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Research article

Exploring the Efficacy Enhancement Mechanism of Qixue Shuangbu prescription after TCM processing for treating chronic heart failure by regulating ERK/Bcl-2/Bax/Caspases-3 signaling pathway

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ABSTRACT

Qixue Shuangbu prescription (QSP) has been used for the treatment of chronic heart failure (CHF) with remarkable curative effect. Processed QSP (PQSP) could significantly improve the treatment of CHF after traditional Chinese medicine (TCM) processing. This study elucidated the underlying efficacy enhancement mechanism of QSP after TCM processing for treating CHF *in vitro* and *in vivo*. The injury of rat cardiomyoblast H9c2 cells was induced by anoxia/reoxygenation to mimic CHF state *in vitro*. Sixty Sprague-Dawley rats were used to established CHF model by intraperitoneally injecting doxorubicin (the accumulative dose 15 mg/kg). Biochemical examinations were performed in serum and cellular supernatant, respectively. Cardiac functions and histopathological changes were evaluated in CHF model rats. The protein and mRNA levels of ERK1/2, Bcl-2, Bax and Caspase-3 were evaluated by Western blot and RT-PCR, respectively. All above results of low dose crude QSP-treated group (L-CQSP), high dose CQSP-treated group (H-CQSP), low dose PQSP-treated group (L-PQSP), high dose PQSP-treated group (H-PQSP) were compared to systematically explore correlations between TCM processing and the efficacy enhancement for treating CHF of PQSP. Compared with the model group, the L-CQSP group showed significant improvement in cardiac function at 8th weeks, while no significant improvement in cardiomyocyte apoptosis and fibrosis. Both H-CQSP, L-PQSP and H-PQSP exerted beneficial therapeutic effects in injured H9c2 cardiomyocytes and CHF model rats. L-PQSP and H-PQSP significantly increased cell viability and the activity of SOD, decreased the activities of LDH, MDA and NO, up-regulated the expression of ERK1/2 and Bcl-2, down-regulated the expression of Bax and Caspase-3 compared to the same dosage of CQSP. The efficacy enhancement mechanism of

Abbreviations: BHT, benazepril hydrochloride tablet; CHF, chronic heart failure; CQSP, crude Qixue Shuangbu prescription; DMEM, Dulbecco's Modified Eagle's Medium; DOX, doxorubicin; EF, ejection fraction; FBS, fetal bovine serum; FS, fractional shortening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; LVEDd, left ventricular end-diastolic dimension; LVEDV, left ventricular end-diastolic volume; LVESd, left ventricular end-systolic dimension; LVESV, left ventricular end-systolic volume; MDA, malondialdehyde; NO, nitric oxide; PQSP, processed Qixue Shuangbu prescription; QSP, Qixue Shuangbu prescription; SOD, superoxide dismutase; TCM, traditional Chinese medicine.

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PQSP after TCM processing for treating CHF was directly related to the regulation of ERK/Bcl-2/Bax/Caspases-3 signaling pathway.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, with immense social and economic consequences. The prevalence of CVD in adults is 48.6 % overall and increases with age in both males and females due to multiple risk factors [1,2]. The occurrence and development of cardiovascular diseases are closely related to cardiomyocyte apoptosis, due to apoptosis exists in physiological and pathological changes of cardiovascular system [3]. Apoptosis, known as programmed cell death, is a pathological process controlled by a series of programs and mediated via extrinsic or intrinsic signal pathways. Chronic heart failure (CHF) is the final result of various cardiovascular diseases. Prevention and treatment of CHF can be achieved by modulating apoptosis-related signaling pathways [4]. Therefore, cardiomyocyte apoptosis plays an important role in treating CHF.

TCM processing is an effective method to enhance therapeutic effects, reduce the toxicity and modify the nature of some Chinese herbal medicines. Based on the first function of TCM processing, it is still a tough mission to explore the mechanism of improving effects. Qixue Shuangbu prescription (QSP) is a classical traditional Chinese medicine (TCM) prescription for the treatment of CHF with remarkable curative effect [5,6]. It is composed of nine Chinese herbs, among which five Chinese medicine decoction pieces must be processed to improve the curative effect guiding by clinical medication requirements of TCM. Our previous study has demonstrated that the clinical effect of processed QSP (PQSP) on CHF was better than crude QSP (CQSP) [5]. However, the efficacy enhancement mechanism of PQSP after TCM processing for treating CHF need to be explored furthermore.

ERK1/2 signaling pathway can be activated after myocardial injury for exerting cardioprotective effects both *in vivo* and *in vitro* [7–9]. ERK1/2 is a key factor and site in determining cell fate and an important signal transduction system mediating cell response when subjected to external stimuli [10]. It is not only closely related to growth and signal transduction of cardiomyocytes, but also plays a crucial role in the occurrence and development of cardiomyocyte apoptosis. Activation of ERK1/2 signaling pathway is benifical for alleviating cardiomyocyte apoptosis after myocardial injury [11].

Caspase families play a vital role in mediating various apoptotic responses, especially in cardiomyocyte apoptosis [12]. Caspases-3 is a key protease in cell apoptosis, the main effector and the most important executor of apoptosis, and is regarded as the necessary pathway for the cascading reaction of apoptotic proteases [13]. Meanwhile, Caspases-3 is the most critical protease downstream in the caspase cascade reaction that executes apoptosis. Bcl-2 and Bax from the Bcl-2 family proteins can inhibit the activation of Caspase and play important roles of anti-apoptosis and anti-oxidation [14]. Pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 are in a balanced state under normal physiological conditions. This balance will be broken after cardiomyocyte apoptosis when stimulating by certain factors.

In this study, we investigated the efficacy enhancement mechanism of PQSP after TCM processing by inhibiting cardiomyocyte apoptosis and modulating ERK/Bcl-2/Bax/Caspases-3 signaling pathway in injured H9c2 cardiomyocytes and CHF model rats. Confirmatory pharmacological and biological experiments were conducted to relevant targets and pathways, which will provide a relevant basis reference for explaining the TCM processing mechanism of PQSP with better effects in the treatment of CHF than CQSP.

2. Materials and methods

2.1. Chemicals and reagents

Nine crude Chinese medicine decoction pieces of QSP were supplied by the Affiliated Taizhou People's Hospital of Nanjing Medical University, and authenticated by Dr. Linwei Chen. The CQSP and PQSP were prepared according to our previous studies [15–17]. Benazepril hydrochloride tablet (BHT) was purchased from Beijing Novartis Pharmaceutical Co., Ltd (Beijing, China). Doxorubicin (DOX) was provided by Meda AS Meiji Pharmaceutical Co., Ltd (Tokyo, Japan). Antibodies against ERK1/2 (9102), *p*-ERK1/2 (9101), Bcl-2 (15071), Bax (2772), Caspase-3 (9662) were obtained from Cell Signaling Technology (Danvers, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-32233) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V-FITC/PI apoptosis detection kit was produced by Beyotime Institute of Biotechnology (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), D-Hank's equilibrium buffer and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Animal handling

One hundred eight-week-old male Sprague-Dawley rats (200 ± 20 g) were obtained from Qinglongshan Animal Farm (Nanjing, China). After one week of acclimatization, DOX was delivered systemically through intraperitoneal injections (2.5 mg/kg dissolved in normal saline) every two days for six times (the accumulative dose 15 mg/kg) to induce CHF model [18], while the control group received orally the equivalent volume of normal saline. All animal procedures were carried out according to the Guide lines for Care and Use of Laboratory Animals and obtained approval from the ethics committee of Nanjing University of Chinese Medicine (IACUC number: 202101A057).

