



Effect of Xin Mai Jia on atherosclerosis in rats

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ABSTRACT. We investigated the therapeutic effect of Xin Mai Jia (XMJ) on atherosclerosis (AS) in rats. Rat models of AS were established by peritoneally injecting vitamin D, feeding a high-fat diet, and inducing balloon injuries in rats. The stomachs of the rats were irrigated continuously for 10 weeks with XMJ. Blood lipid- and hemorheology-related indices of blood samples were detected. Pathological changes in the right common carotid arterial tissues were also determined. The protein expression levels of endothelial nitric oxide synthase, angiotensin-1, and endothelin-1 were determined by western blotting. XMJ reduced cholesterol, triglyceride, and low-density lipoprotein levels as well as blood viscosity, sedimentation, and hematocrit. Furthermore, XMJ alleviated vascular endothelial injury and reduced/eliminated atherosclerotic plaques. In contrast, XMJ significantly increased the endothelium-dependent relaxing response of the AS rat models. The

western blotting results showed that XMJ upregulated endothelial nitric oxide synthase but downregulated angiotensin-1 and endothelin-1. XMJ prevented the development of AS by regulating blood lipid levels, hemorheology, and vascular function.

Key words: Angiotensin-1; Atherosclerosis; Vascular function; Endothelial nitric oxide synthase; Endothelin-1

INTRODUCTION

Atherosclerosis (AS) is an important arteriosclerotic cardiovascular disease. AS lesions originate from the endarterium. In general, symptoms of AS include hypertension, hypercholesterolemia, or diabetes. AS is one of the leading causes of death in developed countries (Linsel-Nitschke and Tall, 2005; Hansson, 2005). However, the pathogenesis of AS is not fully understood, and many risk factors lead to AS. Studies have reported that AS is caused by the co-actions of vascular endothelial lesions, inflammation, and immunological dysfunction.

Pathological studies have shown that AS related to phlegm syndrome can be treated with traditional Chinese medicine. Researchers have explored the mechanism by which AS is prevented by eliminating phlegm and resolving stasis theory using traditional Chinese medicine. Xin Mai Jia (XMJ) is composed of astaxanthin, functional red rice, pueraria isoflavone, soybean isoflavone, bamboo leaf flavones, and resveratrol (Patent No. ZL 2010 1 0536001.x). XMJ can alleviate the symptoms of AS and reduce or eliminate complications after few months; moreover, XMJ can reduce blood lipids, normalize blood pressure, and improve sleep quality. However, the mechanism by which XMJ exerts its therapeutic effects remains unclear. In the present study, XMJ was used to treat rats with AS, after which we determined the changes in blood lipids, hemorheology, endothelial nitric oxide synthase (eNOS), angiotensin-1 (AT-1), and endothelin-1 (ET-1) levels to explore the possible mechanism.

MATERIAL AND METHODS

Animals

Forty-eight male Sprague-Dawley (SD) rats weighing 200-300 g (Laboratory Animal Center of HeNan Province) were used as subjects. All rats were fed with diets and water. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University.

Groups

The SD rats were randomly divided into 8 groups: control group; drug-medium control group; model group; 6 mg·kg⁻¹day⁻¹ lovastatin treatment group; 0.7813 g·kg⁻¹day⁻¹ Zhi-Bituo treatment group (Chengdu Di'ao Group); 0.5934 g·kg⁻¹day⁻¹ low-dose XMJ treatment group; 1.875 g·kg⁻¹day⁻¹ middle-dose XMJ treatment group; and 5.925 g·kg⁻¹day⁻¹ high-dose XMJ treatment group.

AS model

AS rat models were established by feeding high-fat diets, injecting vitamin D3, and inducing balloon injuries in all groups except the normal control group and the positive control group, which were fed basic diets. High-fat diets included 81.5% basic diets, 10% lard, 0.5% sodium cholate, 3% cholesterol, and 5% sugar. The dose of high-fat diets was 150 g/day. Common carotid arterial intima injury was induced after the rats were fed for 4 weeks. The rats were continuously fed with high-fat diets for 10 weeks.

Pathomorphological observation

After the rats were anesthetized, the common carotid artery was obtained and embedded in paraffin. Paraffin sections were then prepared by dehydration, transparency, wax dip, and embedment. The sections were then stained with hematoxylin and eosin and observed under an electron microscope.

