

Original Article

Tongxinluo alleviates adventitial vasa vasorum angiogenesis via regulating oxidative stress and inflammation in early carotid atherosclerosis

Yujie Yin¹, Liuyi Ma², Zhenhua Jia^{1,3}, Qianzhang¹, Hongtao Wang^{2,4}, Cong Wei^{2,4}, Liping Chang^{2,4}, Hongrong Li²

¹Graduate School, Hebei University of Chinese Medicine, Shijiazhuang 050090, Hebei, China; ²Key Laboratory of State Administration of Traditional Chinese Medicine, Shijiazhuang 050035, Hebei, China; ³Department of Cardiology, Affiliated Yiling Hospital of Hebei University of Chinese Medicine, Shijiazhuang 050091, Hebei, China; ⁴New Drug Evaluation Center, Yiling Medical Institute of Hebei Province, Shijiazhuang 050035, Hebei, China

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Abstract: This study was to investigate the effects of Tongxinluo on adventitial vasa vasorum (VV) angiogenesis in early carotid atherosclerosis (AS). A rabbit model of AS was established by high-fat diet combined with encapsulation of left carotid artery. The rabbit models of AS were treated with high (0.6 g/kg/d)-, mid (0.3 g/kg/d)-, and low (0.15 g/kg/d)-dose Tongxinluo, respectively. Serum lipid levels were determined. Serum MDA level, SOD activity, and T-AOC level were assessed. Histological characteristics were detected with H&E staining. mRNA and protein expression levels were evaluated with quantitative real-time PCR and Western blot analysis, respectively. For the rabbit models of AS, Tongxinluo significantly alleviated the pathological changes, down-regulated mRNA and protein expression levels of VEGF and VEGF-R2, and reduced carotid VV angiogenesis. Moreover, compared with the model group, the Tongxinluo treatment reduced serum levels of TC and LDL-C, significantly down-regulated mRNA expression levels of TNF- α and IL-6, and significantly decreased protein expression levels of nuclear NF- κ B, TNF- α , and IL-6. On the other hand, compared with the model group, the mRNA expression levels of NQO1 were up-regulated, and the protein expression levels of nuclear Nrf2 and NQO1 were increased, in the Tongxinluo treatment groups. Tongxinluo could reduce carotid adventitial VV angiogenesis and alleviate early AS lesions, through inhibiting carotid inflammation and oxidative stress injury.

Keywords: Atherosclerosis (AS), vascular adventitia, vasa vasorum (VV) angiogenesis, oxidative stress, inflammation

Introduction

Atherosclerosis (AS) is a chronic inflammatory pathological disease [1]. AS not only violates the large and medium arteries, but also cause changes in the microcirculation system [2], which is a common site of pathology for a variety of poor prognosis cardiovascular diseases [3]. Moreover, AS-induced acute cardiovascular complications, such as stroke and myocardial infarction, are the most important factors for the high incidence and mortality worldwide [4].

In 1986, Ross et al. [5] proposed the *inside-out* hypothesis for AS pathogenesis, and thereafter, studies concerning the disease mechanism have mainly focused on the vascular intima. In recent years, however, increasing investigation

has shown that, in the integral and regular layer structure inside the arterial wall, various exogenous cells in the local outer membrane (such as the mononuclear cells, macrophages, and T-cells) could promote the expression of local inflammatory factors. This *outside-in* pathological hypothesis for AS has been gradually attracting increased attention [6, 7].

In 1876, Koester et al. [8] proposed for the first time that the vasa vasorum (VV), and the VV angiogenesis are mainly distributed in the arterial adventitia of large and medium arteries (such as aorta and coronary artery), as well as in the 1/2-2/3 region outside the carotid media. The related region provides the blood supply and nutrients, and contributes to the excretion of metabolites, maintaining the material metab-

olism and energy balance of the vascular wall. Moreover, Gössl et al. [9] observed a close relationship between VV angiogenesis and AS pathogenesis. The pathological intimal thickening is characterized by vascularization, which originates from VV [10], which could be inhibited by angiogenesis inhibitors [11]. Therefore, it is of great importance to investigate the role of VV angiogenesis in AS pathogenesis.

Tongxinluo is registered and approved by China's State Food and Drug Administration for the treatment of angina pectoris. Ma et al. [12] have shown that Tongxinluo could reduce angiogenesis in atherosclerotic plaques in apo E^{-/-} mice. However, the role of Tongxinluo in early lesions of AS needs to be further explored. In this study, a rabbit model of AS was established by high-fat diet combined with carotid artery encapsulation. The role of VV angiogenesis in the early pathogenic process of AS, and the effects of Tongxinluo, were investigated and analyzed.

