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Original Article

Shenmai Injection Improves Hypertensive Heart Failure by Inhibiting Myocardial Fibrosis via TGF-β1/Smad Pathway Regulation*

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ABSTRACT **Objective:** To study effects of Shenmai Injection on hypertensive heart failure and its mechanism for inhibiting myocardial fibrosis. **Methods:** Salt-sensitive (Dahl/SS) rats were fed with normal diet (0.3% NaCl) and the high-salt diet (8% NaCl) to observe the changes in blood pressure and heart function, as the control group and the model group. Salt-insensitive rats (SS-13BN) were fed with the high-salt diet (8% NaCl) as the negative control group. After modeling, the model rats were randomly divided into heart failure (HF) group, Shenmai Injection (SMI) group and pirfenidone (PFD) group by a random number table, with 6 rats in each group. They were given sterilized water, SMI and pirfenidone, respectively. Blood pressure, cardiac function, fibrosis and related molecular expression were detected by sphygmomanometer, echocardiogram, enzyme linked immunosorbent assay (ELISA), hematoxylin-eosin staining, Masson staining, immunofluorescence and qPCR analysis. **Results:** After high-salt feeding, compared with the control and negative control group, in the model group the blood pressure increased significantly, the left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were significantly reduced, and the serum NT-proBNP concentration increased significantly (all $P<0.05$); furthermore, the arrangement of myocardial cells was disordered, the edema was severe, and the degree of myocardial fibrosis was also significantly increased ($P<0.05$); the protein and mRNA expressions of collagen type Ⅰ (Col Ⅰ) were up-regulated ($P<0.05$), and the mRNA expressions of transforming growth factor β1 (TGF-β1), Smad2 and Smad3 were significantly up-regulated ($P<0.05$). Compared with HF group, after intervention of Shenmai Injection, LVEF and LVFS increased, myocardial morphology was improved, collagen volume fraction decreased significantly ($P<0.05$), and the mRNA expressions of Col Ⅰ, TGF-β1, Smad2 and Smad3, as well as Col Ⅰ protein expression, were all significantly down-regulated (all $P<0.05$). **Conclusion:** Myocardial fibrosis is the main pathological manifestation of hypertensive heart failure, and Shenmai Injection could inhibit myocardial fibrosis and effectively improve heart failure by regulating TGF-β1/Smad signaling pathway.

KEYWORDS myocardial fibrosis, heart failure, Shenmai Injection, transforming growth factor β1/smad, hypertension

Heart failure (HF) is the end stage of various cardiac conditions. HF is a complex syndrome that results in decreased ejection capacity or insufficient ventricular filling due to abnormalities in the structure and function of the heart, and it is characterized by high morbidity, high hospitalization rate, and high mortality rates. The World Health Organization (WHO) considers cardiovascular disease to be the leading cause of death worldwide.[1] HF, in particular, has been characterized as a contemporary epidemic, affecting 1%–2% of the world's adult population.[2] Hypertension is a high-risk factor for HF. It often leads to late-stage HF and seriously affects cardiac function. Therefore, in-depth study of the pathogenesis and prevention methods of

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hypertensive HF is the focus of current research.

In recent years, modern medicine has encountered a choke point in the treatment of hypertensive HF. However, the use of Chinese medicine for cardiovascular diseases has been attracting increasing attention. Shenmai Injection (SMI, 参麦注射液) is a Chinese medicine compound containing red ginseng and *Ophiopogon japonicus* that has a wide range of pharmacological effects. Its main active ingredients are ginsenosides and Ophiopogon saponins, which are effective against hypertension and HF.[3] Studies have found that ginsenosides can regulate the activity of matrix metalloprotein-9 (MMP-9), down-regulate the expression of α-smooth muscle actin and improve heart structure.[4-6] Ophiopogon saponins can down-regulate interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) to inhibit inflammation and effectively reduce myocardial damage.[7] However, their molecular mechanism and targets for improving hypertensive HF have not yet been fully elucidated. It was hypothesized that myocardial fibrosis may be the main pathological manifestation of hypertensive HF, and that the therapeutic effects of Shenmai Injection may be mediated via reducing myocardial fibrosis. Therefore, the present study replicated animal models of hypertension and HF, and Shenmai Injection was used as an intervention. The aim was to observe the changes in myocardial fibrosis and explore the effect of Shenmai Injection on the classical transforming growth factor β1 (TGF-β1)/Smad fibrosis signaling pathway.