Blood fat detection

Approximately, 2 mL blood was extracted from the common carotid artery and centrifuged at 3000 rpm, 730 g for 15 min at 4°C. The supernatant was obtained, and the levels of total cholesterol, triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were detected using the oxidase reaction.

Hemorheology test

Blood viscosity was tested using an LG-R-80F automatic blood rheometer (Beijing gtmsteellex Science Instrument Co., Ltd., Beijing, China).

Vascular endothelial function detection

One part of the common carotid artery was cut to form a vascular circle and then treated with 10^{-6} M norepinephrine to reach the largest shrink tension. Approximately 10^{-8} - 10^{-4} M acetylcholine was added. We recorded vascular tension and observed the effect of the drug on the endothelial-dependent relaxation response.

Western blot

Approximately, 100 mg common carotid artery was collected, and 0.5 mL cold lysis buffer solution was added (lysate: inhibitor phosphatase = 1:5, 1 μ L protease inhibitor and 5 μ L 100 mM phenylmethylsulfonyl fluoride). The resulting solution was homogenized in ice water and centrifuged at 1000 rpm for 5 min at 4°C. The supernatant contained the total protein extract. Protein concentration was evaluated using the bicinchoninic acid method. Protein samples were stored at -80°C. The lysates (50 μ g protein/well) were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 4 h and then blotted onto polyvinylidene fluoride membranes. The blots were subsequently incubated with primary antibodies overnight at 4°C, washed, and incubated for 1.5 h with horseradish peroxidase-conjugated

secondary antibodies at 1:4000 dilutions. After washing, we developed the membranes using an electrochemiluminescence system. Protein expression levels were determined by analyzing the signals captured on the membranes by using the Image-Pro Plus 6.0 analyzer (Media Cybernetics, Inc., Rockville, MD, USA) with β -actin as an internal control.

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA), and all results are reported as mean \pm standard deviation. One-way analysis of variance was performed to compare different groups. Statistical significance was considered to be $P < 0.05$.

RESULTS

Pathomorphological changes

Compared with the control group, the model group exhibited evident endothelial injury as observed under electron microscope. Yellow atherosclerotic plaques were also observed in the model group. XMJ reduced the degree of endothelial injury and promoted plaque disappearance in a dose-dependent manner (Figure 1). Electron microscopy revealed that the integrity of the vascular endothelial cell membrane was retained and no special particles were observed in the nucleus, and a significant difference was observed between the control group and the model group (Figure 2).

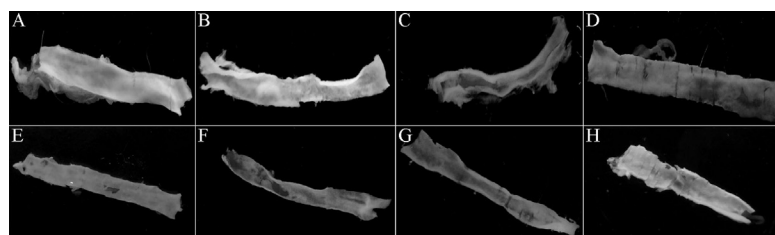


Figure 1. Observation of common carotid artery with eyes. **A.** Normal control group. **B.** Drug-medium control group. **C.** Lovastatin group. **D.** Zhibituo group. **E.** Model group. **F.** Low-dose Chinese medicine group. **G.** Medium-dose Chinese medicine group. **H.** High-dose Chinese medicine group.

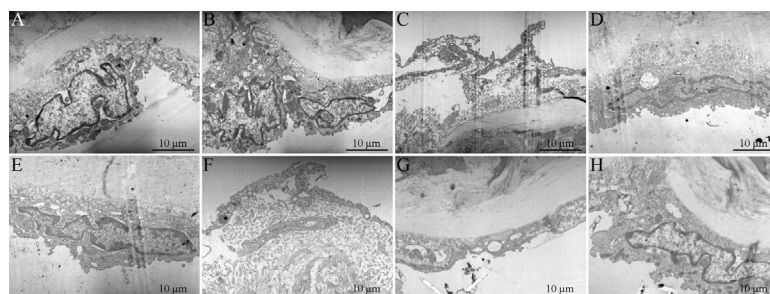


Figure 2. Observation of endothelial cells in the common carotid artery of rats using light microscope (1000X). **A.** Normal control group. **B.** Drug-medium control group. **C.** Model group. **D.** Lovastatin group. **E.** Zhibituo group. **F.** Low-dose Chinese medicine group. **G.** Medium-dose Chinese medicine group. **H.** High-dose Chinese medicine group.

