X indening oral liquid improves cardiac function of rats with chronic cardiac failure via TGF-β1/Smad3 and p38 MAPK pathway

Yunliang Wei, Changsheng Guo, Jingsheng Zhao, Jun Yang, Weiguo Yi**, Hong Liu***, Xinwei Lin*, Zhengchen Zhang[1]*

Departments of Cardiovascular Medicine, *Nephropathy, **Radiology, ***Drug and Equipment the 152nd Central Hospital of PLA, Pingdingshan; Henan-*China*, [1]Department of Drug and Equipment, the 371st Central Hospital of PLA, Xinxiang; Henan-*China*

Abstract

Objective: Xindening oral liquid (Xin) is a widely used traditional Chinese medicine for the treatment of chronic heart failure (CHF). However, the exact mechanisms related to its therapeutic effects against CHF remain unclear. In the present study, we investigate the effects of Xin on cardiac function in CHF rats and the possible mechanisms involved.

Methods: Transverse aortic constriction (TAC) was conducted to induce a CHF rat model in this study. Sixty male Wistar rats were randomly assigned to six groups 28 days after TAC: sham; CHF model; Xin at concentrations of 5 ml/kg, 10 mL/kg, and 20 mL/kg; and QiLi 0.6 g/kg. After four weeks, the rats were treated with Xin (5, 10, or 20 mL/kg/d) for six weeks consecutively. At the end of the study, the cardiac function, heart weight index (HWI) and left ventricular mass index (LVMI), serum level of LDH, B-type natriuretic peptide (BNP), cTnI and CK-MB, and collagen volume fraction were studied. The expression of transforming growth factor-β1 (TGF-β1), drosophila mothers against decapentaplegic protein 3 (Smad3), and p38 mitogen activated protein kinase (p38 MAPK) were detected.

Results: The results showed that Xin treatment significantly improved cardiac function but decreased the serum level of LDH, BNP, cTnI, and CK-MB of CHF rats. In addition, it reduced the HWI, LVMI, and collagen volume fraction compared with the model group. Xin treatment significantly improved cardiac function and attenuated cardiac fibrosis by suppressing the p38 MAPK and TGF-β1/Smad3 signaling pathway in CHF rats.

Conclusion: These results suggested that Xin might be a promising complementary treatment for CHF. More detailed experimental studies will be carried out in our subsequent research. *(Anatol J Cardiol 2017; 17: 367-73)*

Keywords: chronic heart failure; transverse aortic constriction; TGF-1/Smad3; p38 MAPK

Introduction

Chronic heart failure (CHF), a major public health problem worldwide, is characterized by the inability of the heart to maintain a normal cardiac output without invoking maladaptive compensatory mechanisms (1, 2). Most cardiovascular diseases, such as myocardial infarction, hypertension, myocarditis, dilated cardiomyopathy, and congenital heart disease, may lead to heart failure. The incidence of heart failure has increased for more and more obesity, hypertension, and diabetes induced by a subhealth lifestyle, especially in low- and medium-income countries (3–5). For CHF patients, both survival (6) and quality of life have been significantly improved by the use of β-adrenoreceptor blockers and the renin-angiotensin-aldosterone system blocker. Unfortunately, the result of the management of heart failure is still unsatisfying (7). Therefore, searching for new therapeutic strategies to prevent heart failure and cardiac remodeling is in urgent need.

Our laboratory and others have reported that Qili Qiangxin Capsule (QL, a traditional Chinese medicine) can reduce cardiac fibrosis remolding and improve cardiac function (8–10). The use of QL in CHF also has an immunomodulatory effect by decreasing the proinflammatory cytokine TNF-α and increasing the anti-inflammatory cytokine IL-10 (11). In this study, we estimated the cardiac function improvement of Xindening oral liquid (Xin) on heart failure rat model. Xin is a traditional Chinese medicine that consists of ginseng, *Salvia miltiorrhiza*, *Allium macrostemon*, Semen Lepidii Apetali, and Tuckahoe, which exhibit therapeutic effects on CHF patients of all etiologies, including as hypertension or coronary heart disease.

However, the exact mechanisms related to its therapeutic effects against CHF remain unclear. In the present study, we used a transverse aortic constriction (TAC) heart failure model to determine whether Xin can improve cardiac function. We hypothesized that Xin could reduce the expression and activation of cardiac remodeling-related signal pathways, including TGF-β1,

The first two authors contributed equally to this work.

