Effect of chrysanthemum extract on myocardial fibrosis in rats with renovascular hypertension

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Abstract

OBJECTIVE: To investigate the inhibitory effect of chrysanthemum extract on myocardial fibrosis in rats with renovascular hypertension, and explore the possible mechanism underlying this effect.

METHODS: Sixty Wistar rats were randomly divided into six groups: sham operation, model, positive control, and low-, medium-, and high-dose Huai chrysanthemum extract groups (ten rats per group). With the exception of the sham operation group, a renal hypertensive model was established in rats using the “two-kidney, one clip” method. After 6 weeks, low-, medium-, and high-dose groups were intragastrically administered chrysanthemum extract at 1, 2, or 4 g/kg, respectively, once daily for 4 weeks. The positive control group was administered Kato Purly at 50 mg/kg once daily for 4 weeks, while sham operation and model groups received an equal volume of distilled water once daily for 4 weeks. Blood pressure changes were examined before modeling, 6 weeks after modeling, and after 4 weeks of treatment administration. Ventricular remodeling indexes were measured by high frequency echocardiography after 4 weeks of treatment administration. Pathological changes were observed by hematoxylin and eosin, and Masson’s trichrome staining methods. Collagen type I (Col I) and type III (Col III) expression were examined by enzyme-linked immunosorbent assays. Transforming growth factor-β1 (TGF-β1), sma mad 3 (Smad3), Smad7, Ras homolog gene family, member A (RhoA), and Rho-associated protein kinase 1 (ROCK1) protein expression were detected by Western blot.

RESULTS: Compared with the model group, chrysanthemum-administered groups and the positive control group showed significant improvement of arterial blood pressure, echocardiography indicators, and degree of myocardial fibrosis (P < 0.05). In addition, these groups exhibited decreased expression of Col I, Col III, RhoA, ROCK1, TGF-β1, and Smad3, and increased Smad7 expression. Such improvements were most obvious in the high-dose chrysanthemum extract group (P < 0.05).

CONCLUSION: Chrysanthemum extract could effectively reduce myocardial fibrosis in rats with renovascular hypertension by a mechanism that potentially involves inhibition of RhoA/ROCK1 and TGF-β1/Smad signaling pathways.

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Keywords: Chrysanthemum; Hypertension, renovascular; Fibrosis; Ventricular remodeling; Transforming growth factor beta

INTRODUCTION
Huai chrysanthemum, one of the four main medicinal herbs in Henan, is the ancestor of medicinal chrysanthemum and has a long history of cultivation. In particular, Huai chrysanthemum has been widely applied in the clinic for its functions of "smoothing liver and improving eyesight and dispelling wind and dissipating heat". At present, domestic and foreign research on the chemical constituents of Huai chrysanthemum mainly concentrate on its volatile oil, flavonoid, amino acid, trace element, and organic acid components. Modern pharmacology has confirmed that chrysanthemum extract has ideal antihypertensive effects; can increase superoxide dismutase expression in heart, brain, kidney, and other tissues of hypertensive rats; and can reduce vascular endothelial damage. According to Traditional Chinese Medicine, the root cause of hypertension exists in the kidney, while the current problem appears in the liver. The main symptoms of hypertension are hyperactivity of liver-Yin, or deficiency of liver-Yin and kidney-Yin. However, some hypertension patients are overlooked because of their subtle clinical symptoms, which may result in adverse consequences. As hypertension affects over 200 million people, it has become a public health concern. Further, elevated blood pressure is a trigger for many diseases and increases the risk for coronary heart disease, heart failure, and kidney disease. Moreover, studies have confirmed that long-term hypertension can cause myocardial fibrosis, reduce myocardial compliance, affect heart function, and in severe cases initiate the occurrence of heart failure. Chrysanthemum extract has been shown to elicit good antihypertensive effects, but its effect on myocardial fibrosis caused by hypertension is unknown. As such, this study prepared a renovascular hypertensive rat model by the "two-kidney, one clip" method to observe the effects of Huai chrysanthemum extract on ventricular remodeling, myocardial fibrosis, and ras homolog gene family, member A (RhoA) / rho-associated protein kinase 1 (ROCK1) and transforming growth factor-beta 1 (TGF-beta 1) / smad (Smad) signaling pathways in renal hypertensive rats.