Blood fat

XMJ significantly reduced cholesterol, TG, and LDL levels, but increased glutamic acid, HDL, and apolipoprotein levels in a dose-dependent manner ($P < 0.05$, Table 1) compared with the control group.

Table 1. Lipid protein levels in rat plasma (μM , $N = 6$, means \pm SD).

| Group/index | GLU | CHOL | TG | HDL | LDL | HDL/CHOL | APOA-1 | APOB |
|------------------------------|--|---|---|---|---|---|---|---|
| Control | 15.11 \pm 0.65 ^{**} | 0.72 \pm 0.09 ^{**} | 1.13 \pm 0.09 ^{**} | 0.42 \pm 0.08 ^{**} | 0.13 \pm 0.03 ^{**} | 0.51 \pm 0.07 ^{**} | 0.17 \pm 0.04 ^{**} | 0.13 \pm 0.03 ^{**} |
| Drug-medium control | 11.85 \pm 0.54 ^{**} | 0.54 \pm 0.07 ^{**} | 0.42 \pm 0.04 ^{**} | 0.47 \pm 0.07 ^{**} | 0.11 \pm 0.02 ^{**} | 0.56 \pm 0.08 ^{**} | 0.18 \pm 0.05 ^{**} | 0.11 \pm 0.02 ^{**} |
| Model | 5.95 \pm 0.44 ^{#Δ} | 1.13 \pm 0.16 ^{#Δ} | 1.82 \pm 0.12 ^{#Δ} | 0.27 \pm 0.05 ^{#Δ} | 0.28 \pm 0.05 ^{#Δ} | 0.34 \pm 0.06 ^{#Δ} | 0.07 \pm 0.01 ^{#Δ} | 0.02 \pm 0.00 ^{#Δ} |
| Lovastatin | 6.82 \pm 0.52 ^{#Δ} | 0.51 \pm 0.06 ^{#Δ} | 0.48 \pm 0.07 ^{#Δ} | 0.33 \pm 0.06 ^{#Δ} | 0.22 \pm 0.04 ^{#Δ} | 0.43 \pm 0.05 ^{#Δ} | 0.08 \pm 0.01 ^{#Δ} | 0.08 \pm 0.01 ^{#Δ} |
| Zhibituo | 6.95 \pm 0.62 ^{#Δ} | 0.55 \pm 0.08 ^{#Δ} | 0.58 \pm 0.08 ^{#Δ} | 0.35 \pm 0.06 ^{#Δ} | 0.23 \pm 0.03 ^{#Δ} | 0.48 \pm 0.04 ^{#Δ} | 0.09 \pm 0.02 ^{#Δ} | 0.06 \pm 0.01 ^{#Δ} |
| Low-dose Chinese medicine | 5.54 \pm 0.47 ^{#Δ} | 0.48 \pm 0.06 ^{#Δ} | 0.97 \pm 0.07 ^{#Δ} | 0.39 \pm 0.06 ^{#Δ} | 0.19 \pm 0.05 ^{#Δ} | 0.44 \pm 0.05 ^{#Δ} | 0.14 \pm 0.03 ^{#Δ} | 0.05 \pm 0.01 ^{#Δ} |
| Medium-dose Chinese medicine | 8.44 \pm 0.81 ^{#Δ} | 0.44 \pm 0.06 ^{#Δ} | 0.84 \pm 0.06 ^{#Δ} | 0.41 \pm 0.07 ^{#Δ} | 0.15 \pm 0.03 ^{#Δ} | 0.38 \pm 0.04 ^{#Δ} | 0.09 \pm 0.01 ^{#Δ} | 0.09 \pm 0.02 ^{#Δ} |
| High-dose Chinese | 14.23 \pm 0.76 ^{*Δ} | 0.55 \pm 0.07 ^{*Δ} | 0.99 \pm 0.12 ^{*Δ} | 0.32 \pm 0.04 ^{*Δ} | 0.16 \pm 0.02 ^{*Δ} | 0.41 \pm 0.06 ^{*Δ} | 0.11 \pm 0.02 ^{*Δ} | 0.11 \pm 0.03 ^{*Δ} |

* $P < 0.05$ vs the model group; [#] $P < 0.05$ vs the high-dose Chinese medicine group; ^{Δ} $P < 0.05$ vs the normal control group.