The CHF model rats were randomly divided into 6 groups (n = 10): (i) model group (model, normal saline 10 mL/kg/d); (ii) positive

drug group (BHT, 0.86 mg/kg/d) [19]; (iii) low dose CQSP-treated group (L-CQSP, 1.64 g/kg/d) [20]; (iv) high dose CQSP-treated group (H-CQSP, 6.56 g/kg/d); (v) low dose PQSP-treated group (L-PQSP, 1.64 g/kg/d) [20]; (vi) high dose PQSP-treated group (H-PQSP, 6.56 g/kg/d). The control group were orally administered normal saline 10 mL/kg/d. All rats were orally administered with corresponding drugs once a day for 8 weeks. After the last treatment, each group was euthanized under anesthesia, blood and heart tissue samples were collected for the following experiments.

2.3. Preparation of drug-loaded serum

Rats were randomly divided into 6 groups (n = 5) and intragastrically given the corresponding drug once a day for one week: (i) normal serum group (normal saline); (ii) BHT-containing serum group (BHT, 0.86 mg/kg); (iii) L-CQSP-containing serum group (L-CQSP, 1.64 g/kg); (iv) H-CQSP-containing serum group (H-CQSP, 6.56 g/kg/d); (v) L-PQSP-containing serum group (PQSP, 1.64 g/kg); (vi) H-PQSP-containing serum group (H-PQSP, 6.56 g/kg/d). Two hours after the last dosing of drug, blood samples were collected from the abdominal aortas under sodium pentobarbital anesthesia and successive centrifuged at 3000 rpm for 15 min. The serum supernatant was isolated and stored at −80 °C for the subsequent experiments.

2.4. H9c2 cardiomyocytes culture and disposal in vitro

The rat cardiomyoblast H9c2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (GNR5; Shanghai, China). The cells were cultured in DMEM with 10 % FBS at 37 °C in an atmosphere with 5 % CO_2 and plated in 8-well plates at a density of 6×10^3 cells/well. The cells were seeded to 6-well plates and were fully washed with D-Hank's equilibrium buffer, put into the anoxic box, and continuously injected a gas mixture containing N_2 for 2 h. The equilibrium buffer was removed, and the completely flat medium was added and incubated into the CO_2 incubator for 24 h to mimic CHF state. Then the cells were split into 7 groups: (i) normal control group (20 % blank rat serum); (ii) CHF model group (20 % blank rat serum); (iii) positive drug group (20 % BHT-containing serum); (iv) L-CQSP (20 % L-CQSP-containing serum); (v) H-CQSP (20 % H-CQSP-containing serum); (vi) L-PQSP (20 % L-PQSP-containing serum); (vii) H-CQSP (20 % H-PQSP-containing serum). Each group was pretreated with 20 μM according drug-loaded serum for 2 h and all assays were performed in triplicate before the following cell experiments were conducted.

2.5. Cell viability assay

Cell viability was assessed with cell counting kit-8 (CCK-8; Dojindo, Japan) after treating with drug-loaded serum in different experimental groups. In brief, 10 μL of CCK-8 reagent was added to the culture medium for incubating 30 min. The optical density (OD) value was measured at a wave length of 450 nm by a microplate spectrophotometer (Biotech, USA).

2.6. Flow cytometry

The levels of apoptosis were determined by flow cytometry with Annexin V-FITC/PI apoptosis detection kit. After treatment with different drug-loaded serums, cells were harvested by centrifugation, washed with ice-cold PBS three times, and resuspended at a concentration of 1×10^6 cells/mL in binding buffer. According to the manufacturer's instructions, cells were stained with 1 mL Annexin V binding buffer containing 5 μL Annexin V-FITC and 10 μL PI solution for 10 min in the dark at room temperature. Quantitative analysis of apoptotic rate was performed by a flow cytometer (Bection Dikinson, Mountain View, USA).

2.7. Biochemical examinations

Superoxide dismutase (SOD, A001-3), lactate dehydrogenase (LDH, A020-2), malondialdehyde (MDA, A003-1) and nitric oxide (NO, A012-1) test kits were purchased by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Biochemical examinations were performed to detect the activity of SOD, LDH, MDA and NO in cell supernatant and rat serum of each group, respectively. The activity of each index was spectrophotometrically measured by using a commercially biochemical assay kit according to the manufacturer's instructions.

2.8. Echocardiography measurements

Rats were anesthetized with 3 % isoflurane (Jiangsu Hengfengqiang Biotechnology Co., Ltd., Nanjing, China) and fixed on the operating plate in supine position. Cardiac functions were evaluated non-invasively by M-mode echocardiography with an ultrasound imaging system (Vevo 2100, Visual Sonics Inc., Toronto, Canada). The left ventricular end-diastolic dimension (LVEDd, mm), left ventricular end-systolic dimension (LVESd, mm), left ventricular end-diastolic volume (LVEDV, mL) and end-systolic volume (LVESV, mL) were recorded by parasternal long-axis and short-axis echocardiograms for at least 3 consecutive cycles of each rat under stable conditions. Left ventricular ejection fraction (EF, %) and fractional shortening (FS, %) refected the systolic function, which were subsequently calculated with Vevo Analysis software (version 2.2.3).

2.9. Histopathological analysis

Histopathological analysis of the left ventricle samples from each group were performed by Hematoxylin-eosin (HE) and Masson's trichrome (MT) staining to assess the extent of apoptosis. The left ventricle samples from each group were fixed with 10 % formalin and embedded in paraffin wax. Sections (5 μm) were cut with a rotary microtome and mounted on glass slides. Specimens were deparaffinized with xylene and rehydrated through decreasing concentrations of ethanol.

For HE staining, sections were warmed in a dry oven at 55 °C for 10 min, incubated in xylene for 5 min twice at room temperature, rehydrated in a series of decreasing concentrations of ethanol, stained with Harris-modified hematoxylin solution for 3 min, in H_2O for 10 min, briefly in 1 % acetic acid for 30 s, rinsed in H_2O for 5 min, washed in 95 % ethanol for 30 s, stained in eosin alcoholic solution for 1 min, rinsed in H_2O for 5 min, then briefly dehydrated in an ascending ethanol series and followed by xylene.

For MT staining, sections were incubated in celestine blue solution for 5 min, briefly washed with H_2O, incubated in hemalun solution for 5 min, in H_2O for 10 min, in fuchsine acid/Ponceau xylidine for 5 min, briefly washed with H_2O, incubated in 1 % phosphomolybdic acid for 10 min, in aniline blue solution for 5 min, briefly washed with H_2O, incubated in 1 % acetic acid for 1 min, then briefly in an ascending isopropanol series and followed by xylene. Images were acquired by a microscope (ZEISS Axio vert. A1, Germany).

2.10. Real-time PCR (RT-PCR)

Total mRNA was extracted from the samples (H9c2 cardiomyocytes and cardiac tissues) using TRIzol reagent (Nordic Bioscience, Beijing, China). After the synthesis of cDNA, it was subjected to RT-PCR on ABI-7300 system (Applied Biosystem, USA) according to the manufacturer's instructions. The relative amounts of mRNA were determined based on $2^{-\Delta\Delta Ct}$ calculations with GAPDH as the endogenous reference. Primer sequences used in RT-PCR are shown in Supplementary Table 1.

2.11. Western blot analysis

Total protein was extracted from the samples of H9c2 cardiomyocytes and heart tissue, and quantified by bicinchoninic acid (BCA) assay. Equal amounts of protein were run on 10 % SDS-PAGE gels followed by electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Nonspecific antibody binding was blocked with 5 % skimmed milk in Tris-buffered saline with Tween-20 (Sangon Biotech Co., Ltd., Shanghai, China) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with primary antibodies (1:1000) against ERK1/2, *p*-ERK1/2, Bcl-2, Bax, Caspase-3 and GAPDH, respectively. Detection of the primary antibodies were performed by incubation with HRP-conjugated secondary antibodies (1:3000) for 1 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence system (Millipore) and Western signals were analyzed with ImageJ software (version 1.50).

