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Original Article

Huoxin Pill (活心丸) Attenuates Cardiac Fibrosis by Suppressing TGF-β1/Smad2/3 Pathway in Isoproterenol-Induced Heart Failure Rats*

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ABSTRACT **Objective**: To evaluate the effects of Huoxin Pill (活心丸, HXP) on cardiac fibrosis and heart failure (HF) in isoproterenol (ISO)-induced HF rats. **Methods**: Thirty Wistar rats were randomly divided into 5 groups including control, HF, isosorbide mononitrate (ISMN), HXP low (HXP-L), and HXP high (HXP-H) groups (n=6 for each group) according to the complete randomization method. Rats were pretreated with ISMN (5 mg/kg daily), low concentration of HXP (10 mg/kg daily) or high concentration of HXP (30 mg/kg daily) or equal volume of saline by intragastric administration for 1 week, followed by intraperitoneal injection of ISO (10 mg/kg, 14 days), and continually intragastric administrated with above medicines or saline for additional 6 weeks. The effects of HXP treatment on the cardiac function, heart weight index (HWI), pathological changes, and collagen content were further assessed. Moreover, the role of HXP on activation of transforming growth factor-β1 (TGF-β1)/Smads pathway was further explored using immunohistochemistry (IHC) and Western-blot assay. **Results**: HXP treatment significantly alleviated the decrease of ejection fraction (EF) and fractional shortening (FS), while decreased the elevation of left ventricular end-systolic volume (LVESV) in ISO-induced HF rats (P<0.05). Moreover, HXP treatment obviously attenuated the increase of HWI and serum level of creatine kinase MB (CK-MB, P<0.05), as well as pathological changes in ISO-induced HF rats. Further determination indicated that HXP treatment alleviated the elevation of collagen Ⅰ and collagen Ⅲ protein expression in cardiac tissues of ISO-induced HF rats. Furthermore, HXP treatment significantly down-regulated the increase of TGF-β1 and p-Smad2/3 protein expression in cardiac tissues of HF rats (P<0.05), while did not affect the expression of total Smad2/3. **Conclusions**: HXP attenuated heart failure and cardiac fibrosis in ISO-induced HF rats by suppression of TGF-β1/Smad2/3 pathway.

KEYWORDS Huoxin Pill, heart failure, cardiac fibrosis, isoproterenol, TGF-β1/Smad2/3 pathway

Heart failure (HF) is the final stage of various cardiovascular diseases with increasing morbidity and mortality.[1,2] As a major health issue, HF is a complex clinical syndrome, affecting about 5.7 million people in the United States, with greater than 40% and 60% of 5- and 10-year mortality rates.[3,4] Cardiac fibrosis is one of the main manifestations of ventricular remodeling which is an important pathological process of HF.[5] Therefore, inhibition of myocardial fibrosis may be a promising novel therapeutic approach for the treatment of HF.

Cardiac fibrosis is caused by excessive accumulation of extracellular matrix proteins, which contributes to systolic and diastolic dysfunction, ultimately leading to the development of HF.[6] The pathogenesis of cardiac fibrosis is complicated and

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there are still no satisfying treatment methods for cardiac fibrosis.[7] As the most important isotypes of transforming growth factor-β (TGF-β) in cardiac tissue, TGF-β1 promotes cardiac fibrosis by phosphorylating its down-stream mediator Smad2/3, which further translocated into nucleus by binding to Smad4, resulting in promoting transcription of relates genes (including collagen Ⅰ and collagen Ⅲ).[8,9] Moreover, elevation of TGF-β1 and its downstream mediator play an important role during the development of cardiac fibrosis, therefore, inhibition of TGF-β1 pathway using inhibitor of TGF-β receptor obviously attenuate cardiac fibrosis.[10,11] Given the important role of the TGF-β1/Smads signaling pathway in the pathogenesis of fibrosis in myocardial infarction (MI) and HF, it suggests that its mediated fibrosis pathway may be a potential target for patients with myocardial fibrosis in the treatment of HF.[12-14]

