Effect of Sini decoction on angiotensin II, transforming growth factor β, and connective tissue growth factor in rats with myocardial fibrosis-induced banding of the abdominal aorta

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Abstract

OBJECTIVE: To investigate the effect of Sini decoction on rats with myocardial fibrosis induced by banding the abdominal aorta, and explore the mechanism underlying its actions on angiotensin II (Ang II), transforming growth factor-β (TGF-β), and connective tissue growth factor (CTGF).

METHODS: Forty-eight male Sprague-Dawley rats were randomly divided into sham operation, model, Captopril, and Sini decoction groups. The models were established by the partial banding of the abdominal aorta according to Doering's method. Eight weeks later, heart weight indexes were calculated; hemodynamic changes of the hearts were tested; changes in myocardial tissue morphology were observed by Masson staining; and myocardial collagen volume fraction was calculated. Enzyme-linked immunosorbent assay was used to measure the concentration of Ang II in serum. The expression of TGF-β, and CTGF were determined by immunohistochemistry and Western blotting.

RESULTS: Compared with the sham operation group, the heart weight index, collagen volume fraction of the myocardium, serum levels of Ang II, and the expression of myocardial TGF-β, and CTGF in the model group were significantly increased (P < 0.05). Compared with the model group, the heart weight index, collagen volume fraction of the myocardium, serum levels of Ang II, and the expression of myocardial TGF-β, and CTGF in all treatment groups were significantly reduced (P < 0.05).

CONCLUSION: Sini decoction reduced Ang II level and inhibited the expression of myocardial TGF-β, and CTGF, which may explain the mechanism of its protective effect on myocardium with fibrosis.

Keywords: Myocardial fibrosis; Angiotensin II; Transforming growth factor beta1; Connective tissue growth factor; Sini decoction

INTRODUCTION

Myocardial fibrosis is characterized by interstitial fibroblast proliferation and excessive extracellular matrix deposition, and leads to heart failure, arrhythmia, sudden cardiac death and other serious complications. Long-term pressure overload induces ventricular remodeling, including myocyte hypertrophy and interstitial fibrosis. Myocardial fibrosis is one of the most important factors contributing to the transition from compensatory ventricular hypertrophy to heart failure.
Therefore, inhibiting the process of myocardial fibrosis is a crucial issue in clinical treatment. Sini decoction is described in the "Treatise on Febrile Diseases" and is thought to revive the Yang based on the theory of Traditional Chinese Medicine (TCM). A study suggested the Sini decoction prevents myocardial ischemia, protects myocardial cells, antagonizes atherosclerosis, and reduces neointimal proliferation after vascular injury. Our study investigated the Sini decoction efficacy on rats with myocardial fibrosis induced by banding abdominal aorta according to Doering's method, and the possible mechanism involved.

**MATERIALS AND METHODS**

**Drugs and reagents**
The Sini decoction was prepared with a Chinese medicinal formula consisting of Fuzi (Radix Aconiti Carminchaeli) [130801], Ganjiang (Rhizoma Zingiberis) [121201], and Gancao (Radix Glycyrrhizae) [130501], using granules free of frying (Beijing Kang Ren Tang Pharmaceutical Co., Ltd., Beijing, China). Other reagents were Captopril Tablets (20130910, Shanghai Xudong Haipu Pharmaceutical Co., Ltd., Shanghai, China); Ang II Kit (Biotechnology Research Institute of Beijing North, Beijing, China); rabbit anti-TGF-β1 polyclonal antibody (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China); diaminobenzidine (DAB) horseradish peroxidase color development kit (Beyotime Biotechnology Research Institute, Beijing, China); and modified Masson’s trichrome staining kit (Beijing Leagene Biotechnology Co., Ltd., Beijing, China). Urethane was obtained from Shanghai Yika Biological Technology Co., Ltd., Shanghai, China. A silver clip with a diameter of 0.7 mm was made by Shanghai Alcott Biotechnology Co., Ltd., Shanghai, China.