METHODS

Animals

Salt-sensitive (Dahl/SS) and non-salt-sensitive (SS-13BN) male rats weighing 220±20 g of 6-week old were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., China [License No. SCXK (Beijing) 2016-0006] and kept in specific pathogen free (SPF) animal laboratory of Hunan University of Chinese Medicine. Animal studies were performed in accordance with the 'Guiding Principles for Research Involving Animals and Human Beings'.[8] All experimental protocols involving animals and their care were approved by The Experimental Animal Ethics Committee of Hunan University of Chinese Medicine (approval No. 20170906).

Model Establishment

According to the literature,[9-12] a high-salt diet was used to construct a model of HF caused by hypertension. A total of 30 rats, salt-insensitive rats (SS-13BN) served as the negative control group (*n*=6), salt-sensitive rats (Dahl/SS) were divided into the control group (*n*=6) and the model group (*n*=18) using the random number table method. The control group was given a normal diet (0.3% NaCl). The model and negative control groups were given a high-salt diet (8% NaCl) for 20 weeks of intervention. These rats were housed in cages (3 rats per cage), and 60 g of feed were fed to each cage every day, and free drinking water.

Drug Intervention

After successful modeling, the rats in the model group (*n*=18) were randomly divided into a HF group (HF, *n*=6), a Shenmai Injection intervention group (SMI, *n*=6), and pirfenidone group (TGF-β1 inhibitor, as a positive control drug, PFD, *n*=6) using random number table method. All treatments were administered as peritoneal injections and gavage for 15 days, and the dosage was calculated according to surface area of the human and animal body. Rats in the control group and the HF group were intraperitoneally injected with sterile water 6.0 mL/kg, rats in the SMI group were intraperitoneally injected with Shenmai Injection 6.0 mL/kg once a day, with 10 mL/kg distilled water by gavage per day in each group; while rats in the PFD group were injected intraperitoneally 6.0 mL/kg sterile water, gavage 10 mL/kg distilled water containing 100 mg/kg pirfenidone per day. Shenmai Injection was purchased from Yunnan Plant Pharmaceutical Co., Ltd., China (approval No. Sinopharm Standard Z53021720) and pirfenidone was purchased from Beijing Continent Pharmaceutical Co., Ltd. (approval No. Sinopharm Standard H20133376).

Enzyme Linked Immunosorbent Assay

During the experiment, blood was collected under anesthesia (20% urethane, 6 mL/kg, intraperitoneal injection) after the model was established. Some disposable blood collection tubes were used to collect 0.5 mL of blood. The collected blood was allowed to stand for 3 h at room temperature, and the serum was centrifuged at 3,000 r/min for 15 min. After coagulation and separation, the serum was dispensed into 1.5 mL EP tubes and stored in a refrigerator at –20 ℃. The serum N-terminal pro B-type natriuretic peptide (NT-proBNP) content was detected according to the

enzyme linked immunosorbent assay (ELISA) method instructions (CSB-E08752r, Cusabio, China).

Echocardiography

The cardiac function of rats in each group after modeling and intervention was detected by echocardiography with an ultrasound color Doppler diagnostic equipment (S2N, Shenzhen Kaili Technology Co., Ltd., China). After the rats were fasted for 12 h, anesthesia was performed as described above. The anesthetized rats were fixed on the rat board in the supine position, with the hair removed from the chest using a depilatory cream. The left ventricular end-diastolic diameter and left ventricular end-systolic diameter were measured using a color Doppler ultrasound system under the guidance of two-dimensional ultrasound with an M-mode ultrasound array probe through the long-axis section of the left ventricle. The mean of three cardiac cycles was first calculated; subsequently, the left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated according to the Teichholz formula.

Hematoxylin-Eosin Staining

After removing the heart, it was washed in distilled water to remove the blood, and the myocardial tissue was quickly placed in 10% formaldehyde for 24 h, embedded paraffin and cut into 4 μm sections. The sections were placed in an oven at 60 ℃ for 2 h and dewaxed in water. The sections were placed in xylene twice for 10 min each time, and then in 100%, 95%, 85% and 75% ethanol for 5 min, followed by washing in distilled water for 5 min. The sections were then stained with hematoxylin for 10 min, rinsed with distilled water, placed back in phosphate buffered saline (PBS), stained with eosin for 5 min, rinsed again with distilled water, and then dehydrated with gradient alcohol for 5 min in each concentration. The sections were then soaked in xylene again for twice for 10 min each time, sealed with neutral gum and observed under a microscope.

