

Original research article

Guanxinning tablets improve myocardial hypertrophy by inhibiting the activation of MEK-ERK1/2 signaling pathway

Yan Zhang^{1,2}, Yu Huang², Quan-xin Ma², Song-tao Xu³, Liye Shen³, Yan-yun Xu³, Hai-ye Tu³, Min-li Chen², Yi-li Rong^{2*}

¹ Jiaying Hospital of Traditional Chinese Medicine, Jiaxing 314000, China

² Zhejiang Chinese Medical University, Academy of Chinese Medicine & Institute of Comparative Medicine, Hangzhou 310053, China

³ Zhejiang Chinese Medical University, College of Pharmacy, Hangzhou 310053, China

Abstract

Myocardial hypertrophy may lead to heart failure and sudden death. As traditional Chinese medicine, Guanxinning tablets (GXN) have significant pharmacological effects in the prevention and treatment of cardiovascular diseases. However, the anti-cardiac hypertrophy efficacy of GXN and its mechanism of action are still unclear. Therefore, we established a heart failure rat model and isolated primary cardiomyocytes of neonatal rat to observe the protective effect of GXN on heart failure rat model and the intervention effect on myocardial cell hypertrophy, and to explore the possible mechanism of GXN preventing and treating myocardial hypertrophy. The results of *in vivo* experiments showed that GXN could significantly reduce the degree of cardiac hypertrophy, reduce the size of cardiomyocytes, inhibit the degree of myocardial remodeling and fibrosis, and improve cardiac function in rats with early heart failure. The results of *in vitro* experiments showed that GXN was safe for primary cardiomyocytes and could improve cardiomyocyte hypertrophy and reduce the apoptosis of cardiomyocytes in pathological state, which may be related to the inhibition of the over-activation of MEK-ERK1/2 signaling pathway. In conclusion, GXN may inhibit cardiac hypertrophy and improve early heart failure by inhibiting the over-activation of MEK-ERK1/2 signaling pathway.

Keywords: Early heart failure; Guanxinning tablet; MEK-ERK1/2 signaling pathway; Myocardial hypertrophy

Highlights:

- 8-week GXN treatment significantly reduced cardiac hypertrophy in rats with early heart failure.
- GXN was safe, improved cardiomyocyte hypertrophy and reduced apoptosis of cardiomyocytes.
- GXN inhibits the overactivation of MEK-ERK1/2 signaling pathway.

Introduction

Myocardial hypertrophy is a compensatory response to maintain cardiac output under various physiological and pathological stimuli. However, long-term cardiac hypertrophy often leads to cardiac decompensation, leading to heart failure and sudden death, which is a major cause of cardiac morbidity and mortality worldwide (Nakamura and Sadoshima, 2018). Mainstream drugs for the treatment of cardiac hypertrophy are all western medicines, and mainly include beta-receptor blockers and renin-angiotensin-aldosterone system inhibitors. They have defects and shortcomings, such as unsuitable control of optimal dose, and large individual differences in efficacy (McDonagh et al., 2022). Traditional Chinese medicine is characterised by diverse active components, a mild curative effect,

few side effects, and its treatment of both symptoms and root causes (Huang et al., 2020). Guanxinning tablet (GXN) is extracted from *Salvia miltiorrhiza* and *Ligusticum chuanxiong* in 1 : 1 ratio using special technology. It has the effect of promoting blood circulation and removing blood stasis, clearing arteries and nourishing the heart (Chen et al., 2006). Previous studies have shown that GXN has significant pharmacological effects in the prevention and treatment of cardiovascular diseases. For instance, (1) GXN effectively inhibits sympathetic nerve activity to improve myocardial action potential and excitability during myocardial ischemia reperfusion injury in rats, thereby correcting autonomic regulatory dysfunction and enhancing left ventricular function (Hu et al., 2017). (2) GXN can improve the abnormality of hemorheology, increase the plasma NO concentration in rats with blood stasis and Qi deficiency/stagnation, and treat myocardial ischemia in

* **Corresponding author:** Yi-li Rong, Zhejiang Chinese Medical University, Academy of Chinese Medicine & Institute of Comparative Medicine, Hangzhou 310053, China; e-mail: ryl@zcmu.edu.cn
<http://doi.org/10.32725/jab.2023.014>

Submitted: 2022-10-25 • Accepted: 2023-09-15 • Prepublished online: 2023-09-18

J Appl Biomed 21/3: 137–149 • EISSN 1214-0287 • ISSN 1214-021X

© 2023 The Authors. Published by University of South Bohemia in České Budějovice, Faculty of Health and Social Sciences.

This is an open access article under the CC BY-NC-ND license.

dogs (Chen et al., 2005a, b; Hu et al., 2017). (3) Additionally, GXN exhibits a cardioprotective effect by reducing myocardial oxygen consumption and maintaining normal oxygen metabolism and energy supply-demand balance during myocardial ischemia (hypoxia) (Pan et al., 2007). At present, the research and development of anti-myocardial hypertrophy traditional Chinese medicine is mainly in the experimental research stage (Xu et al., 2022; Wang et al., 2017), such as in *Astragalus membranaceus* (Zang et al., 2020), Digitoxinb – monomer extracted from *Digitalis lanata* Ehrh (Bavendiek et al., 2019), QSYQ – a patented Chinese prescription (Shang et al., 2013), while GXN has obtained clinical application (Sun et al., 2019). Therefore, this paper aims to study the anti-myocardial hypertrophy efficacy of GXN and its mechanism of action. GXN is expected to expand its clinical application in the treatment of cardiac hypertrophy, significantly reducing the development time and achieving cost savings in the treatment of this disease with traditional Chinese medicine.

Studies (Li et al., 2020) have shown that the development of pressure-overload induced cardiac hypertrophy is accompanied by apoptosis of cardiomyocytes, resulting in progressive loss of effective systolic functional units, enhanced compensatory function of viable cardiomyocytes, and adaptive hypertrophy of cardiomyocytes. With the progress of myocardial hypertrophy, the increase of myocardial cell apoptosis further reduces the number of myocardial cells; the overall contractility of the myocardium decreases, and the increase of fibrosis reduces the compliance of the left ventricle; the myocardial contractility cannot exert its proper ejection effect, thus forming a vicious circle, resulting in decompensation of myocardial function and heart failure. Apoptosis plays an important role in the process that transforms myocardial hypertrophy into heart failure (Persoon Rothert et al., 2002; Xing et al., 2000; Zhang et al., 2000). Raf/MEK/ERK1/2 has been confirmed to be closely related to cardiac hypertrophy (Lorenz et al., 2009). ERK and its pathway are well-studied members of the MAPK family. Raf/MEK/ERK1/2 is involved in various cellular functions and is closely related to cardiomyocyte apoptosis, providing a potential link between ERK activity and cell survival (Bueno and Molkenkin, 2002).

Based on the above-mentioned research on GXN and the important role of ERK signaling pathway in the occurrence and development of myocardial hypertrophy, it can be suggested that GXN may have an anti-cardiac hypertrophy effect by inhibiting ERK signaling pathway. Animal models are important means of studying diseases. Identifying appropriate animal models that align with diverse etiologies of chronic heart failure holds significant implications for elucidating the pathophysiological mechanisms underlying this condition and exploring the potential application of traditional Chinese medicine in its prevention and treatment. The heart failure model induced by long-term myocardial hypertrophy leading to increased load is often induced by transverse aortic constriction (TAC), isoproterenol (ISO), and Ang II induction methods. TAC (Litwin et al., 1995) causes ventricular wall hypertrophy by elevating pressure load (cardiac afterload), resulting in centripetal hypertrophy and ultimately heart failure. This model offers advantages such as low mortality, precise anatomical ligation points, and high experimental repeatability. It is currently widely used for establishing myocardial hypertrophic chronic heart failure models. Therefore, we utilized the TAC method to establish a rat model of heart failure and observed the protective effect of GXN on these rats, as well as its intervention effect on cardiomyocyte hypertrophy – to provide a theoretical basis for the clinical application of GXN.

Materials and methods

Drug preparation

GXN tablets were provided by Chiatai Qinchunbao Pharmaceutical Co., LTD. (Hangzhou, China). Their preparation has been reported in our previous study (Wang et al., 2020). The tablets were dissolved in sterile water to different concentrations. Captopril tablets (abbreviated as CAP) were purchased from Sino-American Shanghai Squibb Pharmaceuticals, Ltd. No. KTE1452, Shanghai, China, and were also dissolved in sterile water.

Rats

80 SPF male Sprague-Dawley (SD) rats purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Certification No. SCXK [Hu] 2017-0005; Shanghai, China), seven- to eight-weeks-old, and weighing 220 g to 250 g, were adaptively fed for one week in the Laboratory Animal Research Center of Zhejiang Chinese Medical University (Certification No. SYXK [Zhe] 2018-0012; Hangzhou, China). The housing conditions comprised 12 h light/dark cycle, free access to food and water, and constant temperature and humidity. All animal experiment procedures were approved by the Ethical Committee of Zhejiang Chinese Medical University (Approval No. IACUC-201812023-07).

Establishment of TAC induced heart failure rat model

Transverse aortic constriction (TAC) in the rats was performed as follows: rats were anesthetized with 5% isoflurane, their chest coat was shaved, and they were positioned to a 45° oblique fixed table for endotracheal intubation and further secured to the heat preservation table. The small animal anesthesia ventilator was used to assist breathing, with a respiratory ratio of 2 : 1, tidal volume of 30 ml/kg, and respiratory rate of 60 to 65 times/min. 3% isoflurane was inhaled to maintain anesthesia. The thoracic operation area was disinfected with iodophor, the second rib on the left side of the rat was cut, and the chest opened layer by layer. The incision was opened with a stretcher, the thymus was fully exposed. The thymus and aortic arch were separated, the posterior wall of the aortic arch was bypassed with a 3-0 suture package and protruded between the head arm trunk and the left common carotid artery. The self-made “L” needle with an outer diameter of 0.9 mm was placed parallel above the aortic arch. After ligation, the “L” needle was slowly removed, and the thoracic cavity was closed layer by layer to disinfect the incision. The other 6 rats received sham surgery, with the puncture of the aortic arch but no constriction, and the rest of the operation remained unchanged. After the operation, the animals were put into an incubator for warm care until they woke up. 100,000 IU of penicillin was injected intramuscularly for 3 days after the operation. The rats were then routinely fed to 4th week after penicillin injection for 3 days.

