP induced greater vasoconstrictor response. These results suggest that CF-related mechanisms of AVP are more significant in the late-stage than the early-stage endotoxemia, an interesting finding different from that

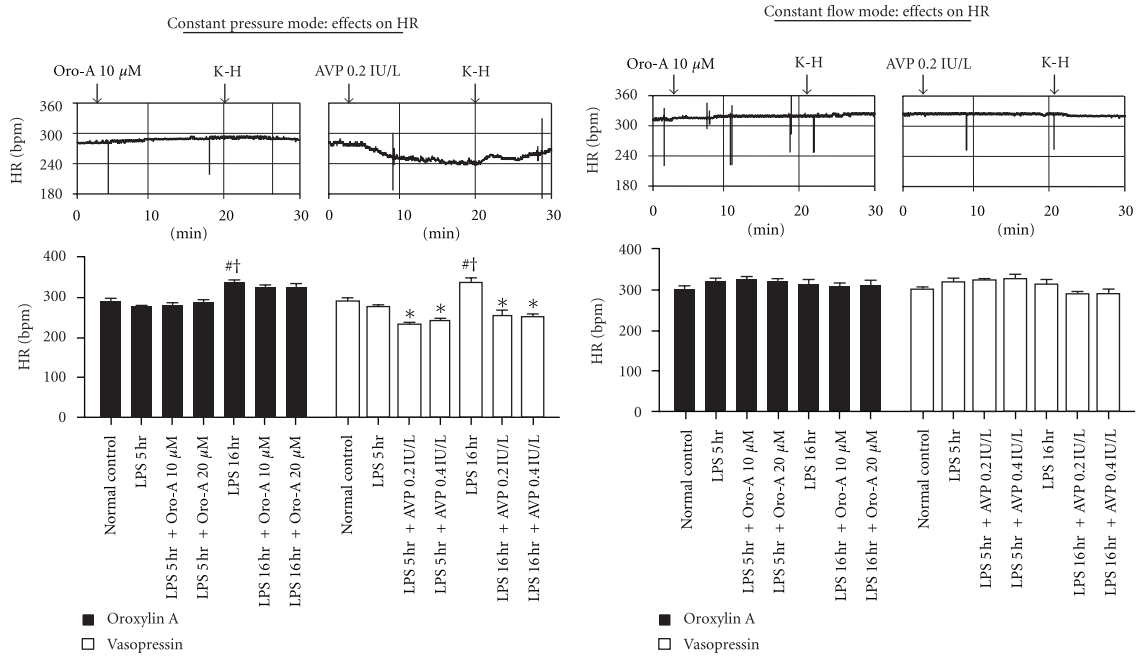

Figure 4: Effects of Oro-A and AVP on the HR of isolated hearts from endotoxemic rats. The representative tracings in (a) and (b) indicate that Oro-A (10 μM) perfusion does not affect the HR in either constant pressure or constant flow heart preparation from 5 hr post-LPS rats. AVP (0.2 IU/L) perfusion, however, decreases HR in constant pressure heart preparation but does not affect that in constant flow heart preparation. The bar chart in (a) summarizes effects of Oro-A (10 and 20 μM, solid bar) and AVP (0.2 and 0.4 IU/L, open bar) on HR in constant pressure heart preparation from 5 hr or 16 hr post-LPS rats. The bar chart in (b) summarizes effects of Oro-A (10 and 20 μM, solid bar) and AVP (0.2 and 0.4 IU/L, open bar) on HR in constant flow heart preparation from 5 hr or 16 hr post-LPS rats. Values of normal control, and 5 hr and 16 hr post-LPS rats are from Table 1. Values are mean ± SEM ($n = 6$ each group). $^{*}P < 0.05$ versus respective LPS groups; $^{\#}P < 0.05$ versus normal control; $^{\dagger}P < 0.05$ versus 5 hr post-LPS group. HR (heart rate).