Masson's Trichrome Staining

Myocardial tissue was embedded in paraffin and cut into sections, which were then placed in an oven at 60 ℃ for 2 h. The sections were dewaxed in water and, after removing excess water by shaking, an appropriate amount of nuclear staining solution was added to cover the entire tissue section for 5 min. The staining solution was rinsed with water, and the sections were soaked in distilled water, followed by soaking for 10 min in PBS to restore the color of the nuclei to blue. Next, excess water was removed from the section by shaking, and an appropriate amount of slurry staining solution was added to cover the entire section. The section was stained for 2 min, and then rinsed with a washing solution. Color separation solution was used for 30 s, then discarded. The counterstaining solution was added in a dropwise manner to cover the entire tissue section, allowed to stain for 8 min, discarded, and the section was rinsed with absolute ethanol. Finally, the sections were blow-dried, cleared, sealed and examined under a microscope (400×).

Immunofluorescence

Sections of myocardial tissue were dewaxed and subjected to heat-induced antigen retrieval, then incubated with rabbit anti-mouse type Ⅰ collagen fiber (Col Ⅰ) polyclonal antibody (1:400, ab34710, Abcam, UK), overnight at 4 ℃. Then the fluorescent secondary antibody was added for incubation at 37 ℃ for 90 min. After washing, the nucleus was stained with DAPI (1:1000, WH1149, Wellbio, China) at 37 ℃ for 10 min. The slides were mounted with buffered glycerol after washing and observed under a fluorescence microscope (400×). Green fluorescence marked as positive signal and blue marked as nuclear staining signal. Quantitative analysis and calculation of mean fluorescence intensity (MFI) were performed using Image-Pro Plus 6.0 software.

Analysis of Immunohistochemical Images

Image-Pro Plus 6.0 (Media Cybernetics, US) was used to calculate the collagen volume fraction (CVF) and the mean fluorescence intensity (MFI) of myocardial tissue Col Ⅰ after Masson's staining.

Quantitative Polymerase Chain Reaction

Myocardial tissue was washed in distilled water to remove the blood, 1 mL TRIzol® (Thermo Fisher Scientific, USA) was added to 50 mg of myocardial tissue and mixed well to extract total RNA, and the concentration was determined with an ultraviolet spectrophotometer. The sequence of the gene of interest was searched on National Center for Biotechnology Information (NCBI), primers were designed using Primer 5 (Sangon Biotech China). Quantitative polymerase chain reaction (qPCR) was performed by SYBR method with total mRNA as template and reverse transcription of cDNA (Reverse

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Table 1. Primer Sequence

mRNA	Forward	Reverse
TGF-β1	5'-ATTCAAGTCAACTGTGGAGCAAC -3'	5'-CGAAAGCCCTGTATTCCGTCT-3'
Col Ⅰ	5'-CTGGCTCTCCTGGTACCCCT-3'	5'-GGACCACGTTCACCACTTGCT-3'
Smad2	5'-TCCATCTTGCCATTCACTCCG-3'	5'-CAAGCTCATCTAATCGTCCTGT-3'
Smad3	5'-CACGCCACACCGAGATCCC-3'	5'-CTCCATCTTCACTCAGGTAGCCA-3'
GAPDH	5'-ACAGCAACAGGGTGGTGGAC-3'	5'-TTTGAGGGTGCAGCGAACTT-3'

Notes: PCR amplification conditions: 95 ℃ for 15 s, 60 ℃ for 60 s, a total of 40 cycles, followed by melting curve analysis: 60 ℃–95 ℃. The expression levels of examined transcripts were compared with that of GAPDH and normalized to the mean value of controls.

transcription kit, UltraSYBR Mixture, DM2000 Plus DNA Marker from Beijing Kangwei Century, China). The primer sequence information is shown in Table 1.

Statistical Analysis

Statistical analysis was performed by SPSS 22.0 (IBM Corp., USA) and all data were presented as mean ± standard deviation ($\bar{x} \pm s$). The data were tested for normality and homogeneity of variance. If satisfied, the *t* test was used for comparison between two groups, and ANOVA was used for comparison between multiple groups. If not satisfied, the rank sum test and Kruskal-Wallis test were used. $P<0.05$ is regarded as a significant difference and statistical pictures were drawn using Prism 9.