Chinese medicine (CM) has been widely used to treat various cardiovascular diseases. Increasing evidence demonstrated that CM could improve the outcome in the treatment of ischemic heart disease.[15] Huoxin Pill (活心丸, HXP) has long been used in China for the clinical treatment of coronary heart disease. Many active components have been identified in HXP, such as chlorogenic acid, tauroursodeoxycholic acid and cinobufagin, which have been shown to possess cardiotonic effects.[16-18] However, the protective effects and underlying mechanisms of HXP on HF and cardiac fibrosis remain unclear. Therefore, the aim of current study was to investigate effects of HXP treatment on HF and cardiac fibrosis, as well as activation of TGF-β1/Smad2/3 pathway, which might provide experimental evidence for the clinical application of HXP on treatment of HF.

METHODS

Materials

HXP (concentrated pill) was purchased from Guangzhou Youcare Biopharmaceutics Co., Ltd. (Guangzhou, China, lot No. Z44021835). Isoproterenol (ISO) was obtained from Sigma-Aldrich (St Louis, MO, USA. Cat No. I5627-5G). Isosorbide Mononitrate Tablets was purchased from Lunan Beite Pharmaceutical Co., Ltd. (Shandong, China, Cat No. H10940039). Rat creatine kinase MB (CK-MB) enayme-linked immunosorbent assay (ELISA) kit (No. MM-0625R1) was purchased from Jiangsu Enzyme Free Industrial Co., Ltd. TGF-β (Cat No. 3711S), Smad2/3 (Cat No. 8685S), p-Smad2/3 (Cat No. 8828S) antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). Collagen Ⅲ (Cat No. #33341) antibody was purchased from SAB (College Park, Maryland, USA). Collagen Ⅰ (Cat No. GTX26308) antibody was provided by GeneTex (Texas, USA). GAPDH (Cat No. A01021) antibody was purchased from Abbkine (California, USA).

Animals

Thirty male Wistar Kyoto rats (age: 4 weeks; weight: 200±20 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were maintained under specific pathogen-free conditions with proper temperature (22 ℃) and humidity, food and water available *ad libitum*. An alternating light/dark cycle was observed every 12 h. All animal studies were conducted in accordance with international ethical guidelines and the US National Institutes of Health Guide concerning the care, and were approved by the Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine.

Experimental Protocol

Thirty rats were randomly divided into 5 groups (n=6 in each group): control, HF, isosorbide mononitrate (ISMN, 5 mg/kg), and HXP low (HXP-L), HXP high (HXP-H) groups according to the complete randomization method. The rats in HF, ISMN, HXP-L and HXP-H were intragastrically administered with saline, ISMN (5 mg/kg daily), low (10 mg/kg daily) or high (30 mg/kg daily) dose of HXP for total 7 weeks, and were intraperitoneally injected with ISO (10 mg/kg) for 14 days at 8th day of treatment. The rats in the control group was intragastrically administered and intraperitoneally injected with equal volume of saline. During the experiment, the body weight was measured once a week. At the end of the experiment, the rats were anesthetized with isoflurane (Shenzhen Ruiwode Life Technology Co., Ltd., China) and sacrificed, and abdominal aortic blood samples were collected, centrifuged (3500×g, 15 min at 4 ℃), and separated the serum, which was stored at –80 ℃ for the following experiments. The hearts were anatomized and weighed to calculate the ratio of heart weight and tibia length (heart weight index: HWI; mg/mm). Removed the apex of the heart tissues and freezed it in liquid nitrogen, stored at –80 ℃ until analysis or fixed in 4% paraformaldehyde for pathological analysis.

Chin J Integr Med 2021 Jun;27(6):424-431

Echocardiography

Transthoracic echocardiography was obtained using Vevo2100 (VisualSonics, Toronto, Canada) equipped with a MS250 sensor. Echocardiographic studies were performed on rats under isoflurane inhalation anesthesia at the end of the experiment. Ultrasound probe was placed on the left sternal border, and morphological and functional parameters such as interventricular septal thickness (IVS) and left ventricular posterior wall thickness (LVPW), left ventricular end systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS) were measured by M-mode tracings. LVEF and LVFS were calculated by Vevo LAB software (FUJIFILM VisualSonics, Inc., Canada) as followes: ejection fraction (EF, %)=(LVEDV–LVESV)/LVEDV × 100%; fractional shortening (FS, %)=(LVDd-LVSd)/LVDd × 100%, all echocardiographic parameters were averaged over three consecutive cardiac cycle measurements.