Grouping and administration

Four weeks after the operation, echocardiography was performed to record ventricular wall thickness and ejection fraction (EF). Blood was taken to separate the serum, and the serum NT-proBNP was detected. Based on the results of echocardiography and serum NT-proBNP detection, 41 selected rats with heart failure after TAC were divided into 4 groups according to the left ventricular mass (LVMass) measured by ultrasound system as the main index, combined with ejection fraction (EF) and body weight. Namely the model

control group (Model, $n = 14$), low-dose GXN group (GXN-L, 600 mg/kg, $n = 9$), high-dose GXN group (GXN-H, 1200 mg/kg, $n = 9$), and positive control group (Captopril, captopril at 12.5 mg/kg, $n = 9$). The six rats that underwent sham surgery became the sham surgery group (Sham). Each group was given corresponding drugs intragastrically once a day for 8 weeks. Six rats in each group were randomly selected for observation, detection, and analysis of the following indexes.

Echocardiography

Every 4 weeks after the start of administration of GXN and Captopril, 3% isoflurane gas was used to induce and maintain anesthesia, and echocardiography was performed. The specific methods were as follows: the rats were anesthetized with isoflurane inhalation, and the left ventricular interventricular septum thickness at end-diastole (IVSd), left ventricular interventricular septum thickness at end-systole (IVSs), left ventricular diameter at end-diastole (LVIDd), left ventricular diameter at end-systole (LVIDs), left ventricular posterior wall thickness at end-diastole (LVPWd), and left ventricular posterior wall thickness at end-systole (LVPWs) were measured using a high-resolution ultrasound imaging system for small animals. The indexes were measured and averaged in 2–3 cardiac cycles.

Histopathological observation

After eight weeks of administration with GXN and Captopril, the rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium solution at a dose of 45 mg/kg, and abdominal aorta blood was taken by laparotomy. After euthanasia, the heart was separated, weighed and recorded. Heart coefficient was calculated as heart weight (g)/rat body weight (kg). The same part of myocardial tissue from each rat was dehydrated, hyalinized, waxed and embedded. After this, it was fixed in 10% neutral formaldehyde solution for 48 h, cut into sections with a thickness of 5 μm , stained with hematoxylin-eosin (HE) and sealed. In addition, the sections were stained with Masson to observe the deposition of collagen fibers in myocardial tissue.

Cell

Isolation of primary myocardial cells from neonatal rats. The neonatal rat thoracic cavity was opened under sterile conditions, then the ventricles were cut into pieces and digested in 0.08% trypsin and 0.04% collagenase II at 37 °C for 10 min. The digestion was terminated with DMEM containing 20% FBS. The supernatant was centrifuged to collect the isolated single cells, resuspended with DMEM containing 20% FBS, and incubated at 37 °C with 5% CO₂. After incubation for 2 h, adherent non-cardiomyocytes were removed, and the remaining cell particles were suspended in DMEM/20% FBS for 24 h. 0.1 mm 5-bromodeoxyuridine was added to inhibit the fibroblasts proliferation for 72 h.

Cell grouping

The cells were seeded in 12-well plates and divided into a blank control group, model control group (isoproterenol ISO, 10 $\mu\text{mol/l}$), GXN low-dose group (GXN 200 $\mu\text{g/ml}$), and GXN high-dose group (GXN 400 $\mu\text{g/ml}$), with 3 parallel wells in each group. ISO was added after GXN intervention for 12 h, the culture medium was discarded after 48 h of incubation, and rinsed twice with PBS quickly.

Cell viability assay

The isolated and cultured neonatal rat primary cardiomyocytes were counted and adjusted to a concentration of $5 \times 10^5/\text{ml}$,

and 100 $\mu\text{l/well}$ was inoculated into a 96-well plate. After being starved in a serum-free medium for 24 h, the cells were divided into groups and administered GXN according to different concentration gradients. 48 h after addition, MTT solution (5 mg/ml) was added at the rate of 20 $\mu\text{l/well}$ for further culture for 4 h. Then the supernatant was discarded, 150 μl DMSO were added to each well, and shaken for 15 min to fully dissolve the crystals. The optical density (OD) at 540 nm was detected by a microplate tester.

Determination of myocardial cell area

According to the methods provided in the literature (Zhang et al., 2015), after phalloidin staining, the cells treated with different concentrations of GXN and ISO were observed and photographed under a confocal microscope, the cytoskeleton were shown in red, and the nucleus in blue. Each group was photographed in 5 fields with 10–15 cells in each field, and the area of every single cell (μm^2) was measured with Image-Pro Plus 6.0 image analysis system.

Flow cytometry assays

Cells treated with different concentrations of GXN and ISO were processed and collected into centrifuge tubes and subjected to flow cytometry using the Annexin V/Propidium Iodide (PI) Apoptosis Kit (BD Bioscience, USA). Cells were washed 3 times with PBS, then resuspended in 1 $\times$ binding buffer. After that, cells were incubated with 5 μl Annexin V-FITC and 10 μl propidium iodide, each for 15 min in the dark (Wang et al., 2009). 300 μl of 1 $\times$ Binding buffer was added and mixed, then the cell suspension was transferred into a 5 ml flow cytometer under dark conditions. Samples were detected by FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA) within an hour. Data were processed using FlowJo 10 (FlowJo, LLC, Ashland, OR, USA).

Quantitative PCR

Total RNA was extracted from both rat myocardial tissue and primary cardiomyocytes using total RNA extraction kit (Shanghai Bao Biological Company, China) according to the manufacturer's recommendations, and reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, Japan). The reverse transcription condition was 37 °C for 15 min (reverse transcription reaction) followed by 85 °C for 5 s (reverse transcriptase inactivation reaction), and finally cooled to 4 °C. The mRNA primers are listed in Table 1. The cDNA was amplified with StepOne Plus Real-Time PCR System (Applied Biosystems, USA). The amplification reaction system was prepared as follows: 10 μl of SYBR® Premix Ex Taq™ (2 $\times$), 1 μl each of forward and reverse primers (20 $\mu\text{mol/l}$), 0.4 μl of Rox, 1 μl of cDNA, and 6.6 μl of ddH₂O. The amplification reaction conditions are as follows: pre denaturation: 95 °C for 3 min; 48 cycles (95 °C 10 s, 60 °C 30 s); dissolution curve: starting from 55 °C, increase by 0.5 °C every 30 s until 95 °C. Periodic threshold method was used as a relative quantitative method for data analysis (Livak and Schmittgen, 2001), and GAPDH was normalized as housekeeping gene.

Determination of myocardial cell protein content

The rat primary cardiomyocytes were inoculated into 12-well plates with 3 parallel wells in each group. Culture medium was discarded after 48 h of treating with different concentrations of GXN and ISO, and the cells were quickly rinsed with PBS solution for three times. 1 ml 0.5 mol/l NaOH was added, and the cells were lysed at 37 °C for 1 h, followed by centrifugation for 15 min (12000 rpm, 4 °C). The extracted supernatant was

Table 1. Primer list

Gene	Primer sequence	Gene ID	Product size (bp)
GAPDH	5'-GGCAAGTTCAATGGCACAGT-3' 5'-TGGTGAAGACGCCAGTAGACTC-3'	24383	151
ANP	5'-ATGGGCTCCTTCTCCATCAC-3' 5'-TCTTCGGTACCGGAAGCT-3'	24602	456
BNP	5'-CCAGATGATTCTGCTCCTGCT-3' 5'-GCATCGTGGATTGTTCTGGA-3'	25105	101
RCAN1	5'-GACCCGCGCGTGTTC-3' 5'-TGTCATATGTTCTGAAGAGGAATC-3	266766	67
BCL2	5'-GACTGAGTACCTGAACCGGCATC-3' 5'-CTGAGCAGCGTCTTCAGAGACA-3'	24224	135
BAX	5'-CGAATTGGCGATGAACTGGA-3' 5'-CAAACATGTCAGCTGCCACAC-3'	24887	109

stored in an ultra-low temperature refrigerator at -80°C . Protein concentration in myocardial cells was measured by BCA method (Olson, 2016; Walker, 1994).

Western blot

50 mg of protein was extracted from both rat myocardial tissue and primary cardiomyocytes using total protein extraction kit. Denatured agent was added and boiled, SDS-PAGE gel was separated, and the membrane was transferred to NC membrane at 120 V for 2 h by constant pressure wet transfer method. After sealing at 37°C for 2 h in 3% bovine serum albumin solution, the proteins in the NC membrane were incubated with primary antibody (purchased from Santa Cruz Biotechnology, p-mek: sc-271914, mek: sc-6250, p-erk: sc-6250, erk: sc-271269, BCL-2: sc-7382, BAX: sc-7480) at 4°C overnight. After rinsing 3 times, the membrane was incubated with fluorescence IRDyeTM800 conjugated secondary antibody at room temperature for 1.5 h and scanned by Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE, United States) to calculate the relative expression levels of MEK-ERK1/2 related signaling pathway proteins. The expression levels of BAX and BCL2 were standardized with GAPDH as the internal reference. P-ERK and p-MEK are calculated by comparing the total protein content of ERK and MEK to calculate the phosphorylation level of the target protein.

Statistical analysis

SPSS 22.0 software was used for statistical analysis. All data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm \text{SD}$). ANOVA analysis of variance was used to evaluate the test results for measurement data, and LSD test was used for pair comparison. Value of $P < 0.05$ was considered statistically significant. Value of $P < 0.01$ was considered extremely statistically significant. The statistical result is rounded to two decimal places for precision.