Materials and methods

Animal grouping and model establishment

In total, 50 New Zealand rabbits (half males and half females), weighing 2.0±0.3 kg, were purchased from Beijing Fuhao Experimental Animal Breeding Center [experimental animal license number: SCXK (Beijing) 2010-0010]. All the animal experimental procedures were approved by the local Animal Use and Care Committee. After 2 weeks of adaptive feeding, the rabbits were randomly divided into the control and model groups, as well as the high-, mid-, and low-dose Tongxinluo groups (n = 10). In the control group, rabbits were fed with normal diet. In the model and treatment groups, early hyperlipidemia rabbit model was established by high-fat diet (i.e., 1% cholesterol, 5% lard, 7.5% cholesterol, and 86.5% basal diet), combined with encapsulation of left carotid artery.

For the encapsulation of left carotid artery, the animals were anesthetized by 3% pentobarbital sodium (1 ml/kg) via the ear vein, and mounted on the operating table. After skin disinfection and preparation, a cut was made on the skin along neck, and the common carotid artery sheath was exposed. The left carotid artery was isolated with ophthalmic tweezers, which

was then encapsulated by the disinfected silicone tube (with the inside diameter of 1.7 mm, outside diameter of 3.2 mm, and length of 2 cm). After local treatment of streptomycin, the wound was sutured layer by layer. Local disinfection was conducted twice daily for 3 consecutive days. The animals were housed in a 12 h/12 h dark/light cycle, with free access to water.

Drug administration

Tongxinluo was provided by Shijiazhuang Yiling Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China), whose active ingredients mainly include ginseng, leech, scorpion, *Eupolyphaga sinensis*, centipede, red peony root, borneol, and other Chinese herbal medicine. For quality control, labeled compounds had been verified and standardized according to the Chinese Pharmacopoeia (2005), and its processing methods were also strictly standardized [13]. For the drug administration, Tongxinluo ultrafine powder was resolved by 0.5% CMC-Na solution in advance. Rabbit models from the high-, mid-, and low-dose Tongxinluo groups were treated with 0.6 g/kg/d, 0.3 g/kg/d, and 0.15 g/kg/d Tongxinluo ultrafine powder, respectively, by gavage, for 4 consecutive weeks.

Blood lipid level determination

Animals were fasted for 12 h before sample collection, followed by injection of 3% pentobarbital sodium (1 ml/kg) via ear vein. Blood samples were collected from the abdominal aorta. After standing for 30 min and centrifugation at 3500 r/min for 10 min, the samples were subjected to the automatic biochemical analyzer (Model 7080; Hitachi, Tokyo, Japan), to detect the serum levels of the total cholesterol (TC), triglyceride (TG), high density lipoprotein HDL-C, and low density lipoprotein LDL-C.

Serum MDA, SOD, and T-AOC level determination

Blood samples were collected as described above. The serum MDA level was then detected with the thiobarbituric acid (TBA) method, SOD was determined by the xanthine oxidase method, and T-AOC was assessed by the chemical colorimetric method, respectively, all according to the manufacturer's instructions.

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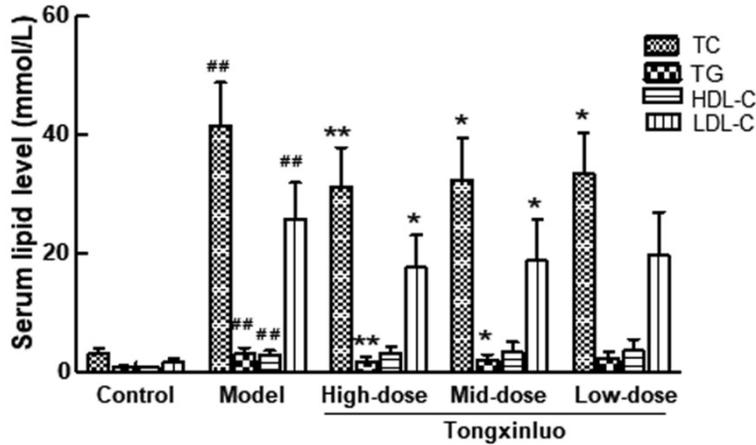


Figure 1. Effects of Tongxinluo on serum lipid levels in early AS. After modeling and drug administration, the serum levels of TC, TG, HDL-C, and LDL-C were detected for the control and model groups, as well as the high (0.6 g/kg/d)-, mid (0.3 g/kg/d)-, and low (0.15 g/kg/d)-dose Tongxinluo treatment groups. Compared with the control group, ^{##}*P* < 0.01; compared with the model group, ^{*}*P* < 0.05, ^{**}*P* < 0.01.