Histopathological Analysis

To observe the degree of inflammatory cell infiltration, myocardial necrosis and cardiac fibrosis, heart tissues were fixed in 4% paraformaldehyde and embedded in paraffin for routine histological processing, and 4 μm sections were prepared for hematoxylin and eosin (HE) staining and Masson trichrome staining.[19] The sections were then examined with an optical microscope (Leica, Germany) and images were taken at 10× and 400× magnification. The collagen volume fraction (CVF) was analyzed by Microscopic Image Analysis Software (Motic Med 6.0) in the infarcted zone. Six separate views (400× magnification) of Masson staining sections were selected and CVF was calculated using the following formula: CVF=collagen area/total visual area × 100%, to assess the degree of cardiac fibrosis.

Determination of Serum Biomarkers

The level of CK-MB in serum was measured by ELISA. The serum of each group was taken from –80 ℃ and centrifuged (1,500 × *g*) for 15 min at 4 ℃, and operated according to the manufacturer's instructions of the ELISA kit.

Immunohistochemistry Analysis

Immunohistochemistry (IHC) analysis was used to detect the expression of collagen type Ⅰ and Ⅲ. The procedure was described briefly as follows: the sectioned cardiac tissues were incubated with rabbit polyclonal antibodies against collagen Ⅰ (1:100) and collagen Ⅲ (1:100) overnight at 4 ℃. Then, tissue slices were washed with 1% PBS 3 times (5 min for each time). Subsequently, horseradish peroxidase (HRP)-polymer conjugated anti-rabbit IgG (Maixin, Fuzhou, China) was used as the secondary antibody. The sections were reacted with diaminobenzidine (DAB) kit (Maixin; Cat No. DAB-2031), and then counterstained for 20 s with hematoxylin. Slides were visualized under a light microscope (400× magnification), and 6 fields of view were randomly selected and the average optical density of positive cells in each field was determined using Microscopic image analysis software (Motic, China).

Western Blot Analysis

Myocardial tissues were homogenized with Western and IP cell lysis buffer (Beyotime, China) containing phenylmethylsulfonyl fluoride (PMSF, Solarbio, China), protease inhibitor cocktail (MCE; Cat No. hy-k0010) and phosphatase inhibitor phosstop (Roche, Basel, Switzerland), followed by centrifuged at 14,000 × *g* for 20 min at 4 ℃. The supernatants were collected as total proteins then measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA, USA), equal amounts of proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel and were transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane (AmershamHybond, GE Healthcare, München, Germany). Membranes were incubated with tris-buffered saline with Tween 20 (TBST) containing 5% skim milk powder (OXOID, UK, No. lp0031b-500), followed by incubation using the primary antibodies for TGF-β (1:1000), Smad2/3 (1:1000), p-Smad2/3 (1:1000), GAPDH (1:5000) overnight at 4 ℃. After incubation, the membranes were incubated with anti-rabbit IgG, HRP-linked antibody (CST, USA) at room temperature for 2 h. Protein expressions were detected with electrochemi-luminescence (ECL) chemiluminescence kit, using ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Image J Software (National Institutes of Health, Bethesda, Maryland, USA) was used for gray value analysis.