Results

Effect on ventricular wall thickness in ultrasonic imaging

Fig. 1 showed that IVSd, IVSs, LVPWd, and LVPWs in the model control group were significantly thicker than those in the sham surgery group at the 4th and 8th weeks after the daily administration of GXL and Captopril ($P < 0.01$) began, sug-

gesting that the model control group had hypertrophy of cardiomyocytes and abnormal cardiac structure. Compared with the model control group, the thickness of the ventricular wall in the treatment group decreased to varying degrees. In the low-dose GXN group, IVSs and LVPWd were significantly thinner at 4 weeks after administration ($P < 0.05$), and IVSd and LVPWs were significantly thinner at 8 weeks after administration ($P < 0.05$). In the high-dose GXN group, IVSs, LVPWd, and LVPWs were significantly thinner at 4 weeks after administration ($P < 0.05$, $P < 0.01$), and IVSd and LVPWs were significantly thinner at 8 weeks after administration ($P < 0.05$, $P < 0.01$). In the positive control group, IVSs was significantly thinner at 4 weeks after administration ($P < 0.05$), and IVSs, LVPWd, and LVPWs were significantly thinner at 8 weeks after administration ($P < 0.05$, $P < 0.01$).

Effect on heart weight and coefficient

Fig. 2 showed that the heart weight and coefficient of rats in the model control group were significantly higher than in the sham surgery group ($P < 0.01$). Compared with the model control group, the heart weight and coefficient of rats in each treatment group decreased to varying degrees after drug intervention, with a significant decrease of heart coefficient in the high-dose GXN group ($P < 0.05$) and a significant decrease of heart weight in the positive control group ($P < 0.05$).

Effect on myocardial histopathology

The results of routine pathological HE staining of myocardial tissue showed that the cardiomyocytes of rats in the model control group were significantly enlarged, accompanied by inflammatory infiltration and interstitial edema, which was significantly different from that in the sham operation group. However, after the intervention of different drugs, the inflammatory infiltration of myocardial tissue decreased, the size of myocardial cells improved to varying degrees, and the edema was not obvious (Fig. 3). After Masson staining of myocardial tissue, collagen fibers were observed to exhibit a blue hue. As shown in Fig. 3A, the myocardial tissue of rats in the sham surgery group was neatly arranged, and there was almost no blue collagen fiber. In the model control group, fibrous tissue hyperplasia was evident, and the cardiomyocytes exhibited loose arrangement with collagen fibers interposed in a reticular pattern. After intervention with different drugs, fibrosis improved to varying degrees.

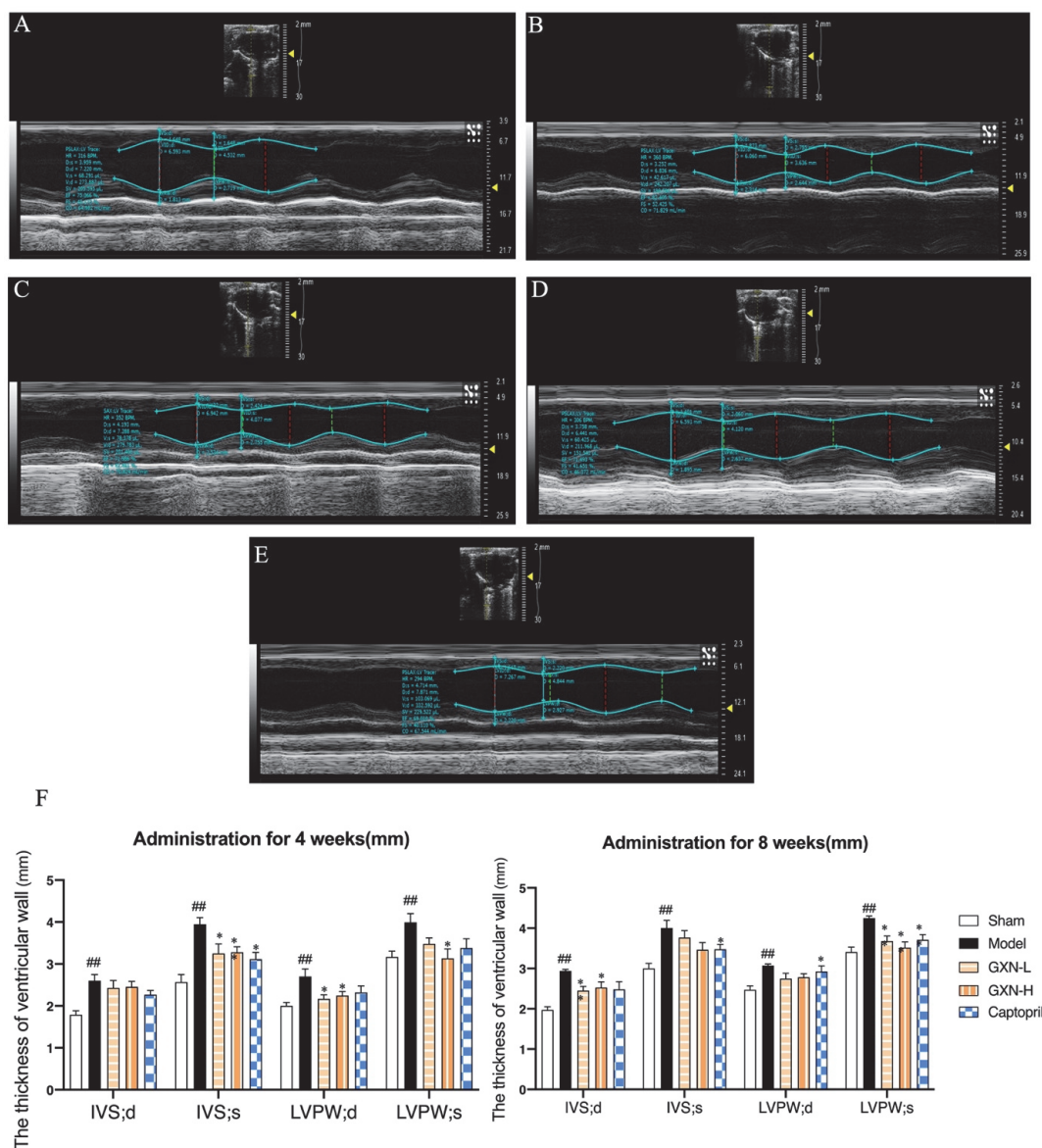


Fig. 1. The effect of GXN on echocardiographic ventricular wall thickness (mm) in rat models with early heart failure. **A-E:** M-ultrasonography. **(A)** Sham: sham-operated group; **(B)** Model: model group; **(C)** GXN-L: GXN low-dose group; **(D)** GXN-H: GXN high-dose group; **(E)** Captopril: positive control group. **(F)** The thickness of ventricular wall after administration for 4 and 8 weeks. Data are shown as mean $\pm$ SEM ($n = 6$, # $P < 0.05$, ## $P < 0.01$ vs sham group; * $P < 0.05$, ** $P < 0.01$ vs model group).

Semi-quantitative analysis of the myocardial area showed that, compared with the sham surgery group, the myocardial cell area of rats in the model control group increased significantly ($P < 0.01$). Compared with the model control group, the myocardial cell area decreased significantly after intervention with different drugs ($P < 0.01$).

Effect on gene expression of cardiac hypertrophy related genes

Compared with the sham group, the mRNA expressions of ANP, BNP, and RCAN1 in the heart tissue of model control group were significantly increased ($P < 0.01$). The mRNA expressions of these three genes related to left ventricular hypertrophy in the high-dose GXN group and positive control group were significantly decreased ($P < 0.05$, $P < 0.01$) compared with the model group (Fig. 4).

Effect of GXN on the proliferation of cardiomyocytes

In normal cultured rat primary cardiomyocytes, GXN had no significant inhibitory effect on cell proliferation in the concentration range of 50 $\mu\text{g/ml}$ –1200 $\mu\text{g/ml}$ ($P > 0.05$). This indicated that when the concentration of GXN was less than 1200 $\mu\text{g/ml}$, there was no significant effect on the proliferation of rat primary myocardial cells (Fig. 5A).

After adding 10 $\mu\text{g/ml}$ of ISO, the proliferation of rat primary cardiomyocytes was not significantly inhibited ($P > 0.05$) and GXN had no significant effect on the proliferation of rat primary cardiomyocytes in the range of 50 $\mu\text{g/ml}$ to 1200 $\mu\text{g/ml}$ ($P > 0.05$), indicating that GXN is relatively safe for cardiomyocytes (Fig. 5B).

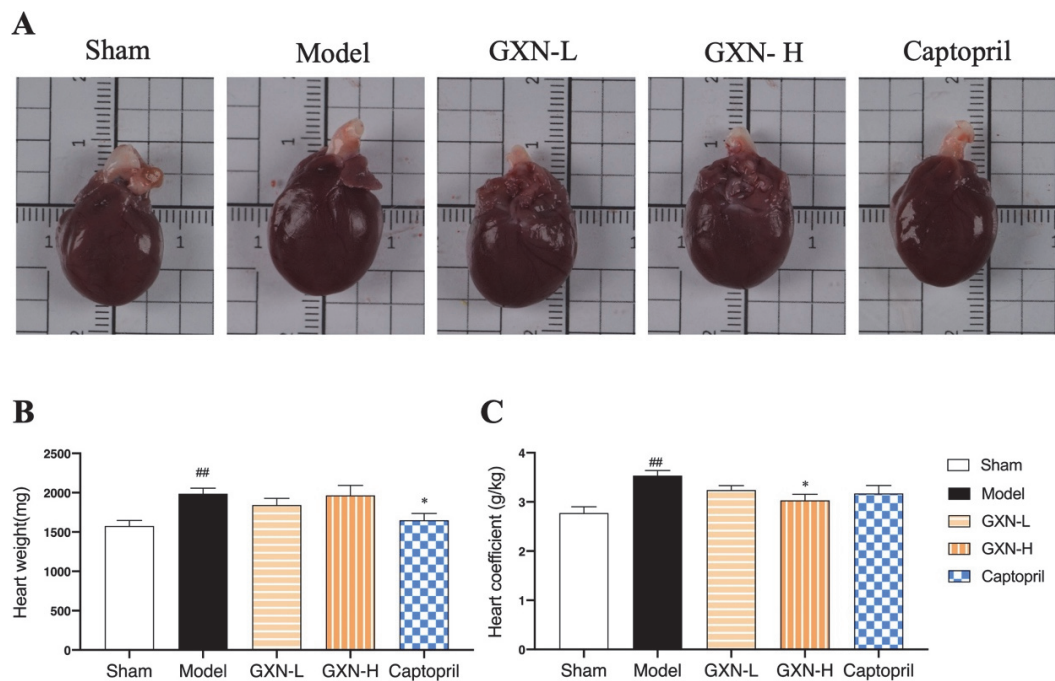


Fig. 2. The effect of GXN on heart weight and coefficient in rat models with early heart failure. **(A)** The appearance of the whole hearts from each group. **(B)** The heart weight of each group. **(C)** The heart coefficient of each group. Sham: sham-operated group; Model: model group; GXN-L: GXN low-dose group; GXN-H: GXN high-dose group; Captopril: positive control group. Data are shown as mean $\pm$ SEM ($n = 6$, $^{##} P < 0.01$ vs sham group; $^{*} P < 0.05$ vs model group).