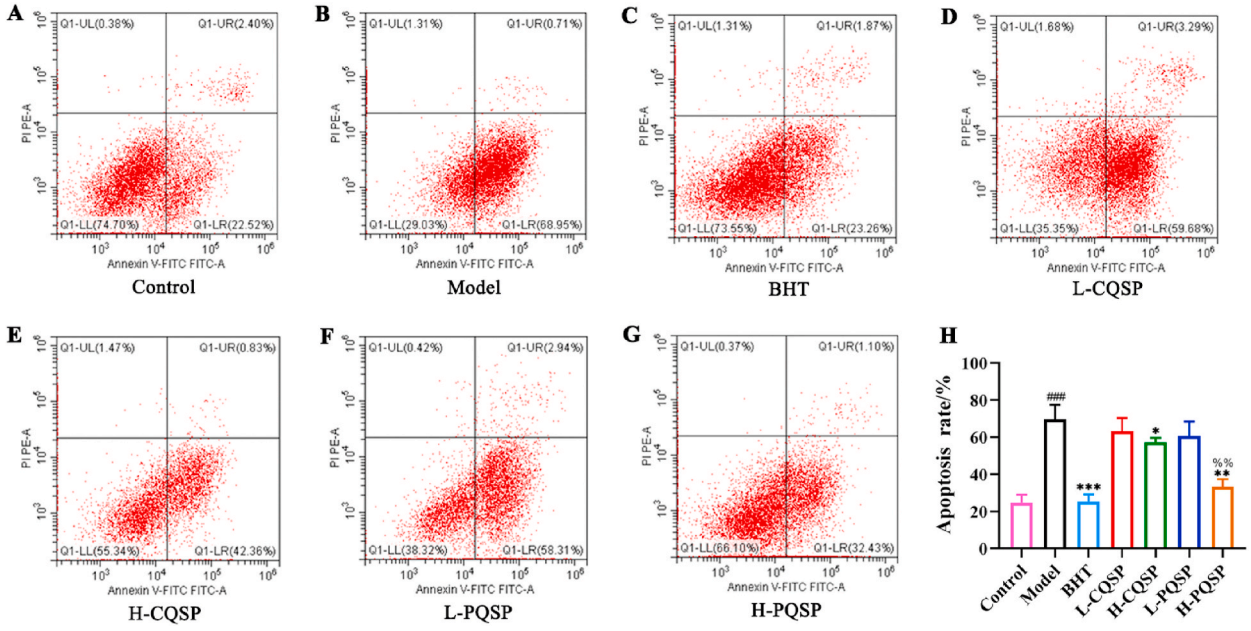

Fig. 1. The apoptotic ratio of H9c2 cardiomyocytes by flow cytometry analysis. $^{###}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs the model group; $^{\%\%}P < 0.01$ vs the H-CQSP group.

2.12. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by GraphPad 9 (GraphPad Software Inc, USA). Differences between groups were conducted through one-way analysis of variance or Student's *t*-test. The data were regarded as statistically significant if $P < 0.05$ or less.

3. Results

3.1. Effects of different drug-loaded serums on cell viability

The viability of cardiomyocytes after treating with different drug-loaded serums were shown in Supplementary Fig. 1. The cell viability was significantly reduced in the model group after myocardial injury compared to the control group. Compared with the model group, the L-CQSP group showed no significant change in cell viability, while the H-CQSP group could significantly increased cell viability. The viability of injured cardiomyocytes was significantly increased in both L-PQSP and H-PQSP groups, and the cell viability increased in a dose-dependent manner.

3.2. Effects of different drug-loaded serums on cardiomyocytes apoptosis

The apoptotic ratio of H9c2 cardiomyocytes was measured by flow cytometry analysis. As shown in Fig. 1B, C, E, G, H, the apoptosis levels were significantly reduced by BHT, H-CQSP and H-PQSP compare to the model group. In the model group, the percentage of apoptotic cells was 69.77 % ± 7.70 % (Fig. 1B and H). The percentage of apoptotic cells were decreased to 57.39 % ± 2.25 % (Fig. 1E and H) and 33.37 % ± 4.01 % (Fig. 1G and H) after treatment with H-CQSP and H-PQSP, respectively. These results suggested that CQSP and PQSP significantly suppressed cardiomyocyte apoptosis.

3.3. Effects on the activities of SOD, LDH, MDA and NO in injured cardiomyocytes

Compared with the control group, the activity of SOD was significantly decreased (Fig. 2A), the activities of LDH, MDA and NO were significantly increased in model group (Fig. 2B–D). The H-CQSP, L-PQSP and H-PQSP groups significantly increased the activity

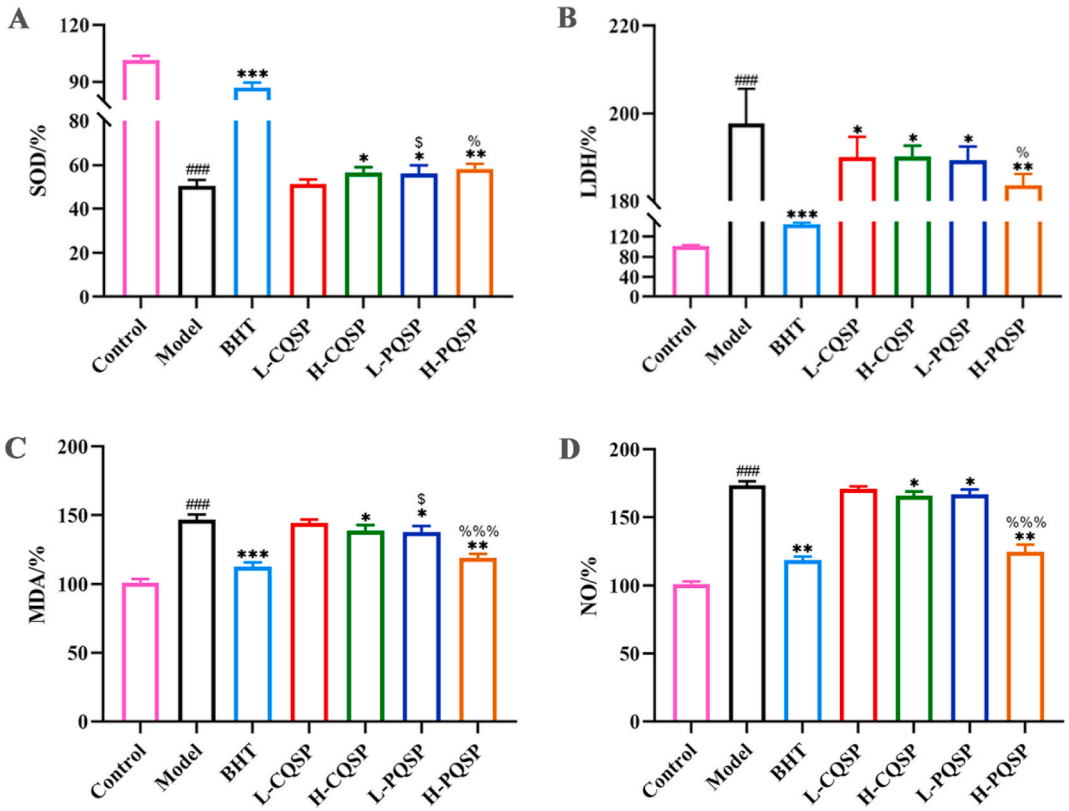

Fig. 2. Activities of SOD (A), LDH (B), MDA (C) and NO (D) in H9c2 cardiomyocytes. $^{\#\#\#}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$vs the model group; $^{\$}P < 0.05$ vs the L-CQSP group; $^{\%}P < 0.05$, $^{\%\%\%}P < 0.001$ vs the H-CQSP group.

of SOD and decreased the activities of LDH, MDA and NO compared with the model group. Both CQSP and PQSP had protective effect on injured cardiomyocytes, but the protective effect of PQSP was higher than that of CQSP at the same concentration.