**Animals**
Forty-eight male Sprague-Dawley rats [8 weeks old; (200 ± 20) g] of Specific Pathogen Free grade were purchased from Liaoning Changle Biotechnology Limited Company [Certificate of quality: SCXK (liao) 2010-0001]. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animal ethics committee of Jinzhou Medical University. All rats were maintained at 22-24 °C with a relative humidity of 50% and normal photoperiods (12 h light/12 h dark). The rats were fed a normal diet and allowed access to water ad libitum in the Laboratory of the First Affiliated Hospital of Jinzhou Medical University.

**Animal modeling and grouping**
Overall, 48 rats were randomly divided into four groups using a random number table as follows: sham operation group, model group, Captopril group, and Sini decoction group with 12 rats per group. The models were established by the partial banding of the abdominal aorta according to Doering’s method. The rats were anaesthetized by intraperitoneal injection with 10% chloral hydrate (3 mL/kg body weight), fixed in an operating frame, shaved and sterilized. Then, the abdominal cavity was opened at 1 cm below the left costal margin, the retroperitoneal soft tissues were separated at the upper edge of the left kidney to expose the abdominal aorta, which was separated from both kidneys. Next, the abdominal aortas were banded with silver clip (0.7 mm diameter), and finally the abdominal cavities were closed in layers. In the sham operation group, the abdominal cavities were only opened and separated from abdominal aortas without constriction. Three days later, all rats were given an intramuscular injection of gentamicin (0.09 mg/kg) to prevent infection, once a day.

**Treatments**
The fourth day after the operation, the sham operation group and model group were orally administered with distilled water (1.5 mL/100 g), the Captopril group was perfused with captopril (100 mg/kg) dissolved in distilled water, and the Sini Decoction group was administered with 3.8 g/kg Sini Decoction [Fuzi (Radix Aconiti Carminchaeli) : Ganjiang (Rhizoma Zingiberis) : Gancao (Radix Glycyrrhizae) = 5 : 3 : 2], containing granules free of frying (based on a human and animal body surface area ratio, this dose was 5 times that of a clinical dose, equivalent to the pharmacological medium dose). All rats were perfused once a day for 8 weeks.

**Hemodynamic measurements**
After 8 weeks, all rats were anesthetized by intraperitoneal injection of 20% urethane (5 mL/kg). The right common carotid artery was separated, and the distal end was ligated. A v-type cut was made at the proximal part and a polyethylene tube of 1 mm diameter connected to the pressure transducer was dipped in liquid paraffin and sent to the left ventricle. Then, the left ventricular pressure curve was determined. The signal of the left ventricular pressure curve was input into a BL-420S biological signal collecting and processing system. The left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP) and maximum rates of rise and decline in left ventricular pressure (± dp/dtmax) were recorded.

**Determination of weight index**
After measuring the cardiac function, the chest was opened and the heart was removed. Then, the residual root of the large vessel was cut, the heart was placed in saline to remove the blood, and then put on filter pa-
per to soak up the water. The heart was then weighed to obtain the heart weight (HW), and the atria and large vessels were removed along the atrioventricular junction. The right ventricular wall was removed, the left ventricle was separated and weighed and the left ventricle weight (LVW), heart weight index (HWI = HW/BW, mg/g, BW: body weight), and left ventricular weight index (LVWI = LVW/BW, mg/g, LVW: ventricle weight) were calculated. Finally, the apex heart tissue was put in 4% paraformaldehyde for immunohistochemistry and optical microscope tests, and the rest was frozen at −80 °C for Western blotting.

**Observation of pathological histology**

Myocardial tissue was put in 4% paraformaldehyde for fixation, dehydrated, made transparent and dipped in paraffin for embedding. Serial coronal sections of the myocardial tissue were cut at 4 μm intervals and stained by masson staining. Sections were then mounted with Neutral Balsam, and pathomorphological changes of the myocardium were observed by optical microscopy. Ten sections were randomly selected from each group and 5 fields were randomly selected from each section. The collagen volume fraction (CVF) was measured by an image analyzer.

