Background: The purpose of the present study was to investigate whether tanshinone IIA (Tan II), one of the major lipophilic components of Salvia miltiorrhiza Bunge, could inhibit the development of elastase-induced experimental abdominal aortic aneurysms (AAAs).

Methods: Male Sprague-Dawley rats (n = 12/group) were randomly distributed into three groups: Tan II, control, and sham. The rats from the Tan II and control groups underwent intra-aortic elastase perfusion to induce AAAs, and the rats in the sham group were perfused with saline. Only the Tan II group received Tan II (2 mg/rat/d). The maximum luminal diameter of the abdominal aorta was measured before and 5, 12, 18, and 24 d after perfusion. The systolic blood pressure was measured twice using the tail cuff technique before administration and death. Aortic tissue samples were harvested at 24 d and evaluated using reverse transcriptase-polymerase chain reaction, Western blot, immunohistochemistry, and Miller’s elastin-Van Gieson staining.

Results: The rats in the control group had significantly increased aortic sizes compared with the sham group after 24 days (P < 0.05), and the Tan II group had a significant reduction in aortic size (Tan II versus control, P < 0.05) without affecting blood pressure (P > 0.05). The overexpression of matrix metalloproteinase-2, metalloproteinase-9, monocyte chemotactic protein-1, and inducible nitric oxide synthase and the depletion of elastic fibers and vascular smooth muscle cells induced by elastase perfusion were significantly decreased by Tan II treatment (P < 0.05).

Conclusions: Tan II inhibited the development of elastase-induced experimental AAAs by suppressing proteolysis, inflammation, and oxidative stress and preserving vascular smooth muscle cells. It could be a new pharmacologic therapy for AAAs.

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1. Introduction

Abdominal aortic aneurysm (AAA) can cause death from aneurysm rupture, which has a mortality rate of up to 90% [1]. This disease has been 1 of the 15 leading causes of death in the United States [2]. Two randomized controlled trials have shown that early surgery offered no survival advantage for small AAAs (diameter less than 5.5 cm) in patients diagnosed with ultrasound surveillance [3,4]. Therefore, surgical repair is the only recommended treatment for patients with large AAAs (diameter 5.5 cm or larger). However, from aneurysm screening programs performed in the United States about 10 years ago, an estimate of 360,000 reported aneurysms were small AAAs (greater than 90%) [5–7]. Those patients could not receive effective therapy, except for “watchful waiting” in clinics. Also, they were more likely to acquire psychological problems and a reduced quality of life [8]. For the slow development of small AAAs, it would take a long period before they reach the threshold size for surgical intervention [9]. Thus, an effective pharmacologic therapy might be a good option to limit or stop the growth of small AAAs.

Tanshinone IIA (Tan IIA) is one of the major lipophilic components extracted from the root of Salvia miltiorrhiza Bunge, which has been used to treat various types of ischemic disease as a traditional Chinese medicine for many years [10]. Recent studies have shown that Tan IIA might have the potential to suppress AAA formation and affect AAA development [10–15]. Therefore, we studied the effect of Tan IIA on experimental AAAs and investigated the possible molecular mechanisms.

2. Methods

2.1. Experimental groups and AAA model

All experiments in our study were conducted in compliance with the Guide for the Care and Use of Laboratory Animals approved by the Ethical Committee of Researches of Nanjing University and were approved by the Committee on the Use and Care of Animals of Medical School of Nanjing University. Six-week-old male rats (SLAC Laboratory Animal, Shanghai, China) weighing 180 to 220 g were used in our study. All rats (n = 12/group) were randomly distributed into three groups: Tan IIA, control, and sham. The rats were housed with a 12-h light/dark cycle and standard diet and water. They were approved by the Ethical Committee of Researches of Nanjing University and were approved by the Committee on the Use and Care of Animals of Medical School of Nanjing University.

2.1.1. Experimental groups and AAA model

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2.2. Drug administration

From the findings of previous reports and experiments, 2 mg Tan IIA with 0.4 mL saline (Shanghai No.1 Biochemical & Pharmaceutical, Shanghai, China) was given to each rat daily by intraperitoneal injection in the Tan IIA group (n = 12) [20]. Tan IIA injection was started 1 d before elastase perfusion until the rats were killed. The same volume of saline was given to the sham (n = 12) and control (n = 12) groups for the same period by intraperitoneal injection.

2.3. Measurement of aortic size using ultrasonography

An ultrasound system (SonoSite, Bothell, WA) containing a linear transducer (25 MHz) was used to demonstrate the dilation of the rat aortas. We measured the maximum inner luminal diameter of aortas. The aortic size was measured before laparotomy and 5, 12, 18, and 24 d after. Two experienced operators who were unaware of the protocols performed the quantitative analysis of the ultrasound data.