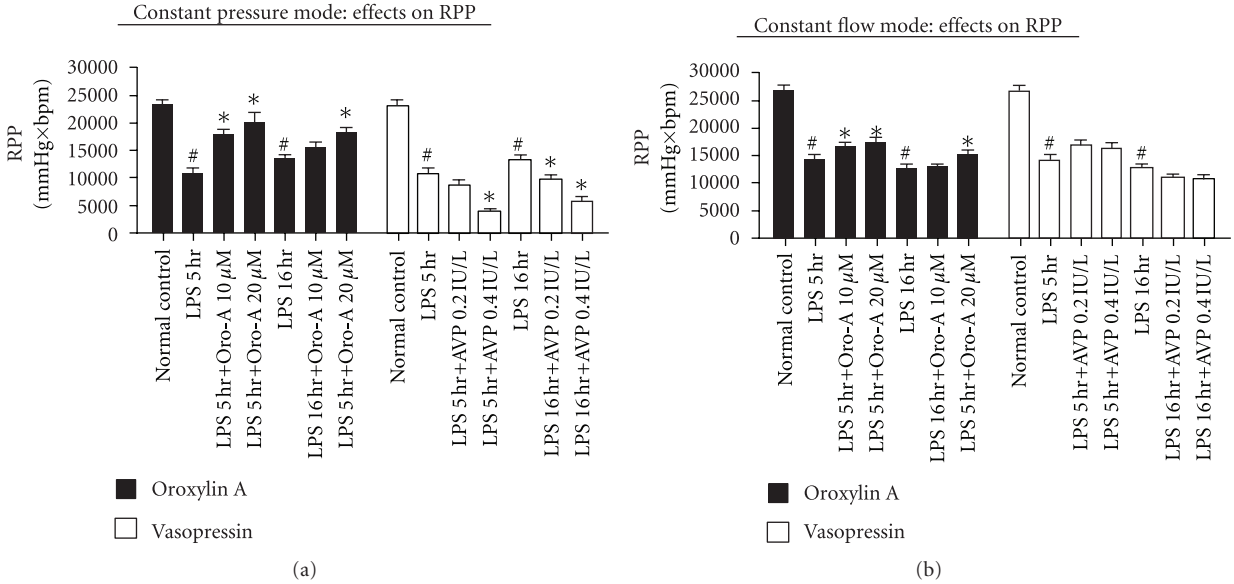

Figure 5: Effects of Oro-A and AVP on the RPP of isolated hearts from endotoxemic rats. (a) summarizes effects of Oro-A (10 and 20 μM, solid bar) and AVP (0.2 and 0.4 IU/L, open bar) on RPP in constant pressure heart preparation from 5 hr or 16 hr post-LPS rats. (b) summarizes effects of Oro-A (solid bar) and AVP (open bar) on RPP in constant flow heart preparation from 5 hr or 16 hr post-LPS rats. Values of normal control and 5 hr and 16 hr post-LPS rats are from Table 1. Values are mean ± SEM ($n = 6$ each group). $^{*}P < 0.05$ versus respective LPS groups; $^{\#}P < 0.05$ versus normal control. RPP (rate-pressure product).

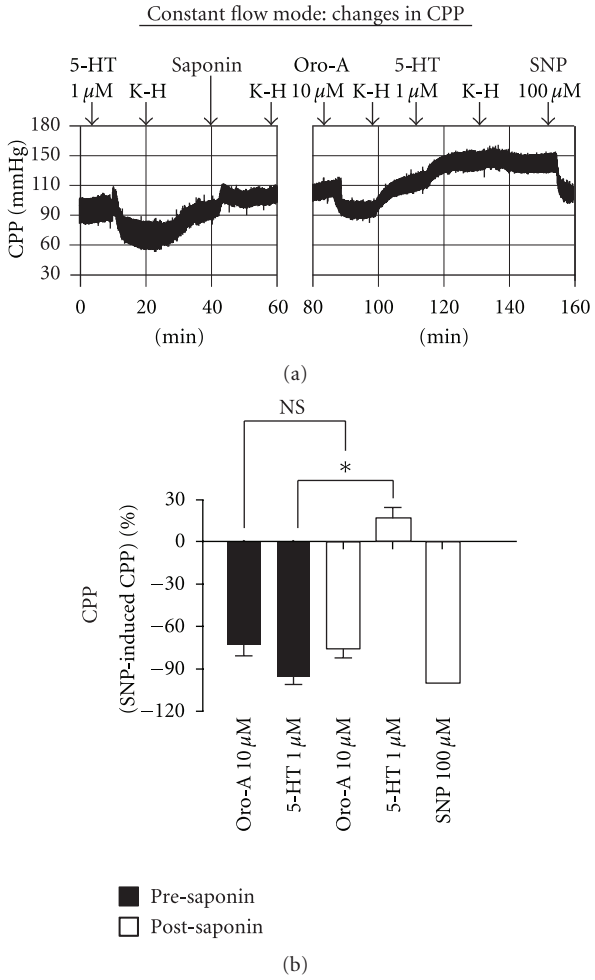

Figure 6: Effects of endothelium-denudation on CPP induced by 5-HT and Oro-A in isolated hearts from normal control rats. A representative tracing in (a) shows that 5-HT (1 μM) decreased CPP. After intravascular infusion with saponin (50 μg/mL) for 15 min, the decreased CPP induced by Oro-A (10 μM) was not affected, while that induced by 5-HT was blocked and converted to an increase. These effects of Oro-A (10 μM) and 5-HT (1 μM) on CPP (percentage of 100 μM SNP-induced) before (solid bar) and after (open bar) saponin are summarized in (b). Values are mean ± SEM ($n = 4$). $^*P < 0.05$ versus presaponin; NS (not significant, $P > 0.05$). 5-HT (5-hydroxytryptamine); SNP (sodium nitroprusside).

found for Oro-A effects. Furthermore, positive correlations between CF and corresponding decrease in LVDP and RPP induced by 0.4, U/L AVP in both early- and late-stage were found. It appears that the consistent negative inotropic effects of AVP may be induced more likely by its dose higher than 0.2, U/L (equivalent to 0.003 U/min) in the early-stage endotoxemia. These results further favor the coronary vascular effects of Oro-A and AVP in modulating cardiac functions. This information may be helpful in managing endotoxemia, particularly, in those with cardiac dysfunction.