Address for correspondence: Jingsheng Zhao, MD, Department of Cardiovascular Medicine
The 152nd Central Hospital of PLA, Pingdingshan, Henan 467000-*China*
Phone: +86 0375-3843114 E-mail: jszhao_cardio@126.com

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Smad3, and p38 MAPK. Those pathways were examined in our study, and the underground mechanism was investigated.

Methods

Vegetal materials

Practolol oral liquid consists of ginseng, *Salvia miltiorrhiza*, *Allium macrostemon*, Semen Lepidii Apetali, and Tuckahoe (the 152nd Hospital of PLA, Pingdingshan, China). Qiliqiangxin capsule (Yiling Pharmaceutical Corporation, Shijiazhuang, China) was dissolved in sterile water at a concentration of 0.3 g/mL QL as the positive control in this study.

Animal experiments

Healthy male Wistar rats (200~250 g) were provided by Beijing Vital River Laboratory Animal Technology Co. Ltd. The animals were fed a standard diet, and water was provided ad libitum. The rats were maintained under a 12-h light and 12-h dark cycle at a temperature of 20±3°C and a humidity of 50±2%. The CHF model was induced by TAC according to previous studies (12). Sodium pentobarbital (40 mg/kg) was intraperitoneally injected to anesthetize rats, and endotracheal intubation was performed. After opening the chest and identifying the transverse aorta, an 8-0 silk suture was placed around the transverse aorta and immediately removed. To create a similar degree of pressure gradient in all rats, a 26-gauge was used and the suture was tied as tightly as possible. For sham control, rats were given a similar surgical procedure without aortic constriction. In clinical, the adult dose of Xin was 1 mL/kg and the equivalent in rats was 10 mL/kg, which was used as moderate dose. In the treatment group, rats were given 5-, 10-, and 20-mL/kg doses of Xin as the low, moderate, and high doses, respectively. Animals were randomly assigned to six groups 28 days after TAC: sham; CHF model; Xin at concentrations of 5 mL/kg, 10 mL/kg, and 20 mL/kg; and QL 0.6 g/kg.

Xin and QL were administered by gavage once a day for six weeks. An equal volume of distilled water was used for the CHF model and sham groups. No rats died during the period of the study, and they were anesthetized and euthanized for physiological, biochemical, and histological studies at the end of the study. This study was carried out in the 152nd Central Hospital of PLA and approved by the 152nd Center Hospital of the People's Liberation Army in accordance with the Guidelines for Experimental Animals, which was issued by Ministry of Science and Technology (Beijing, China).

Cardiac function and hemodynamic measurements

To monitor the cardiac function, a water-filled latex balloon connected to a pressure transducer was inserted into the left ventricle through an incision in the left atrium and inflated to set a left ventricular end-diastolic pressure (LVEDP) (5–10 mm Hg) using a hemodynamic analysis system (Taimeng Co., Chengdu, China). Heart rate (HR), left ventricular systolic pressure (LVSP), and the rate in rise and fall of ventricular pressure (±dp/dtmax) were recorded as previously reported (13, 14).

Heart weight index (HWI) and left ventricular mass index (LVMI) assessment

All the rats underwent cardiac function assessment and were sacrificed. Heart tissues were washed with cold PBS solution after excision. Left ventricle weight (LVW, mg) and heart weight (HW, mg) were weighed after separating the atria, aorta, and adipose tissue. LVMI (mg/g) and HWI (mg/g) were defined as the ratio of the LVW to the body weight and the HW to the body weight, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The levels of lactate dehydrogenase (LDH), B-type natriuretic peptide (BNP), and cardiac troponin I (cTnI) in serum were measured by ELISA according to the manufacture instructions. Creatine kinase MB isoenzyme (CK-MB) level in the plasma was determined by spectrophotometry.