Hemorheology

Hemorheology results indicated that XMJ reduced the vascular viscosity of rat blood plasma in a dose-dependent manner. A significant difference was observed between the control and model groups ($P < 0.05$, Table 2).

Table 2. Viscosity of rat blood plasma ($N = 6$, means \pm SD).

| Group/shear rate | 200 | 30 | 3 | 1 |
|------------------------------|---|---|--|--|
| Control | 3.12 \pm 0.34 ^{**} | 4.43 \pm 0.35 ^{**} | 7.44 \pm 0.54 ^{**} | 12.43 \pm 1.33 ^{**} |
| Drug-medium control | 3.17 \pm 0.18 ^{**} | 3.93 \pm 0.08 ^{**} | 7.29 \pm 0.73 ^{**} | 12.07 \pm 1.95 ^{**} |
| Model group | 4.24 \pm 0.91 ^{#Δ} | 5.45 \pm 1.08 ^{#Δ} | 10.94 \pm 1.80 ^{#Δ} | 18.92 \pm 2.75 ^{#Δ} |
| Lovastatin | 3.42 \pm 0.30 ^{#Δ} | 4.34 \pm 0.10 ^{#Δ} | 8.51 \pm 1.02 ^{#Δ} | 14.56 \pm 2.91 ^{#Δ} |
| Zhibituo | 3.08 \pm 0.28 ^{#Δ} | 3.93 \pm 0.26 ^{#Δ} | 7.73 \pm 0.45 ^{#Δ} | 13.23 \pm 1.14 ^{#Δ} |
| Low-dose Chinese medicine | 3.27 \pm 0.38 ^{#Δ} | 4.12 \pm 0.36 ^{#Δ} | 7.89 \pm 0.31 ^{#Δ} | 13.28 \pm 0.65 ^{#Δ} |
| Medium-dose Chinese medicine | 2.74 \pm 0.26 ^{#Δ} | 3.24 \pm 0.29 ^{#Δ} | 5.34 \pm 0.49 ^{#Δ} | 8.18 \pm 0.86 ^{#Δ} |
| High-dose Chinese | 3.16 \pm 0.47 ^{*Δ} | 3.85 \pm 0.51 ^{*Δ} | 6.81 \pm 0.67 ^{*Δ} | 10.92 \pm 0.83 ^{*Δ} |

* $P < 0.05$ vs the model group; [#] $P < 0.05$ vs the high-dose Chinese medicine group; ^{Δ} $P < 0.05$ vs the normal control group.

Vascular function

XMJ increased endothelial-dependent relaxation in a dose-dependent manner. A significant difference was detected between the control and model groups ($P < 0.05$, Figure 3).

Western blot

Western blot results showed that the expression of eNOS significantly reduced in the model group compared to that in the control group ($P < 0.05$). However, the expression levels of AT-1 and ET-1 were significantly higher ($P < 0.05$) in the control group than that in the model group. XMJ increased the expression of eNOS and decreased the expression levels of AT-1 and ET-1. A significant difference was observed between the model group and the XMJ treatment group ($P < 0.05$, Figures 4-6).

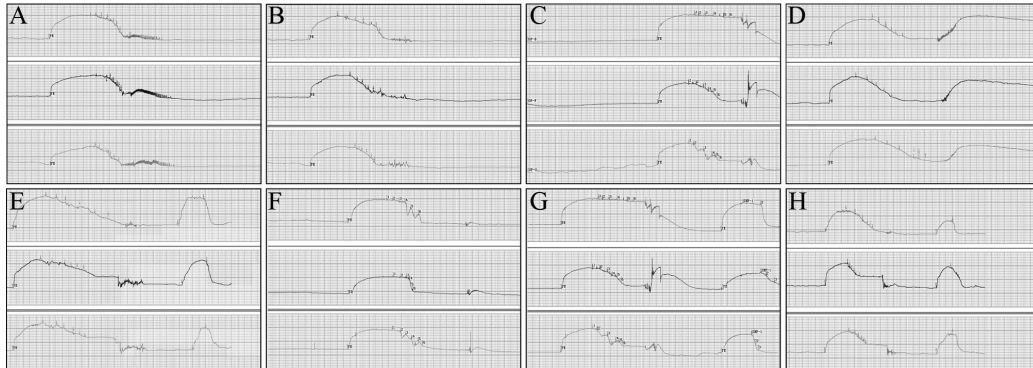


Figure 3. Endothelium-dependent reaction of the common carotid artery of rats (N = 6, means ± SD). **A.** Normal control group. **B.** Drug-medium control group. **C.** Model group. **D.** Lovastatin group. **E.** Zhibituo group. **F.** Low-dose Chinese medicine group. **G.** Medium-dose Chinese medicine group. **H.** High-dose Chinese medicine group.

