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Basic Investigation

Effects of Wenxiao II Decoction on the expression of MCP-1 and VCAM-1 in atherosclerotic rabbits

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Abstract

OBJECTIVE: To observe the effects of different doses of Wenxiao II Decoction on the expression of monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) in an experimental model of atherosclerosis in rabbits and to explore the mechanism by which it alleviates atherosclerosis.

METHODS: Sixty 3-4 month-old New Zealand rabbits of both sexes were randomly divided into six groups: simvastain; model; blank; and high-dose, mid-dose, and low-dose Wenxiao II Decoction groups. Except for those in the blank group, all rabbits were fed a high-cholesterol diet. Carotid atherosclerosis was established by balloon-induced injury to the endothelium of the carotid artery in conjunction with consumption of a high-cholesterol diet. After 8 weeks, all rabbits were killed to evaluate the expression of MCP-1 and VCAM-1 by immunohistochemical staining.

RESULTS: Expressions of MCP-1 and VCAM-1 were significantly decreased in all groups except the blank group compared with the model group ($P<0.05$). When compared with the simvastain group only variation of MCP-1 expression in low-dose group was not appreciable, and the differences were indistinct ($P<0.05$). When comparing among Wenxiao II Decoction groups, MCP-1 expression in the mid- and high-dose groups was significantly lower than that seen in the low-dose group ($P<0.01$), but there were no differences among three dosage groups with respect to VCAM-1 expression ($P>0.05$).

CONCLUSION: These data suggested that high, mid, and low doses of Wenxiao II Decoction can inhibit the expression of MCP-1 and VCAM-1, which may prevent the formation of or stabilize atherosclerotic plaques. There may be a direct relationship between the dosage of Wenxiao II Decoction and its therapeutic efficacy.

Key words: Traditional Chinese Medicine; Wenxiao II Decoction; Atherosclerosis; Inflammatory; Monocyte chemoattractant protein-1; Vascular cell adhesion molecule-1

INTRODUCTION

Atherosclerosis is a chronic inflammatory process [1]. It involves in the formation, progression, rupture, and thrombosis of atherosclerotic plaques[2-4]. Monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and other inflammatory

cytokines have important roles in the development of atherosclerosis[5-6]. They can aggravate inflammation, destabilize plaques, attenuate fibrin caps, and eventually rupture plaques, causing thrombosis. Recent studies have shown that plaque rupture and subsequent intraluminal thrombosis is the most common cause of acute coronary syndrome. Early prevention of plaque rupture might be an effective way to reduce the risk of this catastrophic life-threatening event. Unfortunately, an ideal drug for stabilizing vulnerable plaques has not been developed. Therefore, finding an effective means of interfering with the production of these inflammatory cytokines is of considerable medical significance. Exploration of new drugs with high efficacy and few side effects for stabilizing vulnerable plaques is clearly warranted. Furthermore, our previous experimental results indicated that Wenxiao cereal recipes could inhibit the expression of inflammatory cytokines such as fibrinogen, C-reactive protein, tumor necrosis factor-α (TNF-α), and intercellular adhesion molecule-1 (ICAM-1). Wenxiao cereal recipes were also effective for the regulation of blood lipids[7-8]. The present study was carried out to test the effects of different doses of Wenxiao II Decoction on the expression of MCP-1 and VCAM-1 in atherosclerotic rabbits, and to develop a better understanding of the mechanism underlying its clinical efficacy.

MATERIALS AND METHODS

Experimental animals

Sixty 3-4-month-old New Zealand rabbits of both sexes (2.2-2.5 kg) were purchased from the Shanghai Shengwang Experimental Animal Breeding Co. Ltd. (Shanghai, China). Rabbits were kept in the Experimental Animal Center of the Sixth People's Hospital (affiliated to Shanghai Jiaotong University, Shanghai, China) for 1 week under the following conditions: Room temperature was maintained at 22-24°C and the relative humidity was 50%. Rabbits were exposed to a light-dark cycle with illumination from 7 am to 5 pm. All rabbits had free access to food and water before experimentation.