Morphological examination

Heart tissues were fixed in 10% buffered formalin and embedded in paraffin. The sample was cut into 5-μm slices. Hematoxylin and eosin staining (H&E) and sirius red staining were performed according to previous studies (15). Image-pro plus 6.2 software (Media Cybernetics, Bethesda, MD, U.S.A.) was used to analyze images.

mRNAs detection

Total RNA was extracted from heart tissue by using TRIzol reagent and reverse transcribed to cDNA by cDNA synthesis kit and amplified by quantitative real-time PCR (rt-PCR). The primers used in this study were purchased from GENEWIZ Co., Ltd. (Suzhou, China). The sequences were as follows: TGF-β1(F) 5′-TGCTTCAGC TCCACAGAGAA-3′, TGF-β1(R) 5′-TGGTTG-TAGAGGGCAAGGAC-3′, Smad3(F) 5′-G GCAGGATGTTTCCAGC-TA-3′,Smad3(R) 5′-GCAGTCCACAGACCATGTCA-3′, p38 MAPK (F) 5′-GGGACCTCCTTATAGACGAA-3′, p38 MAPK (R) 5′-GGCACTT-GAATG GTATTTGG-3′, β-actin (F) 5′-AGGGAAATCGTGCGT GA-CAT-3′, β-actin (R) 5′-GAACCGCTCATTGCCGATAG-3′. Each experiment was performed three times. In this study, β-actin was used as an internal control gene to normalize the mRNA levels of TGF-β1, Smad3, and p38 MAPK in ventricular tissues.

Western blots

TGF-β1, drosophila mothers against decapentaplegic protein 3 (Smad3), phosphorylated Smad3, p38 mitogen activated protein kinase (p38 MAPK), and phosphorylated p38 MAPK were quantified by Western blot. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a loading control for western blots in this study. Protein concentration was measured with a microplate reader (Thermo Fisher, MA, U.S.A.). Protein samples from heart tissues were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene

difluoride membranes (MILLIPORE). Blots were probed with anti-TGF-β1 (Santa Cruz Biotechnology), anti-Smad3 (Cell Signaling Technology), and anti-p38 MAPK (Abcam), respectively. Horseradish peroxidase-conjugated anti-immunoglobulin G (Southern Biotechnology) was used as a secondary antibody. The bands were detected by use of Gel Image System (Tanon, Shanghai, China).

Statistical analysis

Continuous data were represented as mean±SD. The Shapiro–Wilk normality test was used for checking the normality of the data. Differences between two groups were analyzed by Student's t-tests. Results involving more than two groups were analyzed by a one-way ANOVA procedure with the Dunnett Significant Difference test used to evaluate differences among means. All data were analyzed with statistical software (SPSS 20; IBM SPSS, Chicago, IL, U.S.A.). A p-value of <0.05 was considered to be statistically significant.

Results

Effects of Xindening oral liquid on cardiac function and hemodynamic measurements

Compared with the sham-operated rats, the LVEDP was significantly increased while the HR and LVSP were significantly decreased in the TAC model group. However, after Xin treatment, the above parameters were improved, showing significantly reduced LVEDP but higher HR and LVSP (Table 1). In comparison with the sham group, the dp/dtmax were reduced in the TAC model group significantly. Xin treatment partially reversed the above changes, leading to increased dp/dtmax. However, the changes in dp/dtmax were not significant. Notably, the effects of Xin were does -dependent (Table 1).

Effects of Xindening oral liquid on cardiac weight indexes

HWI and LVMI in the TAC rats were also improved by Xin treatment. The HWI (Fig. 1a) and LVMI (Fig. 1b) of the TAC rats were obviously greater than that of the sham-operated rats. After the treatment of 20 mg/mL of Xin for six weeks, HWI and LVMI were recovered to 3.186±0.361 mg/g and 2.037±0.239 mg/g from 3.583±0.415 and 2.344±0.193 ($p<0.05$ and $p<0.01$), respectively.

