Effect of Shenfu Qiangxin on the expression of TGF-β/Smads signaling pathway-related molecules in myocardium of rats with heart failure

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Abstract
This study aims to evaluate the effect of Shenfu Qiangxin on TGF-β/Smads signaling pathway-related molecules in myocardial tissue of rats with heart failure. Five rats were selected as sham-operated group, while another 15 rats with heart failure were divided into three groups, including model group, losartan group, and Shenfu Qiangxin group. Rats in losartan group were given losartan intragastric intervention, the rats in Shenfu Qiangxin group were given Shenfu Qiangxin mixture intervention, while rats in another two groups were given equal volume of sterile saline intervention. During the treatment, the levels of B-type brain natriuretic peptide (BNP), lactate dehydrogenase (LDH), free fatty acids (FFA), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and TGF-β/Smads signaling pathway were measured in rats. Compared with model group, the expression of ejection fraction (EF), left ventricular ejection fraction (LVSP), TGF-β 1, Smad2, and Smad3 significantly decreased in sham-operated group, losartan group, and Shenfu Qiangxin group, while left ventricular end-diastolic volume (LVEDV), left ventricular end-diastolic diameter (LVDd), left ventricular end-diastolic pressure (LVEDP), BNP, LDH, FFA, TNF-α, and IL-6 levels increased (P < 0.05). Compared with sham-operated group, the expression of EF, LVSP, TGF-beta 1, Smad2, and Smad3 dramatically decreased in losartan group, Shenfu Qiangxin group, but LVEDV, LVDd, LVEDP, BNP, LDH, FFA, TNF-α, and IL-6 levels increased (P < 0.05). Compared with losartan group, the expression of EF, LVSP, TGF-beta 1, Smad2, and Smad3 upregulated in Shenfu Qiangxin group, while LVEDV, LVDd, LVEDP, BNP, LDH, FFA, TNF-α, and IL-6 levels downregulated (P < 0.05). Consequently, Shenfu Qiangxin could effectively improve the heart function of rats with heart failure, and play an anti-heart failure role by regulating the expression of related molecules of TGF-β/Smads signaling pathway.

Keywords
B-type brain urine natriuretic peptide, cardiac function, heart failure, Shenfu Qiangxin mixture

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Introduction
Heart failure is a kind of common critical conditions in clinical practice, and its prevalence keeps increasing in China in recent years,1,2 severely affecting the life quality of patients. TGF-β could induce the transition of the myocardial cells into the myocardial collagen fibers,3 while Smads is the major signaling factors of the TGF-β receptors, which are critically involved in the formation of the myocardial fibers.4 However, there is still no effective method to inhibit the progress of TGF-β/Smads signaling pathway-related molecules during the treatment of heart failure.

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According to the Chinese traditional medicine (CTM), lack of the vital energy is the pathogenesis of the heart failure, and the key link of the pathogenesis includes the blood stasis in the heart vessels, continuous generation of phlegm, and water-rheum collecting internally. Thus, CTM is considered to be a magic and promising way, to supplementing the qi to nourish yang, which is also the essence of the treatment. Among all, the Shenfu Qiangxin Decoction, originated from the Good Remedies For Women, is made of the red ginseng, monkshood, semen lepidii, and polygonatum odoratum, and has the functions of rescuing the patients from collapse by restoring yang and stopping the collapse by nourishing qi. Shaqura et al. reported that with an excellent performance in treatment of heart failure, Shenfu Qiangxin Decoction could effectively relieve the clinical symptoms of the patient.

In this study, based on this guideline, we added the semen lepidii and radix polygonati officinalis to form the Shenfu Qiangxin Decoction. In the theory of traditional Chinese medicine, both of the semen lepidii and radix polygonati officinalis could promote diuresis, reduce edema, and relieve dyspnea. In fact, it often receives satisfactory clinical results for the patients with the phlegm-fluid accumulation, dyspnea with being unable to lie, and cough with excessive sputum. However, the special molecular mechanism was unclear. Therefore, we probe the effect of the Shenfu Qiangxin Decoction on the expressions of proteins relating to the TGF-β/Smads signaling pathway in the myocardial tissues of heart failure rats.