Histological detection

After blood sample collection, the animal skin was cut open along the center line of neck. The carotid artery was removed at 1 mm from the upper and lower ends of the silicone tube. After washing with saline, the sample was embedded with paraffin, and sliced in to 4- μ m sections. After dewaxing, the section was washed with PBS, and then immersed in hematoxylin for 2 min, followed by treatment of 0.3% hydrochloric acid and Iraq red dye staining for 4 min. Then the section was sealed and observed under light microscope.

Quantitative real-time PCR

Total RNA was extracted with the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), which was then subjected to the cDNA synthesis. Primer sequences were as follows: VEGF, forward 5'-GCGTTCTCAGTGGTGTGTA-3' and reverse 5'-TGGCTTGTCTCCTCTTCTTG-3'; VEGFR-2, 5'-GGTTCAAAGACAACGAGATGC-3' and reverse 5'-TCACACAGCCAAGGACACT-3'; NF- κ B, forward 5'-GGCTTGCGTTCAGATGTTG-3' and reverse 5'-AACCGTCTTGTGCTCTTGA-3'; TNF- α , 5'-AGTAGCAAACCCGCAAGTG-3' and reverse 5'-GCTGAAGAGAACCTGGGAGTA-3'; IL-6, 5'-AACCAGTGGCTGAAGACGA-3' and reverse 5'-TCCAGGAAGTTTGTGAGGC-3'; Nrf2, forward 5'-TTCTTTCGGCAGCATCCT-3' and

reverse 5'-TGGCATTGAGTTCACGC-3'; and NQO1, 5'-CTG-GAGAGTATTTGGGATGA-GA-3' and reverse 5'-AGTTGGGATG-GATTTGCC-3'. Real-time PCR was performed on the ABI 7300 Real-Time PCR System (ABI, Branchburg, NJ, USA), and the reaction conditions were as follows: 94°C for 3 min; 94°C for 45 s, 53°C for 45 s, and 72°C for 45 s, for totally 40 cycles. GAPDH was used as internal reference.

Western blot analysis

Tissue was lysed with 1 ml lysis. After protein content determination, the protein sample was subjected to SDS-PAGE. The sample was then electronically transferred onto

the PVDF membrane. After blocking, the membrane was incubated by mouse monoclonal anti-VEGF-A primary antibody (1:1000 dilution; ab1316, Abcam, UK), goat polyclonal anti-VEGFR-2 primary antibody (1:1000 dilution; sc-48161, Santa Cruz Biotechnology, USA), and goat polyclonal anti-Nrf2 primary antibody (1:1000 dilution; ab121035, Abcam, UK), and goat polyclonal anti-NQO1 primary antibody (1:1000 dilution; sc-16464, Santa Cruz Biotechnology, USA), and goat polyclonal anti-NF- κ B primary antibody (1:1000 dilution; sc-1190, Santa Cruz Biotechnology, USA), and goat polyclonal anti-IL-6 primary antibody (1:1000 dilution; ab9770, Abcam, UK), and monoclonal anti-TNF- α primary antibody (1:1000 dilution; sc-12744, Santa Cruz Biotechnology, USA), respectively, at 4°C overnight. The membrane was then treated with horseradish peroxidase-labeled secondary antibody at room temperature for 2 h. Protein bands were detected by the chemiluminescence method. Protein expression was determined the GAPDH was the internal reference.

Statistical analysis

Data are expressed as mean \pm SD. SPSS 19.0 software was used for statistical analysis. ANOVA was performed for group comparison. *P* < 0.05 was considered as statistically significant.

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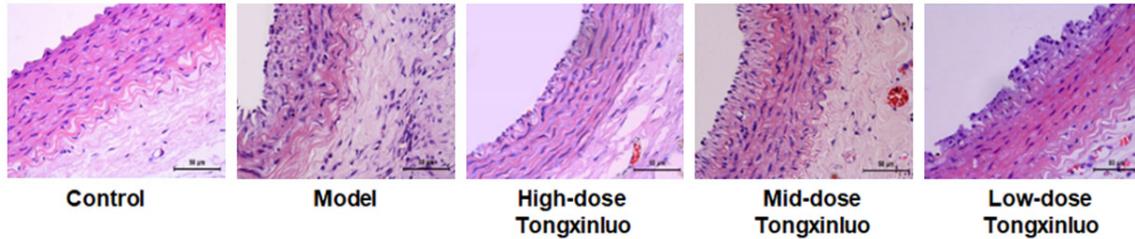


Figure 2. Effects of Tongxinluo on histological changes in early AS. Histology was detected with the H&E staining in the rabbits from the control, model, high-, mid-, and low-dose Tongxinluo groups (200 \times).

