Original Article

Application of the traditional Chinese medicine XinMaiJia to the treatment of atherosclerosis in rats

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Abstract: Background: Atherosclerosis is the physiological basis of several types of cardiovascular and cerebrovascular diseases. The traditional Chinese traditional herb XinMaiJia (XMJ) has blood lipid-regulating and anti-lipid peroxidation effects. Purpose: To investigate the therapeutic effects and possible mechanism of XMJ on atherosclerosis in rats. Methods: Intraperitoneal injection of Vitamin D, high-forage feeding, and balloon injury were adopted to establish the atherosclerosis model in rats. The rats were administrated with high-, medium-, and low-dosage Zhibituo and XMJ as well as a control medicine. The blood plasma levels of lipids, relevant inflammatory factor levels, and oxidative stress indicators were determined. The expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in vascular smooth muscle cells was determined. Results: XMJ reduced the levels of plasma cholesterol, triglyceride, and low-density lipoprotein in atherosclerotic rats and increased the levels of glutamate, high-density lipoprotein, and apolipoprotein. After treatment with XMJ, the plasma levels of nitric oxide and superoxide dismutase markedly increased, and the level of malondialdehyde greatly decreased compared with those in the model group (P < 0.05). In addition, XMJ reduced the plasma levels of intercellular adhesion factor-1, vascular cell adhesion factor-1, interleukin-1, interleukin-6, matrix metalloproteinase-2, and tissue inhibitor of metalloproteinase-2, while reducing the content of cytokine nuclear factor-kappa B and expression of LOX-1. Conclusion: XMJ may have anti-inflammatory and antioxidative effects on atherosclerosis in rats.

Keywords: XinMaiJia, atherosclerosis, blood lipid, LOX-1

Introduction

Atherosclerosis (AS) is the physiological basis of several kinds of cardiovascular and cerebrovascular diseases [1] that can severely damage a human's health. In the complex pathogenesis of AS, hyperlipidemia is by far the most important and dangerous factor [2]. Oxidized low-density lipoprotein (OX-LDL) is one of the most important pathogenic factors. Lipoprotein receptor-1 (LOX-1) is the main receptor of OX-LDL and can adjust most of the biological functions of OX-LDL. Thus, LOX-1 is considered to be a promising new target for treating AS.

Preliminary test results in humans indicate that the monomer of the Chinese traditional herbs used in this experimental research, XinMaiJia (XMJ) (patent number: ZL 2010 1 0536001.x, now in capsule preparation), has obvious blood lipid-regulating and anti-lipid peroxidation effects. The progression of AS has been shown to be prevented by XMJ in all previous research. To determine the effects of XMJ on the blood lipid profile of rats with AS, the expression level of LOX-1, relevant inflammatory factors, and oxidative indicators in vascular tissue were determined. We used a rat model with AS to determine the effects of XMJ on reducing inflammation and anti-oxygenation, to analyze the possible mechanism through which XMJ has anti-AS effects, and to provide experimental evidence for its medicinal development and clinical application.

Materials

Animals

This research used 48 healthy male Sprague Dawley (SD) rats that were 4-5 months old and weighed 200-300 g [SCXK (YU) 202-0002, Experimental Animal Center in Henan province].
The rats were fed conventional rat forage and purified tap 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 used protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xinxiang Medical University.

**Experimental groups and drug treatments**

The SD rats were divided into eight groups at random (n = 6): (1) a normal control group; (2) a control group of drug medium (XMJ, anthoxanthin, functional monascus, isoflavones of pueraria, bamboo leaf flavonoid, and resveratrol, bought from Beijing Tong Ren Tang Co., Ltd., 1.875 g/kg/day, intragenic administration); (3) an AS model group; (4) a group fed with lovastatin (lovastatin, bought from American Sigma Corporation, 6 mg/kg/day, intragenic administration); (5) a group fed with Zhibituo (Zhibituo, bought from Chengdu Dijiu Hong Pharmaceutical Industry Co., Ltd., 0.7813 g/kg/day, intragenic administration); (6) group given a low dose of Chinese medicine (0.5934 g/kg/day, intragenic administration); (7) a group given medium dose of Chinese medicine (1.875 g/kg/day, intragenic administration); and (8) a group given high dose of Chinese medicine (5.925 g/kg/day, intragenic administration).

**Preparation of AS model**

Mixed modeling was adopted in which a high-fat diet with balloon injury and VitD₃ was used. For both the normal and drug medium control groups, the diet given was basic forage, and the rest of the groups had the same diet as in the AS model. Rats were given an intraperitoneal injection with 30,000 units VitD₃/200 mg for the first time, and then they had high-fat forage.