Materials and methods

Materials

Research animals: A total of 23 Sprague-Dawley rats (130.5 ± 10.5 g) were provided by the Experimental Animal Center of Hebei Medical University. All rats were fed in separated cages. For the feeding environment, the room temperature was set at 25°C ± 1°C, relative humidity at 54% ± 3%, and ventilation at 8–12 times/h. All rats had the free access to the standard food, and experiments were conducted only after 1 week of feeding. The protocol relating to the animal experiments had been approved by the Ethic Committee of the University. Major reagents: Primary and secondary goat anti-mouse antibodies of TGF-β1, Smad2, Smad3, and Smad7 (Invitrogen, USA); PBST suffer (Tiangen Biotech Co., Ltd., Beijing, China); losartan (MSD, Hangzhou, China; Approval No. of SFDA: J20180054).

Methods

Establishment and grouping of the heart failure models

Of the 23 rats, 5 rats were selected as the sham operation group, while the remaining rats were prepared for the establishment of the heart failure models according to the methods of the literatures. Rats were weighed, and the according to the weight, rats were anesthetized by using the 3% pentobarbital sodium; thereafter, rats in anesthesia were fixed on the operation table, and in the middle of abdomen, an incision in length of 2 cm was made; the abdominal aorta was bluntly dissected, and along the dissected aorta, the branch of the renal artery was found; at about 2 mm above the branch, the branch was ligated by using the No. 7 needle and No. 4 suture, thereby forming the 50% stenosis in the abdominal aorta. In this operation, rats received the abdominal injection of penicillin (100,000 units), and the abdomen was closed. For rats in the sham operation group, they only underwent the blunt dissection of abdominal aorta, without any stenosis.

Drug intervention

Composition of the Shenfu Qiangxin Decoction: red ginseng, 5 g; monkshood, 5 g; radix polygonati officinalis, 15 g; and semen lepidii, 10 g (Beijing Tongrentang Pharma Co., Ltd., Beijing, China). Compositions were mixed and boiled in water twice, first boil lasting for 1.5 h and second boil for 0.5 h, and finally being concentrated into the decoction at a concentration of 2 g/mL. The heart failure models were established successfully in 15 rats, which were later divided into the model group, losartan group, and Shenfu Qiangxin group, with 5 rats in each group. In the losartan group, losartan was given by gavage at a dose of 10 mg/kg; in the Shenfu Qiangxin group, rats took the Shenfu Qiangxin decoction for intervention at a dose of 15 g/kg; those in the model group and the sham
operation group received the septic normal saline in the same volume for intervention. Intervention was performed once every 4 days, consecutively for 3 weeks, during which rats had the free access to the water and food.

**Detection of the heart functions**

After 3 weeks of the intervention, the echocardiography was adopted to detect the indexes of the heart functions, including the left ventricular ejection fraction (LVEF), left ventricular end-diastolic volume (LVEDV), left ventricular end-diastolic diameter (LVDd), and left ventricular end-diastolic pressure (LVEDP).

**HE staining**

Following the experiments, all rats were executed by decapitation to collect the samples of the heart tissues which were later preserved in the 4% paraformaldehyde (PFA) for fixation. Fixed tissues were then prepared for the paraffin sections, and these sections were stained in the 0.5% hematoxylin-eosin for 10 min, and then rinsed in the tap water. Thereafter, sections were dehydrated in the ethanol in gradient concentrations, and mounted in the neutral balsam.

**Measurement of the levels of BNP, LDH, FFA, TNF-α, and IL-6 by enzyme-linked immunosorbent assay (ELISA)**

Antigen was dissolved in the 50 mM carbonate-coated buffer to prepare a solution at 10–20 μg/mL, and then aliquoted into the 96-well plate (100 μL/well) and preserved at 4°C overnight. Carbonate-coated buffer was then discarded, and then wells were washed in PBST three times. In each well, 150 μL bovine serum albumin was added, and blocked at 37°C for 1 h. Later, wells were washed in PBST three times, and in each well, 100 μL serum in different dilutions was added, and the samples for control were also added for incubation for 1 h at 37°C. Following five washes in PBST, 100 μL diluted horseradish peroxidase (HRP)–labeled secondary antibody was added for 1 h of incubation at 37°C. After five washes in PBST, samples were incubated with the color development reagent for 20 min, and the A405 absorbance was measured in a microplate reader to analyze the levels of BNP, LDH, FFA, TNF-α, and IL-6.