Drugs

Wenxiao II Decoction was prepared by Tianyin Pharmaceutical (Chengdu, China; production series number, 20090809). It was composed of the extracts of leeches, earthworms, salvia, paeonol, turmeric root tuber, and pinellia at a ratio of 3:3:3:3:3:2 by mass. Simvastatin (commercial name, zocor) was purchased from Merck Sharp & Dohme (Whitehouse Station, NJ, USA; production series number 2009014). By scaling down the recommended daily dosage for adult humans to the body weight of rabbits[9], we determined that the mid-dose of Wenxiao II Decoction for rabbits should be 575 mg/kg/d of the extracts of leeches, earthworms, salvia, paeonol and turmeric root tuber, and to 383.33 mg/kg/d of pinellia extract. We established the ratio of high:medium:low doses at 4:2:1. The daily dose for rabbits in the simvastatin group was 0.77 mg/kg/d.

Animal feed

A high-cholesterol diet (1% cholesterol, 5% egg yolk powder, and 3% lard mixed with 91% standard feed) was prepared by the Experimental Animal Center of the Sixth People's Hospital of Shanghai.

Reagents and instruments

Balloon catheters (2.5 mm × 10 mm) were purchased from Cordis Corporation (Bridgewater, NJ, USA). Diaminobenzidine Color Kits were purchased from Sigma – Aldrich (St Louis, MO, USA). Anti-rabbit VCAM-1 monoclonal antibody was purchased from Boster Biological Technology (Fremont, CA, USA). MCP-1 (1: 250 dilution) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Optical microscope was purchased from Olympus Scientific Equipment Group (Tokyo, Japan). Medical analysis software and IMS Cell Image Analysis Systems were purchased from Shanghai Shenteng Information Technology Co., Ltd. (Shanghai, China).

Modeling and grouping

After being fed a standard diet (150 g/d) for 7 days, no abnormalities were observed in any experimental rabbits. Animals were randomly divided into a blank group (10 rabbits) and a model group (50 rabbits). Both groups had free access to water. The blank group continued with the 150 g/d standard diet, and the model group was fed 100 g/d of a high-cholesterol diet and 50 g/d of the standard diet. One week later, all rabbits underwent balloon-induced injury to the endothelium of the carotid artery[10-13], and continued to feed on the high-cholesterol diet until the end of the eighth week. Two rabbits were randomly selected and killed, and their internal carotid arteries collected 1-2-cm distal from the bifurcation of the internal and external carotid arteries. These specimens were made into paraffin sections for observation under a light microscope. This procedure confirmed that there were different degrees of atherosclerosis on the walls of the carotid arteries: the atherosclerosis model had been established.

After the establishment of the atherosclerosis model, the 10 rabbits in the blank group survived, and 7 rabbits in the model group died. One died due to an anesthetic accident, 2 of surgical bleeding, 1 of postoperative infection, one of diarrhea-related complications, 2 of unknown causes, and 2 were killed to confirm the establishment of the model.

Thereafter, the atherogenic diet was replaced with a regular diet. The remaining 41 rabbits were randomly di-

vided into five groups for treatment: high dose (n=8), moderate dose (n=8), and low dose (n=8) of Wenxiao II Decoction; simvastatin (n=8); and control (n=9).

Drug administration and specimen collection

The three dosage groups and simvastatin group were given the drugs to be tested. Drugs were dissolved in distilled water, mixed, and then administered via the intragastric route each day. The blank group was given equal volumes of physiological (0.9%) saline solution. Each group was fed the standard diet, and had free access to water, during these 8 weeks.