2.4. Histologic studies

All rats were killed 24 d after the operation. The excised aorta was fixed in 10% neutral-buffered formalin and processed using routine paraffin embedding. Aortic tissue cross-sections (5 μm) were stained with hematoxylin and eosin and Miller’s elastin-Van Gieson according to standard procedures. The percentage of the surface area occupied by the elastin-Van Gieson–stained elastic fibers was quantified using the morphometry system MacScope, version 2.2 (Mitani, Tokyo, Japan).

2.5. Immunohistochemical staining

Mouse monoclonal antibodies for matrix metalloproteinase (MMP)-9 (ab58803, Abcam, Cambridge, MA) and MMP-2 (ab3518, Abcam) were used to analyze the local expression of matrix-degrading proteinases in the aorta walls 24 d after operation. Also, mouse monoclonal antibody for α-smooth muscle cell (SMC) actin (ab7817, Abcam) was used to analyze the depletion of medial SMCs. Immunohistochemical staining was performed using an immunoperoxidase avidin-biotin complex system. After blocking the activity of endogenous peroxidase, the sections were incubated in the primary antibodies (1:100) overnight at 4 °C. Next, according to the manufacturer’s specifications (Vectorstain Elite ABC Kit, Vector Laboratories, Burlingame, CA), we incubated the sections with biotinylated anti-mouse IgG antibody for 30 min (Vector
2.6. Quantitative real-time reverse transcriptase-polymerase chain reaction

The expression of MMP-2 and MMP-9 mRNA in the aortic tissues was determined using quantitative real-time reverse transcriptase-polymerase chain reaction (PCR). Total mRNA was extracted from the aorta using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA produced by reverse transcription using oligo-(dT) primer 3’ and 0.2% Tween-20 at room temperature. The membranes were transfected onto polyvinylidene difluoride membranes at 300 mA, and incubated for 1 h in Tris-buffered saline, 5% nonfat milk, 0.05 M Tris, 0.15 M NaCl, and 0.05% Tween-20. The membranes were electrophoresed at 80 V, transferred in sodium dodecyl sulfate-polyacrylamide gel at 300 mA for 30 s at 72°C, and stained with Ponceau S. The membranes were visualized using an ECL plus chemiluminescent kit (Amersham Biosciences), according to the manufacturer’s instructions, and exposed to X-ray film (Kodak, Rochester, NY). The results for each sample were normalized to the concentration of β-actin mRNA. PCR amplification was performed under the following conditions: denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. Primers of MMP-2, MMP-9, and β-actin were designed according to published rat sequences, and the amplified fragments were 135 bp, 228 bp, and 136 bp, respectively. The primer sequences were as follows: MMP-2 primer (GenBank reference no. NM031054, 135 bp), sense primer: 5’-CTGTAACCTGAGTAG CAGTGTG-3’, antisense primer: 5’-CACGCTAGTCCGATTGTA-3’; MMP-9 primer (GenBank reference no. NM031055, 228 bp), sense primer: 5’-TTCAAGAGCGTCCGTATT-3’, antisense primer: 5’-CTCCTGAGCCTAGCCTCAATTA-3’; and β-actin primer (GenBank reference no. NM031144, 136 bp), sense primer: 5’-GCAGAAGGAG ATTACTGCCCCT-3’, antisense primer: 5’-GCTGATCCACATCTG CTGGGAT-3’.

2.7. Western blot analysis

Aortic tissues were obtained and homogenized 24 d after the operation, and the total proteins were extracted from the frozen aorta tissue. The samples (70 μg) were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel at 80 V, transferred onto polyvinylidene difluoride membranes for 300 mA, and incubated for 1 h in Tris-buffered saline, 5% nonfat milk, and 0.2% Tween-20 at room temperature. The membranes were then incubated for 24 h at 4°C with different antibodies, including mouse monoclonal antibodies for MMP-9 (1:2000 dilution; Abcam), MMP-2 (1:2000 dilution; Abcam), and inducible nitric oxide synthase (iNOS) (1:4000 dilution; Abcam), rabbit polyclonal antibody for monocyte chemoattractant protein-1 (MCP-1) (1:5000 dilution; Abcam). They were washed in Tris-buffered saline and 0.1% Tween-20 and then incubated for 2 h at room temperature with sheep antimouse IgG secondary antibody for MMP-2, MMP-9, and iNOS (1:5000; Amersham Biosciences, Uppsala, Sweden), and goat anti-rabbit IgG for MCP-1 (1:5000; Amersham Biosciences). The membranes were visualized using an ECL plus chemiluminescent kit (Amersham Biosciences), according to the manufacturer’s instructions, and exposed to X-ray film (Kodak, Rochester, NY). β-Actin levels were used to standardize protein loading. To quantify and compare the levels of proteins, the density of each band was measured using densitometry (Shimazu, Kyoto, Japan).