**Measurement of the expressions of the proteins in relation to the TGF-β/Smads signaling pathway by Western blot**

The tissue samples were homogenized and placed in the buffer for extraction of proteins, and the concentration was determined by bicinchoninic acid assay (BCA). Protein samples (50 μg) were loaded in the gel for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and then transferred electrically onto the PVDF (polyvinylidene difluoride) membrane. The unoccupied sites on the membrane were blocked in 5% skimmed milk for 1 h in a dark place. After the membrane was washed in the TBST, proteins on the membrane were probed by incubation with the corresponding dilutions of the primary antibodies (TGF-β1, Smad2, Smad3, and Smad7) in dilution of 1:1000 at 4°C overnight. Following several washes in TBST, the immunoblots were incubated in the diluted secondary antibodies (TGF-β1, Smad2, Smad3, and Smad7) in dilution of 1:5000 at 37°C for 1 h. Then, membrane was washed in the TBST and on the membrane, enhanced chemiluminescence reagent was added to detect the bands which were later analyzed by using the software, with GAPDH as the internal reference.

**Statistics**

SPSS 21.0 software was utilized for data analysis. Measurement data were presented as means ± standard deviation, and the intergroup comparison was carried out by the F test, while comparison between either two groups was performed by the independent sample t test. P < 0.05 suggested that the difference had the statistical significance.

**Results**

**General condition of the rats**

The results showed that rats in the sham operation group had no difficulties in activity and responses, and were in the regular breathe and diet, without any edema. However, rats in the model group had coarse, dark yellow hair and a significant reduction in activity and diet, with the significant signs of
edema and hollow when pressed. Furthermore, rats in the losartan group had the smooth hair with luster, regular activity, and diet. And they responded bluntly, while the breath was short without any evident edema. To date, the rats in the Shenfu Qiangxin group showed the smooth hair with luster, regular activity, and diet, while the breath was only slightly short, and the edema was remarkably mitigated.

Comparison of the indexes in relation to the heart functions of the rats

Compared to the model group, sham operation group, and losartan group, the LVEF and left ventricular systolic pressure (LVSP) decreased significantly, with evident increases in LVEDV, LVDd, and LVEDP in the Shenfu Qiangxin group ($P < 0.05$, Figure 1(a)–(d)). When compared with the sham operation group and losartan group, rats in the Shenfu Qiangxin group manifested the significant decrease in the LVEF and LVSP, but increase in the LVEDV, LVDd, and LVEDP ($P < 0.05$, Figure 1(a)–(d)). To date, rats in Shenfu Qiangxin group still had higher LVEF and LVSP, but lower LVEDV, LVDd, and LVEDP, compared to the losartan group also showed that rats in the ($P < 0.05$, Figure 1(a)–(d)).

Pathological observations

In the sham operation group, no degradation was identified in the myocardial fiber tissues of rats, without any necrosis or atrophy of the myocardial cells, and all myocardial cells were evenly stained in the clear lines, while the transverse striation was well-aligned, with intact membrane, boundaries between cells were visible but there was no infiltration of inflammatory cells. In the model group, rats had the swollen myocardial cells, with the rupture of myocardial fibers and no transverse striation, and there was severe edema between the myocardial cells, evident hyperplasia in the fibrous tissues and severe infiltration of the inflammatory cells. In the losartan group, the swelling myocardial cells were...
somehow mitigated, but the transverse striation was vague, and myocardial cells had severe edema, with significant hyperplasia in fibrous tissues and infiltration of inflammatory cells; in the losartan group, mitigation was identified in the swelling myocardial cells of the rats, but the transverse striation remained vague, with slight infiltration of the inflammatory cells; in the Shenfu Qiangxin group, rats had the clear lines of the myocardial cells, with significant mitigation in the swelling myocardial cells, but no significant hyperplasia of the fibrous tissues or infiltration of the inflammatory cells (Figure 1(e)).