3.4. Expression levels of related mRNAs in injured cardiomyocytes

The expression levels of ERK1, ERK2, Bcl-2, Bax and Caspase-3 in injured cardiomyocytes were analyzed using RT-PCR. The expression levels of ERK1, ERK2 and Bcl-2 were significantly up-regulated (Fig. 3A–C) and the expression levels of Bax and Caspase-3 were significantly down-regulated (Fig. 3D and E) in H-CQSP, L-PQSP and H-PQSP groups than that of model group. Compared to L-CQSP, mRNA expressions of ERK1 and Bcl-2 were significantly up-regulated and mRNA expressions of Bax and Caspase-3 were significantly down-regulated in the L-PQSP group. Furthermore, the up-regulation effects on ERK1 and down-regulation effects on Bax and Caspase-3 were more significant in the H-PQSP group than the H-CQSP group.

3.5. Expression levels of related proteins in injured cardiomyocytes

As shown in Fig. 4A, Supplementary Figs. 2A–F, the H-CQSP and H-PQSP groups significantly increased the expressions of ERK1/2, *p*-ERK1/2 and Bcl-2 (Fig. 4B–D), reduced the expressions of Bax and Caspase-3 compared with the model group (Fig. 4E and F). L-PQSP significantly increased the expressions of ERK1/2, *p*-ERK1/2 and Bcl-2, reduced the expressions of Bax compared with the model group. However, the levels of *p*-ERK1/2, Bcl-2, Bax and Caspase-3 were not significant affected by L-CQSP-containing serum. These results suggested that QSP-containing serum inhibited apoptosis of H9c2 cells by down-regulating the expression of Bax and Caspase-3, up-regulating Bcl-2 and Bcl-2/Bax.

3.6. Effects on cardiac functions in CHF rats

4-week disposal-free feeding after DOX induced signifcant CHF manifestations, evidenced by reduction in EF and FS (Fig. 5E and F), and augmentation in LVEDd, LVESd, LVEDV and LVESV at 4th, 6th and 8th weeks versus the control group (Fig. 5A–D), respectively. The values of EF were significantly lower in the model group, compared with those in group L-CQSP 6th and 8th weeks, and H-CQSP, L-PQSP and H-PQSP at 4th, 6th and 8th weeks, respectively (Fig. 5E). Similar decrease in FS was found in the model group compared with those in group L-CQSP at 6th and 8th weeks, and H-CQSP, L-PQSP and H-PQSP at 4th, 6th and 8th weeks, respectively (Fig. 5F).

Similar results could be found in H-CQSP, L-PQSP and H-PQSP groups that LVEDd, LVESd, LVEDV and LVESV were significantly improved compared with those in the model group at 6th and 8th weeks, respectively (Fig. 5A–D). These parameters were significantly improved in L-CQSP group only at 6th and 8th weeks. Above results suggested that H-CQSP, L-PQSP and H-PQSP could significantly improved heart parameters and cardiac functions of CHF model rats in varying degrees.

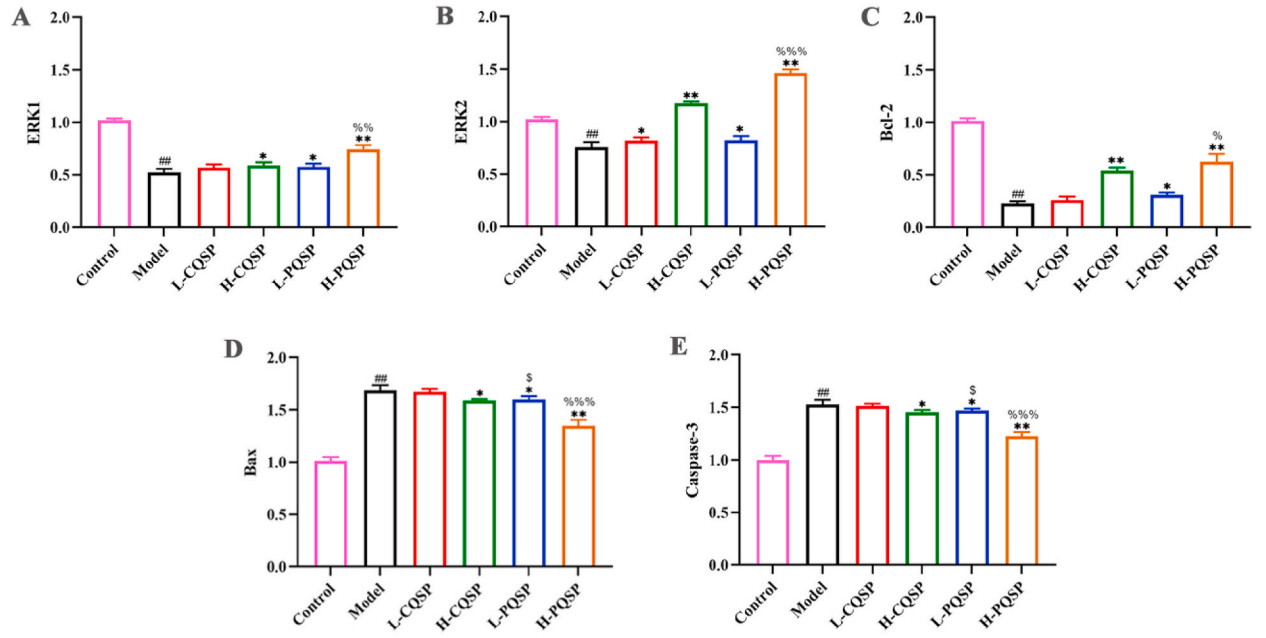

Fig. 3. Expression of cell apoptosis-associated mRNAs in H9c2 cardiomyocytes. Quantitative analysis of ERK1 (A), ERK2 (B), Bcl-2 (C), Bax (D), Caspase-3 (E). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs the model group; $^{\$}P < 0.05$ vs the L-CQSP group; $^{\%}P < 0.05$, $^{\%\%}P < 0.01$, and $^{\%\%\%}P < 0.001$ vs the H-CQSP group.