RESULTS

Blood Pressure and Cardiac Function

According to the blood pressure before and after modeling, there is no significant difference in blood pressure between Dalh salt-sensitive rats (control) and SS-13BN salt-insensitive rats (negative control) before and after modeling. However, compared with the control and negative control groups, the systolic and diastolic blood pressure of the rats in the model group continued to increase and far exceeded normal levels ($P<0.05$), which is one of the signs of successful modeling (Figures 1A to 1H). LVEF and LVFS were calculated using echocardiography. Consistent with expectations, there was no significant change in the control and negative control groups, but a significant reduction was observed in the model group ($P<0.05$, Figures 1I and 1J). Nevertheless, the LVEF value of the model group is still exceeded 70%, which is in line with the characteristics of hypertension with preserved ejection fraction. Moveover, according to the serum NT-proBNP value, the model group was significantly higher than the control group and the negative control group ($P<0.05$), and the average value exceeded 450 pg/mL (Figure 1K). It is believed that Dalh rats in the model group have successfully replicated the hypertensive HF model.

Shenmai Injection Improves Cardiac Function

Intervene with Shenmai Injection or PFD in rats with

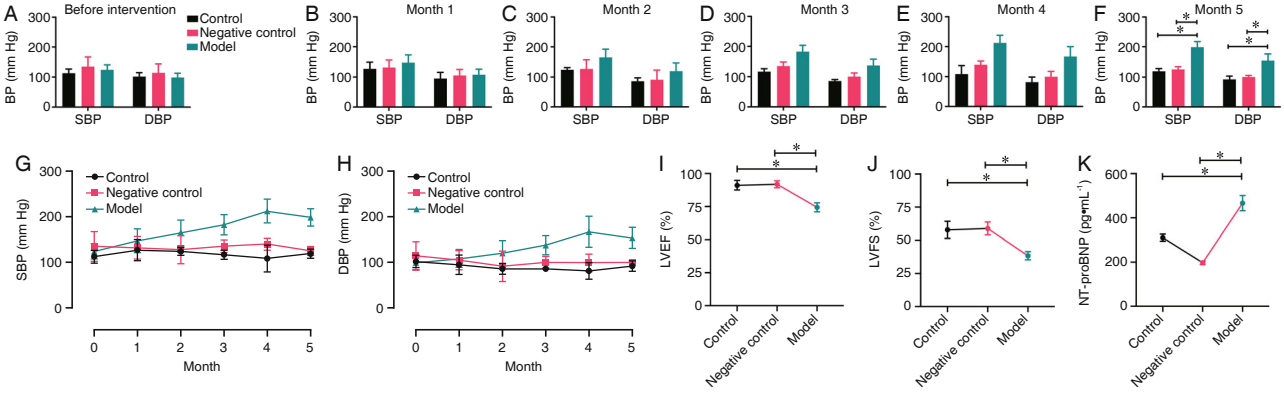

Figure 1. Changes in Blood Pressure and Cardiac Function of Rats in Each Group ($\bar{x} \pm s$, $n=6$)

Notes: Figures A to H show monitoring of systolic blood pressure (SBP) and diastolic blood pressure (DBP) from pre-intervention to 5th month in each group. Before the intervention (A), compared with the control group and the negative control group, there was no significant difference in blood pressure in the model group. However, at the 5th month (F), the systolic and diastolic blood pressure in the model group were higher than those in the control group and negative control group, $^{*}P<0.05$. In addition, echocardiography was used to detect LVEF (I) and LVFS (J) and found that the LVEF and LVFS in the model group were significantly lower than those in the control group and negative group, $^{*}P<0.05$. The serum NT-proBNP was detected by ELISA, and it was found that the average concentration of the model group had exceeded 450 ng/mL, which was significantly higher than that of the control group and the negative control group, $^{*}P<0.05$.

hypertensive HF, it turns out that the LVEF and LVFS of the HF group were significantly reduced compared with the control group (P<0.05). Secondly, the LVEF and LVFS of the SMI group were significantly increased compared with the HF group and PFD group (P<0.05). Additionally, compared with the HF group, there is no significant change in the PFD group (Figure 2).