The high-fat forage formula for the rats was basic forage (1.5%), lard (10%), sodium cholate (0.5%), cholesterol (3%), and white sugar (5%). From their diet, the rats obtained 150 g of fat a day. After four weeks, the rats were given an intima injury operation in the common carotid artery, in which a two diameter sacculus was inserted into the mid-piece of the nick aorta to expand the sacculus. After the traction was repeated three times, an injury was induced to the intima in the nick aorta. Then the rats continued to be fed the high-fat forage for ten weeks.

**Observation of patho-morphology**

After anesthesia and the taking of blood, the 1.5 cm nick aorta was removed and observed by eye. Then a picture was taken of the nick aorta before it was placed in 10% neutral formalin. It was dehydrated, transparentation was performed, was dipped in wax, and was embedded to obtain a paraffin section. After hematoxylin-eosin staining and immunohistochemistry, the nick aorta was placed under a light microscope (400×) and electron microscope (10,000×) for further observation.

**Determination of blood lipid**

The 2 ml of blood taken from the nick aorta was quickly put into a test tube with heparin and separated under the conditions of 4°C and 300 r/min in a centrifuge for 15 min. Then the supernatant was taken to determine the oxides and to identify the content of total cholesterol and triglyceride, and then the method of chemical modification was employed to determine the HDL and LDL contents.

**ELISA**

Using blood taken from the nick aorta, we used ELISA (American Sigma Corporation) to determine according to the manufacturer's directions.

**Immunohistochemistry**

The cut sections were de-waxed and incubated in 3% H₂O₂ for 5-10 min. Antigen was performed hot-fix for 30 min at 92-98°C. The blood lipid was immobilized for 20 min and LOX-1 (Sigma, USA) was added at a 1:100 diluted concentration. Meanwhile, in the negative control, phosphate buffered saline (PBS) was added instead of the LOX-1 and kept in the refrigerator for one night. Then, the samples were heated in an oven at 37°C for 1 h. The horseradish enzyme was added, labelled strep-avidin operating fluid, and incubated in an oven at 37°C for 30 min. Then 3,3’-diaminobenzidine coloration was performed, followed by counter-staining with hematoxylin, dehydration, transparency, and sealing of the cut sections.

**Western blot**

The nick aorta (100 mg) was taken and added to 0.5 ml of cold lysis buffer (with 5 μL of phos-
Phatase inhibitors, 1 μL of protease inhibitor, and 5 μL of 100 mmol/L phenylmethyl sulfonyl fluoride to 1 ml cold Lysis buffer). Then, after homogenizing in an ice bath, the samples were separated by centrifugation at 4°C and 10,000 rpm for 5 min. The holoprotein extract was the supernatant. The bicinchoninic acid assay was used to determine the concentration of the holoprotein extract. The rest of the protein was diluted through the use of 4× upper buffer solution so that it had the same concentration. The samples were placed in 100°C water for 10 min and stored at -80°C. 50 μL of the upper buffer solution was used to perform the sodium dodecyl sulfate polyacrylamide-gel electrophoresis under a constant voltage (spacer gel 80 v, separation gel 100 v). Then, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane. It was blocked with 5% skimmed milk powder for 90 min and incubated with the LOX-1 primary antibody at 4°C for one night. Then, the membrane was warmed to 37°C for 60 min, washed with Tris-buffered saline plus Tween 20 (TBST), and incubated with a LOX-1 secondary antibody. Then, the membrane was washed, and enhanced chemiluminescence was used to image the membrane by exposing it to an X-ray.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., IL, USA). Comparisons among different groups were conducted using one-way or two-way ANOVA followed by Newman-Student’s t-test. A P-value of less than 0.05 was considered to be statistically significant.