**Immunohistochemical staining**

The heart sections were deparaffinized and degraded in distilled water. Antigens were unmasked in 0.01 M Citrate Buffer (Sun Biomedical Technology Co., Ltd., China) by microwave, and endogenous peroxidase activity was quenched by 3% hydrogen peroxide (Sun Biomedical Technology Co., Ltd., China) for 15 min at room temperature. The sections were incubated with the primary antibody in humidified boxes at 4 °C overnight (TGF-β, 1: 300; CTGF 1: 500) at 4 °C overnight. After washing with PBS containing 0.5% Tween 20 (PBST) (Sun Biomedical Technology Co., Ltd., China) 3 times, the membranes were incubated at room temperature for 1 h with secondary antibody at 1 : 5000 dilution on a shaker. Bound peroxidase was detected using ECL. Expression levels of TGF-β, and CTGF were measured following the instructions of ImageJ Software.

**Western blotting**

The myocardial tissue was placed in an EP tube, the lysis solution added and the homogenates centrifuged at 4 °C for 20 min before collection. Protein levels in the supernatants were determined by the Bicinchoninic acid (BCA) method. The protein extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Laemmli discontinuous buffer system. Proteins were electro-blotted onto a polyvinylidene difluoride (PVDF) membrane, and then incubated with primary antibody (TGF-β, 1: 300; CTGF 1: 500) at 4 °C overnight. After washing with PBS containing 0.5% Tween 20 (PBST) (Sun Biomedical Technology Co., Ltd., China) 3 times, the membranes were incubated at room temperature for 1 h with secondary antibody at 1 : 5000 dilution on a shaker. Bound peroxidase was detected using ECL. Expression levels of TGF-β, and CTGF were measured following the instructions of ImageJ Software.

**Statistical analysis**

Data were processed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All continuous data were expressed as the mean ± standard deviation ( x ± s ). Data were tested for normality and homogeneity of variance. Differences between groups were tested with one-way analysis of variance, and post hoc analysis was conducted with the least significant difference test. P < 0.05 was considered statistically significant.

**RESULTS**

In the process of modeling, one rat in the sham operation group died of intestinal obstruction, one in the Captopril group died of wound dehiscence, rats in the model group had slow responses and thin hair without luster. The symptoms of rats in the model group gradually worsened and two rats in the model group died. The mental state and activity change in all treatment groups were less than in the model group, while rats in the Captopril group had various degrees of cough. In addition, compared with the Captopril group, rats in the Sini decoction group had thick and shiny hair without cough. At the end of the eighth week, 44 rats had survived.

**Hemodynamic and weight index changes**

Table 1 shows the hemodynamic and weight index changes in all groups after 8 weeks. The LVSP and ± dp/dtmax levels in the model group were significantly lower than in the sham operation group, and the LVEDP, HWI and LVWI levels was significantly higher than sham operation group ( P < 0.05). Compared with the model group, the LVSP and ± dp/dtmax levels were significantly increased but the LVEDP, HWI and LVWI levels were significantly decreased in the other treatment groups ( P < 0.05).
Immunohistochemical staining of TGF-β1
Expression of TGF-β1 and CTGF in myocardium (Table 1).

The mean CVF in the model group was higher than in the sham operation group (P < 0.05), and compared with the model group, the mean CVF level was significantly decreased in all treatment groups (P < 0.05) (Table 2).

Histopathological changes of cardiac muscle
Masson staining showed blue collagen fibers (arrow), and red myocardial cells and fibers. Collagen fibers were mainly distributed around blood vessels and there were few among myocardial cells in the sham operation group, while collagen fibers around blood vessels were thicker. A large number of collagen fibers among the myocardial cells were disordered in the model group. Compared with the model group, numbers of collagen fibers were significantly decreased in all treatment groups (Figure 1).

Table 1 Hemodynamics and weight index changes in all groups (\( \bar{x} \pm s, n=10 \))