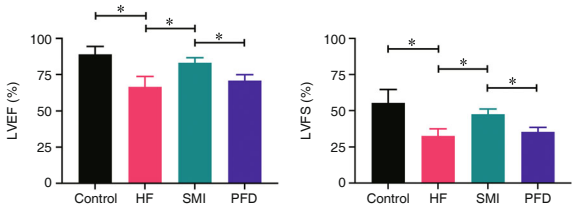

Figure 2. Comparison of Cardiac Function among Groups after Intervention ($\bar{x} \pm s$, n=6)

Note: *P<0.05

Shenmai Injection Inhibits Myocardial Fibrosis

According to HE staining, the intercalated discs and striations of cardiomyocytes in the control group were clear, and the size and morphology of cardiomyocyte nuclei were normal. However, in the HF group, the myocardial structure of the rats was disrupted, the intercalated discs were not clear, the striations had disappeared, and the myocardium appeared edematous. In addition, the structure of myocardial cells in the SMI and PFD groups were clearer, and the cellular edema was less obvious (Figure 3A).

By Masson staining, it was found that the degree of myocardial fibrosis in the HF group was more extensive. However, compared with the HF group, the degree of myocardial fibrosis in the SMI and PFD groups were improved. Compared with control group, the CVF of the HF group was significantly increased (P<0.05). Compared with HF group, it in the SMI group was significantly decreased (P<0.05, Figures 3B and 3C).

The positive proportion of Col Ⅰ in the HF group was more than the control group, while the positive proportion of Col Ⅰ in the SMI group and the PFD group were lower than that in the HF group (Figure 3D). Calculated MFI found that the HF group was significantly higher than the control group (P<0.05), and the SMI and PFD groups were significantly lower than HF group (P<0.05, Figure 3E). Furthermore, qPCR was also used to detect the relative expression of Col Ⅰ mRNA. The

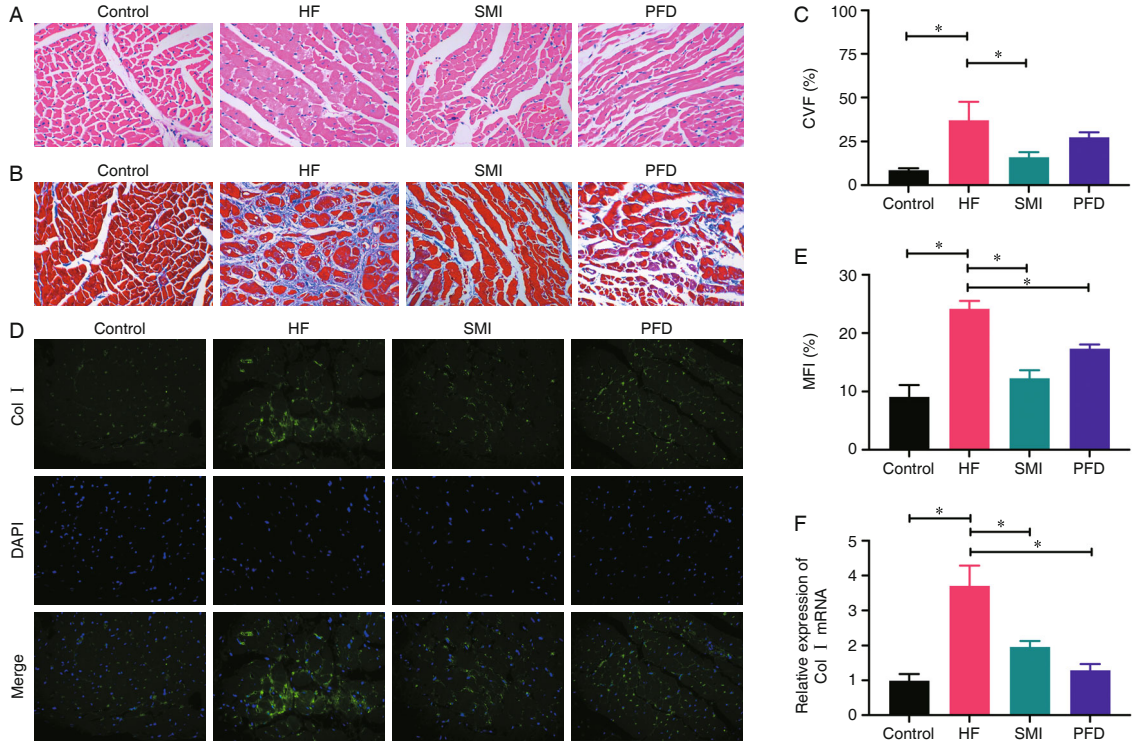

Figure 3. Myocardial Tissue Staining and Collagen Fiber Expression Detection ($\bar{x} \pm s$)