MATERIALS AND METHODS

Drugs and preparation
Huai chrysanthemum (Qinhai Medicine Research Institute, Wujie, Taiwan) was smashed, screened over 60 mesh, and soaked in ethanol solvent for 30 min (material: liquid = 1:100). After heating to 80 degrees, the mixture was refluxed for 2 h and extracted for 3 times. Extracts were combined and stored at 4°C.

Reagents
Enzyme-linked immunosorbent assay (ELISA) kits for collagen type I (Col I, No. AB82354) and type III (Col III, No. AB82362) were purchased from Xinbosheeng (Yiwu, China). Masson Staining Kit (No. PT0003) was from Bogu (Shanghai, China). Antibodies included rabbit anti-ROCK1 (No. 21850-1-AP, Proteintech, Rosemont, IL, USA), rabbit anti-RhoA (WL01216, Wanle Biotechnology, Shenyang, China), anti-TGF-B1 (No. 34710C, Abcam, Cambridge, UK), and anti-Smad3 (No. AE99293Rag) and anti-Smad7 (No. AE99297Rag) from Bioworld Technology (St. Louis Park, MN, USA).

Instruments
A VIVID E9 color doppler ultrasound (GE Healthcare, Chicago, IL, USA), which is used in clinical practice and small animal testing, was employed with an ML6-15 superficial probe (GE Healthcare). A BX43 optical microscope (Olympus, Tokyo, Japan), WD-9405B horizontal rocking bed (LiuYi, Beijing, China), WD-9413B gel imaging system (LiuYi), Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Hercules, CA, USA), and VE-180 vertical plate electrophoretic device (Tian Energy Technology, Shanghai, China) were also used.

Establishment of renal hypertensive rat model
The rat abdominal cavity was fully exposed through a mid-abdominal incision. The left renal artery was bluntly dissected along the renal vein. Next, an acupuncture needle (0.25-mm diameter) was placed parallel to the long axis of the renal artery, and then ligated along the surgical line. Subsequent removal of the acupuncture needle formed a unilateral renal artery stenosis. In the sham operation group, the same operation was performed without placement of the acupuncture needle and blood vessel ligation. After surgery, penicillin sodium (3 × 10⁴ U/d) was injected to prevent infection, and animals were allowed to eat and drink normally.

Experimental animals and groups
Sixty adult Wistar male rats, weighing (220 ± 15) g, were purchased from Henan Traditional Chinese Medicine College Experimental Animal Center (China), clean grade (Grade II). Rats were maintained at an appropriate temperature (22 °C) in a well-ventilated laboratory with ample light (14 h/d), suitable humidity (40%-55%), and routine diet. After 1 week of feeding, rats were randomly divided into six groups: sham operation, model, positive control, and low-, medium-, and high-dose chrysanthemum extract groups. With the exception of the sham operation group, the renal hypertensive model was established in rats using the "two-kidney, one clip" method. Six weeks after surgery, rats whose blood pressure increased to 20 kPa were identified as successfully modeled. After 6 weeks, low-

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medium-, and high-dose chrysanthemum extract groups were intragastrically administered 1, 2, or 4 g/kg chrysanthemum extract, respectively, once daily for 4 weeks. The positive control group was administered Kato Pury at 50 mg/kg once daily for 4 weeks, while sham operation and model groups received an equal volume of distilled water once daily for 4 weeks.

**Blood pressure measurement**

Rats were placed in a familiar environment for measurement of systolic blood pressure in tail arteries using a noninvasive blood pressure meter. Each rat was measured three times and the average value was obtained. Blood pressure was measured before modeling, 6 weeks after modeling, and 4 weeks after treatment administration.

**Cardiac echocardiography**

After 4 weeks of treatment administration, ventricular remodeling indexes were measured by high-frequency transthoracic echocardiography with a GE Vivid E9 color doppler ultrasound and ML6-15 probe (13-MHz frequency, 3.5-cm image depth). Rats were anesthetized using an intraperitoneal injection of 10% chloral hydrate (0.2 g/kg). Under two-dimensional ultrasound guiding, M-ultrasound was used to determine the sternum of the left ventricular long-axis ventricular septal thickness (IVSd), left ventricular diastolic diameter (LVIDd), and end diastolic left ventricular posterior wall thickness (LVPWd). Each measurement value is reported as an average value of four consecutive cardiac cycles.