The greater effects Oro-A and AVP on changing LVDP and RPP in the constant-pressure (Figures 3(a) and 5(a)) than those in the constant-flow (Figures 3(b) and 5(b)) heart preparations are consistent with the explanation by the reported "Gregg"s phenomenon' [39] or the "garden-hose effect" [40], in which the increase of heart contractility was a result of elevated CF in the constant-pressure heart preparations.

Oro-A-induced coronary relaxation with increased CF is endothelium independent (Figure 6). Since endotoxemia may render coronary endothelial dysfunction [41], endothelium-independent vasodilation induced by Oro-A, therefore, becomes an interesting and clinically important mechanism. This is supported by the report that Oro-A represses the phorbol-12-myristate-13-acetate (PMA, a protein kinase C/PKC activator)-induced translocation of PKC-δ [42] which is present in the vascular smooth muscle cells [43]. Incidently, physiological concentrations of AVP, which constricts vascular smooth muscle by directly acting on V_{1a} receptors [44] leading to activation of PKC [45]. These results provide additional evidence justifying the different vascular responses induced by Oro-A and AVP.

Furthermore, an increased HR was observed in the constant-pressure, but not the constant-flow, heart preparations of the late-stage endotoxemic rats (Figure 4(a)). The HR in both early-stage and late-stage endotoxemia was not affected by Oro-A, but was significantly decreased by AVP. The rate of contraction of isolated atrial preparations from endotoxemic hearts also was significantly increased in the early-stage endotoxemia and remained increased until late-stage endotoxemia. These results are similar to those reported by others [29, 46, 47]. The rate of contractions in both stages was not affected by Oro-A or AVP, suggesting that both agents at the concentrations used do not directly affect these tissues or exhibit nonspecific effects. Again, the decreased HR by AVP only in the constant-pressure heart preparations but not in the constant-flow heart preparations suggests the involvement of indirect mechanism or secondary to decreased CF via vasoconstriction. This latter suggestion is likely, since Oro-A and AVP did not affect the contractile force of depressed atrial and ventricular strips. In this regard, Oro-A may not be beneficial for cardiogenic shock due to mechanical dysfunction of the heart.

Sepsis is a systemic inflammatory response of endotoxemia. In severe sepsis and septic shock patients with depressed cardiac function may have higher mortality than those without cardiac dysfunction [5]. Although the LPS-treated animal model may have its limitations in representing sepsis in human, it, however, can be useful to help determination of possible pathophysiology of endotoxemia [48]. Likewise, isolated hearts in Langendorff preparations are widely used to study mechanisms of myocardial functions in health and disease [49]. Our present findings, therefore, provide interesting information indicating that Oro-A improves, while AVP worsens the cardiac functions of endotoxemic rats. Although examination of effects of these two agents in a more clinically relevant model is needed, it is reasonable to suggest, based on the present animal studies, that Oro-A is a potentially favorable candidate for managing endotoxemic