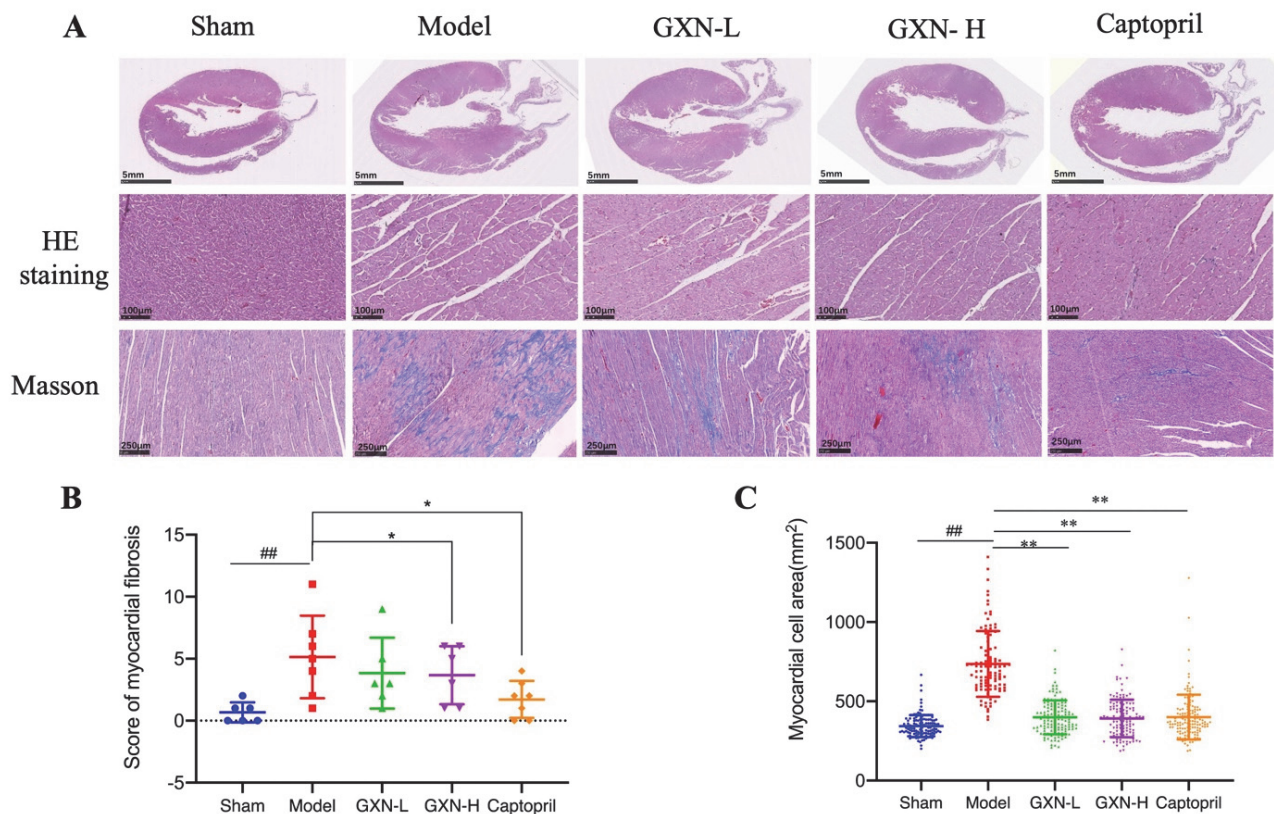


Fig. 3. The effect of GXN on cardiac pathology and cardiomyocyte size in rat models with early heart failure. **(A)** HE staining and Masson staining of the heart. **(B)** The score of myocardial fibrosis. **(C)** Cardiomyocyte size. Sham: sham-operated group; Model: model group; GXN-L: GXN low-dose group; GXN-H: GXN high-dose group; Captopril: positive control group. Data are shown as mean $\pm$ SEM ($n = 6$, $^{##} P < 0.01$ vs sham group; $^{*} P < 0.05$ vs model group).

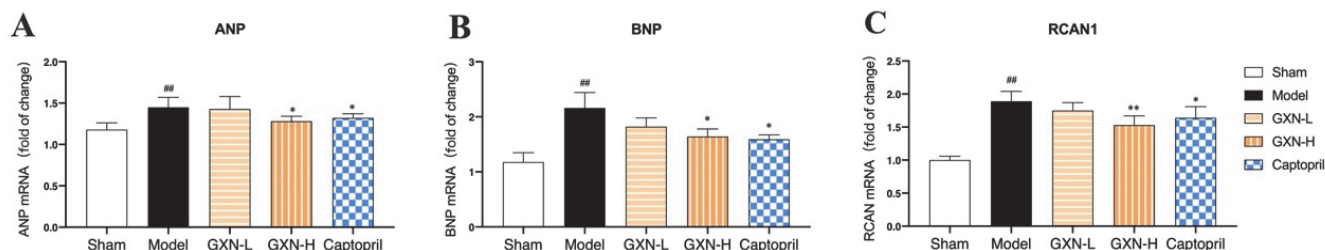


Fig. 4. The effect of GXN on the mRNA expression of cardiac hypertrophy related genes. **(A)** ANP mRNA expression. **(B)** BNP mRNA expression. **(C)** RCAN1 mRNA expression. Sham: sham-operated group; Model: model group; GXN-L: GXN low-dose group; GXN-H: GXN high-dose group; Captopril: positive control group. Data are shown as mean $\pm$ SEM ($n = 6$, ^{##} $P < 0.01$ vs sham group; ^{*} $P < 0.05$ vs model group, ^{**} $P < 0.01$ vs model group).

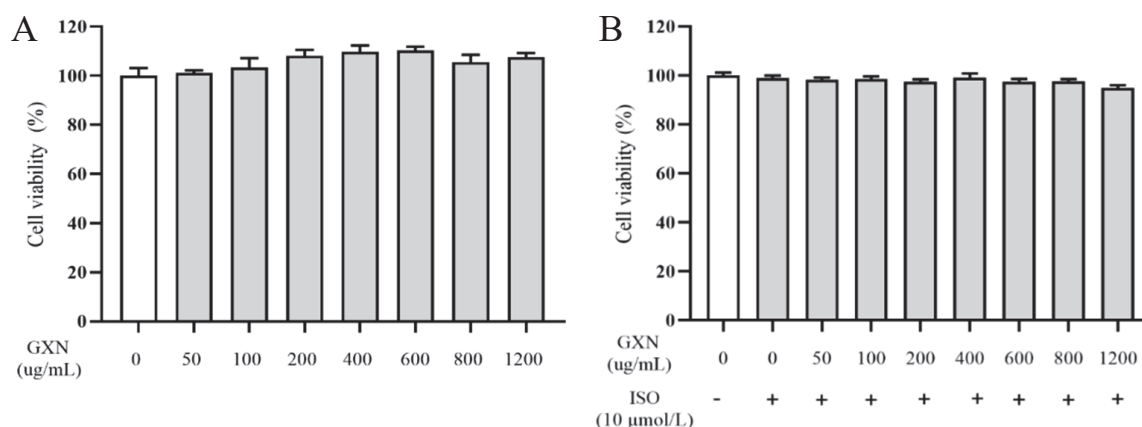


Fig. 5. Effect of GXN on the proliferation of primary cardiomyocytes. **(A)** Effect of GXN on the proliferation of primary cardiomyocytes. **(B)** Effect of GXN on the proliferation of primary cardiomyocyte after stimulation. Data are shown as mean $\pm$ SEM.

Effect of GXN on myocardial cell size

Compared with the blank control group, the cross section of cardiomyocytes was significantly increased after ISO stimulation at 10 $\mu\text{g}/\text{mL}$. Compared with the model control group, there was no significant difference in the cross-sectional size of cardiomyocytes treated with 200 $\mu\text{g}/\text{mL}$ GXN, while the cross-sectional size of cardiomyocytes treated with 400 $\mu\text{g}/\text{mL}$ GXN was significantly decreased ($P < 0.01$) – Fig. 6A, B.

Effect of GXN on cardiomyocyte apoptosis

The early and late apoptosis rates of cardiomyocytes in the blank control group were 0.73% and 0.66% respectively. In the model control group, the early apoptosis rate was 1.07%, and the late apoptosis rate was 5.15%. After treatment with different concentrations of GXN, the apoptosis rate of cardiomyocytes decreased to varying degrees. Among them, after 200 $\mu\text{g}/\text{mL}$ GXN pre-protection, the early apoptosis rate of cardiomyocytes after ISO stimulation was 2.38%, and the late apoptosis rate was 4.64%; 400 $\mu\text{g}/\text{mL}$ GXN pre-protection had an early apoptosis rate of 1.86% and a late apoptosis rate of 2.10% (Fig. 7).