**Histology**

After echocardiography examination, rats were sacrificed immediately. Under aseptic conditions, the left rib of the rat was cut by ophthalmic scissors. After the heart was fully exposed, it was removed. A portion of the ventricular muscle tissue was retained and put into fixative solution (4% poly Formaldehyde Solution). After ethanol dehydration, paraffin embedding, serial sectioning, dewaxing, xylene processing for transparency, hematoxylin and eosin (HE), and Masson’s trichrome staining were performed to observe pathological changes of rat myocardium by light microscopy. The collagen volume fraction (CVF) of Masson staining images was analyzed with ImageJ software as: CVF = collagen area / total area. The remainder of ventricular muscle tissues were used to prepare tissue homogenates for detection of protein expression.

**ELISA and Western blotting**

ELISA assays were used to examine Col I and Col III expression in myocardial tissue according to the manufacturer’s instructions. After rats were sacrificed, the left ventricular myocardium was clipped and triturated using a cold grinding method to extract total protein, which was subsequently measured for concentration using a bicinechnonic acid method and prepared as a sample solution. Twenty microliters of each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, blocked with skimmed milk, and then incubated with primary antibody (anti-TGF-β1, anti-Smad3, anti-RhoA, anti-ROCK1) at a 1:1000 dilution overnight at 4 °C. The following day, membranes were washed, incubated with a secondary antibody at 1:1000 for 2 h at room temperature, washed again, processed with an enhanced chemiluminescence substrate, and imaged. Relative expression of the target protein was equal to the gray value of the target protein divided by the gray value of β-actin.

**Statistical analysis**

Data were processed by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation ( x̄ ± s ), and a paired t-test was conducted to examine statistically significant differences between groups. P < 0.05 was set as the significance level.

**RESULTS**

**Survival rate and model success rate of experimental rats**

The survival rate and model success rate of each group is shown in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Operation (n)</th>
<th>Operation success (n)</th>
<th>Model success (n)</th>
<th>Operation success rate (%)</th>
<th>Model success rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Model</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Low dose</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>90</td>
<td>70</td>
</tr>
<tr>
<td>Medium dose</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>90</td>
<td>70</td>
</tr>
<tr>
<td>High dose</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Positive control</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Notes: sham operation and model groups received an equal volume of distilled water, once daily for 4 weeks; low-dose group was administered chrysanthemum extract at 1 g/kg, once daily for 4 weeks; medium-dose group was administered chrysanthemum extract at 2 g/kg, once daily for 4 weeks; high-dose group was administered chrysanthemum extract at 4 g/kg, once daily for 4 weeks; the positive control group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks.
**Chrysanthemum extract reduced tail artery blood pressure in renal hypertensive rats**

Compared with the sham operation group, tail artery blood pressure of the model group was significantly increased \((P < 0.05)\). Compared with the model group, blood pressure was significantly decreased after treatment with low-, medium-, or high-dose chrysanthemum extract, and in the positive control group \((P < 0.05\), Table 2).

**Chrysanthemum extract alleviated anatomical changes of heart tissue**

Compared with the sham operation group, IVSd and LVPWd in the model group were significantly thickened, and LVIDd was significantly increased \((P < 0.05)\). Compared with the model group, the degree of thickening of IVSd and LVPWd was significantly alleviated, and LVIDd was significantly decreased in low-, medium-, and high-dose chrysanthemum extract groups, and in the positive control group \((P < 0.05\); Figure 1, Table 3).

**Chrysanthemum extract alleviated changes in the texture and appearance of heart tissue**

Sham operation group rats had no obvious abnormalities in heart appearance. In the model group, no significant changes were observed in the color of hearts, but the ventricles were obviously enlarged, and the texture of the ventricular muscle was hard with poor elasticity. The appearance of hearts in low-, medium-, and high-dose chrysanthemum extract groups, and the positive control group was improved with increased elasticity compared with the model group; although, hearts were still somewhat less flexible compared with the sham operation group.

**Chrysanthemum extract reduced histological changes and collagen disruption in heart tissue**

HE staining results were observed with light microscopy. Compared with the sham operation group, ventricular muscle fibers of model group rats were thickened, the intervals of cardiac muscle fibers were broadened, the nucleus arrangement was disturbed, and a large number of lymphocytic embellish phenomena could be seen. However, in low-, medium-, high-dose chrysanthemum extract groups, and the positive control group, most myocardial fibers were arranged in an orderly manner, the intercellular space was closely arrayed, and only a few myocardial cells presented karyopyknosis.