Effects of Xindening oral liquid on BNP, LDH, cTnI, and CK-MB

The results in Figure 2 showed that BNP, LDH, cTnI, and CK-MB in the TAC model group increased significantly compared with the sham group, which indicated cardiac injury. ALL doses of Xin treatment inhibited the level of BNP and LDH significantly in a dose-dependent way (Fig. 2a, b, $p<0.05$ or $p<0.001$). Mean-

Table 1. Effects of Xin on cardiac function and hemodynamic measurements

Groups	HR beats/min	LVSP mm Hg	LVEDP mm Hg	dp/dtmax mm Hg/s	–dp/dtmax mm Hg/s
Sham	358.4±18.8	166.6±13.1	50.2±12.3	1711±157	1588±177
Model	334.5±24.4*	144.2±23.9*	65.5±17.7*	1497±265*	1440±176
Xin 5 mL/kg	336.1±24.3	154.8±14.7	57.4±13.9	1550±215	1503±231
Xin 10 mL/kg	353.8±18.9	164.6±20.5	59.0±17.3	1595±307	1534±208
Xin 20 mL/kg	344.2±21.0	164.0±17.6#	61.0±26.0	1591±227	1502±156
QL	356.0±18.3#	165.3±5.6#	56.0±11.2	1655±105	1540±118

HR - heart rate; LVEDP - left ventricular end-diastolic pressure; LVSP - left ventricular systolic pressure. ±dp/dtmax the rate in rise and fall of ventricular pressure. *P<0.05 versus sham, #P<0.05 versus model

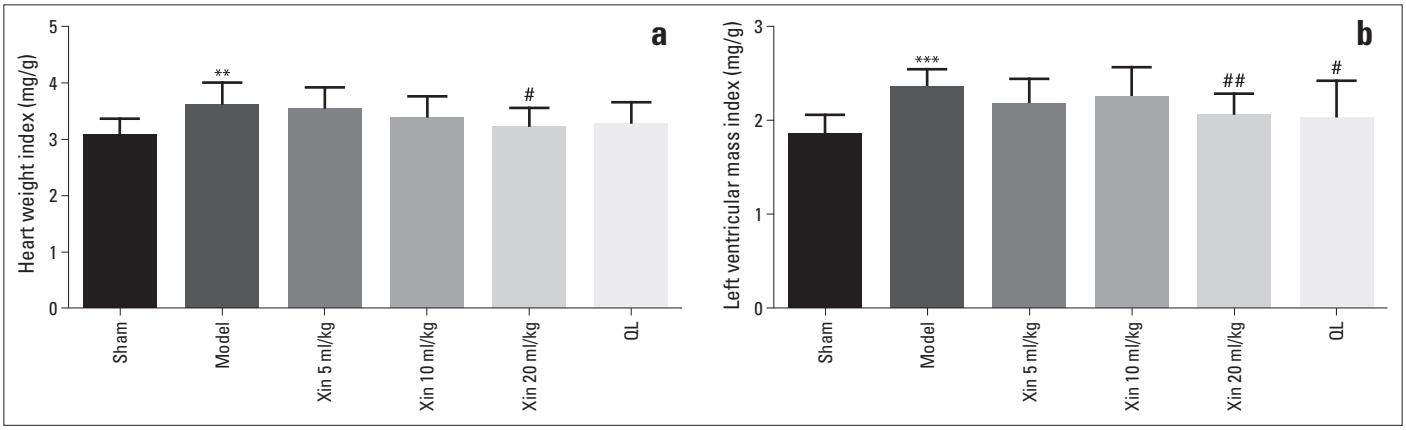

Figure 1. Effects of Xin on HWI (a) and LVMI (b) in CHF rats. Six weeks after the TAC or sham operation, heart weigh index (HWI, A) and left ventricle mass index (LVMI, B) were measured to investigate the cardiac remodeling in each group. Xin at 20 mL/kg decreased the HWI and LVMI induced by TAC. ** P<0.01, *** P<0.001 vs. the sham; # P<0.05, ## P<0.01 vs. the model (n=10)