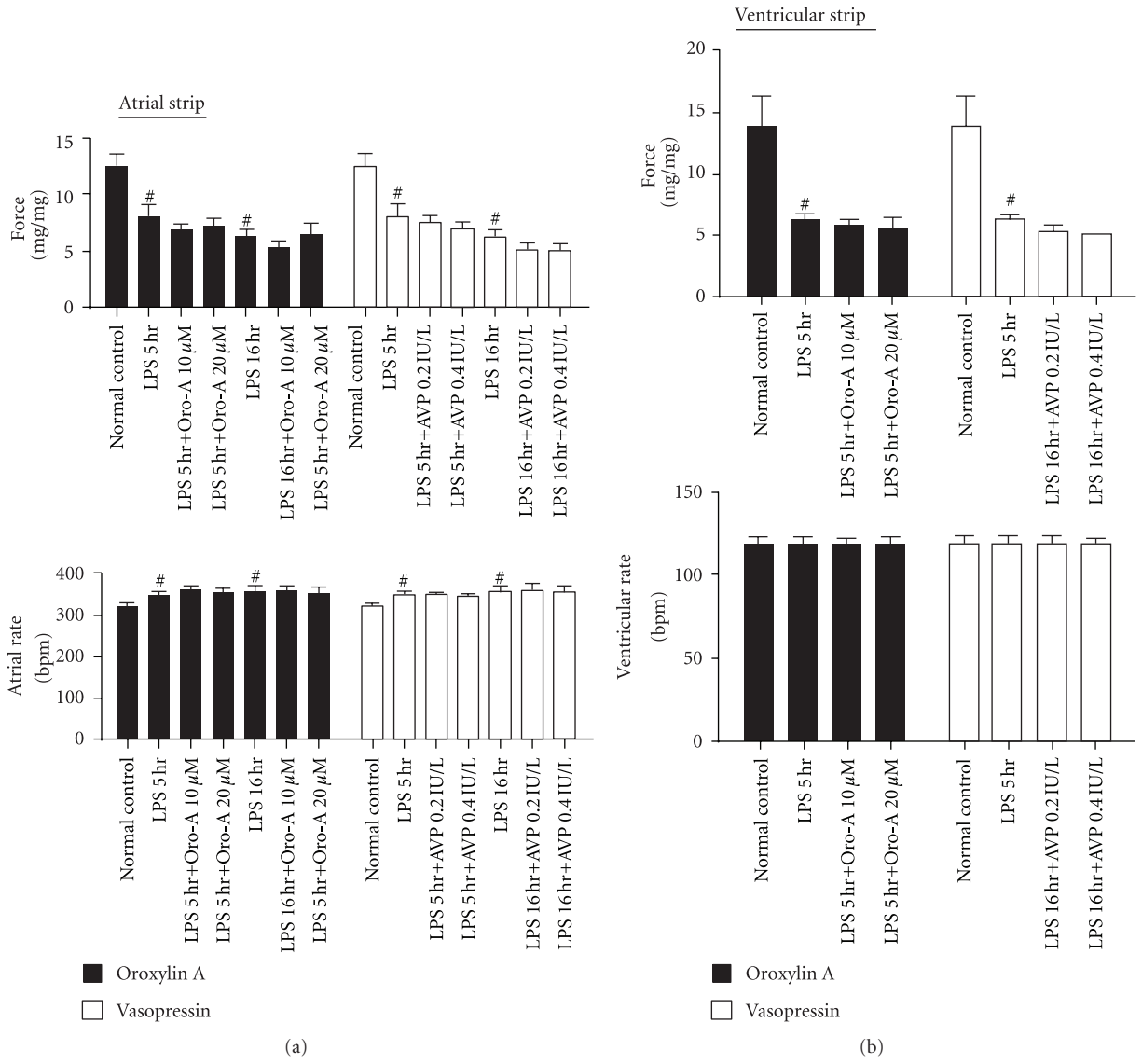

Figure 7: Effects of Oro-A and AVP on force and rate of isolated atrial and ventricular strips from endotoxemic rats. (a) summarizes effects of Oro-A (10 and 20 μM; solid bars) and AVP (0.2 and 0.4 IU/L; open bars) on force and rate of the isolated atrial strips from 5 hr and 16 hr post-LPS rats. (b) summarize effects of Oro-A (10 and 20 μM; solid bar) and AVP (0.2 and 0.4 IU/L; open bars) on electrically-paced force and rate of the ventricular strips from 5 hr post-LPS rats. Values of normal control, and 5 hr or 16 hr post-LPS rats are from Table 1. Values are mean ± SEM ($n = 5 \sim 6$ each group). $^{\#}P < 0.05$ versus normal control.

patients associated with cardiac dysfunction. For this same group of patients, however, use of AVP should be cautious.

It should be noted that our preliminary studies demonstrated that Oro-A post-treatment significantly reversed the LPS-induced systemic hypotension to normal ranges with significantly increased survival rate of the endotoxemic animals. Also, isolated mesenteric and tail arteries from LPS-induced septic rats constricted exclusively upon application of Oro-A. These results suggested that Oro-A did not further aggravate hypotension in endotoxemic shock. In this regard, coronary vessels seem to react differently from other systemic resistant vessels in response to Oro-A. The exact mechanism for the interesting and important difference remains to be fully determined.

In summary, results of the present study indicate that Oro-A exerts a protective action with positive inotropy on isolated endotoxemic hearts via improved CF. In contrast, AVP further aggravates LPS-induced negative inotropy with decreased CF. Oro-A is an interesting candidate for providing not only a therapeutic strategy in treating endotoxemia or severe sepsis complicated with cardiac dysfunction, but also a new insight in understanding the pathophysiology of the LPS-induced cardiac dysfunction.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by grants from National Science Council of Taiwan (NSC95-2320-B-320-013-MY2, NSC99-2320-B-320-008, and NSC 100–2320-B-320-007-MY2) and Tzu Chi University (TCIRP98005-01Y1, TCIRP98005-01Y2, TCIRP98005-02Y1, TCIRP98005-02Y2, and TCRPP99006).

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