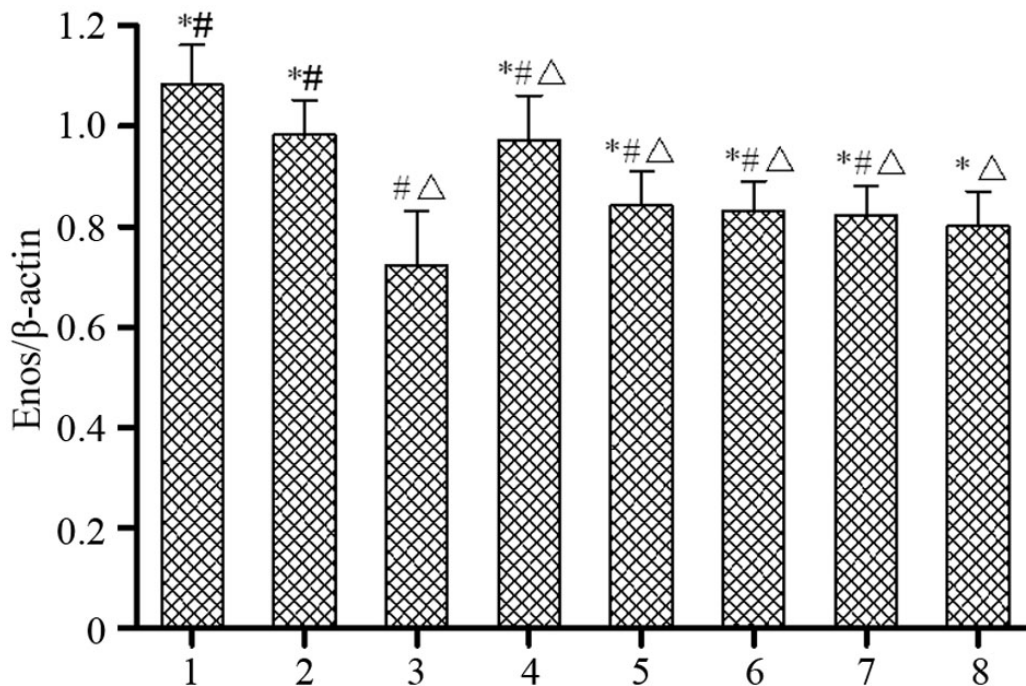


Figure 4. Expression of endothelial nitric oxide synthase (eNOS) in the common carotid artery of rats (N = 6, means ± SD). *Column 1:* Normal control group. *Column 2:* Drug-medium control group. *Column 3:* Model group. *Column 4:* Lovastatin group. *Column 5:* Zhibituo group. *Column 6:* Low-dose Chinese medicine group. *Column 7:* Medium-dose Chinese medicine group. *Column 8:* High-dose Chinese medicine group. (*P < 0.05 vs the model group; #P < 0.05 vs the high-dose Chinese medicine group; ΔP < 0.05 vs the normal control group).