Comparisons of the levels of BNP, LDH, FFA, TNF-α, and IL-6 in rats

In comparison with the model group, sham operation group, and losartan group, rats in the Shenfu Qiangxin group experienced increase in the TGF-β1, Smad2, and Smad3, but decrease in Smad7 \((P < 0.05)\). When compared with the sham operation group, the levels of the TGF-β1, Smad2, and Smad3 were lower in the losartan group and the Shenfu Qiangxin group, but the Smad7 levels were increased \((P < 0.05)\). However, the levels of the TGF-β1, Smad2, and Smad3 in the losartan group were significantly lower than those in the Shenfu Qiangxin group, while the Smad7 was higher \((P < 0.05, \text{Figure 3(a)})\). Consistently, the gray value of the proteins’ expression showed similar tendency \((P < 0.05, \text{Figure 3(b)})\).

Discussion

This study showed that after the intervention of the Shenfu Qiangxin decoction, EF and LVSP were elevated, with declines in LVEDV, LVd, and LVEDP, suggesting that this decoction could ameliorate the heart function of the rats and inhibit the progression of heart failure. However, its related mechanism remains unknown to us.

BNP is a key factor in clinical evaluation of the heart failure, and LDH activity reflects the necrosis of cells. The attack of the heart failure triggers the variation in the energy metabolism in myocardium, in which the abnormal metabolism of the glucose and lipid results in the disorder in the metabolic cycle, increased accumulation of FFA, and aggravation of energy metabolism, severely damaging the functions of mitochondria.\(^9\) The results of this study consistently indicated that Shenfu Qiangxin could reduce BNP, LDH, and FFA, suggesting that this decoction could mitigate the necrosis of cells, increase the oxidation of the FFA, and improve the lipid metabolism to ameliorate the energy metabolism of the myocardium in rats.

Relevant studies have shown the widespread distribution of TNF-α and IL-6 in the heart tissues of patients with heart failure, which could decrease the myocardial contractility and induce the ventricular remodeling, thus promote the apoptosis of the myocardial cells.\(^10\) Our results also showed that the levels of the TNF-α and IL-6 were decreased in
the Shenfu Qiangxin group, suggesting that Shenfu Qiangxin decoction can decrease the infiltration of the inflammatory cells in myocardium of rats to suppress the inflammation.

Recently, increasing studies indicated that the TGF-β/Smads-pathway play a vital role in the pathogenesis of heart failure. In fact, TGF-β had been identified as a major contributor to tissue fibrosis and myofibroblast proliferation in various organ systems. In addition, the TGF-β antagonism could suppress the fibrotic processes, which is benefit for the patients with heart failure. Furthermore, Smads is known as the substrate of the TGF-β receptors and a reliable downstream protein of TGF-β. Smad2, Smad3, and Smad7 are members of the Smads family, in which Smad2 and Smad3 act as the signals to activate the TGF-β1 into the nuclei. Smad7 is first generated in the nucleus, and after the activation of TβRI, would be transferred from the nucleus into the cytoplasm to bind to the membrane receptors competitively, then avoid the phosphorylation of Smad2 and Smad3 and regulate negatively in the signal transduction of TGF-β1. In this study, the Western blot results also indicated that the levels of TGF-β1, Smad2, and Smad3 were decreased in rats in the Shenfu Qiangxin group, but with an elevation in Smad7, suggesting that in the signal transduction of TGF-β1, Shenfu Qiangxin decoction could down-regulate the expressions of Smad2 and Smad3 while promote the expression of Smad7, thereby inhibiting the progression of heart failure.

In conclusion, Shenfu Qiangxin decoction can ameliorate the heart function of rats with heart failure by regulating the expressions of the proteins in relation to the TGF-β/Smads signaling pathway to improve the heart failure. Consequently, Shenfu Qiangxin is a promising medicine for the treatment of heart failure. In this study, we evaluate the therapy effects of Shenfu Qiangxin decoction in rats with heart failure for only 3 weeks, but its long-term effects and drug safety was still unclear. Therefore, in the near further, we will design clinical trial to further explore its clinical application value.

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