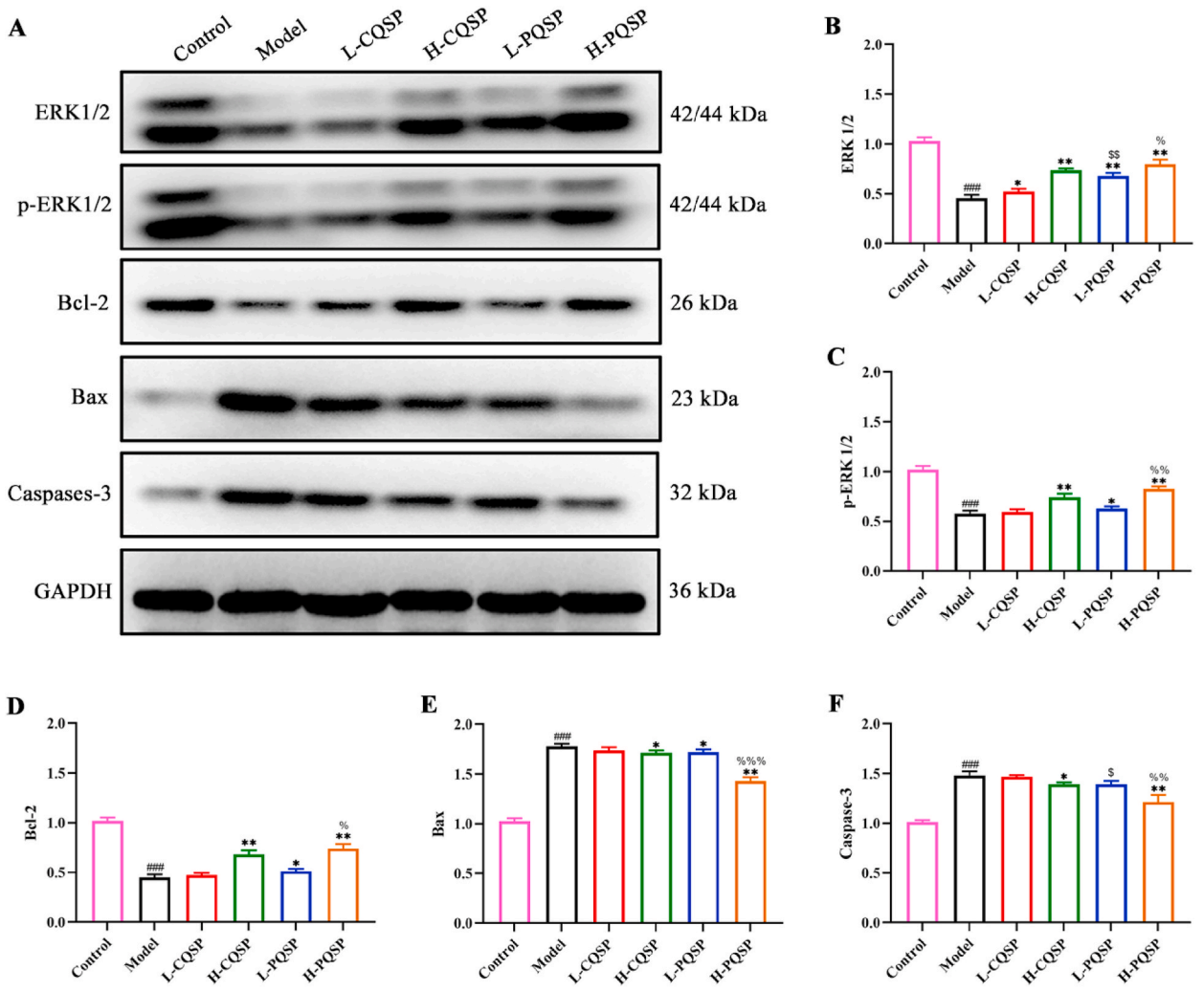

Fig. 4. Expression of cell apoptosis-associated proteins in H9c2 cardiomyocytes. (A) Western blotting of ERK1/2, *p*-ERK1/2, Bcl-2, Bax and Caspase-3. Quantitative analysis of ERK (B), *p*-ERK (C), Bcl-2 (D), Bax (E), Caspase-3 (F). $^{\#\#\#}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs the model group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs the L-CQSP group; $^{\%}P < 0.05$, $^{\%\%}P < 0.01$, and $^{\%\%\%}P < 0.001$ vs the H-CQSP group.

3.7. Effects on the activities of SOD, LDH, MDA and NO in CHF rats

The activity of SOD was significantly decreased (Fig. 6A) and the activities of LDH, MDA and NO were significantly increased (Fig. 6B–D) in model group compared to the control group. Compared with the model group, the H-CQSP, L-PQSP and H-PQSP groups significantly increased the activity of SOD and decreased the activities of LDH, MDA and NO. Both CQSP and PQSP had protective effect on injured cardiac tissues, but the protective effect of PQSP was higher than that of CQSP at the same concentration.

3.8. Expression levels of related mRNAs in CHF rats

The expression levels of ERK1, ERK2 and Bcl-2 were significantly up-regulated (Fig. 7A–C) and the expression levels of Bax and Caspase-3 were significantly down-regulated (Fig. 7D and E) in H-CQSP, L-PQSP and H-PQSP groups than that of model group. Compared to L-CQSP, mRNA expressions of ERK2 and Bcl-2 were significantly up-regulated and mRNA expressions of Bax and Caspase-3 were significantly down-regulated in the L-PQSP group. Furthermore, the up-regulation effects on Bcl-2 and down-regulation effects on Bax and Caspase-3 were more significant in the H-PQSP group than the H-CQSP group.

3.9. Expression levels of related proteins in CHF rats

As shown in Fig. 8A, Supplementary Figs. 3A–F, the H-CQSP and H-PQSP groups significantly increased the expressions of ERK1/2, *p*-ERK1/2 and Bcl-2 (Fig. 8B–D), reduced the expressions of Bax and Caspase-3 compared with the model group (Fig. 8E and F). L-PQSP

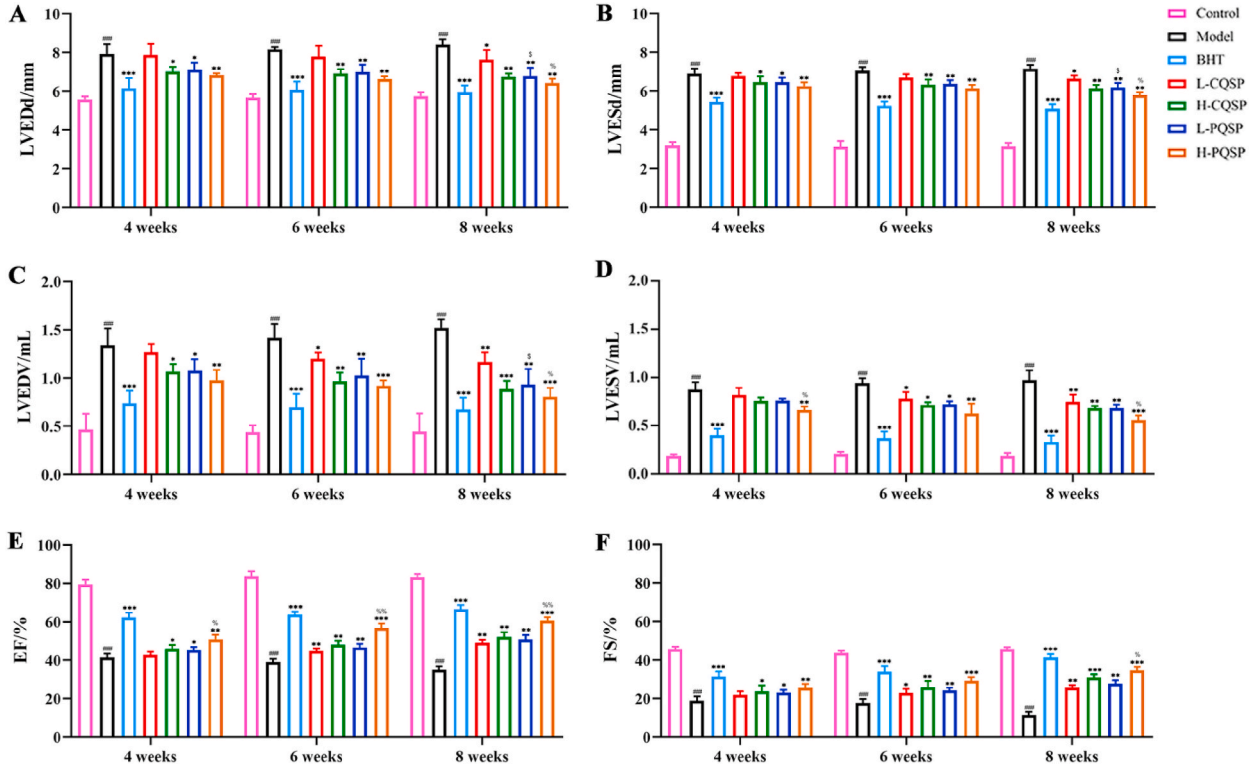

Fig. 5. Comparisons of cardiac function parameters in different groups. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs the model group; $^{\$}P < 0.05$ vs the L-CQSP group; $^{\%}P < 0.05$, $^{\%\%}P < 0.01$ vs the H-CQSP group.

significantly increased the expressions of ERK1/2 and Bcl-2, reduced the expressions of Caspase-3 compared with the model group. However, the levels of *p*-ERK1/2, Bax and Caspase-3 were not significant affected by L-CQSP-containing serum. These results suggested that QSP-containing serum inhibited myocardial apoptosis by down-regulating the expression of Bax and Caspase-3, up-regulating Bcl-2 and Bcl-2/Bax.