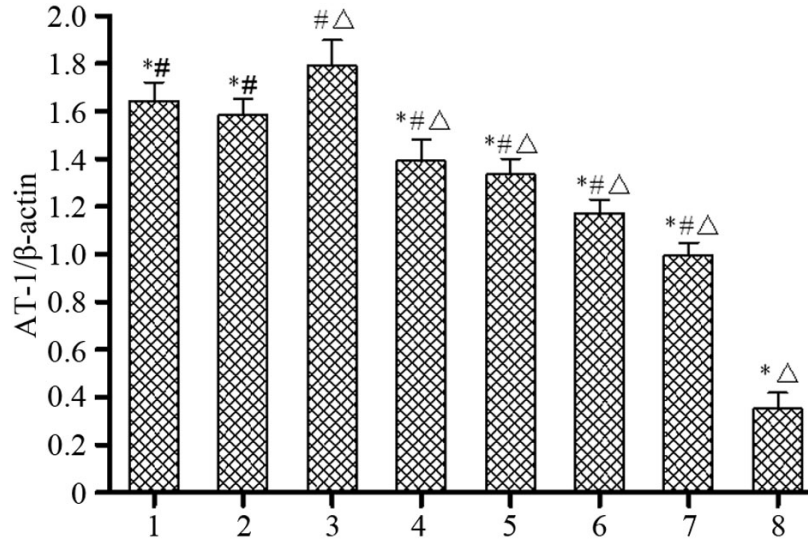


Figure 5. Expression of angiotensin-1 (AT-1) in the common carotid artery of rats (N = 6, means ± SD). *Column 1:* Normal control group. *Column 2:* Drug-medium control group. *Column 3:* Model group. *Column 4:* Lovastatin group. *Column 5:* Zhibituo group. *Column 6:* Low-dose Chinese medicine group. *Column 7:* Medium-dose Chinese medicine group. *Column 8:* High-dose Chinese medicine group. (*P < 0.05 vs the model group; #P < 0.05 vs the high-dose Chinese medicine group; ΔP < 0.05 vs the normal control group).

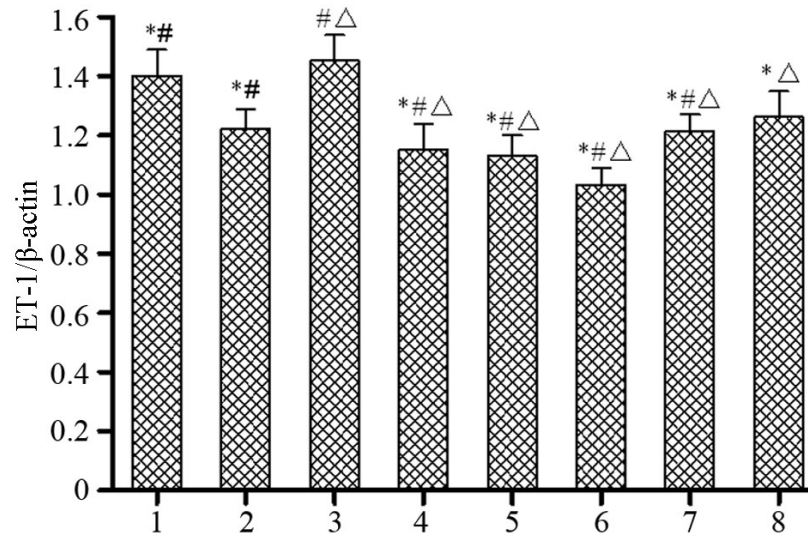


Figure 6. Expression of endothelin-1 (ET-1) in the common carotid artery of rats (N = 6, means ± SD). *Column 1:* Normal control group. *Column 2:* Drug-medium control group. *Column 3:* Model group. *Column 4:* Lovastatin group. *Column 5:* Zhibituo group. *Column 6:* Low-dose Chinese medicine group. *Column 7:* Medium-dose Chinese medicine group. *Column 8:* High-dose Chinese medicine group. (*P < 0.05 vs the model group; #P < 0.05 vs the high-dose Chinese medicine group; ΔP < 0.05 vs the normal control group).

DISCUSSION

AS is an important pathological basis for cardio-cerebral vascular diseases. This condition is also considered to be a target for exploring treatment measures for cardiovascular and cerebrovascular diseases and studying the mechanisms of cardio-cerebral vascular diseases. Atherogenesis is the developmental process of atheromatous plaques. Numerous risk factors lead to AS, and high blood lipid levels and hemorheology promote AS. Other studies have reported that hyperlipidemia is an independent risk factor for AS and cardio-cerebral vascular diseases (Nordestgaard et al., 2010; Zurek et al., 2013). Fatty deposits damage the endothelium in the artery by triggering dyslipidemia, thereby upregulating the expression of cytokines to form foam cells. Foam cells and platelets encourage the migration and proliferation of smooth muscle cells and macrophages, which in turn ingest lipids, are replaced with collagen, and transform into foam cells. Epidemiological research confirmed that hypertriglyceridemia is a risk factor of AS. AS is positively related to total cholesterol, TG, and LDL levels and is considered to be an independent risk factor of coronary heart disease. However, HDL directly or indirectly transfers cholesterol to the liver to decrease cholesterol deposits in the artery wall. The results of the present study indicated that XMJ reduced total cholesterol, TG, and LDL levels, but increased HDL level, indicating that XMJ promoted blood fat reduction.