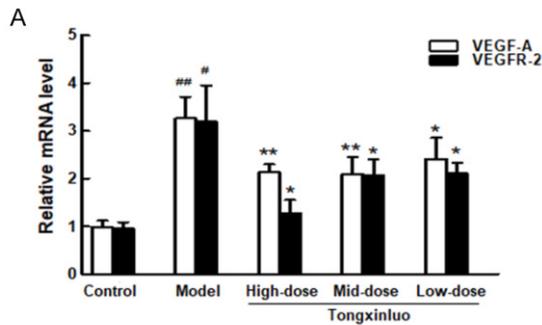
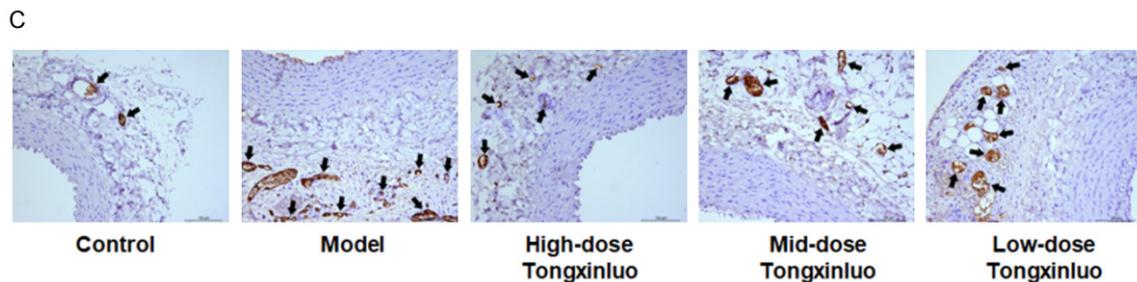
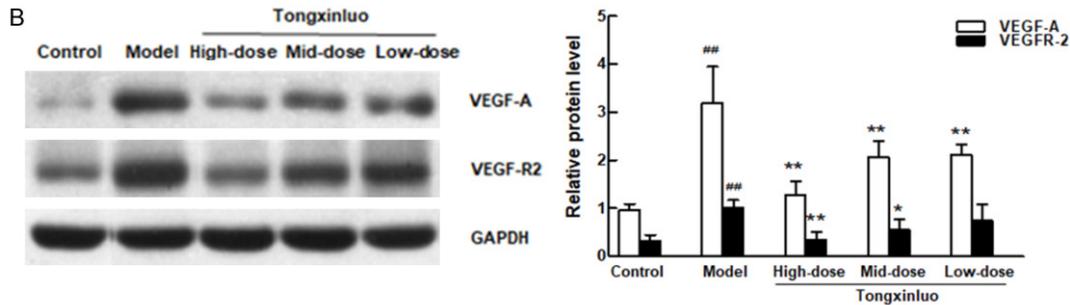


Figure 3. Effect of Tongxinluo on adventitial VV angiogenesis in early carotid AS. A. Nuclear mRNA expression levels of VEGF-A and VEGF-R2 in the carotid artery wall were detected with real-time PCR. B. Nuclear protein expression levels of VEGF-A and VEGF-R2 in the carotid artery wall were detected with Western blot analysis. C. Expression of CD34 was detected with immunohistochemistry (200 \times). Compared with the control group, [#] $P < 0.05$, ^{##} $P < 0.01$; compared with the model group, ^{*} $P < 0.05$, ^{**} $P < 0.01$.



Results

Tongxinluo inhibits early atherosclerotic lesion in rabbits

During model establishment and drug administration, no significant differences were observed in the response, food intake, and body weight among all the groups (data not shown). The serum levels of TC, TG, HDL-C, and LDL-C

were first investigated. Our results showed that, compared with the model group, the serum levels of TC, TG, and LDL-C were significantly decreased in the high- and mid-dose Tongxinluo Groups ($P < 0.05$), and significantly decreased serum TC level was observed in the low-dose Tongxinluo group ($P < 0.05$). However, no significant difference in the serum HDL-C level was observed in the treatment groups, as compared with the model group (Figure 1).