3.10. Histopathological examination in CHF rats

HE (Fig. 9A) and MT (Fig. 9B) staining were performed to observe the effect of CQSP and PQSP on the pathological changes in the myocardial tissues. The contorl group showed intact myocardial cells, the myocardial fibrosis was arranged neatly, tightly, and crisscrossed with each other. The model group exhibited disordered myocardial cells, dissolved nuclear pyknosis, myocardial fibrosis swelling, and extensive inflammatory cell infiltration. The degree of myocardial injury in the BHT, H-CQSP and H-PQSP groups was significantly reduced, infiltration of few inflammatory cells and relatively neatly arranged myocardial fibrosis were observed. These results indicated that H-CQSP and H-PQSP could significantly improve myocardial fibrosis and reverse the pathological changes of myocardial tissue in CHF rats.

4. Discussion

In the present study, we compared the efficacy and elucidated the mechanisms between CQSP and PQSP in treating CHF *in vitro* and *in vivo*. Both H-CQSP, L-PQSP and H-PQSP improved cardiac function, alleviated cardiomyocyte apoptosis and fibrosis. Moreover, PQSP significantly up-regulated the expression of ERK1/2 and Bcl-2, down-regulated the expression of Bax and Caspase-3 compared to the same concentration of CQSP. These results indicated that PQSP displayed better effects in the treatment of CHF by regulation of ERK/Bcl-2/Bax/Caspases-3 signaling pathway.

Our results showed that cardiomyocyte apoptosis was significantly increased in injured H9c2 cells and CHF model rats. SOD, LDH and MDA are important markers of myocardial injury, the levels of SOD and MDA reflect the severity of cell lipid membrane injury and oxidative stress. Both CQSP and PQSP significantly suppressed cardiomyocyte apoptosis and improved myocardial injury, while the positive effect of PQSP was higher than that of CQSP at the same concentration. These results suggested that TCM processing mechanism of PQSP with better effects in the treatment of CHF was related to inhibiting myocardial cell apoptosis and improving myocardial injury, thereby improving cardiac function.

The ERK1/2 signaling pathway plays an important role in the development of cardiomyocyte apoptosis. When ischemia, hypoxia

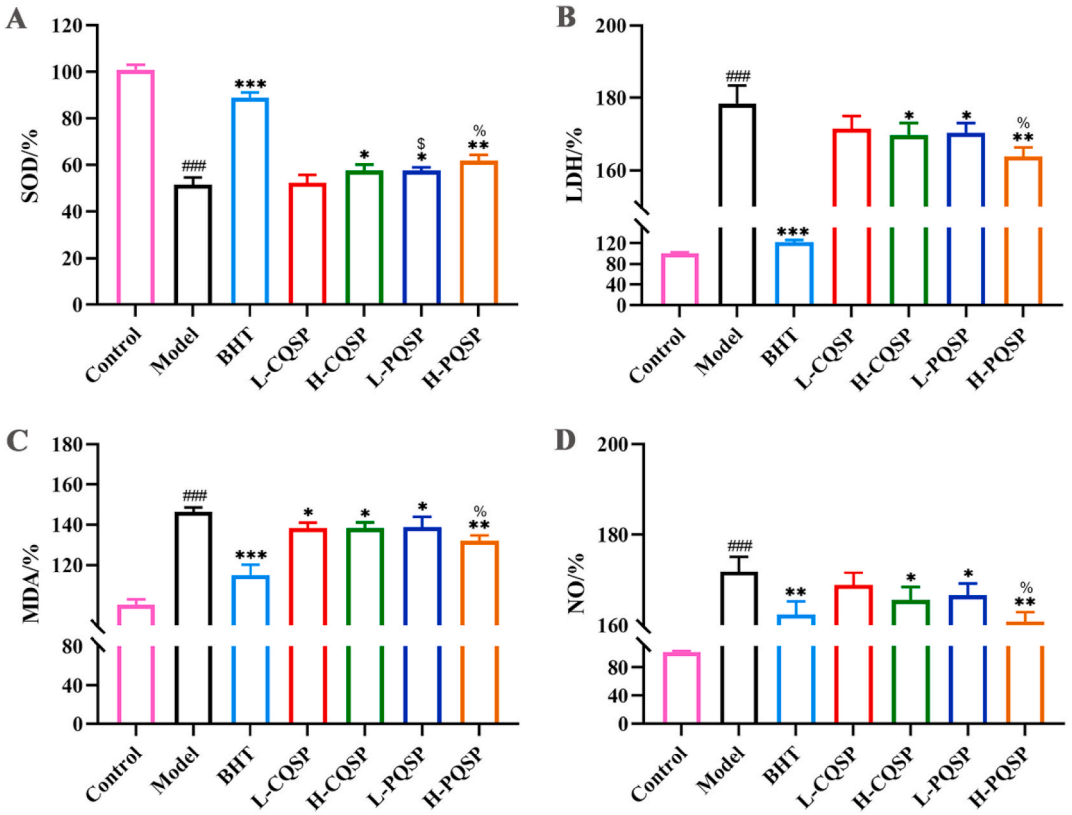

Fig. 6. Activities of SOD (A), LDH (B), MDA (C) and NO (D) in cardiac tissues. $^{\#\#\#}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs the model group; $^{\$}P < 0.05$ vs the L-CQSP group; $^{\%}P < 0.05$ vs the H-CQSP group.