Statistical Analysis

All the values obtained from the experimental results were expressed as mean ± standard deviation ($\bar{x} \pm s$). Using SPSS software (version 23.0, SPSS, Inc., Chicago, USA), independent Student's *t* or one-

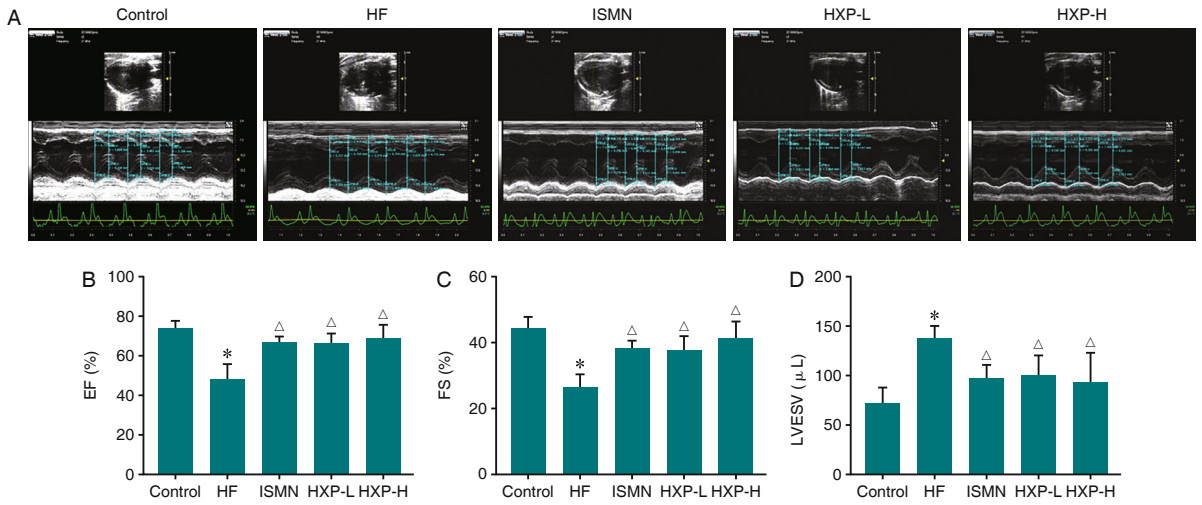

Figure 1. HXP Improves Cardiac Function after HF in Rats (n=6, $\bar{x} \pm s$)

Notes: (A) The representative echocardiographic images of rats in each group after treatment for 7 weeks. Densitometric analysis of the data demonstrated a significantly improvement of cardiac function, including preserving (B) EF, (C) FS and (D) LVESV after HXP and ISMN treatment. *P<0.05 *vs*. control group, △P<0.05 *vs*. HF group

way analysis of variance (ANOVA) was performed to compare difference between two groups or among three and more groups, when conforming to a normal distribution. Non-parametric test was used to compare difference between two groups when not consistent with normal distribution. P<0.05 indicated that the difference was statistically significant.

RESULTS

HXP Improves Heart Function in HF Rats

As showed in Figures 1A–1C, compared with the control group, injection of ISO reduced EF and FS in the ISO-induced HF rats (P<0.05), which were increased after both ISMN and HXP treatment compared with the HF group (P<0.05). On contrast, ISMN or HXP treatment obviously attenuated the elevation of the LVESV in ISO-induced HF rats (Figures 1A and 1D, P<0.05). Furthermore, determination of HWI revealed that HXP and ISMN treatment significantly attenuated adverse cardiac structural changes and reduced HWI (Figures 2A and 2B, P<0.05) in ISO-induced HF rats.

HXP Alleviates Cardiac Injury in HF Rats

HE staining showed that the cardiac tissue of rats in the control group exhibited normal organized architecture, whereas extensive necrotic myocardial tissue with infiltration of inflammatory cells and moderate myocardial cell edema were observed in cardiac tissues of HF rats. However, HXP and ISMN treatment significantly attenuated necrosis and infiltration of inflammatory cells (Figure 3A). Moreover,