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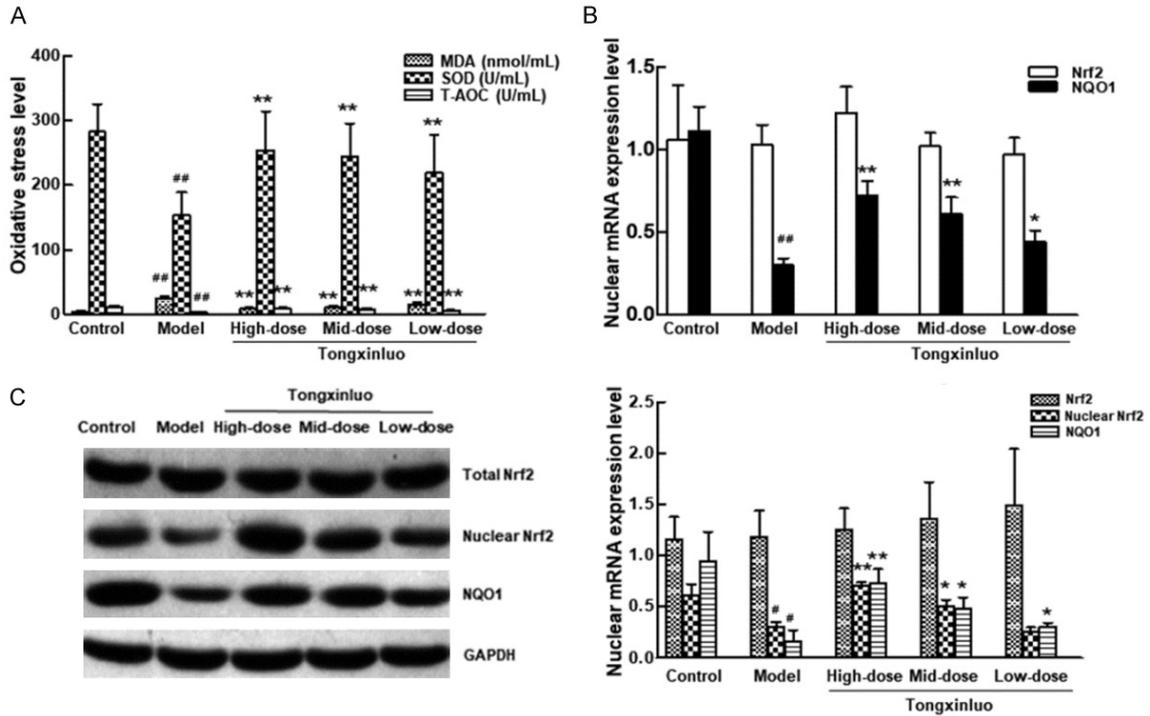


Figure 4. Effects of Tongxinluo on oxidative stress in early AS. A. Effects of Tongxinluo on serum MDA level, SOD activity, and T-AOC level in early AS. B. Nuclear mRNA expression levels of Nrf2 and NQO1 in the carotid artery wall were detected with real-time PCR. C. Nuclear protein expression levels of Nrf2 and NQO1 in the carotid artery wall were detected with Western blot analysis. Compared with the control group, [#]P < 0.05, ^{##}P < 0.01; compared with the model group, ^{*}P < 0.05, ^{**}P < 0.01.