2.8. Statistical analysis

All values are presented as the mean ± standard error of the mean. Statistical analysis was performed using SPSS, version 16.0 (SPSS, Chicago, IL). Multiple comparisons were analyzed using one-way analysis of variance followed by Bonferroni’s post hoc test. The threshold for significance was P < 0.05.

3. Results

3.1. Blood pressure

At 24 d after intra-aortic perfusion, the systolic blood pressure in all three groups was not significantly different (Table 1). This result indicated that Tan IIA (2 mg/rat/d) did not affect the blood pressure (P > 0.05).

3.2. Development of elastase-induced AAAs

AAA was induced by elastase perfusion. Next, the aortic size was measured using ultrasonography before perfusion and 5, 12, 18, and 24 d afterward (Fig. 1A1, A2). Compared with the sham group, we found that the aortic size of the control group was significantly increased (P < 0.05; Fig. 1A3), which suggested that the AAA model was successfully established. However, the aortic size of the Tan II A group was obviously smaller than that of the control group (P < 0.05; Fig. 1A3). Therefore, Tan IIA inhibited the progression of the AAAs.

3.3. Histologic changes in aortic walls

The aortas of the rats were harvested after 24 d. On hematoxylin and eosin staining, it was found that the control group had a thinner aortic wall and more mural thrombus than other groups (Fig. 2A1, A2). Elastin-Van Gieson staining showed observable degeneration of elastic lamellae in the control group (control versus sham, P < 0.05; Fig. 2B1). In contrast, no remarkable degeneration of the medial elastic lamellae was seen in the Tan IIA or sham groups (Tan IIA versus control, P < 0.05; Fig. 2B2).

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Before administration (mm Hg)</th>
<th>24 d After perfusion (mm Hg)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (12)</td>
<td>113 ± 3</td>
<td>112 ± 3</td>
<td>0.304</td>
</tr>
<tr>
<td>Control (12)</td>
<td>113 ± 4</td>
<td>115 ± 3</td>
<td>0.287</td>
</tr>
<tr>
<td>Tan IIA (12)</td>
<td>112 ± 4</td>
<td>113 ± 4</td>
<td>0.226</td>
</tr>
</tbody>
</table>

P value

Tan IIA = tanshinone IIA.
3.4. Expression of MMP-2 and MMP-9

Activated MMP-2 and MMP-9 in the aortic walls were analyzed using immunohistochemical staining (Fig. 3). The strongest staining of the activated MMP-2 or MMP-9 subunits was detected in the control group (Fig. 3A1, A2). The staining was weaker in the Tan IIA group than in control group but stronger than in the sham group. Western blotting showed that both MMP-2 and MMP-9 within the aortic segment were overexpressed in the control group (control versus sham, $P < 0.05$; Fig. 3B2). However, the expression of MMP-2 and MMP-9 was significantly downregulated in the Tan IIA group (Tan IIA versus control, $P < 0.05$; Fig. 3B2). Compared with the sham group, the expression of MMP-9 in the Tan IIA group was not significantly different ($P = 0.165$; Fig. 3B2); however, the expression of MMP-2 was still significantly greater ($P < 0.05$; Fig. 3B2). The mRNA expression analysis by quantitative real-time reverse transcriptase-PCR had a similar result (control versus sham, $P < 0.05$; Tan IIA versus control, $P < 0.05$; Fig. 3C).
3.5. **Depletion of vascular SMCs**

Immunohistochemical staining revealed a marked depletion of medial vascular SMCs (VSMCs) in the control group but preservation of VSMCs in the Tan IIA group (Fig. 4).

3.6. **Production of MCP-1 and iNOS**

The expression of MCP-1 and iNOS in the aortic walls was increased in the control group after 24 d (control versus sham, \( P < 0.05 \); Fig. 5A2). However, they were obviously lower in Tan IIA group (Tan IIA versus control, \( P < 0.05 \); Fig. 5A2).