At the end of the eighth week after the implementation of drug intervention, all animals were deprived of food and water for 12 h, and then anaesthetized with pentobarbital sodium (1.1-1.3 mL/kg). Blood samples were collected and the serum centrifuged with 3000 rpm for 15 min at room temperature. Rabbits were killed under sterile conditions so that their internal carotid arteries could be extracted 1-2 cm distal from the bifurcation of the internal and external carotid arteries. Ophthalmic scissors were used to cut along the long axis of the carotid arteries, where white or light-yellow fatty streaks and plaques could be seen with the naked eye. Specimens were gently rinsed with 0.9% saline solution and placed in 4% neutral formaldehyde solution for fixation. They were preserved at －40°C.

Staining and imaging

MCP-1 and VCAM-1 were detected by immunohistochemical staining. Experimental procedures were strictly in accordance with the manufacturers' instructions enclosed with the kits. One section was randomly selected from each specimen and observed under ×250 magnification under an optical microscope (Olympus BX51), purchased from Olympus Scientific Equipment Group (Tokyo, Japan). Five views were taken, and IMS Cell Image Analysis software used to calculate the ratio of positive expression area to total area (%).

Statistical analyses

Data were analyzed with the statistical software package SPSS 11.5 (SPSS, Chicago, IL, USA). Measurement data are the mean±standard deviation. The statistical significance of differences between groups of data was tested by One-way ANOVA. All P values are two-sided. P<0.05 was considered significant.

RESULTS

General health status

During the intervention phase, 1 rabbit from the mid-dose group died of pneumonia, 1 from the model group died of gangrene necrosis, 1 from the blank died of diarrhea-related complications, and 1 in the simvastatin group died of an unidentified cause.

Comparison of the expression of MCP-1 and VCAM-1 among groups after intervention

The expression of MCP-1 and VCAM-1 for all three dosages of Wenxiao II Decoction and for simvastatin was significantly decreased compared with that seen in the model group (P<0.05). When compared with the simvastatin group, the expression of MCP-1 in the low-dose Wenxiao II Decoction group was significantly increased (P<0.05). However, there were no significant differences among the simvastatin group and medium- or high-dose groups of Wenxiao II Decoction with regard to MCP-1, or in the three dosages of the Wenxiao II Decoction groups with respect to VCAM-1 (P> 0.05). The expression of MCP-1 in the mid- and high-dose Wenxiao II Decoction groups was lower than that in the low-dose Wenxiao II Decoction group (P<0.05). VCAM-1 expression in the mid- and high-dose Wenxiao II Decoction groups was lower than that in the low-dose group, but this difference was not significant (P>0.05) (Table 1 and Figures A-L).

Table 1 Comparison of the expression of MCP-1 and VCAM-1 ($\bar{x}$ ±s)

Group	N	MCP-1 (%)	VCAM-1 (%)
High dose	8	2.68±0.30$^{*▲}$	2.72±0.55*
Mid dose	7	2.98±0.28$^{*▲}$	2.88±0.55*
Low dose	8	3.89±0.59$^{*△}$	3.28±0.57*
Simvastatin	7	2.66±0.34*	2.68±0.61*
Model control	8	4.83±0.54	5.03±0.88

Notes: Compared with the model group, $^{*}P$<0.05; compared with the simvastatin group, $^{△}P$<0.05; compared with the low-dose Wenxiao II Decoction group, $^{▲}P$<0.05.

DISCUSSION

The Wenxiao II Decoction comprises extracts of leeches, earthworms, salvia, paeonol, turmeric root-tuber, and pinellia. According to TCM, the leech extract is the "king of medicines"; it can break up dead and coagulated blood in the body. ZHANG Zhong-jing said "Most of the blood-breaking medicines work at the *Qi* level. Only the salty leech can go deep into blood without damaging the *Qi*; it can break up blood imperceptibly. It is a truly good medicine." Earthworm extracts can invigorate blood circulation. They are very mobile and can reach many parts of the body through the meridians. They are considered to be primary medicines.