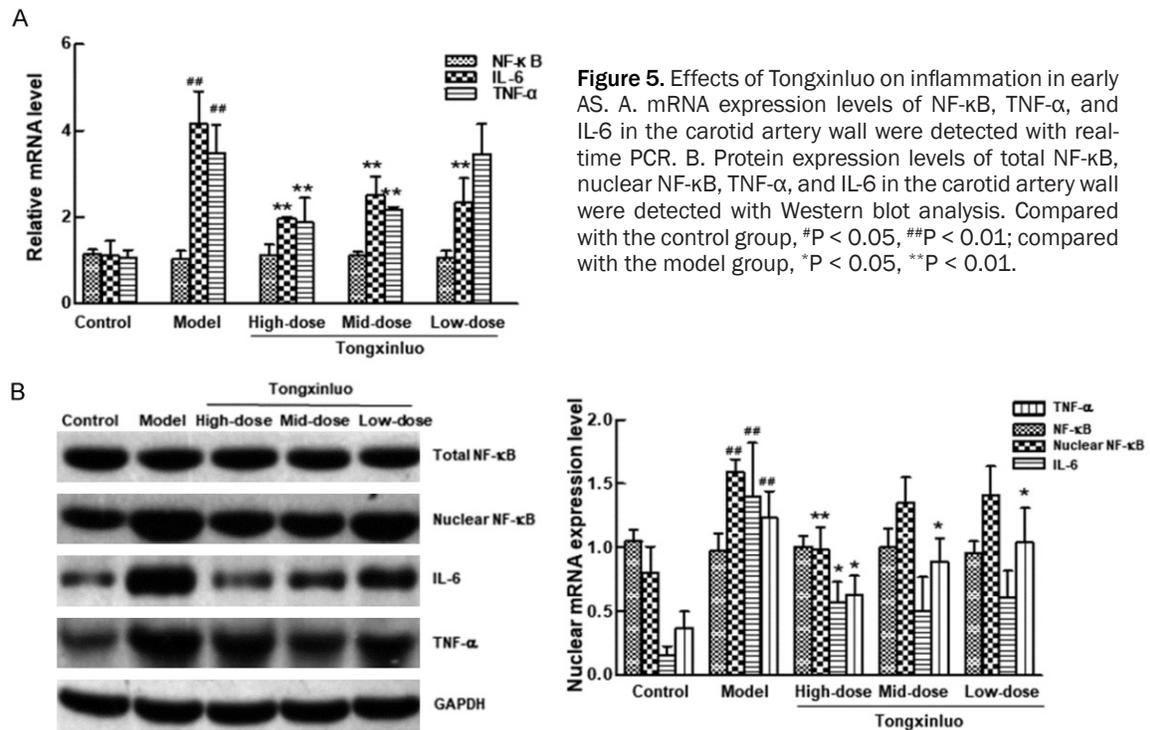


Figure 5. Effects of Tongxinluo on inflammation in early AS. A. mRNA expression levels of NF-κB, TNF-α, and IL-6 in the carotid artery wall were detected with real-time PCR. B. Protein expression levels of total NF-κB, nuclear NF-κB, TNF-α, and IL-6 in the carotid artery wall were detected with Western blot analysis. Compared with the control group, [#]P < 0.05, ^{##}P < 0.01; compared with the model group, ^{*}P < 0.05, ^{**}P < 0.01.

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To investigate the effects of Tongxinluo on histology in the rabbit models, H&E staining was performed. In the model group, there was obvious vascular intimal hyperplasia, mainly with mononuclear cells, and the mesangial space was widened, with irregularly arranged smooth muscle and elastic fibers. However, in the high- and mid-dose Tongxinluo groups, intimal hyperplasia in the carotid artery was alleviated, with reduced subcutaneous foam cells, and normally arranged medial smooth muscle (**Figure 2**). Taken together, these results suggest that Tongxinluo could inhibit the early atherosclerotic lesions in these rabbit models.

Tongxinluo inhibits early adventitial VV angiogenesis in carotid AS

The effects of Tongxinluo on the VV angiogenesis in the early pathogenesis of carotid AS were next investigated. As shown in **Figure 3**, compared with the model group, the mRNA and protein expression levels of vascular endothelial growth factor-A, VEGF-A and its receptor, VEGF-R2 were significantly down-regulated in the high- and mid-dose Tongxinluo groups. Moreover, the expression levels of CD34 in the carotid artery were significantly decreased in the high- and mid-dose Tongxinluo groups, compared with the model group. These results suggest that Tongxinluo might inhibit carotid AS via reducing adventitial VV angiogenesis.

Tongxinluo exerts antioxidant effects in early AS pathogenesis

The antioxidant effects of Tongxinluo in carotid AS rabbit models were then investigated. Our results showed that, compared with the control group, the serum MDA level was significantly elevated, while the SOD activity and T-AOC level were all significantly reduced in the model group. However, compared with the model group, the serum MDA level was significantly decreased, while the SOD activity and T-AOC level were significantly increased in all the Tongxinluo-treated groups (**Figure 4**). Moreover, no significant difference was observed in the mRNA expression of Nrf2 among all these groups. However, compared with the control group, the mRNA expression level of NQO1 was significantly decreased in the model group, which could be significantly up-regulated by the high-, mid-, and low-dose Tongxinluo treatments (**Figure 4**). Furthermore, there were no

significant differences in the total Nrf2 protein expression levels among these groups. However, compared with the model group, the nuclear Nrf2 protein expression levels were significantly elevated in the high- and mid-dose Tongxinluo groups (**Figure 4**). These results suggest that Tongxinluo could exert antioxidant effects in the early pathogenesis of AS.