4. **Discussion**

Tan IIA is one of the major lipophilic components from *Salvia miltiorrhiza Bunge*, which has been used in Asia for hundreds of years. Because of the effects of anti-atherosclerosis and vasodilation, it has been widely used in the treatment of peripheral artery disease and the prevention of myocardial infarction. Recent experimental studies have demonstrated that Tan IIA has some other vascular protective effects, such as inhibiting the expression of MMPs [10,12], anti-inflammation [11,18,19], attenuating oxidative stress [14,15,19], and preventing the loss of VSMCs [12,13]. Thus, we supposed that Tan IIA could suppress the development of AAAs.
Fig. 3 — Effects of Tan IIA on expression of MMP-2 and MMP-9. (A1) Immunohistochemical staining for MMP-2 (40×) in aortic tissue; (A2) Immunohistochemical staining for MMP-9 (40×) in aortic tissue; (B1) Western blot results of MMP-2 and MMP-9 in aneurysm wall. (B2) Quantitative analysis of MMP-2 and MMP-9 expression by densitometry. (C) mRNA expression of MMP-2 and MMP-9 measured using real-time reverse transcriptase-PCR. All data displayed as mean ± standard error of mean for transcript levels for MMP-2 and MMP-9 as normalized to β-actin (n = 12). *P < 0.05 versus sham; **P < 0.05 versus control.
The elastase-induced AAA in our experiments is a standard small animal aneurysm model. Compared with other AAA-induced methods, it achieves pathologic characteristics similar to those of human AAAs [20]. The animal surgery was performed according to a previously published protocol, with some modifications [21]. Previous studies have indicated that AAA formation correlated with the pump pressure and elastase concentration [21]. We increased the perfusion pressure to 2 atm with an elastase concentration of 8.16 U/mL to shorten the period of anesthesia and allow the operator to suture the incision in the bifurcation of the abdominal aorta in time. These modifications resulted in successful AAA models.

AAA is a complex multifactorial disease with an unknown etiology. It occurs after the development of an inflammatory response, destruction of elastin and collagen, the loss of VSMCs, and other histologic changes [21–26]. Among these characteristics, chronic inflammation is prominent and could lead macrophages and lymphocytes to infiltrate into the outer aortic wall [27]. The recruited macrophages could secret MMPs and pro-inflammatory cytokines, contributing to the development of AAAs [28]. Increased expression of MMPs (especially MMP-2 and MMP-9) could promote elastin degradation, which is considered to cause the direct enlargement of AAA [24,29]. In addition, some of the pro-inflammatory cytokines such as MCP-1 could induce the migration of monocytes to the aneurysm walls [18]. These infiltrated monocytes would further secret MMPs and pro-inflammatory cytokines and continue to deteriorate the pathologic process [19,30]. Our results showed that MMP-2, MMP-9, and MCP-1 were highly expressed in the aortic walls of AAA rats. In accordance with previous studies of atherosclerotic and inflammatory animal models, our studies showed that, along with inhibiting the overexpression of MMP-2, MMP-9, and MCP-1, Tan IIA could attenuate the inflammatory response and downregulate the expression of MMP-2 and MMP-9 in an AAA model.

Apoptosis of VSMCs is another major pathologic feature of AAA. It impairs the reparation for connective tissues and reduces the capacity for anti-pressure. The loss of VSMCs is generally caused by two mechanisms, one associated with inflammation. Previous research has shown that Fas ligand and inflammatory cytokines (e.g., interleukin-6) from infiltrated inflammatory cells play a crucial role in the depletion of medial VSMCs [21,31]. The other mechanism is related to the activation of nuclear factor-κB (NF-κB) caused by oxidative stress [32]. In our study, the loss of VSMCs in the control group was observed by immunohistochemical staining. Some investigators have reported that Tan IIA was able to inhibit the migration of human aortic SMCs and the proliferation of VSMCs [12,13]. Our study has demonstrated that Tan IIA treatment can preserve the medial level of VSMCs in the rat aortic wall. Therefore, it might inhibit the development of AAAs by preventing the loss of VSMCs.

Fig. 4 – Effects of Tan IIA on the preservation of VSMCs. Immunohistochemical staining for α-SMC actin in aorta tissues (400×).

Fig. 5 – (A1) Western blot results of MCP-1 and iNOS expression. (A2) Quantitative analysis of MCP-1 and iNOS expression by densitometry (n = 12 per group, *P < 0.05 versus sham, **P < 0.05 versus control).
and animal models [19,33]. Previous studies have suggested that nitric oxide synthesized from iNOS can damage the arterial wall by producing reactive oxidative stress. Thus, the expression of iNOS could promote oxidative vascular injury in AAAs [34—36]. According to our study, the elastase-induced AAAs had overexpressed iNOS, and the Tan IIA-treated rats did not share this phenomenon. Thus, it can be inferred that Tan IIA might inhibit the development of AAAs by downregulating the expression of iNOS and attenuating oxidative stress.

5. Conclusions

We found that Tan IIA could significantly inhibit the expansion of elastase-induced experimental AAAs. The possible mechanisms include downregulating the expression of MMP-2, MMP-9, and MCP-1, preventing the loss of VSMCs, suppressing the infiltration of monocytes, and decreasing oxidative stress by inhibiting iNOS synthesis. Our findings have shown that it could be a new potential drug for AAA therapy.

Acknowledgment

The authors thank Dr. Wei Wang and Zhu Cheng-yan for technical assistance with the duplex ultrasound examination of the rat aorta. This work was supported by grants from Nature Science Foundation of Jiangsu Province, China (grant BK2009035).

References