Results

Pathomorphology change of common carotid artery

By comparing the model group and the normal control group, it was possible to observe the endothelial injury and the yellow atherosclerotic plaques with the naked eye. XMJ reduced the vascular endothelial injuries suffered by the rats but also enhanced the continuity and flexibility of the endothelium. In addition, XMJ reduced the atherosclerotic plaques and even made them disappear. Although lovastatin could also relieve the vascular endothelial injury in the model rats, strengthen the flexibility of the rats’ endothelium, and lessen the atherosclerotic plaques, it had certain negative effects on the continuity of the rats’ endothelium. As can be seen from Figure 1, Zhibituo was
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inferior. With the observation from the light microscope, it was easy to see that XMJ could make the model rats’ vascular endothelium relatively smooth, cause the endothelial nuclear staining to be relatively well-distributed, make cell spaces relatively distinct, and put the endothelial muscular layer into relative order. However, XMJ should be used on the basis of a certain dose (Figure 2). From the electron microscope, it could be seen that the rats fed XMJ had complete vascular endothelial cell membranes and complete organelles without proliferation when compared to the model group. In addition, there were no special metachromatic granules in their cell membranes. The rats that were given lovastatin had complete vascular endothelial cell membranes, and there were no special metachromatic granules in their cell membranes. They had complete organelles, but there was some proliferation present. The rats fed with Zhibituo had complete vascular endothelial cell membranes and complete organelles. In addition, there were no special metachromatic granules in their cell membranes (Figure 3).

Effects of XMJ on the blood lipid level

XMJ decreased the plasma cholesterol level in the rats with AS; triglyceride and low-density lipoprotein levels were also reduced. Moreover, XMJ increased the content of glutamic acid, high-density lipoprotein, and apolipoprotein.

Figure 2. The observation of common carotid artery with light microscope (400×) (A: The normal control group; B: The drug-medium control group; C: The model group; D: The lovastatin group; E: The Zhibituo group; F: The low-dose XMJ group; G: The medium-dose XMJ group; H: The high-dose XMJ group).
Compared with the model group, the XMJ group was significantly different ($P < 0.05$). By comparing the lovastatin group and the model group, there were also differences ($P < 0.05$). There were also differences between the Zhibituo group and model group. There was a certain dose-dependence for the protective effects of XMJ. The overall effect was superior to the lovastatin and Zhibituo groups. However, not all the results from the data represent significant differences ($P < 0.05$) (Table 1).

**Effects of XMJ on blood plasma and the relevant inflammatory factors in vascular tissues**

XMJ can obviously decrease the content of the rat plasma and the content of intercellular adhesion factor-1 (ICAM-1), vascular cell adhesion factor-1 (VCAM-1), interleukin-1 (IL-1), and interleukin-6 (IL-6). In addition, it increased the content of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 while decreasing cytokine nuclear factor-kappa B, all of which were dose-dependent effects. There were significant differences between the XMJ group and the model group ($P < 0.05$) (Tables 2 and 3).

**Effects of XMJ on blood plasma and oxidative index in vascular tissues**

The results showed that the rat plasma content as well as nitric oxide and superoxide dismutase contents in the vascular tissues all
increased and that the malondialdehyde content decreased in the presence of XMJ. Thus, this group is distinctly different from the model group (P < 0.05) (Tables 4 and 5).

**Immunohistochemical staining**

LOX-1 was labeled in the common carotid artery tissue sections from each group (Figure 4). Via comparison with the normal control group, it can be seen that the LOX-1 in the model group was increased (P < 0.05). Compared with the model group, XMJ significantly decreased the content of LOX-1 (P < 0.05), and this effect was dose-dependent.

**Western blot**

The expression of LOX-1 was determined by western blot (Figure 5). From the statistical results and comparison with the normal control group, the expression of LOX-1 from the model group as increased (P < 0.05). Compared to the model group, XMJ decreased LOX-1. Even more,
The oxidative index of rat plasma (mean ± SE) 

<table>
<thead>
<tr>
<th>Group</th>
<th>NO (µmol/L)</th>
<th>SOD (kU/g prot)</th>
<th>MDA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>23.2±3.36</td>
<td>65.6±4.51</td>
<td>1.3±0.23</td>
</tr>
<tr>
<td>Drug-medium control</td>
<td>22.39±2.02</td>
<td>55.98±5.31</td>
<td>1.26±0.18</td>
</tr>
<tr>
<td>Model</td>
<td>7.76±0.89</td>
<td>17.65±2.19</td>
<td>4.38±0.33</td>
</tr>
<tr>
<td>Zhibituo</td>
<td>13.38±1.68</td>
<td>26.59±2.37</td>
<td>3.69±0.33</td>
</tr>
<tr>
<td>Low-dose XMJ</td>
<td>14.33±1.14</td>
<td>21.41±2.39</td>
<td>3.54±0.26</td>
</tr>
<tr>
<td>Medium-dose XMJ</td>
<td>9.65±0.67</td>
<td>22.08±3.27</td>
<td>3.29±0.47</td>
</tr>
<tr>
<td>High-dose XMJ</td>
<td>16.69±1.37</td>
<td>38.47±3.31</td>
<td>2.54±0.41</td>
</tr>
</tbody>
</table>

Note: *P < 0.05 compared with the model group; #P < 0.05 compared with the High-dose XMJ group; ΔP < 0.05 compared with the Normal control group. NO, nitric oxide; SOD, superoxide dismutase; MDA, malonaldehyde.