Extracts of salvia and paeonol are believed to cool the blood, invigorate the blood circulation, break up dead blood cells, and expel heat and toxins from the blood. They are considered to be auxiliary medicines. Pinellia can expel dampness and phlegm as well as diminish the aggregation of pathogenic materials in the body. The fast pace and competitive pressures of modern life can

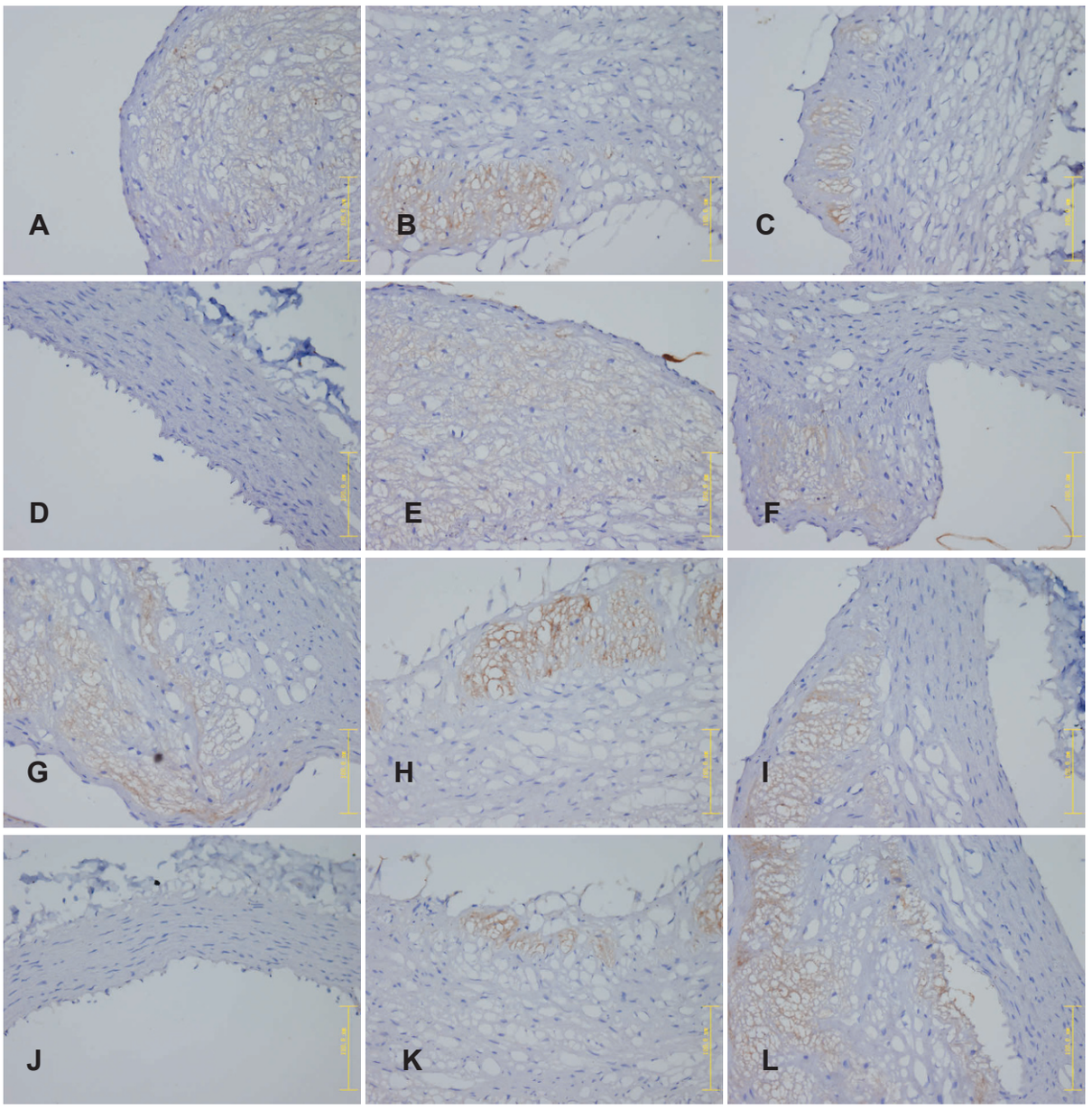

Figure A-L: A brown color indicates positive expression. The model group had the most intense color, and the expression of VCAM-1 in the high dose of Wenxiao II Decoction group and simvastatin group was the weakest. High-dose group (Figure A and Figure G); mid-dose group (Figure B and Figure H); low-dose group (Figure C and Figure I); simvastatin group (Figure D and Figure J); model group (Figure E and Figure K); blank group (Figure F and Figure L).