Notes: (A) HE staining results (400×): it can be seen that the myocardial cell structure of the control group is clear, the structure of myocardial cells in HF group is blurred, and the cell edema is obvious. Compared with HF gourp, the SMI and PFD groups improved slightly. (B) As a result of Masson staining (400×), the fibrosis level was found to be significant in the HF group and lower in the SMI and PFD groups. (C) CVF was calculated according to the Masson staining figure (n=3). (D) Immunofluorescence of Col Ⅰ in each group (400×). (E) Image-Pro Plus 6.0 software was used to analyze the mean fluorescence intensity of Col Ⅰ in the myocardial tissue of each group of rats (n=3). (F) The relative expression of Col Ⅰ mRNA in myocardial tissues of rats in each group was detected by qPCR (n=4). *P<0.05

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results demonstrated that the relative expression of Col Ⅰ mRNA in the HF group was significantly higher than that in the control group (P<0.05). Whereas, compared with the HF group, it significantly decreased following intervention with Shenmai Injection or pirfenidone (P<0.05, Figure 3F).

Shenmai Injection Regulates mRNA Expression in TGF-β1/Smad Pathway

Detection of mRNA expression levels of TGF-β1/Smad signaling pathway by qPCR. It was found that the relative expression levels of TGF-β1, Smad2 and Smad3 mRNA in the HF group were significantly higher than in the control group (P<0.05). However, compared with the HF group, the relative expressions of TGF-β1, Smad2 and Smad3 mRNA in the SMI group and the PFD group were significantly reduced (P<0.05, Figure 4).

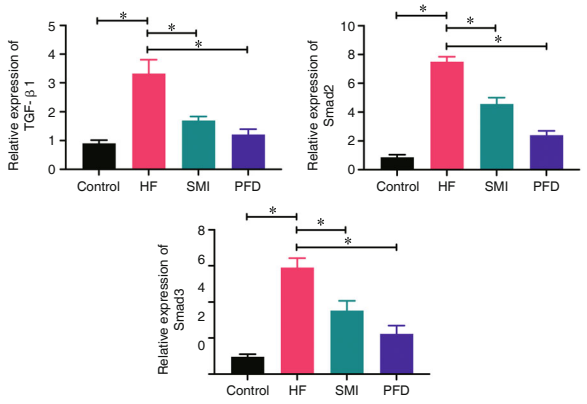

Figure 4. mRNA Expression in TGF-β1/Smad Pathway ($\bar{x} \pm s$, n=4)

Note: *P<0.05

DISCUSSION

Hypertension is a common cardiovascular disease that is a high-risk factor for HF.[13,14] The majority of patients with HF have a history of hypertension, and chronic hypertension eventually leads to HF. Long-term hypertension will cause long-term pressure overload on the heart. When the heart is subjected to pressure overload over a prolonged period of time, diastolic dysfunction and left ventricular hypertrophy may develop, which will further reduce the systolic function and eventually develop into systolic HF or diastolic HF with ejection fraction retention.[15]

Modern medicine considers myocardial remodeling as the main factor accompanying the occurrence and development of HF, which causes morphological changes such as myocardial hypertrophy, myocardial cell apoptosis and myocardial fibrosis, leading to decreased cardiac function and, eventually, HF. The animal model of hypertensive HF in this study showed that normal rats and SS-13BN rats were not sensitive to high-salt diet for 20 weeks. However, the condition of Dalh/SS rats after 20 weeks of high-salt diet is not optimistic, blood pressure continues to rise, and hypertension develops. In addition, LVEF and LVFS are significantly reduced, and the serum concentration of HF marker NT-proBNP is significantly increased, which indicates that the heart function is significantly reduced. Hypertensive HF has been established as reported in the literature.[16-20] The pathological manifestations are structural disorders of myocardial cells, increased expression of Col Ⅰ, and massive proliferation of fibrous tissue. These suggest that myocardial fibrosis may be the key to the heart function of hypertensive HF.