<table>
<thead>
<tr>
<th>Group</th>
<th>LVSP (mm Hg)</th>
<th>LVEDP (mm Hg)</th>
<th>+dp/dtmax (mm Hg)</th>
<th>-dp/dtmax (mm Hg)</th>
<th>HWI (mg/g)</th>
<th>LVWI (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>125.69±13.96</td>
<td>4.75±1.81</td>
<td>3679.10±376.24</td>
<td>3323.20±332.19</td>
<td>2.61±0.29</td>
<td>2.17±0.19</td>
</tr>
<tr>
<td>Model</td>
<td>85.30±17.05</td>
<td>10.92±2.96</td>
<td>2381.90±369.94</td>
<td>1987.50±315.06</td>
<td>3.74±0.49</td>
<td>2.82±0.35</td>
</tr>
<tr>
<td>Captopril</td>
<td>105.89±15.24</td>
<td>7.47±1.88</td>
<td>2990.10±355.24</td>
<td>2557.60±334.38</td>
<td>2.99±0.36</td>
<td>2.43±0.24</td>
</tr>
<tr>
<td>Sini</td>
<td>105.05±17.20</td>
<td>7.65±1.88</td>
<td>2827.90±389.51</td>
<td>2480.00±385.85</td>
<td>3.18±0.39</td>
<td>2.49±0.30</td>
</tr>
</tbody>
</table>

Notes: sham group was treated with distilled water (1.5 mL/100 g). Model group was treated with distilled water (1.5 mL/100 g). Captopril group was treated with Captopril (100 mg/kg) dissolved in distilled water. Sini group was treated with Sini decoction. *P < 0.05 compared with sham group; †P < 0.05 compared with model group; ‡P > 0.05 compared with Captopril group.

Ang II in serum
The mean Ang II level in the model group was higher than that in the sham operation group (P < 0.05), and compared with the model group, the mean Ang II level was significantly decreased in all treatment groups (P < 0.05) (Table 2).

Table 2 Ang II and CVF levels in all groups (\( \bar{x} \pm s, n=10 \))

<table>
<thead>
<tr>
<th>Group</th>
<th>Ang II (pg/mL)</th>
<th>CVF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>79.8±3.2</td>
<td>4.4±0.9</td>
</tr>
<tr>
<td>Model</td>
<td>103.5±5.1†</td>
<td>14.8±1.0‡</td>
</tr>
<tr>
<td>Captopril</td>
<td>88.6±4.8§</td>
<td>8.2±0.6§</td>
</tr>
<tr>
<td>Sini</td>
<td>85.3±4.0§</td>
<td>8.0±0.8§</td>
</tr>
</tbody>
</table>

Notes: sham group was treated with distilled water (1.5 mL/100 g). Model group was treated with distilled water (1.5 mL/100 g). Captopril group was treated with Captopril (100 mg/kg) dissolved in distilled water. Sini group was treated with Sini decoction. *P < 0.05 compared with sham group; †P < 0.05 compared with model group; ‡P > 0.05 compared with Captopril group. Ang II: angiotensin II; CVF: collagen volume fraction.

Histopathological changes of cardiac muscle
Masson staining showed blue collagen fibers (arrow), and red myocardial cells and fibers. Collagen fibers were mainly distributed around blood vessels and there were few among myocardial cells in the sham operation group, while collagen fibers around blood vessels were thicker. A large number of collagen fibers among the myocardial cells were disordered in the model group. Compared with the model group, numbers of collagen fibers were significantly decreased in all treatment groups (Figure 1).

Expression of TGF-β1 and CTGF in myocardium
Immunohistochemical staining of TGF-β1 and CTGF are shown in Figures 2 and 3, respectively. Brown granules (arrow) were positively expressed. The expression levels of TGF-β1 and CTGF in the model group were significantly lower than in the sham operation group (P < 0.05). Compared with the model group, the expression levels of TGF-β1 and CTGF were significantly decreased in all treatment groups (P < 0.05). The integrated optical density (IOD) levels of TGF-β1 and CTGF are shown in Table 3.

Western blotting of TGF-β1 and CTGF
Western blotting of TGF-β1 and CTGF are shown in
Figure 4. The expression levels of TGF-β and CTGF in the model group were significantly higher than in the sham operation group (P < 0.05). Compared with the model group, the expression levels of TGF-β and CTGF were significantly decreased in all treatment groups (P < 0.05). The RD value is the ratio of the expression levels of TGF-β and CTGF to β-actin.

Figure 3 Immunohistochemical staining of CTGF in rat myocardial tissues
A: sham operation group; B: model group; C: Captopril group; D: Sini decoction group (× 400). Sham group was treated with distilled water (1.5 mL/100 g). Model group was treated with distilled water (1.5 mL/100 g). Captopril group was treated with Captopril (100 mg/kg) dissolved in distilled water. Sini group was treated with Sini decoction.