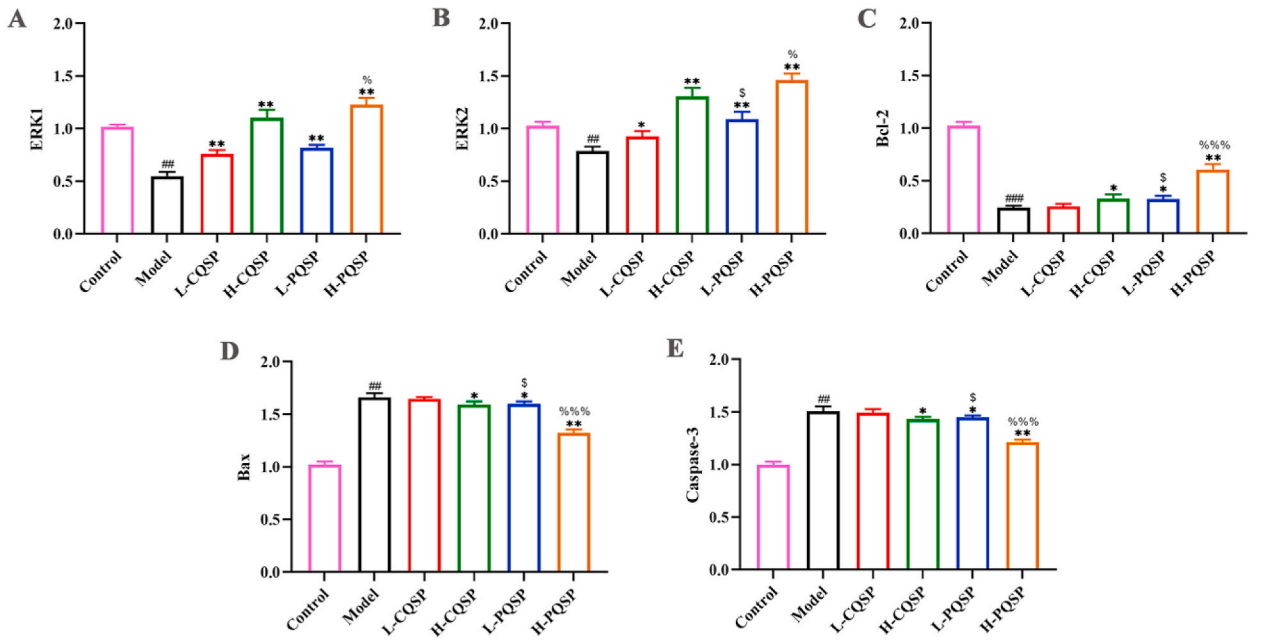

Fig. 7. Expression of cell apoptosis-associated mRNAs in cardiac tissues. Quantitative analysis of ERK1 (A), ERK2 (B), Bcl-2 (C), Bax (D), Caspase-3 (E). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs the model group; $^{\$}P < 0.05$ vs the L-CQSP group; $^{\%}P < 0.05$, $^{\%\%\%}P < 0.001$ vs the H-CQSP group.

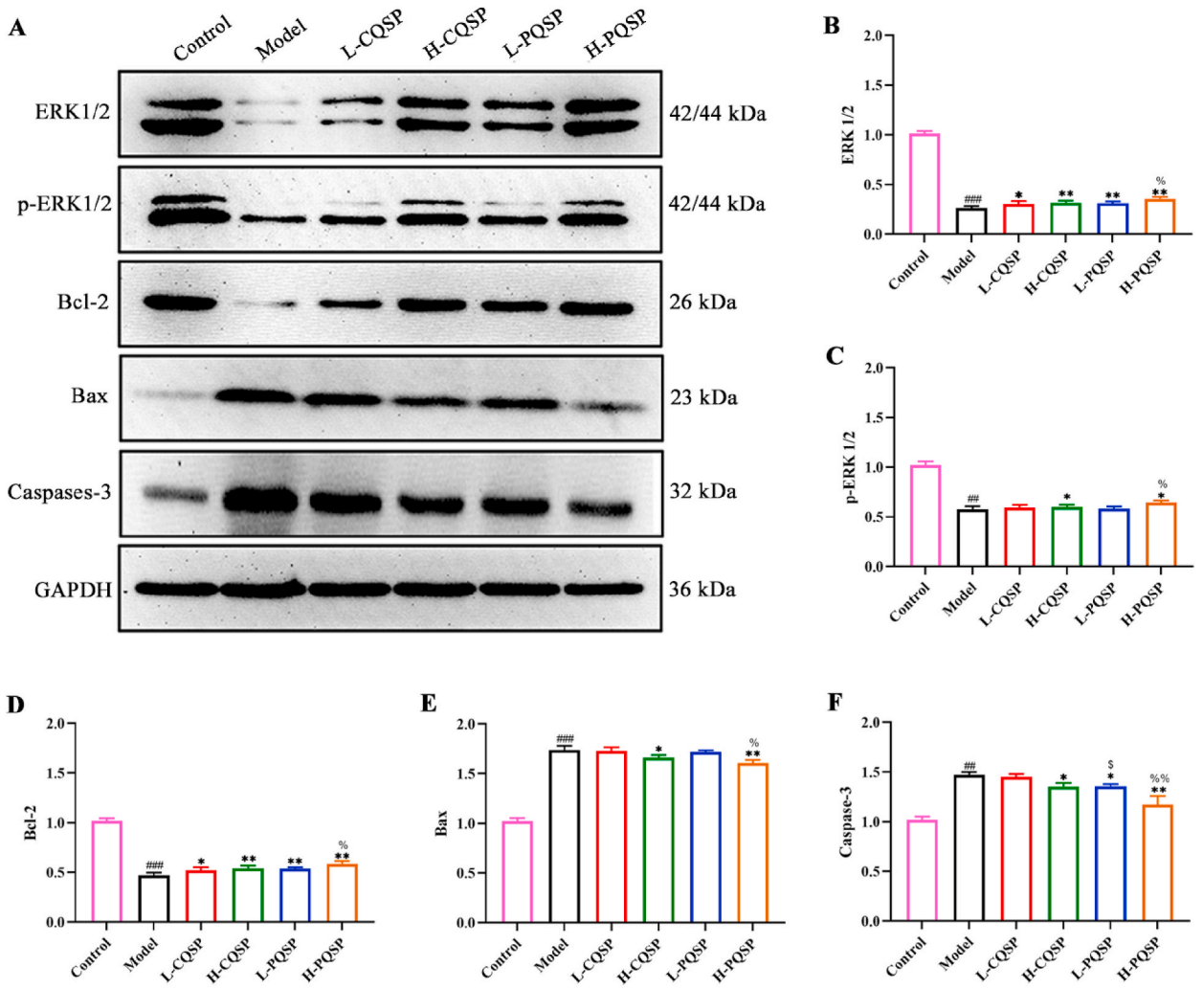

Fig. 8. Expression of cell apoptosis-associated proteins in cardiac tissues. (A) Western blotting of ERK1/2, *p*-ERK1/2, Bcl-2, Bax and Caspase-3. Quantitative analysis of ERK (B), *p*-ERK (C), Bcl-2 (D), Bax (E), Caspase-3 (F). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs the model group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs the L-CQSP group; $^{\%}P < 0.05$, $^{\%\%}P < 0.01$ vs the H-CQSP group.

and reperfusion induced myocardial cell injury, cardiomyocyte apoptosis was significantly increased and the expression activity of ERK1/2 pathway was reduced *in vitro* [21]. If the ERK1/2 signaling pathway can be activated in a timely and effective manner during myocardial injury, the increased phosphorylation of ERK1/2 can inhibit the extensive cardiomyocyte apoptosis [22]. *p*-ERK1/2 can enters the nucleus and acts on transcription factors, thereby promoting the transcription and expression of related genes or proteins and playing the role of cell proliferation and differentiation. As an upstream regulatory kinase, ERK1/2 can activate nuclear factor κB, then regulating the expression of downstream anti-apoptotic genes Bcl-2 and Bcl-xL, while reducing the phosphorylation of pro-apoptotic proteins Bad and promoting the degradation of Bad and Bax, inhibiting the activity of caspase-3, alleviating cardiomyocyte apoptosis, thereby exerting a myocardial protective effect [23].

It has been suggested that the survival ability of cardiomyocytes after apoptosis is determined by the ratio of Bcl-2 to Bax [24]. When Bcl-2 is expressed at high levels, the Bcl-2/Bax ratio is up-regulated, which inhibiting cardiomyocyte apoptosis. On the contrary, when Bax is expressed at high levels, the Bcl-2/Bax ratio is down-regulated, which promoting cardiomyocyte apoptosis. This study shown that myocardial injury mainly increased Bax and decreased Bcl-2 levels, resulting in the obvious down-regulation of the Bcl-2/Bax ratio. Both H-CQSP and H-PQSP not only increased Bcl-2, but also decreased Bax, leading to a much higher Bcl-2/Bax ratio than that in the model group. In addition, H-PQSP significantly inhibited myocardial cell apoptosis by up-regulation of the Bcl-2/Bax ratio for better therapeutic effect than H-CQSP.