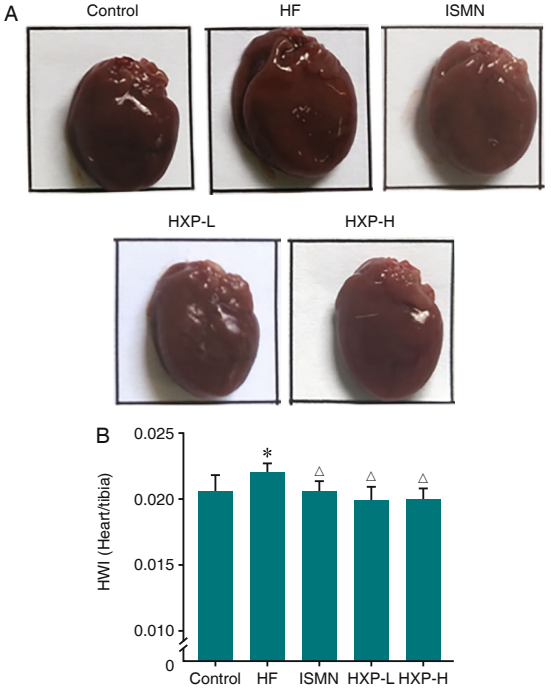

Figure 2. HXP Improves Cardiac Structure after HF in Rats (n=6, $\bar{x} \pm s$)

Notes: (A) The representative images of hearts of rats in each group. (B) The ratio of heart weight and tibial length of rats in each group. *P<0.05 *vs*. control group, △P<0.05 *vs*. HF group

compared with the control group, the level of CK-MB was increased in the HF group (P<0.05), and it was decreased after the treatment of HXP or ISMN compared with the HF group (Figure 3B, P<0.05).

HXP Attenuates Cardiac Fibrosis in HF Rats

Masson's trichrome staining revealed that ISO injection significantly aggravated the degree of cardiac

Chin J Integr Med 2021 Jun;27(6):424-431

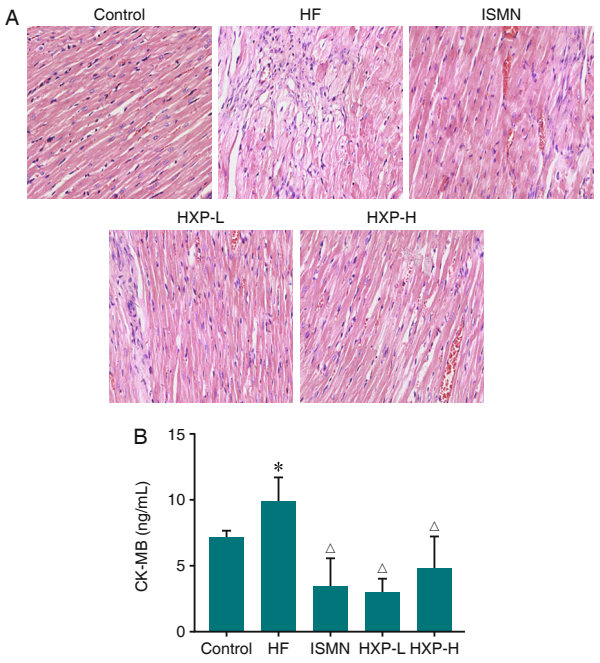

Figure 3. HXP Alleviates Cardiac Injury in HF Rats (*n*=6, $\bar{x} \pm s$)

Notes: (A) Cardiac tissues of rats from each group were stained with HE, representative images were taken at a magnification of ×400. (B) The serum levels of CK-MB in rats from each of 5 groups were detected by ELISA analysis. *P<0.05 *vs*. control group, △P< 0.05 *vs*. HF group