easily render liver *Qi* stagnant. This stagnant liver *Qi* tends to "fire up" if not soothed. Enflamed *Qi* can readily burn away *Yin* fluid, causing the blood to become viscous and stagnate. Extracts of turmeric root tubers can soothe the liver *Qi* and stimulate blood circulation while simultaneously cooling inflamed blood. According to TCM, the combination of these components causes the Wenxiao II Decoction to stabilize and diminish plaques by stimulating blood circulation, soothing liver *Qi*, dispelling heat, and reducing stagnancy.

Atherosclerosis is the common pathological basis of cardio-cerebrovascular diseases. Ross stated that atherosclerosis is an inflammatory disease[1]. It has the pathological characteristics of a chronic inflammatory process, and its development is associated with inflammatory responses. Research shows that the main monocyte chemoattractant in this process is MCP-1[14]. MCP-1 binds to chemokine receptor-2, activating the mitogen-activated kinase cascade. Mononuclear cells in the blood then migrate to the space beneath the endothelium and become activated as macrophages. These macrophages phagocytize lipids and form foam cells. MCP-1 also stimulates the production of other cytokines (e.g., TNF-α, interleukin-1 (IL-1), IL-6) and the activation of matrix metalloproteinases[15]. This leads to the development of atherosclerosis, plaque instability, as well as acute cardiovascular and cerebrovascular events. Hence, some scholars believe that MCP-1 can be used as a non-invasive means of detecting the degree of plaque inflammation[16]. The level of MCP-1 increases with the progression of atherosclerosis, so the plasma

level of MCP-1 may be useful for evaluating the efficacy of treatments for atherosclerosis[17,18].

VCAM-1 belongs to the immunoglobulin supergene family, and is mainly expressed in activated endothelial cells, monocytes, and epithelial cells. The expression of VCAM-1 aggravates vascular endothelial injury at the start of atherosclerosis[19]. As atherosclerosis progresses, it mainly promotes monocyte movement and T-lymphocyte activation, which infiltrate into the nidus. This promotes interactions among cells and promotes infiltration of monocytes into the subendothelium. These actions transform monocytes into macrophages, who phagocytose lipids and form foam cells. On the one hand, VCAM-1 provides lymphocyte interactions with antigens and triggers local immune responses. On the other hand, VCAM-1 releases various cytokines which may cause the transformation and proliferation of vascular smooth muscle cells, which gradually form fibrous plaques[20,21] and eventually impact to the stability of atherosclerotic plaques.

Statins are regarded to be the most effective plaque-stabilizing drugs available. We found that the expression of MCP-1 and VCAM-1 decreased in the simvastatin group as well as in the three Wenxiao II Decoction dosage groups (P<0.05) compared with the model group. Further statistical analyses showed that the expression of MCP-1 and VCAM-1 in the three Wenxiao II Decoction dosage groups was higher compared with that seen in the simvastatin group, but no significant differences were found except for MCP-1 in the low-dose Wenxiao II Decoction group (P<0.05). Comparison with the three dosage groups revealed that high- and mid-doses of Wenxiao II Decoction could more significantly decrease MCP-1 expression, and no other differences were found. These findings suggest that Wenxiao II Decoction may stabilize atherosclerotic plaques at all doses.

The present study suggested that Wenxiao II Decoction can invigorate the blood circulation, soothe *Qi*, clean-up blood vessels, and expel phlegm and heat in the body. However, inhibition of the expression of inflammatory cytokines may be the relevant mechanism underlying its efficacy in the treatment of atherosclerosis.

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