**Table 2** Tail arterial blood pressure values of rats in each group (kPa, ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pre-modeling</th>
<th>Post-modeling</th>
<th>Post-therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>9</td>
<td>16.5±0.4</td>
<td>16.3±0.6</td>
<td>16.8±1.0</td>
</tr>
<tr>
<td>Model</td>
<td>8</td>
<td>16.5±1.8</td>
<td>22.5±1.3</td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>7</td>
<td>16.2±1.6</td>
<td>22.8±2.7</td>
<td></td>
</tr>
<tr>
<td>Medium dose</td>
<td>7</td>
<td>16.6±2.0</td>
<td>23.6±4.3</td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>9</td>
<td>16.4±1.5</td>
<td>24.0±3.4</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>8</td>
<td>15.9±1.3</td>
<td>23.5±2.4</td>
<td></td>
</tr>
</tbody>
</table>

Notes: sham operation and model groups received an equal volume of distilled water, once daily for 4 weeks; low-dose group was administered chrysanthemum extract at 1 g/kg, once daily for 4 weeks; medium-dose group was administered chrysanthemum extract at 2 g/kg, once daily for 4 weeks; high-dose group was administered chrysanthemum extract at 4 g/kg, once daily for 4 weeks; the positive control group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks. *P < 0.05, compared with the control group; **P < 0.05, compared with the model group.

Figure 1 Chrysanthemum extract alleviated anatomical changes of heart tissue

A: sham operation group; B: model group; C: low-dose group; D: medium-dose group; E: high-dose group; F: positive control group. Sham operation group and model group received an equal volume of distilled water, once daily for 4 weeks; low-dose group was administered chrysanthemum extract at 1 g/kg, once daily for 4 weeks; medium-dose group was administered chrysanthemum extract at 2 g/kg, once daily for 4 weeks; high-dose group was administered chrysanthemum extract at 4 g/kg, once daily for 4 weeks; the positive control group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks.
The expression of Col I and Col III levels were significantly decreased in low-, medium-, and high-dose chrysanthemum extract groups ($P < 0.05$, Table 4).

**Expression of RhoA, ROCK1, TGF-β1, Smad3, and Smad7 in ventricular muscle tissue of rats in each group**

Compared with the sham operation group, expression of RhoA, ROCK1, TGF-β1, and Smad3 in ventricular muscle of model group rats was significantly increased, while Smad7 expression was significantly decreased ($P < 0.05$). Compared with the model group, low-, medium-, and high-dose chrysanthemum extract groups exhibited significantly decreased expression of RhoA, ROCK1, TGF-β1, and Smad3, but significantly increased expression of Smad7 ($P < 0.05$, Figure 4, Table 5).

**DISCUSSION**

This study observed that chrysanthemum extract had a certain antihypertensive effect on hypertensive rats by measuring tail artery blood pressure. Moreover, chry-
Compared with the control group, the model group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks; low-dose group was administered chrysanthemum extract at 1 g/kg, once daily for 4 weeks; medium-dose group was administered chrysanthemum extract at 2 g/kg, once daily for 4 weeks; high-dose group was administered chrysanthemum extract at 4 g/kg, once daily for 4 weeks; the positive control group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks.

Table 4 Expression of Col I and Col III in ventricular muscle tissue of rats in each group (±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Col I</th>
<th>Col III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>9</td>
<td>74±9</td>
<td>51±7</td>
</tr>
<tr>
<td>Model</td>
<td>8</td>
<td>143±14</td>
<td>857±9</td>
</tr>
<tr>
<td>Low dose</td>
<td>7</td>
<td>114±10</td>
<td>80±6</td>
</tr>
<tr>
<td>Medium dose</td>
<td>7</td>
<td>97±8</td>
<td>69±8</td>
</tr>
<tr>
<td>High dose</td>
<td>9</td>
<td>83±5</td>
<td>60±6</td>
</tr>
<tr>
<td>Positive control</td>
<td>8</td>
<td>80±9</td>
<td>63±4</td>
</tr>
</tbody>
</table>

Notes: sham operation and model groups received an equal volume of distilled water, once daily for 4 weeks; low-dose group was administered chrysanthemum extract at 1 g/kg, once daily for 4 weeks; medium-dose group was administered chrysanthemum extract at 2 g/kg, once daily for 4 weeks; high-dose group was administered chrysanthemum extract at 4 g/kg, once daily for 4 weeks; the positive control group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks. Col: collagen. *P < 0.05, compared with the control group; **P < 0.05, compared with the model group.