Hyperlipidemia is the pathological basis of AS and causes changes in hemorheology. Therefore, AS may be prevented or treated by understanding hemorheological changes. Studies have demonstrated that blood fat and raises blood viscosity are closely related to AS (Ruggiero et al., 2013) Our results showed that XMJ effectively treated AS by reducing blood fat and viscosity.

Vascular endangium lesions caused lipid deposits to reduce arterial elasticity, after which the narrow cavity induced cardiovascular symptoms (Dong et al., 2012). Thus, lipid deposits may be reduced and vascular elasticity can be recovered. In the present study, XMJ significantly increased the endothelium-dependent relaxation of the model rats ($P < 0.05$) in a dose-dependent manner. This result indicated that XMJ could be used to treat AS and improved vascular elasticity.

The mechanism by which XMJ improved vascular elasticity should be further examined. AS represents endothelium dysfunction, including nitric oxide (NO) dyssynthesis mediated by the endothelium, which is due to the disequilibrium of ET and NO. The expression levels of eNOS, ET-1, and AT-1 were detected by Western blotting. NO is catalyzed by NOS in order to bring about vascular relaxation response. The biological effect of NO can be observed through the study of NOS, since NO cannot be easily measured. Under physiological conditions, NO is generated by eNOS in the vascular system. NOS can also extend the blood vessel, regulate blood pressure, inhibit platelet aggregation (Huang et al., 2006), prevent smooth muscle cell proliferation, and suppress adhesion between endothelial cells and monocytes (Mujynya-Ludunge et al., 2005; Gkaliagkousi and Ferro, 2011). Moreover, eNOS, a key enzyme in the NO/cyclic guanosine monophosphate signal pathway, regulates vascular tone and protects endothelial cell function by coupling Ca^{2+} and calmodulin. NO is catalyzed and released by eNOS to enter near smooth muscle cells and activate guanylate cyclase, which catalyzes cyclic guanosine monophosphate to activate the calcium pump to reduce intracellular free calcium and promote smooth muscle relaxation. Cyclic guanosine monophosphate also promotes smooth muscle relaxation by inhibiting protein kinase activity (Murad, 2006). Studies have shown that NO not only functions as a vascular relaxing factor, but also maintains

vascular wall structure (Ozüm et al., 2008). Low NOS expression reduces NO levels. NOS antagonists significantly accelerated AS development in some animal experiments (Zancan et al., 1999; Sweazea and Walker, 2011). In the present study, eNOS expression decreased in the AS model group. This result suggested that NO reduction is crucial in AS development. XMJ appeared to increase eNOS expression in model rats.

ETs are proteins that constrict blood vessels and increase blood pressure (Yanagisawa et al., 1988). These proteins are released by injured vascular endothelial cells. ET level is positively correlated with the vascular lesion counts of AS (Nakaki et al., 1989; Lerman et al., 1991). Studies have found that high plasma ET levels are related to vascular cell lesions (Naruse et al., 1991; Ray et al., 1993) during AS development. Reriani et al. (2010) also confirmed that an ET-1 receptor antagonist significantly improved the endothelial function of patients with coronary AS, showing that endogenous ET is important in early atherogenesis. Angiotensin II is an important molecule in the rennin-angiotensin system and increases blood pressure. Early studies reported the presence of a positive feedback control mechanism between ET and angiotensin II (Emori et al., 1989; Dohi et al., 1999). Thus, both ET and angiotensin II can function as markers to evaluate the degree of vascular lesion. In the present study, the expression levels of ET-1 and AT-1 were significantly higher in the AS model group than that in the control group.

Low biological NO activity causes vasoconstriction and dysfunction of the endothelium by increasing ET expression (Bourque et al., 2011). An imbalance between NO and ET destroys the arterial endothelium and causes endothelial dysfunction. Furthermore, the most important and earliest characteristic of endothelial dysfunction is the loss of vascular endothelium-dependent function, which was observed in our study. XMJ appeared to increase eNOS expression and decrease ET-1 and AT-1 expression levels. The balance between NO and ET was then recovered, which may be a mechanism for treating AS.

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