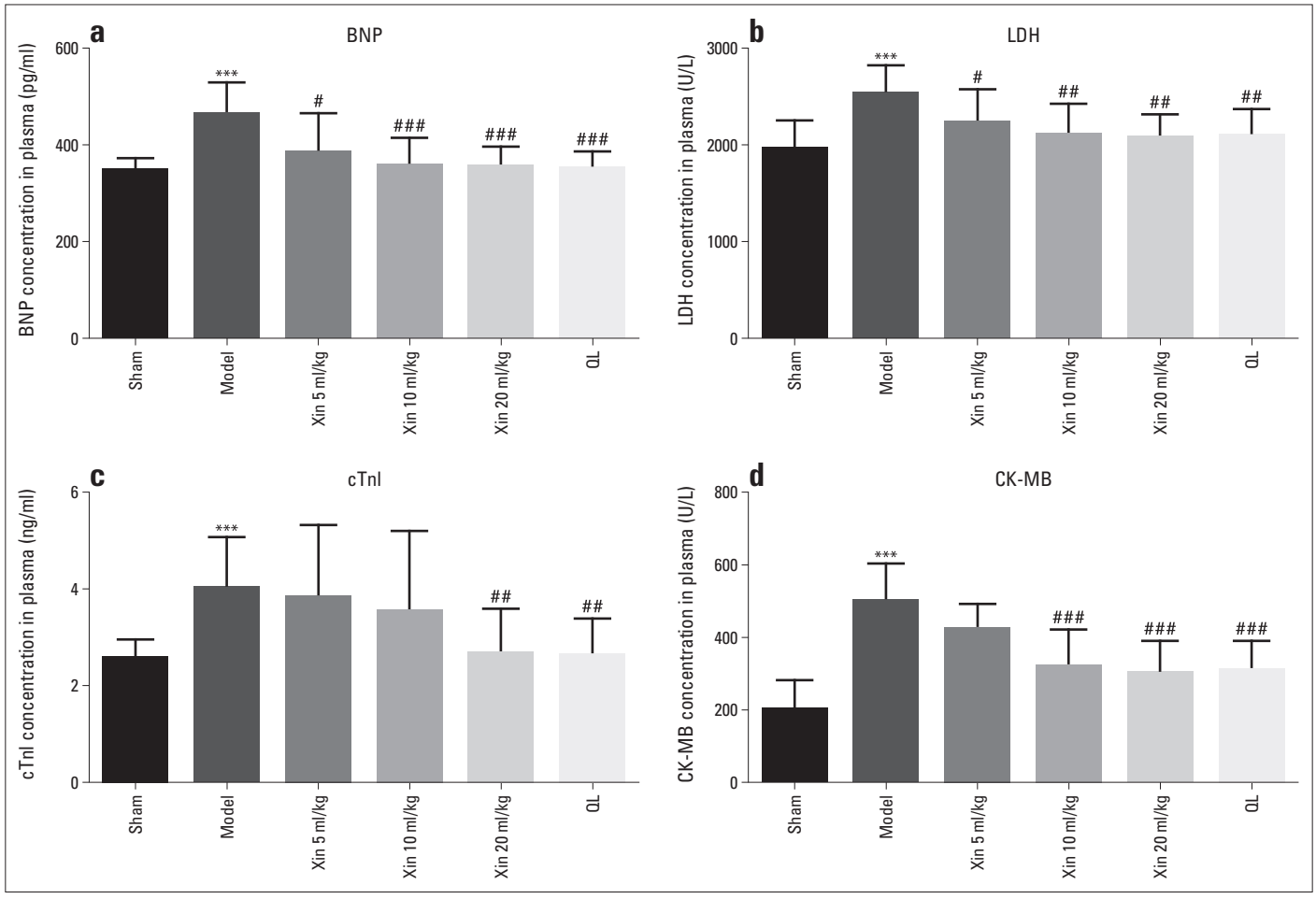

Figure 2. Effects of Xin on BNP (a), LDH (b), cTnI (c), and CK-MB (d) contents determined via ELISA assay. Six weeks after the TAC or sham operation, the plasma level of heart failure-related bio-marker including BNP (a), LDH (b), cTnI (c), and CK-MB (d) were determined by ELISA assay in each group of rats. Xin decreased the levels of BNP, LDH, cTnI, and CK-MB in a dose-dependent way. ***P<0.001 vs. the sham; #P<0.05, ##P<0.05, ###P<0.001 vs. the group model (n=10)

while, Xin also suppressed cTnI and CK-MB content, whereas the effects of a high dose of Xin were significantly stronger (Fig. 2c, d, p<0.01 or p<0.001).

The increased HWI, LVMI, and BNP levels combined with the changes of cardiac and hemodynamic function showed that the CHF model was successfully established.

Effects of Xindening oral liquid on cardiac fibrosis in rats

The myocardial tissue from the sham-operated rats were arranged in order (Fig. 3a). In the TAC model group, the myocardial tissue was arranged irregularly and infiltrated with inflammatory cells (Fig. 3b). Consistent with the protective effects of Xin on cardiac function and serum biomarkers, it also can significantly reduce inflammatory cells (Fig. 3c–e) in myocardial tissues.