Effect of GXN on mRNA of apoptosis-related genes in cardiomyocytes

Compared with the blank control group, the mRNA expression of BCL2 gene in the model control group was significantly

decreased ($P < 0.01$), and the mRNA expression of BAX gene was significantly increased ($P < 0.01$). After 200 $\mu\text{g}/\text{mL}$ GXN pre-protection, the mRNA expression of BCL2 gene was increased with no significant statistical difference ($P > 0.05$); BAX mRNA expression was significantly decreased ($P < 0.05$). After 400 $\mu\text{g}/\text{mL}$ GXN pre-protection, BCL2 mRNA expression was significantly increased ($P < 0.01$), and BAX mRNA expression was significantly decreased ($P < 0.01$) – Fig. 8.

Effects of GXN on the expression of proteins associated with MEK/ERK1/2 signaling pathway in cardiomyocytes

Compared with the normal control group, the phosphorylated expressions of MEK and ERK1/2 in the model control group were significantly increased ($P < 0.01$), and the phosphorylated expressions of these two proteins were significantly decreased after high dose GXN pre-protection compared with the model control group ($P < 0.05$). After high dose GXN preliminary protection, the expression of BCL2 protein was significantly increased ($P < 0.05$), while that of BAX protein was significantly decreased ($P < 0.05$). The results (Fig. 9) were consistent with RT-PCR results (Fig. 8).

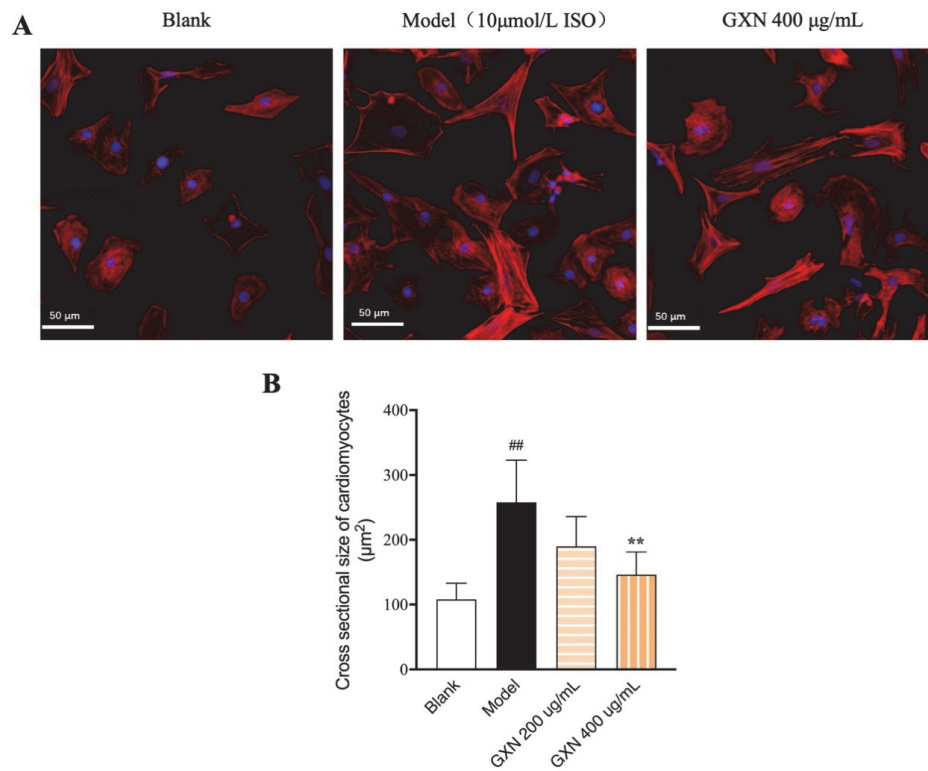


Fig. 6. The effect of GXN on cross-sectional size of cardiomyocytes in rat models with early heart failure. **(A)** Cardiomyocytes labeled with phalloidin. **(B)** Cross-sectional size of cardiomyocytes. Data are shown as mean $\pm$ SEM ($n = 6$, ^{##} $P < 0.01$ vs sham group; ^{**} $P < 0.01$ vs model group).

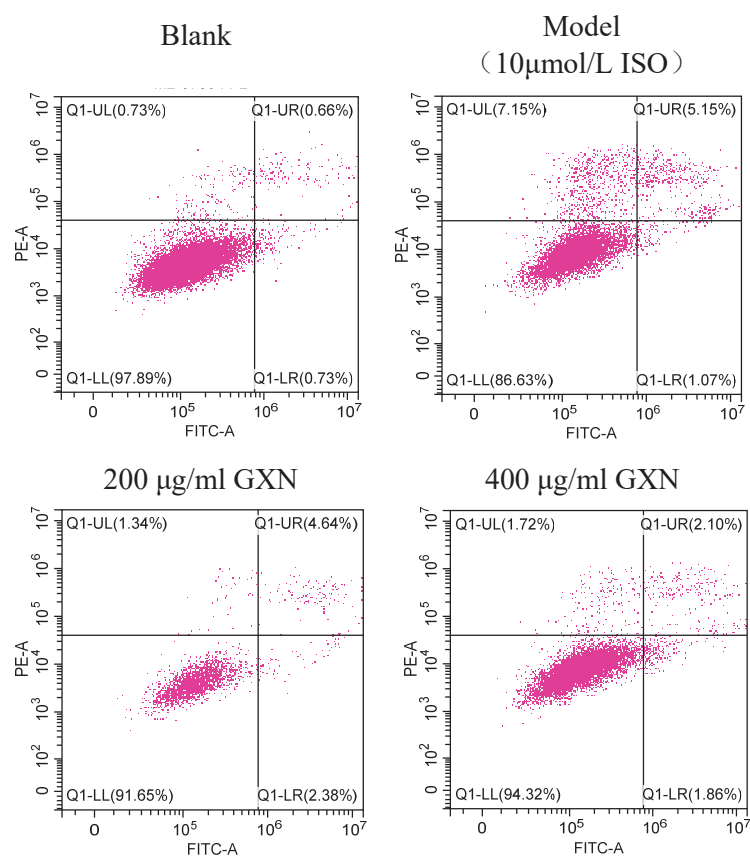


Fig. 7. The effect of GXN on cardiomyocyte apoptosis

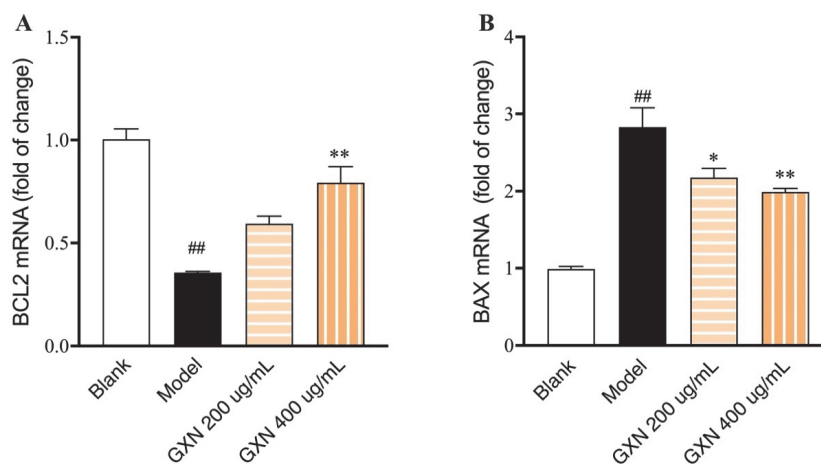


Fig. 8. The effect of GXN on the mRNA expression of apoptosis-related genes in cardiomyocytes. **(A)** BCL2 mRNA expression. **(B)** BAX mRNA expression. Data are shown as mean $\pm$ SEM ($n = 6$, ^{##} $P < 0.01$ vs sham group; ^{*} $P < 0.05$ vs model group, ^{**} $P < 0.01$ vs model group).