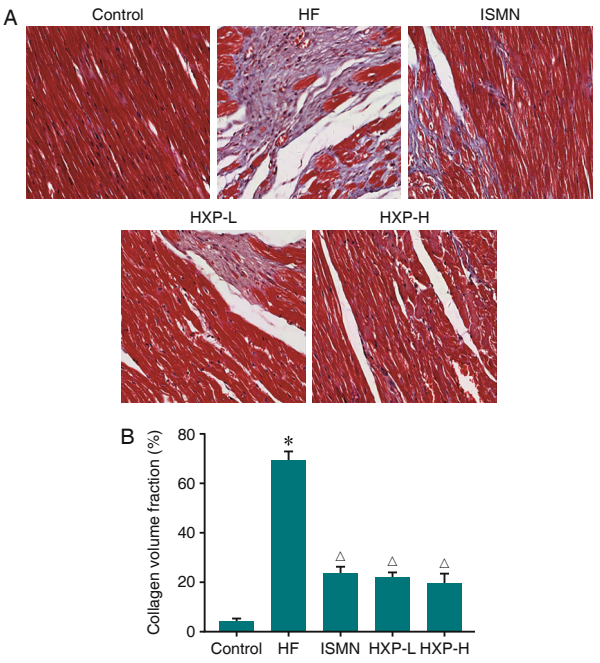

Figure 4. HXP Attenuates Collagen Content in Cardiac Tissues of HF Rats (*n*=6, $\bar{x} \pm s$)

Notes: Masson trichrome staining was used to detect the collagen content of cardiac tissues of rats from each of 5 groups. (A) The representative images were taken at a magnification of ×400. (B) The degree of cardiac interstitial fibrosis in cardiac tissues of rats from each of 5 groups were analyzed. *P<0.05 *vs*. control group, △P< 0.05 *vs*. HF group

interstitial fibrosis in the HF group (P<0.05), which were obviously attenuated after HXP or ISMN treatment compared with the HF group (Figures 4A and 4B, P<0.05). There was a marked increase on expressions of collagen Ⅰ (Figures 5A and 5B) and collagen Ⅲ (Figures 5C and 5D) in ISO-induced HF rats, which was robustly attenuated after HXP or ISMN treatment

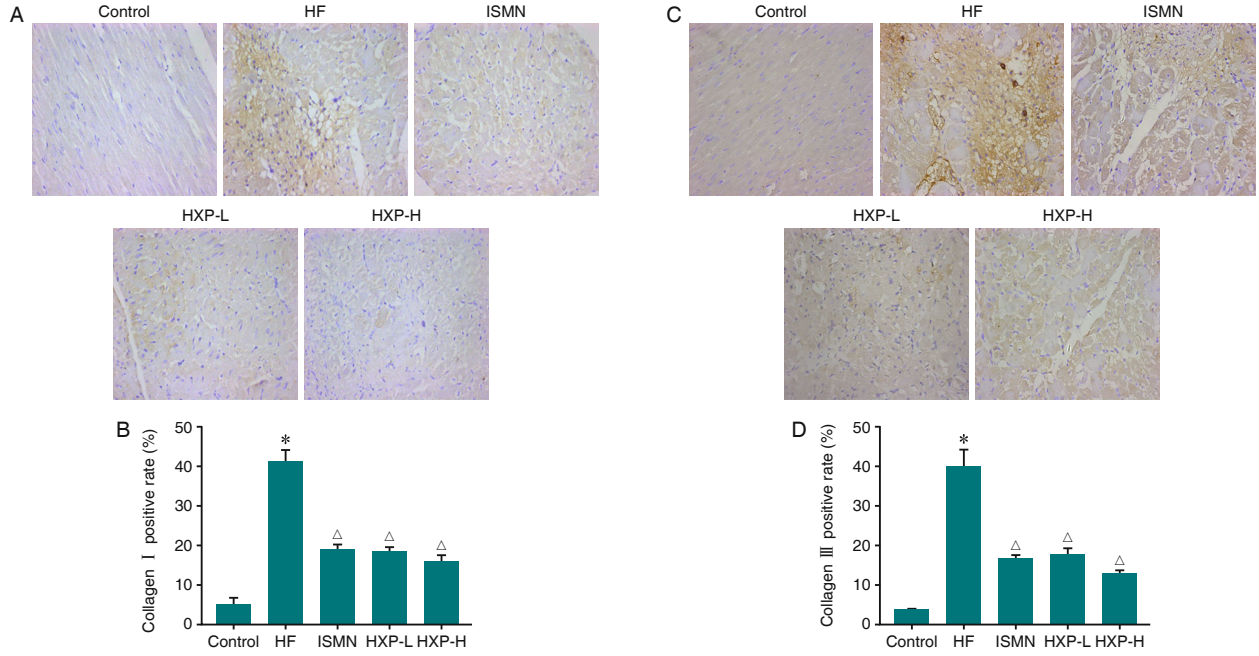

Figure 5. HXP Decreases Collagen Ⅰ and Collagen Ⅲ Protein Expression in HF Rats (*n*=6, $\bar{x} \pm s$)