Sirius red staining is a well-established way to assess the cardiac fibrosis. Collagen fibers were stained red, and myocardial tissues were stained yellow. Myocardial tissue from the sham-operated rats was stained with a few stained collagen fibers (Fig. 4a). For the TAC rats, the collagen fibers increased significantly compared with that for the sham-operated rats (Fig. 4b). Collagen fibers (Fig. 4c–e) were also reduced in myocardial tissues under the treatment of Xin in a dose-dependent way.

Effects of Xindening oral liquid on mRNA expression of TGF-β1, Smad3 and p38 MAPK

rt-PCR was conducted to detect the mRNA expression of TGF-β1, Smad3, and p38 MAPK in this study (n=10). The results in Figure 5 suggested that the expressions of TGF- β1 (1.00±0.20 vs. 7.50±1.68, p<0.01), Smad3 (1.00±0.25 vs. 1.75±0.32, p<0.01), and p38 MAPK (1.00±0.23 vs. 4.68±1.12, p<0.01) mRNA were obviously higher in the TAC rats than that in the sham-operated rats. On the other hand, the over-expression of TGF- β1, Smad3, and p38 MAPK in the TAC rats were significantly inhibited by Xin (Fig. 5). Likewise, the effects of Xin were dose-dependent.

Effects of Xindening oral liquid on protein expression and activation of TGF-β1, Smad3, and p38 MAPK

Consistent with the results of mRNA expression, the protein levels of TGF- β1, Smad3, and p38 MAPK were significantly increased in the TAC model group than in the sham group (Fig. 6).

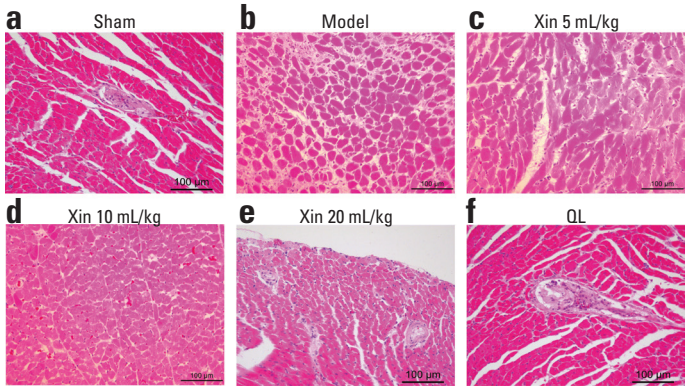

Figure 3. Effects of Xin on histopathology of myocardial tissues in rats represented by HE staining (100×). The myocardial tissues of each group (a, sham; b, Model; c, Xin 5 mL/kg; d, Xin 10 mL/kg; e, 20 mL/kg, f, QL) were harvested six weeks after the treatment. HE staining was carried out to determine the cardiac tissue remolding in each group. Xin improved the cardiac tissue disorder caused by TAC