Table 5. Oxidative index of rat plasma (mean ± SE) 

<table>
<thead>
<tr>
<th>Group</th>
<th>NO (µmol/L)</th>
<th>SOD (kU/g prot)</th>
<th>MDA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6.22±0.65</td>
<td>48.43±4.43</td>
<td>4.32±0.23</td>
</tr>
<tr>
<td>Drug-medium control</td>
<td>6.32±0.54</td>
<td>46.39±5.22</td>
<td>4.39±0.27</td>
</tr>
<tr>
<td>Model</td>
<td>2.36±0.57</td>
<td>23.32±3.47</td>
<td>7.33±0.57</td>
</tr>
<tr>
<td>Zhibituo</td>
<td>3.29±0.62</td>
<td>33.26±4.29</td>
<td>6.45±0.74</td>
</tr>
<tr>
<td>Low-dose XMJ</td>
<td>4.47±0.57</td>
<td>37.38±3.88</td>
<td>6.11±0.54</td>
</tr>
<tr>
<td>Medium-dose XMJ</td>
<td>3.28±0.39</td>
<td>29.44±2.14</td>
<td>6.78±0.64</td>
</tr>
<tr>
<td>High-dose XMJ</td>
<td>5.21±0.47</td>
<td>39.67±3.53</td>
<td>5.48±0.38</td>
</tr>
</tbody>
</table>

Note: *P < 0.05 compared with Model group; #P < 0.05, compared with High-dose XMJ group; ΔP < 0.05 compared with Normal control group. NO, nitric oxide; SOD, superoxide dismutase; MDA, malonaldehyde.

Discussion

In recent years, the significant role of oxidative stress in AS has attracted much attention, leading to the formation of the Oxidative-Stress Theory. OX-LDL has the following characteristics: causes endothelial cell injury and promotes platelet adhesion, aggregation, and thrombosis. OX-LDL is recognized and devoured by phagocytes; thus, gathering cholesteryl esters intracellularly and bringing about the pathological changes of AS-foam cells at its early stage, impairing the flexible function of the endothelium and promoting smooth muscle cell to synthesize collagen tissues and other tissues. It has been confirmed that OX-LDL is present in the AS plaques, especially in unstable ones, through the use of immunostaining [5, 6]. Only after LDL has acquired the oxidative ornament in the body can it exacerbate AS. Therefore, to delay AS as long as possible, oxidative stress should be prevented as long as possible [7-10]. LOX-1 is the main receptor of OX-LDL in endothelial cells, which can induce endothelial cells to take in cytotoxic OX-LDL. After LOX-1 has combined with OX-LDL, it can activate the extracellular signal-regulated kina-

there were significant differences between the groups (P < 0.05).
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...in blood vessels, and finally cause AS and other vascular diseases. Some researchers have shown [11-15] that OX-LDL up-regulates the expression of VCAM-1, ICAM-1, E-selectin and monocyte chemoattractant protein-1 through the receptor of LOX-1 to urge monocytes to gather together with endothelial cells. The Oxidative Stress Theory explicitly emphasizes the important function of reactive oxygen species in the treatment of AS and regards LOX-1 as the key cause of OX-LDL AS.
In this study, it was found that expression of LOX-1 in the common carotid artery of rats could be up-regulated by a high-fat diet. The present study revealed that LOX-1 is a new target for AS treatments. It has shown that the occurrence of AS is linked to increasing expression of LOX-1 and that drugs can prevent AS at an early stage. This effect can be gained by decreasing total cholesterol and by reducing the expression of LOX-1. Previous research has shown that AS can be prevented via the use of drugs to lower the expression of LOX-1 [16-21]. This study confirmed that XMJ can lower the expression of LOX-1 under the stimulation of hyperlipemia, which could block the vascular injury induced by OX-LDL and eventually inhibit the development of atherosclerosis in patients with hyperlipemia.

The experimental results showed that XMJ can effectively decrease the blood lipid level in rats with AS, regulate the plasma, decrease inflammatory factors and oxidative stress as well as lower the expression of LOX-1 in the common carotid artery. These results illustrate the potential anti-inflammatory and antioxidant mechanism of XMJ and to provide experimental evidence for the development of XMJ for clinical application.

Acknowledgements

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Disclosure of conflict of interest

None.

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