Tongxinluo reduces inflammatory responses in early stage of AS

The effects of Tongxinluo on the inflammation levels in the early stage of AS were also investigated. Our results showed that, compared with the control group, the mRNA expression levels of TNF- α and IL-6 were significantly elevated, without significant changes in NF- κ B mRNA expression, in the model group. However, compared with the model group, mRNA expression levels of IL-6 and TNF- α were significantly reduced in the high-, mid-, and low-dose Tongxinluo groups (**Figure 5**). Moreover, compared with the control group, the nuclear protein expression levels of NF- κ B, TNF- α , and IL-6 were significantly elevated in the model group. However, compared with the model group, the nuclear protein expression levels of NF- κ B and TNF- α were significantly reduced in the high-dose Tongxinluo group, while IL-6 protein expression levels significantly declined in all the high-, mid-, and low-dose Tongxinluo groups (**Figure 5**). These results suggest that Tongxinluo treatment could reduce inflammatory responses in early stage of AS.

Discussion

Previous studies have shown that the adventitial VV angiogenesis represents a potential factor in the pathogenesis of atherosclerosis (AS) [10, 11, 14, 15]. However, there is no VV angiogenesis in the normal myocardium of mice and human beings [16]. VV angiogenesis mainly occurs in the arterial wall with more than 29 layers or in the adventitia and medial layer with the lumen diameter of > 0.05 mm [17]. Under physiological conditions, the amount and structure of VV in the arterial wall are relatively stable. However, under pathological conditions, inflammation and invasion of oxidative products could promote VV angiogenesis and increase lipid accumulation, participating in the AS pathogenic processes. Previous studies on carotid VV angiogenesis have been mainly

focusing on the formation and rupture of atherosclerotic plaques [18]. However, before plaque formation, adventitial VV angiogenesis could pathologically thicken the intima [10]. Kwon et al. [19, 20] have re-constructed the spatial distribution of blood vessels in organs with the Micro-CT technology, and they show the abnormal hyperplasia of adventitial VV angiogenesis before the development of coronary artery diseases in high-cholesterol diet-fed pig models. Moreover, the ratio of primary and secondary VV has been reversed, resulting in irregular microvascular network with increased and disordered density [19, 20]. In this study, our results showed that high-fat diet combined with encapsulation of left carotid artery could induce in the thickening of neck carotid artery intima in rabbit models, accompanied with significantly elevated TC, TG, and LDL-C levels, in line with the characteristics of early AS lesions. Carotid VV angiogenesis at the initial stage of AS further demonstrates its relationship with the disease pathogenic processes.

The severity and distribution of VV in the pathogenesis of AS is regulated by a number of angiogenic factors, in which the vascular endothelial growth factor (VEGF) family plays a prominent role. VEGF is expressed on a relatively low level in the normal tissues, while highly expressed in embryonic and angiogenic tissues. Studies have shown that hypoxia could activate the compensatory mechanism of angiogenesis by up-regulation VEGF [21]. Importantly, VEGF family members are effective factors promoting mitogenesis and endothelial cell migration [22, 23]. VEGF-A, a major subtype of VEGF [24], can be detected at various stages of human coronary AS, which promotes the AS process in apo E/apo B¹⁰⁰-defect mice and rabbit atherosclerosis processes. In addition, expression of VEGF-A in unstable plaques is significantly higher than in the stable plaques of the carotid artery from patients receiving carotid artery intimal thrombectomy [25]. VEGF-A could specifically bind to the tyrosine kinase receptor VEGFR-2 expressed in vascular endothelial cells [26], initiate the phosphorylation of tyrosine residues in cytoplasm, and activate the downstream signaling enzymes, therefore regulating the vascular endothelial proliferation, migration, differentiation, and vascular permeability, and ultimately result in the formation of

a capillary network. However, the severity and distribution of VV in the arterial wall depend on a variety of pathological factors.

Oxidative stress refers to the disturbed imbalance between the oxidation system (production of reactive oxygen species, ROS) and the anti-oxidant system (preventing and repairing the oxidative damages) in the body. Under pathological conditions, increased and/or decreased intracellular and extracellular ROS production could lead to oxidative damages in the body, involved in pathological angiogenesis [27, 28]. Nrf2 is an endogenous anti-oxidative stress transcriptional regulator that guards against oxidative damage elicited by injury or inflammation [29]. Under oxidative stress, uncoupled from Keap1, Nrf2 regulates the expression levels of various antioxidant proteins, phase II detoxification enzymes, and transporters via inducing antioxidant response element (ARE) in the body [30]. NADPH quinone oxidoreductase 1 (NQO1) is the important target gene for protecting blood vessels in the Nrf2/ARE pathway, which is an intracellular protective reductase, protecting cells from external quinone and oxidative damages and playing a key role in the responses to the oxidative stress [31]. In this study, oxidative stress markers were detected, and our results show that MDA expression is up-regulated in the early stage of AS, while the expression levels of antioxidant-related factors, i.e., Nrf2, NQO1, SOD, and T-AOC, are down-regulated. Based on these findings, decreased expression of anti-oxidative factors in early AS pathogenesis could activate ROS and participate in the VV angiogenesis. Ross et al. [5] has defined AS as a chronic inflammatory process and suggested the "injury-response" theory. Recent studies have shown that vascular inflammation is closely related to cell metabolism in AS lesions, inducing hypoxia, microvascular neovascularization, bleeding, and plaque rupture.