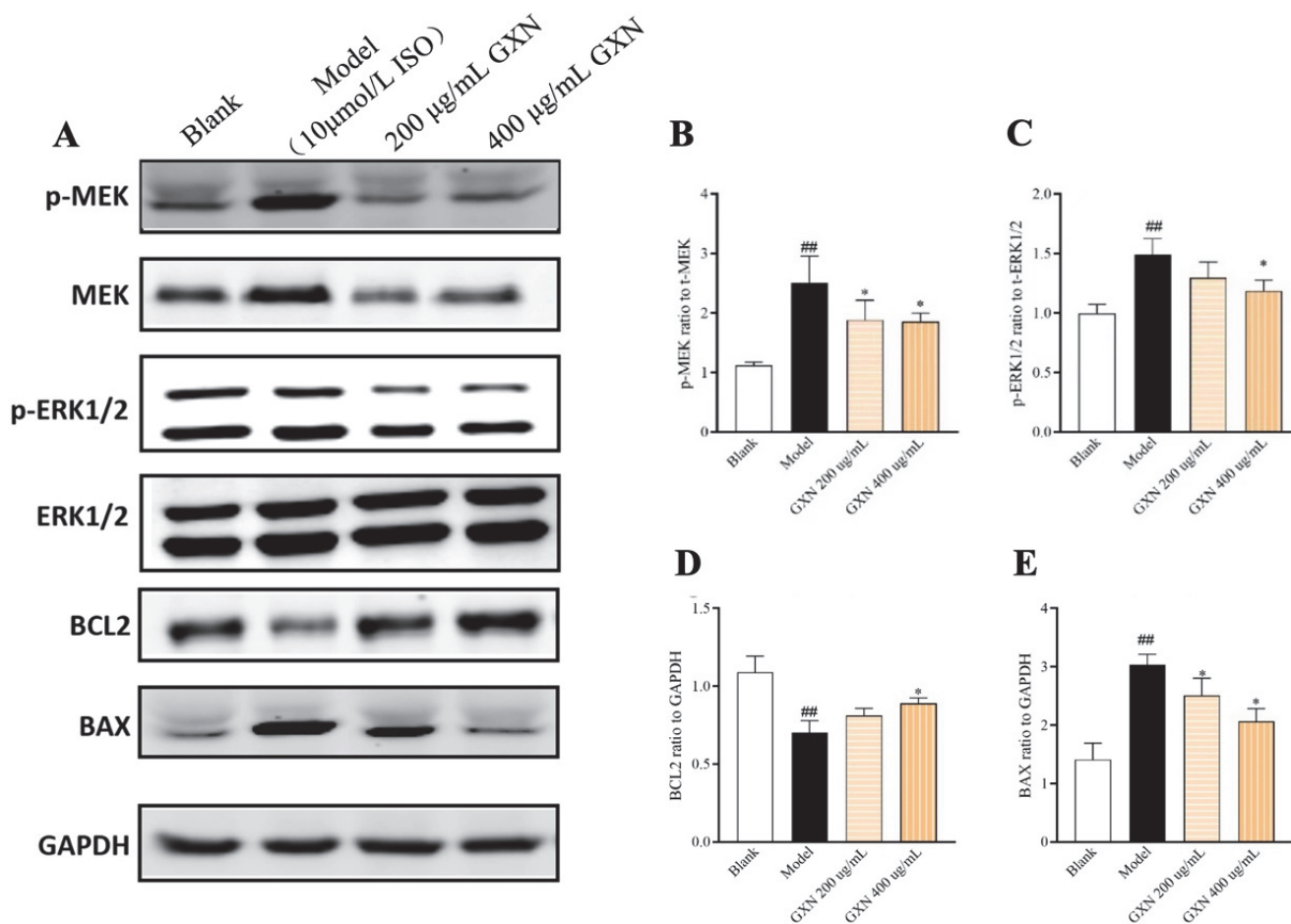


Fig. 9. The effect of GXN on expression of MEK-ERK1/2 signaling pathway proteins. **(A)** The effect of GXN on expression of MEK-ERK1/2 signaling pathway proteins evaluated by western blot. **(B)** Quantitative analysis chart of p-MEK ratio to total MEK. **(C)** Quantitative analysis chart of p-ERK1/2 ratio to total ERK1/2. **(D)** Quantitative analysis chart of BCL2 ratio to GAPDH. **(E)** Quantitative analysis chart of BAX ratio to GAPDH. Data are shown as mean $\pm$ SEM ($n = 6$, ^{##} $P < 0.01$ vs sham group; ^{*} $P < 0.05$ vs model group, ^{**} $P < 0.01$ vs model group).

Discussion

Cardiac hypertrophy is an important pathological feature in the early stages of heart failure, and apoptosis plays an important role in transforming cardiac hypertrophy into heart failure. In this study, left ventricular posterior pressure overload was induced by aortic arch stenosis in rats to establish a rat model of early congestive heart failure. It was found that GXN could significantly reduce the degree of cardiac hypertrophy, reduce the size of cardiomyocytes, inhibit myocardial remodeling and fibrosis, and improve cardiac function in rats with early heart failure. Meanwhile, primary cardiomyocytes of neonatal rats were isolated, and GXN showed no cytotoxicity, and could improve the hypertrophy of cardiomyocytes and reduce the apoptosis of cardiomyocytes under pathological conditions. The mechanism of the above effects may be related to the inhibition of MEK-ERK1/2 signaling pathway activation. Compared with other traditional Chinese medicine that is still in research and development stages, GXN has great advantages and is of great significance in promoting the modernization of traditional Chinese medicine research.

Currently, the main western drugs used clinically for anti-myocardial hypertrophy preparations include angiotensin II receptor antagonists (Panico et al., 2019) valsartan, telmisartan, angiotensin-converting enzyme (ACE) inhibitors (Messerli et al., 2018) benazepril and enalapril, β adrenergic receptor blockers (Watanabe et al., 2016) metoprolol, Bisoprolol, Ca^{2+} ion channel antagonists (Xu et al., 2020) Norvasc, diltiazem, etc. The primary pharmacological effects of these drugs include positive inotropic action, diuresis, vasodilation, and antiarrhythmic activity. In the initial stage of treatment, these drugs often have an alleviating and controlling effect on the disease (Savage et al., 2022), but long-term use of these drugs can cause serious damage to liver and kidney function and the gastrointestinal tract, and some of them lose their efficacy completely due to drug resistance (Zhang et al., 2018). Moreover, they usually contribute to the hardening of blood vessels and destroy the blood balance mechanism after use. As these drugs have their own toxic side effects, using them in combination is more limited for many patients with chronic heart failure (McDonagh et al., 2022; Messerli et al., 2018). Among the traditional Chinese medicines (TCM) used in clinical practice, the most studied is Shengmai Yin (SMY) (Kan et al., 2022), which is a common prescription for clinical multi-system diseases. It is composed of three kinds of TCM, *Codonopsis pilosula*, *radix ophiopogonis*, and *Schisandra chinensis*, and has the effect of invigorating qi and nourishing Yin. It is widely used in the treatment of cardiovascular, central nervous system and endocrine system diseases. Modern pharmacological studies have shown that SMY has a protective effect on myocardial muscle and improves cardiac function (You et al., 2020). It also has a protective effect in rat models of ISO-induced myocardial hypertrophy (Ming et al., 2022). Yiqifumai Injection (YQFM) potentially alleviates myocardial hypertrophy and apoptosis in patients with chronic heart failure (Zhao et al., 2018). Shensongyangxin (SSYX) attenuates angiotensin II-induced cardiomyocyte hypertrophy and reduces cardiac hypertrophy and fibrosis in stress-overloaded mice, and its mechanism is related to the inhibition of Akt and TGF β /Smad signaling pathways (Shen et al., 2016).

Incorporating traditional Chinese medicine treatment in conjunction with conventional western medicine not only aids in ameliorating clinical symptoms of patients with chronic heart failure, enhance activity tolerance, improve the quality

of life, but also improves the long-term prognosis of some patients, providing a new way and choice for the treatment of patients with chronic heart failure.

Myocardial remodeling is the basic mechanism for the development of heart failure, and its occurrence is mainly due to changes in myocardial structure and function (Ponikowska et al., 2022). IVSd, IVSs, LVPWd, LVPWs are important indicators to measure cardiac function and reflect changes in cardiac structure. The results of this experiment showed that GXN can correct cardiac structural abnormalities in time, and thereby improve cardiac function. Cardiac coefficient is a direct index to evaluate the degree of myocardial hypertrophy, which is the ratio of heart weight/body weight (Zhang et al., 2021); it is also a comprehensive reflection of cardiac remodeling in the process of heart failure. The results are in line with each other, suggesting that the occurrence of myocardial compensatory effect in the heart failure model rats and evident degree of myocardial hypertrophy; while GXN can effectively reduce the degree of myocardial hypertrophy in model rats, and its effect has a certain correlation with the dose.

HE staining was used to evaluate the morphology of cardiomyocytes, and Masson staining can effectively evaluate the degree of myocardial fibrosis. Myocardial fibrosis is an essential feature of myocardial remodeling and an influential factor in the development and progression of heart failure (Iyer et al., 2022). Pathological images showed that GXN can alleviate myocardial cell injury, improve cardiac function, and reduce myocardial fibrosis degree in rats with heart failure to a certain extent, and the degree of improvement increases the higher the dose.

Drug toxicity is an essential factor restricting drug research and development. It is also the source of toxic and side effects caused by drug clinical application. The MTT results showed that GXN had no obvious cytotoxic effect on primary rat cardiomyocytes in the concentration range of 50 μ g/ml-1200 μ g/ml, which provided the most basic safety preconditions for the subsequent research and development of GXN. Phalloidin can selectively bind to actin microfilaments, thereby showing the distribution and local orientation changes of cytoskeletal filamentous actin. The results show that after 48 hours of ISO induction, the surface area of rat cardiomyocytes increased significantly and the number of myofibrillar sarcomere increased – indicating that the model of cardiomyocyte hypertrophy was successfully established.

Due to the long-term effect of pre- and post-cardiac overload, hypertrophic cardiomyocytes are more sensitive to oxidative damage caused by ischemia, hypoxia and ROS, and are superimposed on inflammatory factors, high levels of Ca^{2+} , NO and other stimulatory factors, resulting in an increase in cytoplasmic density and cell membrane permeability, nuclear pyknosis and fragmentation, and DNA degradation, leading to the occurrence of cardiomyocyte apoptosis (Hu et al., 2022). In this paper, the apoptosis of cardiomyocytes was observed by flow cytometry, and it was found that GXN had a certain inhibitory effect on the apoptosis process in hypertrophic cardiomyocytes. Bcl2 and Bax belong to the Bcl2 gene family. Bcl2 is an apoptosis inhibitory gene. Bax not only antagonizes the apoptosis inhibition of Bcl2, but also has the function of promoting apoptosis (Wang et al., 2022). The results of this paper showed that GXN can reverse the changes in mRNA and protein levels of Bax and Bcl2 induced by ISO treatment, which further confirmed the role of GXN in antagonizing cardiomyocyte apoptosis.

MEK-ERK1/2 signaling pathway is involved in cardiomyocyte apoptosis, and the activity of MAPK is involved in car-

diomyocyte hypertrophy. ERK is a well-studied member of the MAPK family, and its pathway MEK-ERK1/2 is involved in various cellular functions. Many studies have shown that the activity of MEK-ERK1/2 signaling pathway promotes cardiomyocyte hypertrophy and compensatory cell proliferation, and prolonged overwork leads to massive apoptosis of cardiomyocytes (Gu et al., 2016; Ko et al., 2013). In neonatal rat ventricular myocytes, the phosphorylation levels of MEK and ERK1/2 induced by oxidants are significantly increased, and the phosphorylation levels of these two proteins are significantly decreased after pre-protection by GXN, suggesting that the ERK pathway is involved in the anti-apoptotic signaling pathway of neonatal cardiomyocytes. The anti-apoptotic effect of the regulatory protein Bcl-2 relies on the phosphorylation of serine residues between its BH3 and BH4 domains, and the MEK/ERK1/2 signaling pathway is involved in the phosphorylation of Bcl-2, providing a potential link between ERK activity and cell survival (Shi et al., 2010). It can be concluded that inhibiting the excessive activation of MEK/ERK1/2 signaling pathway can improve the hypertrophic state of cardiomyocytes, thereby slowing down the further development of cardiomyocytes into apoptosis and the progression from cardiac hypertrophy to heart failure.