Caspase-3 is the most important executor of apoptosis, and the expression of Caspase-3 is positively correlated with apoptosis. In this study, *in vivo* and *in vitro* experimental results both showed that H-CQSP, L-PQSP and H-PQSP inhibited cardiomyocyte apoptosis by down-regulating caspase-3 expression. In addition, Caspase-3 can inhibits the expression of Bcl-2, and in contrast Bcl-2 also causes a decrease in the levels of Caspsae-3 [25]. These results further suggest that the efficacy enhancement of PQSP after TCM processing for

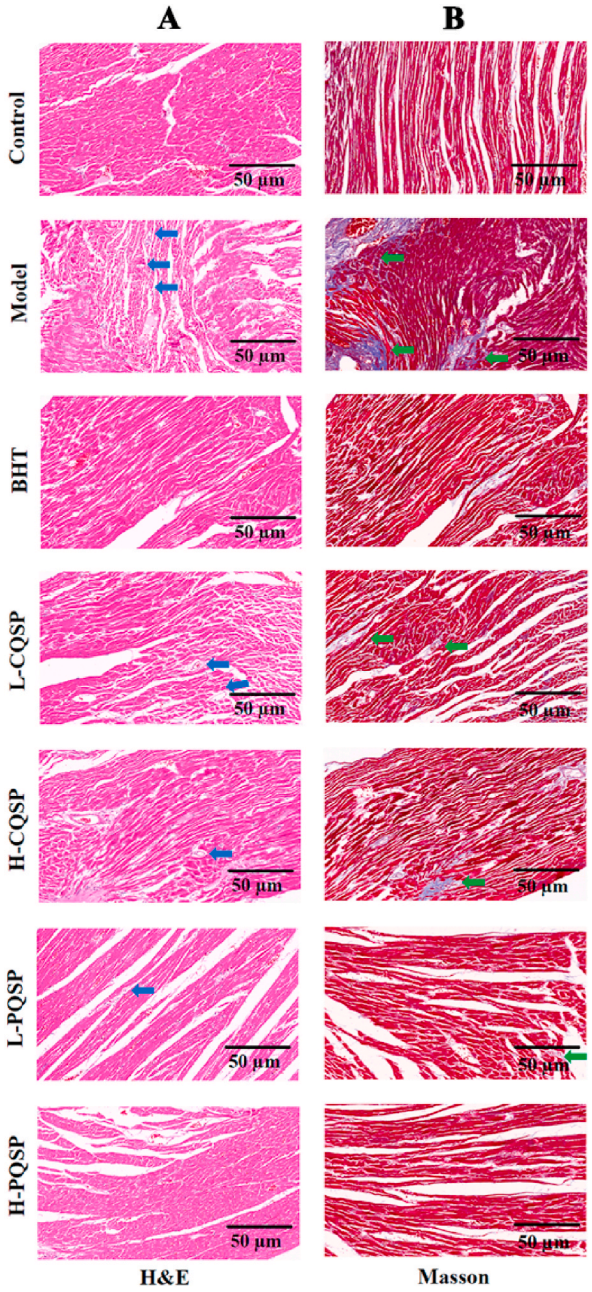

Fig. 9. Histological images of heart tissues from each group. The breakage of myocardial fibrosis and hyperplasia of collagen fibers were highlighted with the blue and green arrows, respectively.

treating CHF may be achieved by inhibiting apoptosis though ERK/Bcl-2/Bax/Caspases-3 signaling pathway.

TCM processing is an important way to promote therapeutic effects and modify the nature. Five Chinese medicine decoction pieces of PQSP have been processed with different adjuvants. Huang Qi was processed by stir-frying with honey, Dang Gui, Bai Shao and Huang Jing were processed by stir-frying with yellow rice wine, He Shou Wu was processed by steaming with black bean extract. Based on the TCM theory, different adjuvants might partake in physical or chemical transformation to alter pharmacological effects and exert the pharmacokinetic behavior for the enhanced therapeutic effect. In the previous study, we have detected differences in chemical compositions between CQSP and PQSP [26]. There were 101 common peaks of compounds in both CQSP and PQSP, in which 31 analytes increased and 38 analytes reduced in PQSP. The pharmacokinetic parameters of main bioactive components indicated longer lasting time elimination, better bioavailability and absorption for enhancing the effect in the treatment of CHF in the PQSP group than that of the CQSP group [17,26].

In recent years, TCM has been successfully employed to treating CHF. According to the TCM theory, heart Qi and Yang deficiencies

are the fundamental causes for CHF. Blood stasis, phlegm formation, and fluid retention are the pathological changes secondary to CHF. Thus, tonifying Qi, warming Yang, activating blood circulation play a vital role in the treatment of CHF. QSP has the effects of tonifying Qi and blood, warming Yang and dispersing knot, activating blood and clearing collaterals. Emerging studies suggested that TCM could prevent and treat myocardial injury by anti-apoptosis, inhibiting inflammatory reaction, blocking calcium channel and scavenging oxygen free radicals [27]. The mechanism of new Shengmai decoction improve cardiac function and inhibit myocardial cell apoptosis by reducing the expression of Caspase-3 and Bax, increasing the expression of Bcl-2, up-regulating the ratio of Bax/Bcl-2 [28].

Ren Shen is preferred in the treatment of CHF due to its unique effects on reinforcing vital energy. Ginsenosides are the primary components of Ren Shen, have strong clinical effect in various cardiovascular diseases such as CHF, ischemic heart disease, and arrhythmia [29]. It has been found that ginsenosides possessed significantly pharmacological activities of anti-apoptosis, anti--oxidation, reducing calcium overload and clearing of oxygen free radicals [30,31]. Ginsenoside Rb1 preconditioning significantly decreased infarct size and cardiomyocyte apoptosis, increased the levels of LDH and Bcl-2, and activated caspase-3 protein expression [32].

Huang Qi is a major medicinal herb widely used in TCM formulas, which has been proved to be effective against CHF [33]. Modern pharmacological research has shown that the major components in Huang Qi can protect myocardial cells, enhance myocardial contractility, regulate immunity, and improve circulation [34]. Astragaloside IV (AS-IV) is a main component of Huang Qi, has been shown to confer protective effects against many cardiovascular diseases [35]. AS-IV could significantly increased caspase-3 activity, up-regulated Bcl-2 expression, prevented apoptosis, and thereby alleviated myocardial injury [36].

Based on the review of the existing literature, this study was the first to analyze the underlying efficacy enhancement mechanism of QSP after TCM processing for treating CHF in injured H9c2 cells and CHF model rats from the signaling pathway perspective. These results of our study will provide valuable data support for the clinical application of PQSP. However, the current study has some limitations. For example, we solely employed two doses of CQSP and PQSP, and have not verify the results in models of specific genes knockout or overexpression. Next studies would be carried out to silence cell apoptosis-associated genes for further verifying the efficacy enhancement mechanism of PQSP after TCM processing for treating CHF in the most effective dosage by ERK/Bcl-2/Bax/Caspases-3 signaling pathway.

5. Conclusion

The present study had provided the mechanism and pharmacodynamics evidence for QSP in the treatment of CHF *in vivo* and *in vitro*. PQSP was more significantly inhibited cardiomyocyte apoptosis, up-regulated the expression of ERK1/2 and Bcl-2, down-regulated the expression of Bax and Caspase-3 than CQSP, thereby improving cardiac function. These results elucidated the efficacy enhancement mechanism of PQSP after TCM processing for treating CHF by suppressing apoptosis via the ERK/Bcl-2/Bax/Caspases-3 signaling pathway.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the ethics committee of Nanjing University of Chinese Medicine (IACUC number: 202101A057).

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CRediT authorship contribution statement

Qin Wang: Validation, Software, Resources, Methodology, Data curation. **Yong Jiang:** Software, Resources, Formal analysis, Conceptualization. **Shun Xie:** Supervision, Software, Resources, Project administration, Formal analysis, Conceptualization. **Linwei Chen:** Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30476.

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