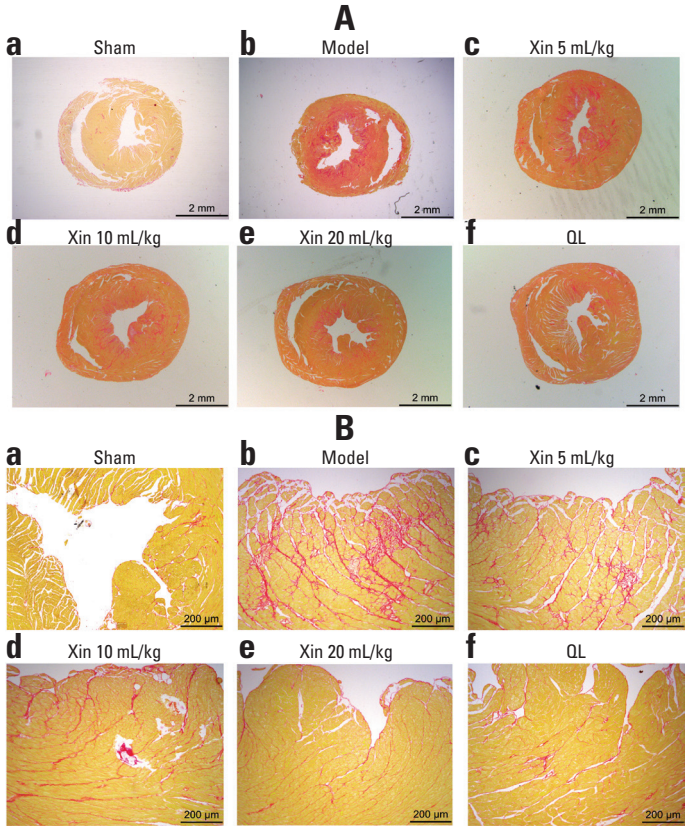

Figure 4. Effects of Xin on collagen fibers of myocardial tissues in rats represented by sirius red staining (A, 10×; B, 100×). After six weeks of treatment, sirius red staining was carried out to determine the cardiac fibrosis in each group (a, sham; b, Model; c, Xin 5 mL/kg; d, Xin 10 mL/kg; e, 20 mL/kg, f, QL). Xin inhibited the cardiac collagen genesis caused by TAC

In addition, the p-Smad3 and p-p38 MAPK were also reduced in the Xin group, indicating that Xin could inhibit the activation of Smad3 and p38 MAPK pathway. Compared with the TAC model group, Xin treatment markedly decreased the protein expressions of TGF- β1, Smad3, and p38 MAPK in myocardial tissues in rats in a dose-dependent way (n=10).

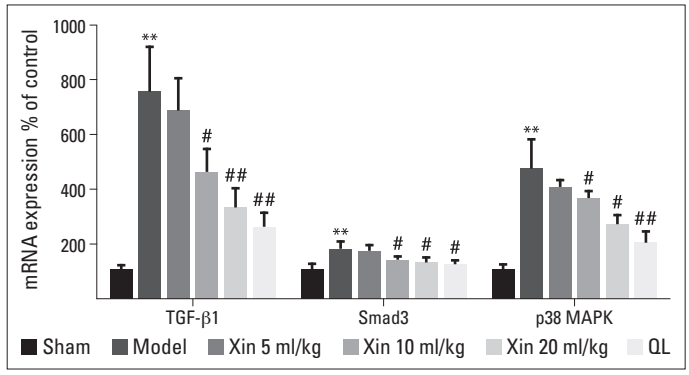

Figure 5. Effects of Xin on TGF-β1/Smad3 and p38 MAPK pathways and TLR4. Heart tissue was harvest after six weeks of treatment, and the expression and activation of heart failure-related proteins were examined by Western blot. Xin inhibited the protein expression of TGF-β1, Smad3, and p38 MAPK in a dose-dependent way (n=10)

Discussion

In this study, we employed a TAC mouse model of heart failure to examine the effects of Xin. The results suggested that Xin seemingly had positive effects on the cardiac structure and function of rats with CHF. The TAC model was chosen to induce an initial compensatory cardiac remodeling and lead to pressure overload-induced heart failure. The TAC model has been extensively used to study signaling pathways involved in cardiac hypertrophy and heart failure in cardiovascular disease studies (16).

After the TAC procedure, rats developed CHF exhibited obvious cardiac tissue fibrosis and significant increase in TGF- β1, Smad3, and p38 MAPK expression and activation in heart tissues. Therapy with Xin reduced heart tissue collagen deposition. Xin reduced heart TGF- β1, Smad3, and p38 MAPK protein expression and activation. These data suggest some direct beneficial effects of Xin on cardiac structure fibrosis, remolding, and inflammation response-related pathways in rats with CHF.

Xin is a traditional Chinese medicine that consists of ginseng, *Salvia miltiorrhiza*, *Allium macrostemon*, Semen Lepidii Apetali, and Tuckahoe, which has similar herbs to Qili Qiangxin (QL) capsule. According to previous studies, QL capsule has been proven to be effective and safe for the treatment of CHF (17). Pharmacological studies have found that QL contains a number of active substances such as ginseng saponin, astragalus saponin, flavonoids, cardenolide, and phenolic acid, which have been proven to have positive inotropic, vasodilation, anti-inflammation, and anti-fibrosis effects. As for Xin, *Salvia miltiorrhiza* and ginseng comprise the main active constituents that have effects on invigorating the heart QI and lung QI. *Salvia miltiorrhiza* can accelerate blood circulation and relieve congestion. Semen Lepidii Apetali can relieve asthma and disperse lung edema. The use of these herbs demonstrated that Xin has protective effects on the heart. In this study, we found excessive collagen deposition and upregulated TGF-β1, Smad3, and p38 MAPK protein expression in the heart of rats with CHF; both were reversed by Xin

therapy, suggesting that Xin may have cardiac protective effects on chronic heart failure.