Myocardial fibrosis is the key factor underlying the transition of cardiac function from the compensatory to the decompensated phase. It is the pathological basis of hypertensive HF, and it is also the key to control the cardiovascular complications of hypertension. The severity of myocardial fibrosis is the main factor determining the development and prognosis of target organ damage in hypertensive HF.[21] The TGF-β1/Smad signaling pathway is a classical myocardial fibrosis-related signaling pathway. TGF-β1 induces the conversion of cardiac fibroblasts into myofibroblasts by regulating Smad2/3 activation. In addition, it promotes the synthesis of extracellular matrix components, such as collagen and fibronectin, promoting the development of myocardial fibrosis.[22-24] In this experiment, the mRNA expression of factors of the TGF-β1/Smad signaling pathway in myocardial tissue of hypertensive HF rats was significantly upregulated, which explained that the activation of TGF-β1/Smad, a key signaling pathway for pro-fibrosis, is one of the typical features of hypertensive HF.

Chinese medicine is characterized by multiple targets and low toxicity and side effects. Shenmai Injection, a traditional Chinese medicine, was approved by the China Food and Drug Administration for the treatment of HF in 1995,[25,26] and it has been widely used in clinical practice.[27,28] In the present study, it was found that Shenmai Injection can improve heart function, enhance pumping ability, reduce NT-proBNP level, and improve the degree of HF. In addition, Shenmai Injection can reduce and improve the microstructure of myocardial cells, reduce excessive collagen deposition. Shenmai Injection can improve the level of myocardial

fibrosis in hypertensive HF, but the underlying molecular mechanism remains unclear. TGF-β1/Smad is an important molecular signaling pathway for fibrosis and is known as the "key" of fibrosis.[29] Therefore, we speculate that the mechanism of Shenmai Injection in improving myocardial fibrosis in hypertensive HF may be related to its regulation of TGF-β1/Smad signaling pathway. It turns out that both Shenmai Injection and pirfenidone can effectively inhibit the expression of related mRNA in the TGF-β1/Smad signaling pathway, thereby reducing the degree of myocardial fibrosis associated with hypertensive HF, which provides a basis and a reference for its clinical application. However, the efficacy of pirfenidone and Shenmai Injection is still different. Shenmai Injection can inhibit TGF-β1/Smad signaling pathway, improve myocardial fibrosis and cardiac function. But pirfenidone has no obvious effect on improving cardiac function, which may be related to their different molecular mechanisms. At the same time, this also shows that Shenmai Injection, as a traditional Chinese medicine, has multi-functional and multi-target effects.

Current research on HF has progressed from single-drug to combination treatment interventions, from drug to nutritional supplements,[30] from late treatment to early care interventions,[31] and from focusing solely on the heart to considering other tissues and organs,[32-34] along with a comprehensive exploration of prevention and treatment strategies for HF.[35] In recent clinical studies, Shenmai Injection has been explored from different perspectives, and it is found that it cannot only regulate myocardial energy metabolism, but also reduce the expression of the HF marker NT-proBNP, as well as reduce inflammation.[36,37] The present study revealed the regulatory effect of Shenmai Injection on the TGF-β1/Smad signaling pathway and confirmed the significance of Shenmai Injection in delaying or reversing myocardial fibrosis. Along with the clinical advantages of Chinese medicine, including multiple targets and lower toxicity, these findings provide a direction for future exploration of integrated Chinese and Western medicine.

A limitation of the present study was that only the mRNA expression changes of related factors in the TGF-β1/Smad pathway by Shenmai Injection were discussed, whereas protein expression detection was not performed. The detection of protein expression of related factors, such as phosphorylated Smad2/3, is in progress, and the results will be presented in subsequent studies. Moreover, in this study, there is still a lack of research on the mechanism of Shenmai Injection, and why it can regulate the expression of mRNA of TGF-β1/Smad pathway related factors, which is worthy of further discussion.

In conclusion, the present study demonstrated that the degree of myocardial fibrosis is closely associated with the severity of hypertension and HF. Shenmai Injection can inhibit myocardial fibrosis by regulating the TGF-β1/Smad signaling pathway and exerts a beneficial effect on hypertensive HF, which provides an experimental basis for future clinical treatment.

Availability of Data and Materials

The data and materials used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest

All authors declare that there is no conflict of interests.

Author Contributions

Hu SY conceived the study and wrote most parts of the manuscript. Zhou Y completed most of the experiments and testing work. Hu ZX provided methodological support in the study design and revised the manuscript. Zhong SJ, Yang M, Huang SM, Li L and Li XC searched and analyzed some literature. All authors have read and approved the manuscript before submission. All authors reviewed the manuscript.

Consent for Publication

All authors confirm that the work described has not been published before, that its publication has been approved by all co-authors.

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