CTGF: connective tissue growth factor.

Table 3IOD values of TGF-β and CTGF in rat myocardial tissues (x ± s, n = 10)

<table>
<thead>
<tr>
<th>Group</th>
<th>TGF-β</th>
<th>CTGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>36±7</td>
<td>51±11</td>
</tr>
<tr>
<td>Model</td>
<td>85±14†</td>
<td>181±25†</td>
</tr>
<tr>
<td>Captopril</td>
<td>51±10†</td>
<td>90±16†</td>
</tr>
<tr>
<td>Sini</td>
<td>50±11±</td>
<td>92±17±</td>
</tr>
</tbody>
</table>

Notes: sham group was treated with distilled water (1.5 mL/100 g). Model group was treated with distilled water (1.5 mL/100 g). Captopril group was treated with Captopril (100 mg/kg) dissolved in distilled water. Sini group was treated with Sini decoction. TGF-β: transforming growth factor-β; CTGF: connective tissue growth factor.

Table 4 TGF-β and CTGF expression levels of rat myocardial tissues (x ± s, n = 10)

<table>
<thead>
<tr>
<th>Group</th>
<th>TGF-β (× 10^6)</th>
<th>RD (TGF-β)</th>
<th>CTGF (× 10^6)</th>
<th>RD (CTGF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.24±0.318</td>
<td>0.152±0.038</td>
<td>0.828±0.140</td>
<td>0.10±0.017</td>
</tr>
<tr>
<td>Model</td>
<td>2.95±0.707</td>
<td>0.370±0.090</td>
<td>3.65±0.300</td>
<td>0.46±0.038</td>
</tr>
<tr>
<td>Captopril</td>
<td>2.09±0.282</td>
<td>0.257±0.034</td>
<td>1.79±0.231</td>
<td>0.22±0.028</td>
</tr>
<tr>
<td>Sini</td>
<td>1.997±0.299</td>
<td>0.246±0.037</td>
<td>1.802±0.271</td>
<td>0.22±0.033</td>
</tr>
</tbody>
</table>

Notes: sham group was treated with distilled water (1.5 mL/100 g). Model group was treated with distilled water (1.5 mL/100 g). Captopril group was treated with Captopril (100 mg/kg) dissolved in distilled water. Sini group was treated with Sini decoction. TGF-β: transforming growth factor-β; CTGF: connective tissue growth factor.

DISCUSSION

The current study reveals that Sini decoction significantly improved the left ventricular diastolic function and that the energy metabolism of ischemia myocardium induced by acute myocardial ischemia protected myocardial cells through the inhibition of apoptosis and stabilization of mitochondria, to prevent heart failure. Moreover, Sini decoction downregulated the expression of TGF-β and inhibited myocardial fibrosis induced by isoproterenol in rats. This study constricted the abdominal aorta of rats to model myocardial fibrosis, which is the last stage of heart failure. In rats, after-loading of the heart was increased, hemodynamic parameters were changed, and the renin angiotensin aldosterone system was activated, consistent with clinical pathological changes in pressure overload induced during heart failure. These features are also in line with the pathogenesis of Yang deficiency and water overflow in Traditional Chinese Medicine.

The experimental results suggest that the area of myocardial collagen was significantly increased, cardiac function was decreased, Ang II levels were increased, and the expressions of TGF-β and CTGF were increased in the model group. Treatment with captopril and Sini decoction reduced the myocardial collagen area, improved cardiac function, decreased Ang II levels, and decreased TGF-β and CTGF expressions. This indicated that the treatments reduced Ang II levels, and downregulated the expressions of TGF-β and CTGF to resist myocardial fibrosis. There was no significant
difference in benefit between the Captopril and Sini decoction groups. However, compared with the Captopril group, rats in Sini decoction group had thick and shiny hair without cough symptoms. In conclusion, pressure overload leads to myocardial fibrosis, which might be closely associated with elevated Ang II levels, and increased expressions of TGF-β and CTGF. Sini decoction alleviates myocardial fibrosis and the mechanism probably involves a reduction in the levels of Ang II and decreasing the expressions of TGF-β and CTGF.

REFERENCES