Notes: IHC was performed to determine the expression of collagen Ⅰ (A, B) and collagen Ⅲ (C, D) in cardiac tissues of rats from each of 5 groups. (A, C) The representative immunohistochemical images were taken at a magnification of ×400. (B, D) The percentage of collagen Ⅰ and collagen Ⅲ positively stained cells was analyzed. *P<0.05 *vs*. control group, △P<0.05 *vs*. HF group

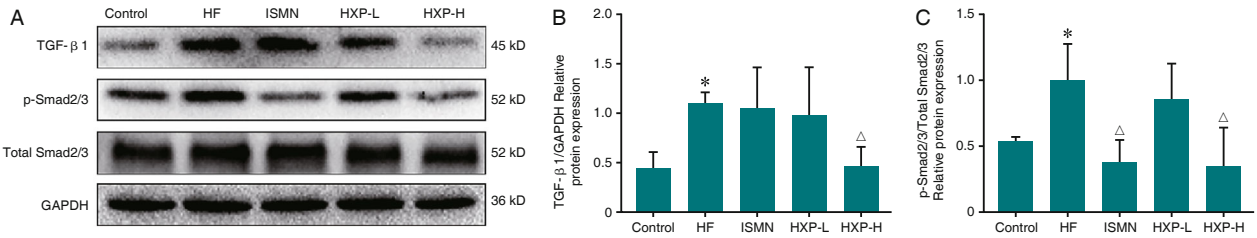

Figure 6. HXP Suppresses TGF-β1/Smad2/3 Signaling Pathway in HF Rats (n=3, $\bar{x} \pm s$)

Notes: Western-blot was performed to determine the expression of TGF-β1 and its downstream effectors. (A) The representative images of Western blot were shown. The expression of TGF-β1/GAPDH (B) and p-Smad2/3/total Smad2/3 (C) were analyzed by quantitation of the bands using ImageJ software. $^{*}P<0.05$ *vs*. control group, $^{\triangle}P< 0.05$ *vs*. HF group

compared with the HF group (Figures 5A–5D, $P<0.05$).

HXP Suppresses TGF-β1/Smad2/3 Signaling Pathway in HF Rats

As shown in Figure 6, the protein expression of TGF-β1 (Figures 6A and 6B) and p-Smad2/3 (Figures 6A and 6C) was obviously up-regulated in the HF group, however, which were down-regulated after treatment of HXP compared with the HF group ($P<0.05$). In addition, ISMN treatment obviously reduced the elevated Smad2/3 phosphorylation in cardiac tissues of HF rats ($P<0.05$), while HXP and ISMN had not affect the expression of total Smad2/3 (Figures 6A and 6C, $P>0.05$).

DISCUSSION

Ischemic heart disease with atherosclerosis is the most important cause of HF in developed countries.[20] The complex pathogenesis of HF and the limitations of current treatment methods require us to further improve its level of prevention and treatment. It has been reported that CM has outstanding advantages in the treatment of HF and is an ideal alternative therapy.[21,22] The current study revealed that HXP treatment significantly attenuated ISO induced cardiac dysfunction, elevation of CK-MB level in serum, and cardiac pathological changes of HF rats. Moreover, HXP treatment obviously alleviated the elevation of collagen content and expression of collagen Ⅰ, collagen Ⅲ, as well as TGF-β1 and its downstream effectors in cardiac tissues of HF rats. These studies suggested that HXP treatment attenuated ISO-induced cardiac fibrosis and HF by suppression of TGF-β1/Smad2/3 pathway.