NF- κ B is the central link of inflammatory responses, which is mainly present in monocytes, vascular endothelial cells, and smooth muscle cells. NF- κ B is usually bound to the inhibitory protein I κ B, which activates a variety of active factors, moving from the cytoplasm into the nucleus and regulating the gene expression concerning inflammation and immune responses [32]. Activated NF- κ B could regulate

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the expression of TNF- α and IL-6, which could promote the further activation of NF- κ B and promote the progression of AS [33]. Moreover, NF- κ B is closely linked with the inflammatory responses, foam cell formation, vascular smooth muscle proliferation, and cell apoptosis. NF- κ B is responsible for transcription of a variety of inflammatory cytokines and chemokines. TNF- α acts on vascular endothelial cells, promotes the expression of cell adhesion molecules, and induces jagged-1 expression to promote angiogenesis via NF- κ B in endothelial cells [34]. IL-6 is another important cytokine involved in body regulation. NF- κ B activation is closely related to these two cytokines. In this study, our results show elevated expression of NF- κ B, TNF- α , and IL-6 in carotid AS, suggesting that inflammatory responses might contribute to VV angiogenesis at the early stage of AS. In addition, oxidative stress and inflammation may interact. NF- κ B-promoted ROS production is achieved, at least partially, by activating NADPH oxidase [35]. Nrf2 activation can reduce the activation of AS-sensitive parts of endothelial cells under inflammatory responses [36]. Oxidative stress and inflammatory factors up-regulate the mRNA and protein expression levels of VEGF/VEGFR-2, promote the VV angiogenesis at the early stage of AS, and increase the local blood flow in carotid artery. These alterations contribute to the invasion of inflammation and oxidative stress-related factors into the media, endometrium, and even plaque, eventually leading to AS development.

Tongxinluo is a representative prescription based on meridian theory of traditional Chinese medicine. Many studies have shown that Tongxinluo has a variety of effects in cardiovascular diseases, including blood lipid lowering, anti-inflammatory, anti-oxidative, and apoptosis inhibiting effects [37-39]. More interestingly, in the mouse model of myocardial infarction, Tongxinluo promotes angiogenesis in ischemic myocardium [40]. In the apo E^{-/-} mouse model of AS, Tongxinluo can enhance the plaque stability by inhibiting microvascular neoplasia [41]. These findings suggest that the regulating effects of Tongxinluo could not be simply defined as inhibition or promotion. However, there are few studies concerning the effects of Tongxinluo on the adventitial VV at the early stage of AS. This study aimed at investigating the effects of Tongxinluo on the VV angiogene-

sis, oxidative stress, and inflammatory responses at the early stage of AS.

This study has some limitations in experimental design and procedures. Whether there were similar changes in the expression levels of some other genes has not been involved in the coronary arteries, abdominal aorta, and other major arteries. Moreover, there might be other factors implied in the regulation of angiogenesis in the early pathogenesis of AS. Further in-depth studies are still needed to address these issues.

In conclusion, our results show that high-, mid-, and low-dose Tongxinluo inhibits the early pathological changes in AS, and that VV neovascularization could be also inhibited. Moreover, Tongxinluo could down-regulate the oxidative stress- and inflammation-related factors, i.e., MDA, NF- κ B, TNF- α , and IL-6, while up-regulating the levels of antioxidant factors, i.e., Nrf2, NQO1, SOD, and T-AOC. Furthermore, Tongxinluo might reduce the inflammation in the carotid artery via inhibiting NF- κ B nuclear translocation, and improve the antioxidant capacity via promoting Nrf2 nuclear translocation, therefore inhibiting the adventitial VV angiogenesis and delaying the disease process of AS.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhenhua Jia, Department of Cardiology, Affiliated Yiling Hospital of Hebei University of Chinese Medicine, 385 Xinchibei Road, Shijiazhuang 050091, Hebei, China. Tel: +86-311 6670 3020; E-mail: 963895141@qq.com; jiatcm@163.com

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