So far, over 61 chemical constituents have been reported from GXN (Chen et al., 2011; Ruan et al., 2012), respectively extracted from *Salvia miltiorrhiza* and *Ligusticum chuanxiong*. The chemical components from *Salvia miltiorrhiza* can be divided into two types: water-soluble and fat-soluble. Salvianolic acid B accounted for the largest proportion of water-soluble components, including Danshensu, salvianolic acid A, rosmarinic acid, protocatechualdehyde and purple oxalic acid. Fat-soluble components included tanshinone I, tanshinone IIA, cryptotanshinone, etc. Chemical components derived from *Ligusticum chuanxiong* include phenolic acid, phthalide, and alkaloids. Ferulic acid is the recognized active ingredient in *Ligusticum chuanxiong*, the content of which is higher than 0.1%. The active ingredient of phthalide includes butylphthalide, senkyunolide I and ligustilide, etc. The alkaloids are represented by ligusticazine. However, in recent years, studies have confirmed that the content of ligustrazine in *Ligusticum chuanxiong* is less than 0.0001%, and it is no longer considered as the main active ingredient in *Ligusticum chuanxiong* (Zhang, 2017). Among these components, salvianolic acid B (Liu et al., 2014), Danshensu (Tang et al., 2011), tanshinone IIA (Zhang et al., 2022), and ferulic acid (Luo et al., 2022) have been widely studied and confirmed to be related to the anti-myocardial hypertrophy effect of GXN, suggesting that the mechanism of anti-myocardial hypertrophy effect of GXN may be the result of their combined action.

In conclusion, GXN may inhibit cardiac hypertrophy and improve early heart failure by inhibiting the over-activation of MEK-ERK1/2 signaling pathway. This study provides a new mechanism for understanding the protective effects of GXN on cardiovascular disease and provides experimental evidence for wider clinical application of GXN.

Conclusion

GXN can effectively reduce the degree of cardiac hypertrophy and the size of cardiomyocytes, inhibit the degree of myocardial remodeling and fibrosis, and improve cardiac function in rats with early heart failure. GXN can also improve the hypertrophy of primary rat cardiomyocytes and reduce the apoptosis of cardiomyocytes in pathological state. These therapeutic

effects of GXN may be related to the inhibition of overactivation of MEK/ERK1/2 signaling pathway.

Authors' contributions

Yan Zhang obtained funding, contributed to the conception/design, data acquisition, data analysis/interpretation of data, and drafted and revised the manuscript. Quan-xin Ma was responsible for conceptualization, design, data analysis, and manuscript preparation. Yi-li Rong designed the experiment, polished the language, and revised the manuscript. Song-tao Xu and Li-ye Shen helped with the establishment of the animal model and daily administration, and collected the urine samples. Yan-yun Xu and Hai-ye Tu helped with the culture of primary cardiomyocytes. Jia-jun Shi helped with the western-blot. Min-li Chen helped us revise the manuscript. All authors reviewed and approved the manuscript.

Funding

The project received support from the Jiaying Science and Technology Planning Project (No. 2019AY32023), Jiaying Key disciplines of Traditional Chinese Medicine support class – Chinese Pharmacy, (NO. Jiawei [2023] 65), Jiaying Hospital of Traditional Chinese Medicine “Teaching Excellence” Special Program (NO. 2021JXZY019) and Research Project of Zhejiang Chinese Medical University (No. 2021JKZKTS031B).

Ethical aspects and conflict of interest

The authors have no conflict of interest to declare.

References

- Bavendiek U, Berliner D, Dávila LA, Schwab J, Maier L, Philipp SA, et al. (2019). Rationale and design of the DIGIT-HF trial (DIGitoxin to Improve ouTcomes in patients with advanced chronic Heart Failure): a randomized, double-blind, placebo-controlled study. *Eur J Heart Fail* 21(5): 676–684. DOI: 10.1002/ehf.1452.
- Bueno OF, Molkentin JD (2002). Involvement of extracellular signal-regulated kinases 1/2 in cardiac hypertrophy and cell death. *Circ Res* 91(9): 776–781. DOI: 10.1161/01.res.0000038488.38975.1a.
- Chen ML, Lv JM, Pan YM, Ying HZ, Guan MW, Zhang JB, et al. (2005a). Effect of Guanxinling Tablet in Acute Myocardial Ischemia in Beagle Dogs. *J Zhejiang Chin Med Univ* 29(4): 54–56. DOI: 10.16466/j.issn1005-5509.2005.04.034.
- Chen ML, Shou QY, Pan YM, Zhang JB, Sang R, Guan MW, et al. (2005b). Inhibitive and protective effects of guanxinling tablets on platelet aggregation and vascular endothelium in qi stagnation and blood stasis rat. *Chin J Clin Pharm Ther* 10(5): 586–589. DOI: 10.3969/j.issn.1009-2501.2005.05.024.
- Chen ML, Wang H, Lin L, Shou QY, Zhou WM, Wang DJ, et al. (2006). Effect of Guanxinling Tablet on Hemorrheology in Aged Rats. *Lab Anim Comp Med* 26(4): 231–234. DOI: 10.3969/j.issn.1674-5817.2006.04.007.
- Chen X, Lou Z, Zhang H, et al. (2011). Identification of multiple components in Guanxinling injection using hydrophilic interaction liquid chromatography/time-of-flight mass spectrometry and reversed-phase liquid chromatography/time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 25(11): 1661–1674. DOI: 10.1002/rcm.5003.
- Gu J, Hu W, Song ZP, Chen YG, Zhang DD, Wang CQ, et al. (2016). Rapamycin Inhibits Cardiac Hypertrophy by Promoting Autophagy via the MEK/ERK/Beclin-1 Pathway. *Front Physiol* 7: 104. DOI: 10.3389/fphys.2016.00104.
- Hu C, Dai F, Wang J, Jiang L, Wang D, Gao J, et al. (2022). Peroxiredoxin-5 Knockdown Accelerates Pressure Overload-Induced Cardiac Hypertrophy in Mice. *Oxid Med Cell Longev* 2022: 5067544. DOI: 10.1155/2022/5067544.