Cardiac fibrosis is the basic mechanism leading to the development of HF, which is the most serious stage of cardiovascular disease.[23] Therefore, it is urgent need to explore novel strategies for prevention of cardiac fibrosis and HF. CM, could improve the outcome in the treatment of ischemic heart disease.[15] As a traditional Chinese formula, HXP has long been used in China for the clinical treatment of coronary heart disease. However, the pharmacologic actions of HXP on HF remain unclear, we therefore assessed the effects of HXP on cardiac fibrosis and HF in ISO-induced HF rats. As a β-adrenergic agonist, ISO injection obviously decreased EF and FS, while increased LVESV, heart weight, serum level of CK-MB, and also induced classic cardiac pathological changes, which suggested successful construction of HF model,[24-26] and therefore were used to evaluate the pharmacologic actions of HXP. Further assessment of cardiac function demonstrated that HXP treatment obviously attenuated the decrease of EF, FS and increase of LVESV. Consistently, ELISA analysis indicated that HXP treatment significantly reduced the increase of CK-MB level in serum of HF rats. The improvement of HXP on heart injury was further confirmed by HE staining. These studies suggested that HXP could ameliorate the impairment in cardiac function in HF rats, which highlight the therapeutic efficiency of HXP on HF treatment, and provide the experimental basis for the clinic use of HXP on HF treatment. However, the pathogenesis of HF is very complex, and involves apoptosis, inflammation, myocardial remodeling,[27] therefore, the effects of HXP on those processes should be further explored in future study.

As a common pathological feature of cardiac remodeling following HF,[28] cardiac fibrosis is characteristic by excessive accumulation of extracellular matrix proteins, contributing to systolic and diastolic dysfunction, and ultimately leading to the development of HF.[6] Therefore, the inhibition of cardiac fibrosis is an important potential strategy for the prevention and treatment of HF.[29] Given the complicated pathogenesis

of cardiac fibrosis and lack of treatment methods,[7] we therefore assessed the effect of HXP on cardiac fibrosis and found that HXP treatment obviously attenuated the elevation of collagen content, expression of collagen Ⅰ and collagen Ⅲ in cardiac tissues of HF rats. These results indicated that HXP treatment obviously ameliorated ISO-induced cardiac fibrosis in HF rats, which might be one of underlying mechanisms of HXP on attenuating cardiac remodeling during HF.

TGF-β1 has been identified as a key regulator of cardiac fibrosis, which has wide-ranging effects, including increase collagen and matrix protein production, maintain fibroblast viability, and inhibit production of metalloproteinase.[30,31] By binding with its receptor in the plasma membrane, TGF-β1 induces phosphorylation of Smad2/3 transcription factor which mediate canonical signaling. Phosphorylated Smad2/3 combines with Smad4 in the cytoplasm and translocate to the nucleus to induce transcriptions of fibrosis related genes, including collagen Ⅰ and collagen Ⅲ.[9,32] These studies suggested that TGF-β1 mediated cardiac fibrosis may be a potential target for patients with cardiac fibrosis in the treatment of HF.[12-14] In our study, HXP treatment significantly down-regulated the expression of TGF-β1 and p-Smad2/3. The results demonstrated that HXP treatment attenuated ISO-induced activation of TGF-β1/Smad2/3 pathway in cardiac tissues of HF rats. However, due to the characteristics of multiple components and multiple targets of the CM, the underlying mechanism of HXP treatment on attenuation cardiac fibrosis and HF should be further investigated using omics and bioinformatics analysis.

In summary, HXP treatment attenuated ISO-induced cardiac fibrosis and HF, by suppression of TGF-β1/Smad2/3 pathway. However, the complex pathogenesis of HF and cardiac fibrosis, as well as underlying mechanisms of HXP on cardioprotection should be further addressed in future study.

Conflict of Interest

HXP in this study was presented by Youcare Biopharmaceutics Co., Ltd., and LI Qi is the marketing director of the company. There is no potential conflict of interest among other authors.

Author Contributions

Chu JF and Peng MZ conceived and designed the experiments. Peng MZ, Yang ML, Shen AL and Zhou XL created the models. Lu Y and Shen ZQ performed the experiments. Huang B analyzed the data. Li Q contributed reagents/materials/analysis tools. Peng MZ drafted the manuscript. Chu JF and Peng J revised the manuscript.

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