Figure 3 Masson trichrome staining of ventricular muscle in rats from each group (light microscope, × 400)
A: sham operation group; B: model group; C: low-dose group; D: medium-dose group; E: high-dose group; F: positive control group. Sham operation and model groups received an equal volume of distilled water, once daily for 4 weeks; low-dose group was administered chrysanthemum extract at 1 g/kg, once daily for 4 weeks; medium-dose group was administered chrysanthemum extract at 2 g/kg, once daily for 4 weeks; high-dose group was administered chrysanthemum extract at 4 g/kg, once daily for 4 weeks; the positive control group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks.

Figure 4 Expression of RhoA, ROCK1, TGF-β1, Smad3, and Smad7 in ventricular muscle tissue of each group
A: sham operation group; B: model group; C: low-dose group; D: medium-dose group; E: high-dose group; F: positive control group. Sham operation and model groups received an equal volume of distilled water, once daily for 4 weeks; low-dose group was administered chrysanthemum extract at 1 g/kg, once daily for 4 weeks; medium-dose group was administered chrysanthemum extract at 2 g/kg, once daily for 4 weeks; high-dose group was administered chrysanthemum extract at 4 g/kg, once daily for 4 weeks; the positive control group was administered Kato Pury at 50 mg/kg, once daily for 4 weeks.

Chrysanthemum extract effectively improved ventricular hypertrophy of hypertensive rats, as confirmed by Doppler echocardiography, indicating that chrysanthemum extract can effectively inhibit the occurrence of ventricular remodeling.

Myocardial fibrosis refers to the abnormal deposition of collagen fibers in myocardial tissue, resulting in a marked increase in collagen concentration or CVF, which is an important cause of heart disease caused by hypertension. Indeed, it has been reported that myocardial tissue collagen content can be increased to 2-3 times normal levels, thus leading to abnormal ventricular filling or decline of ventricular compliance. Moreover, when collagen content was increased four-fold, cardiac ejection fraction was obviously decreased. Masson staining verified that the degree of myocardial fibrosis in hypertensive rats was significantly improved after treatment with chrysanthemum extract. Importantly, expression of collagen in the myocardium is an important index of myocardial fibrosis, which has the characteristics of thick collagen fibers, low elasticity, and strong anti-stretch, which is used to maintain the strength of the ventricular wall. Col III, which accounts for about 11% of total protein in the myocardium, has characteristics including thin fibers and high extensibility and elasticity, which are directly related to
the elasticity of ventricular walls. Our results showed that chrysanthemum extract can effectively reduce Col I and Col III expression in myocardial tissue of hypertensive rats, as was confirmed by the results of Masson trichrome staining, which explains the inhibitory effect of chrysanthemum extract on myocardial fibrosis in hypertensive rats from the point of protein expression. To explore the mechanism underlying the inhibitory effect of chrysanthemum extract on myocardial fibrosis of hypertensive rats, Western blotting was used to quantify expression of RhoA/ROCK and TGF-β1/Smad signaling pathway components in the myocardial tissue of rats in each group. The RhoA/ROCK signaling pathway, which acts as a molecular switch, is up-stream of many signaling pathways and participates in the pathophysiological processes of many cardiovascular diseases, such as cardiomyocyte apoptosis, myocardial hypertrophy, and ventricular remodeling. In addition, ROCK1 is a downstream kinase of RhoA, which can directly stimulate the differentiation of precursor cells into cardiac fibroblasts, and regulate the differentiation of fibroblasts into myofibroblasts, which more strongly secrete extracellular matrix.

The TGF-β1/Smad signaling pathway is closely related to myocardial fibrosis. Indeed, it has been confirmed that upregulation of TGF-β1 expression can promote Smad3 phosphorylation, increase the rate of TGF-β1/Smad signaling pathway by a mechanism that may involve inhibition of TGF-β1/Smad signaling. Thus, whether chrysanthemum extract can inhibit other fibrosis signaling pathways needs further clarification.

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