- Hu F, Rong YL, Zhu KY, Lu H, Chen C, Chen ML, Pan YM (2017). Study on the protective effect of Guanxinling Tablet on myocardial ischemia reperfusion injury in rats. *Chin J Comp Med* 27(5): 76–82. DOI: 10.3969/j.issn.1671-7856.2017.05.017.
- Huang Y, Ni N, Hong Y, Lin X, Feng Y, Shen L (2020). Progress in Traditional Chinese Medicine for the Treatment of Migraine. *Am J Chin Med* 48(8): 1731–1748. DOI: 10.1142/S0192415X2050086X.
- Iyer NR, Le TT, Kui MSL, Tang HC, Chin CT, Phua SK, et al. (2022). Markers of Focal and Diffuse Nonischemic Myocardial Fibrosis Are Associated With Adverse Cardiac Remodeling and Prognosis in Patients With Hypertension: The REMODEL Study. *Hypertension* 79(8): 1804–1813. DOI: 10.1161/HYPERTENSIONAHA.122.19225.
- Kan M, Wang J, Ming S, Sui X, Zhang Z, Yang Q, et al. (2022). Investigating the Mechanism of Shengmai Yin (*Codonopsis pilosula*) in the Treatment of Heart Failure Based on Network Pharmacology. *Comb Chem High Throughput Screen* 25(13): 2191–2202. DOI: 10.2174/1386207325666220221093415.
- Ko SY, Lin IH, Shieh TM, Ko HA, Chen HI, Chi TC, et al. (2013). Cell hypertrophy and MEK/ERK phosphorylation are regulated by glyceraldehyde-derived AGEs in cardiomyocyte H9c2 cells. *Cell Biochem Biophys* 66(3): 537–544. DOI: 10.1007/s12013-012-9501-8.
- Li W, Yang J, Lyu Q, Wu G, Lin S, Yang Q, Hu J (2020). Taurine attenuates isoproterenol-induced H9c2 cardiomyocytes hypertrophy by improving antioxidative ability and inhibiting calpain-1-mediated apoptosis. *Mol Cell Biochem* 469(1–2): 119–132. DOI: 10.1007/s11010-020-03733-7.
- Litwin SE, Katz SE, Weinberg EO, Lorell BH, Aurigemma GP, Douglas PS (1995). Serial echocardiographic-Doppler assessment of left ventricular geometry and function in rats with pressure-overload hypertrophy. Chronic angiotensin-converting enzyme inhibition attenuates the transition to heart failure. *Circulation* 91(10): 2642–2654. DOI: 10.1161/01.cir.91.10.2642.
- Liu M, Ye J, Gao S, Fang W, Li H, Geng B, et al. (2014). Salvianolic acid B protects cardiomyocytes from angiotensin II-induced hypertrophy via inhibition of PARP-1. *Biochem Biophys Res Commun* 444(3): 346–353. DOI: 10.1016/j.bbrc.2014.01.045.
- Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* 25(4): 402–408. DOI: 10.1006/meth.2001.1262.
- Lorenz K, Schmitt JP, Vidal M, Lohse MJ (2009). Cardiac hypertrophy: targeting Raf/MEK/ERK1/2-signaling. *Int J Biochem Cell Biol* 41(12): 2351–2355. DOI: 10.1016/j.biocel.2009.08.002.
- Luo M, Lin HC, Wen ZQ, Chen PP, Shi WL, Li YY, et al. (2022). Sodium Ferulate Inhibits Rat Cardiomyocyte Hypertrophy Induced by Angiotensin II Through Enhancement of Endothelial Nitric Oxide Synthase/Nitric Oxide/Cyclic Guanosine Monophosphate Signaling Pathway. *J Cardiovasc Pharmacol* 80(2): 251–260. DOI: 10.1097/FJC.0000000000001277.
- McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Böhm M, et al. (2022). 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: Developed by the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). With the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail* 24(1): 4–131. DOI: 10.1002/ehf.2333.
- Messerli H, Bangalore S, Bavishi C, Rimoldi SF (2018). Angiotensin-Converting Enzyme Inhibitors in Hypertension: To Use or Not to Use? *J Am Coll Cardiol* 71(13): 1474–1482. DOI: 10.1016/j.jacc.2018.01.058.
- Ming S, Kan M, Liu L, Zhang Z, Liu X, Liu Y, et al. (2022). Protective Effect of Shengmai Yin in Myocardial Hypertrophy-Induced Rats: A Genomic Analysis by 16S rDNA. *Evid Based Complement Alternat Med* 2022: 3188292. DOI: 10.1155/2022/3188292.
- Nakamura M, Sadoshima J (2018). Mechanisms of physiological and pathological cardiac hypertrophy. *Nat Rev Cardiol* 15(7): 387–407. DOI: 10.1038/s41569-018-0007-y.
- Olson BJ (2016). Assays for Determination of Protein Concentration. *Curr Protoc Pharmacol* 73:A.3A.1–A.3A.32. DOI: 10.1002/cpph.3.
- Pan YM, Ying HZ, Chen ML, Xu JQ, Zhou WM, Wang H, et al. (2007). Effects of Guanxinling Tablet on Myocardial Oxygen Consumption in Dogs. *Lab Anim Comp Med* 27(3): 190–194. DOI: 10.3969/j.issn.1674-5817.2007.03.011.
- Panico K, Abrahao MV, Trentin-Sonoda M, Muzzi-Filho H, Vieyra A, Carneiro-Ramos MS (2019). Cardiac Inflammation after Ischemia-Reperfusion of the Kidney: Role of the Sympathetic Nervous System and the Renin-Angiotensin System. *Cell Physiol Biochem* 53(4): 587–605. DOI: 10.33594/000000159.
- Persoon-Rothert M, van der Wees KG, van der Laarse A (2002). Mechanical overload-induced apoptosis: a study in cultured neonatal ventricular myocytes and fibroblasts. *Mol Cell Biochem* 241(1–2): 115–124. DOI: 10.1023/a:1020860209333.
- Ponikowska B, Iwanek G, Zdanowicz A, Urban S, Zymlinski R, Ponikowski P, Biegus J (2022). Biomarkers of Myocardial Injury and Remodeling in Heart Failure. *J Pers Med* 12(5). DOI: 10.3390/jpm12050799.
- Ruan M, Li Y, Li X, Luo J, Kong L (2012). Qualitative and quantitative analysis of the major constituents in Chinese medicinal preparation Guan-Xin-Ning injection by HPLC-DAD-ESI-MS(n). *J Pharm Biomed Anal* 59: 184–189. DOI: 10.1016/j.jpba.2011.10.007.
- Savage P, Cox B, Linden K, Coburn J, Shahmohammadi M, Menown I (2022). Advances in Clinical Cardiology 2021: A Summary of Key Clinical Trials. *Adv Ther* 39(6): 2398–2437. DOI: 10.1007/s12325-022-02136-y.
- Shang H, Zhang J, Yao C, Liu B, Gao X, Ren M, et al. (2013). Qi-shen-yi-qi dripping pills for the secondary prevention of myocardial infarction: a randomised clinical trial. *Evid Based Complement Alternat Med* 2013: 738391. DOI: 10.1155/2013/738391.
- Shen DF, Wu QQ, Ni J, Deng W, Wei C, Jia ZH, et al. (2016). Shensongyangxin protects against pressure overload-induced cardiac hypertrophy. *Mol Med Rep* 13(1): 980–988. DOI: 10.3892/mmr.2015.4598.
- Shi L, Wang S, Zangari M, Xu H, Cao TM, Xu C, et al. (2010). Over-expression of CKS1B activates both MEK/ERK and JAK/STAT3 signaling pathways and promotes myeloma cell drug-resistance. *Oncotarget* 1(1): 22–33. DOI: 10.18632/oncotarget.105.
- Sun MY, Miao Y, Jin M, Dong YR, Liu SR, Wang ML, Gao R (2019). Effect and Safety of Guanxinling Tablet (冠心宁片) for Stable Angina Pectoris Patients with Xin (Heart)-Blood Stagnation Syndrome: A Randomized, Multicenter, Placebo-Controlled Trial. *Chin J Integr Med* 25(9): 684–690. DOI: 10.1007/s11655-019-3069-8.
- Tang Y, Wang M, Le X, Meng J, Huang L, Yu P, et al. (2011). Antioxidant and cardioprotective effects of Danshensu (3-(3,4-dihydroxyphenyl)-2-hydroxy-propanoic acid from *Salvia miltiorrhiza*) on isoproterenol-induced myocardial hypertrophy in rats. *Phytomedicine* 18(12): 1024–1030. DOI: 10.1016/j.phymed.2011.05.007.
- Walker JM (1994). The bicinchoninic acid (BCA) assay for protein quantitation. *Methods Mol Biol* 32: 5–8. DOI: 10.1385/0-89603-268-X:5.
- Wang L, Li Z, Wang C, Yang Y, Sun L, Yao W, et al. (2009). E-cadherin decreased human breast cancer cells sensitivity to staurosporine by up-regulating Bcl-2 expression. *Arch Biochem Biophys* 481(1): 116–122. DOI: 10.1016/j.abb.2008.10.021.
- Wang L, Wang S, Jia T, Sun X, Xing Z, Liu H, et al. (2022). Dexmedetomidine prevents cardiomyocytes from hypoxia/reoxygenation injury via modulating tetramethylcytosine dioxygenase 1-mediated DNA demethylation of Sirtuin1. *Bioengineered* 13(4): 9369–9386. DOI: 10.1080/21655979.2022.2054762.
- Wang ML, Yang QQ, Ying XH, Li YY, Wu YS, Shou QY, et al. (2020). Network Pharmacology-Based Approach Uncovers the Mechanism of GuanXinNing Tablet for Treating Thrombus by MAPKs Signal Pathway. *Front Pharmacol* 11. DOI: ARTN 65210.3389/fphar.2020.00652.
- Wang Y, Wang Q, Li C, Lu L, Zhang Q, Zhu R, et al. (2017). A Review of Chinese Herbal Medicine for the Treatment of Chronic Heart Failure. *Curr Pharm Des* 23(34): 5115–5124. DOI: 10.2174/1381612823666170925163427.

- Watanabe H, Iwanaga Y, Miyaji Y, Yamamoto H, Miyazaki S (2016). Renal denervation mitigates cardiac remodeling and renal damage in Dahl rats: a comparison with beta-receptor blockade. *Hypertens Res* 39(4): 217–226. DOI: 10.1038/hr.2015.133.
- Xing H, Zhang S, Weinheimer C, Kovacs A, Muslin AJ (2000). 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. *EMBO J* 19(3): 349–358. DOI: 10.1093/emboj/19.3.349.
- Xu L, Chen L, Gu G, Wang Y, Xu Y, Zhong Y (2022). Natural products from traditional Chinese medicine for the prevention and treatment of heart failure: progress and perspectives. *Rev Cardiovasc Med* 23(2): 60. DOI: 10.31083/j.rcm2302060.
- Xu X, Ruan L, Tian X, Pan F, Yang C, Liu G (2020). Calcium inhibitor inhibits high glucose-induced hypertrophy of H9C2 cells. *Mol Med Rep* 22(3): 1783–1792. DOI: 10.3892/mmr.2020.11275.
- You Y, Luo L, You Y, Lin Y, Hu H, Chen Y, et al. (2020). Shengmai Yin formula modulates the gut microbiota of spleen-deficiency rats. *Chin Med* 15: 114. DOI: 10.1186/s13020-020-00394-y.
- Zang Y, Wan J, Zhang Z, Huang S, Liu X, Zhang W (2020). An updated role of astragaloside IV in heart failure. *Biomed Pharmacother* 126: 110012. DOI: 10.1016/j.biopha.2020.110012.
- Zhang (2017). Research progress of *Salvia miltiorrhizae* Chuanxiong and its formulation Guanxining Preparation in the treatment of cardiovascular and cerebrovascular diseases. *Chin Tradit Pat Med* 39(05): 1018–1024. DOI:10.3969/j.issn.1001-1528.2017.05.029.
- Zhang B, He P, Lu Y, Bian X, Yang X, Fu X, et al. (2015). HSF1 Relieves Amyloid-beta-Induced Cardiomyocytes Apoptosis. *Cell Biochem Biophys* 72(2): 579–587. DOI: 10.1007/s12013-014-0505-4.
- Zhang D, Gaussion V, Taffet GE, Belaguli NS, Yamada M, Schwartz RJ, et al. (2000). TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. *Nat Med* 6(5): 556–563. DOI: 10.1038/75037.
- Zhang M, Chen Y, Chen H, Shen Y, Pang L, Wu W, Yu Z (2022). Tanshinone IIA alleviates cardiac hypertrophy through m6A modification of galectin-3. *Bioengineered* 13(2): 4260–4270. DOI: 10.1080/21655979.2022.2031388.
- Zhang X, Zhang MC, Wang CT (2018). Loss of LRRC25 accelerates pathological cardiac hypertrophy through promoting fibrosis and inflammation regulated by TGF-beta 1. *Biochem Bioph Res Co* 506(1): 137–144. DOI: 10.1016/j.bbrc.2018.09.065.
- Zhang Y, Cai S, Ding X, Lu C, Wu R, Wu H, et al. (2021). MicroRNA-30a-5p silencing polarizes macrophages toward M2 phenotype to alleviate cardiac injury following viral myocarditis by targeting SOCS1. *Am J Physiol Heart Circ Physiol* 320(4): H1348–H1360. DOI: 10.1152/ajpheart.00431.2020.
- Zhao Y, Li Y, Tong L, Liang X, Zhang H, Li L, et al. (2018). Analysis of microRNA Expression Profiles Induced by Yiqifumai Injection in Rats with Chronic Heart Failure. *Front Physiol* 9: 48. DOI: 10.3389/fphys.2018.00048.