Effects of Xin on the TGF-β1/Smad3 pathway were detected to explore the possible mechanisms related to the cardiac protective effect of Xin for CHF in this study. TGF-β1 has been reported to play an important role in myofibroblast proliferation and tissue fibrosis. Effects of TGF-β in heart failure have been explored by several studies, and studies have shown that TGF-β has protective effects against heart failure by inhibiting fibrosis (18). Smad3 is a transcription factor that acts as a main downstream signal transducer of TGF-β1. Smad3 play an important role in promoting type I and type III collagen gene expression, which contribute much to the fibrosis. Besides, the p38 MAPK signaling pathway are involved in the process of inflammation (19). Both clinical and animal studies have found that sustained p38 MAPK activation is associated with chronic heart and cardiac remodeling (20). In our study, TGF-β1 expression in heart tissues of rats with CHF was increased, and the level of Smad3 and p38 MAPK were also markedly increased; all of these were reversed by Xin therapy. Significant activation of TGF-β1/Smad3 and p38 MAPK signaling pathway in heart tissues after CHF can lead to myofibroblast proliferation and a marked upregulation of type I collagen expression. The suppression of Xin on the TGF-β1/Smad3 and p38 MAPK signaling pathway contributed to its attenuation of cardiac remodeling in CHF.

Study limitations

Although the results of this study indicated that Xin may alleviate myocardial fibrosis and improve cardiac function in TAC-induced cardiac remodeling rats, some limitations should be taken into consideration. First, only three different drug concentrations of Xin were studied. As a result, we could not determine the optimal therapeutic dosage of Xin in the treatment of cardiac remodeling. Besides, we failed to study the optimal period of Xin treatment because no variant-time (6, 8, 12 weeks or more) subgroups were set. Moreover, inflammatory cytokines and parameters of immune function such as lymphocyte counts and interleukin-6 levels were not detected. More detailed experimental studies will be performed in our subsequent research.

Conclusion

In conclusion, Xin could improve cardiac function, decrease serum biomarkers, and alleviate cardiac fibrosis in CHF rat induced by TAC. The protective effects are related to the suppression of the p38 MAPK and TGF-β1/Smad3 signaling pathways. These results suggested Xin may be a promising complementary treatment option for CHF patients. Further studies are needed.

Author contributions and acknowledgments

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Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

Authorship contributions: Concept – J.Z.; Design – Y.W., C.G.; Supervision – J.Z.; Fundings – J.Z.; Materials – Y.W., C.G.; Data collection &/or processing – J.Y., W.Y., H.L., X.L., Z.Z.; Analysis &/or interpretation – J.Y., W.Y., H.L., X.L., Z.Z.; Literature search – Y.W., C.G.; Writing – J.Y., W.Y., H.L., X.L., Z.Z.; Critical review – J.Z.

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Correction

The Editor-in-Chief would like to issue an erratum statement to correct the article "Extracorporeal cardiopulmonary resuscitation for refractory cardiac arrest in children after cardiac surgery," written by Ersin Erek, Selim Aydın, Dilek Suzan, Okan Yıldız, Fırat Altın, Barış Kırat, İbrahim Halil Demir, and Ender Ödemiş, and published online ahead of print in the Anatolian Journal of Cardiology. Doi:10.14744/AnatolJCardiol.2016.6658 that was need to be defined.

The original version of the article incorrectly identified hospital to which some of the study patients belonged. Twenty patients were operated on by the Istanbul Mehmet Akif Ersoy Thoracic and Cardiovascular Surgery Research and Training Hospital Department of Cardiovascular Surgery, not Acıbadem Atakent Hospital, as initially stated.

This error does not affect the conclusion of the paper in any way but the earlier, affilation of the first author's institution would like this statement.

The Editor-in-Chief would like to apologize to the readers for the error and any inconvenience caused.