Regular Article

Qiliqiangxin attenuates oxidative stress-induced mitochondrion-dependent apoptosis in cardiomyocytes via PI3K/AKT/GSK3β signaling pathway

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Summary

Qiliqiangxin capsule (QLQX) is a well-known traditional Chinese medicine that exhibits cardioprotective effects in heart failure patients. However, it remains unclear whether and by which mechanism QLQX attenuates oxidative stress-induced mitochondria-dependent myocardial apoptosis. In vivo, SD rats received left anterior descending coronary artery ligation for 4 weeks to establish a model of heart failure after acute myocardial infarction, and then were treated with QLQX for another 4 weeks. We evaluated cardiac function, oxidative stress injury, as well as the expressions of mitochondria-dependent apoptosis and its signaling factors. The results indicated that QLQX protected cardiac function and attenuated oxidative stress-induced myocardial apoptosis. Meanwhile, QLQX elevated the Bcl-2 expression, declined the expressions of Bax, cytochrome c, apoptotic protease activating factor-1 (Apaf-1), cleaved-caspase9 and cleaved-caspase3, and up-regulated the ratios of phospho-AKT/AKT and phospho-GSK3β/GSK3β. In vitro, H9c2 cardiomyocytes were pretreated with QLQX, then exposed to H2O2 for 24 h. QLQX promoted the proliferation of H9c2 cardiomyocytes induced by H2O2 and reversed oxidative stress damage. Moreover, QLQX inhibited the apoptosis rate and the pro-apoptosis protein expressions, but improved the Bcl-2 expression as well as the ratios of phospho-AKT/AKT and phospho-GSK3β/GSK3β. Meanwhile, it further ameliorated mitochondrion-related apoptosis by inhibiting the mitochondrial fission, mitochondrial permeability transition pore (MPTP) opening, and mitochondrial membrane potential (MMP) decline in H9c2 cardiomyocytes induced by H2O2. In addition, all the effects of QLQX on H2O2-induced mitochondria-dependent apoptosis could be blocked by the PI3K inhibitor, LY294002. We conclude that QLQX may ameliorate oxidative stress-induced mitochondria-dependent apoptosis in cardiomyocytes through PI3K/AKT/GSK3β signaling pathway.

Keywords

heart failure; oxidative stress; qiliqiangxin capsule; myocardial apoptosis.
Acute myocardial ischemia (AMI) has long been a severe cardiovascular disease closely associated with chronic heart failure (CHF). Accumulating evidence has suggested that oxidative stress injury post-AMI plays an important role in the pathological course of CHF. In the failing heart, there is a severe accumulation of reactive oxygen species (ROS) and free radicals caused by cardiac injury, which are in an imbalanced state with the antioxidants or free radical-scavenging enzymes such as superoxide dismutase (SOD). The imbalance could lead to oxidative stress and toxicity to cardiomyocytes, causing impairment of the mitochondrial structure and function and triggering mitochondrion-mediated apoptosis. Thereafter, myocardial apoptosis resulting from oxidative stress would aggravate ventricular remodeling, causing or exacerbating CHF. Therefore, it is of great significance for the treatment of CHF to inhibit oxidative stress-induced mitochondrion-dependent apoptosis in cardiomyocytes.

The phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is vital in mediating cell survival and apoptosis, which could phosphorylate the downstream signaling, such as glycogen synthase kinase-3β (GSK3β), to maintain mitochondrial homeostasis and inhibit mitochondria-mediated apoptosis. A previous study has shown that activation of the PI3K/AKT/GSK3β pathway could inhibit cardiomyocyte apoptosis by up-regulating Bcl-2 expression and down-regulating the expressions of Bax and cleaved-caspase3, thereby reducing the myocardial infarction area and improving cardiac function. It has been also found that regulation of GSK3β activity could inhibit dynamin-related protein 1 (Drp1)-mediated mitochondrial fission, mitochondrial permeability transition pore (MPTP) opening, and mitochondrial membrane potential (MMP) decline, to attenuate early mitochondria-dependent apoptosis. Therefore, the PI3K/AKT/GSK3β pathway plays an important role in mitochondrion-dependent apoptosis.

Qiliqiangxin capsule (QLQX), a traditional Chinese herbal compound preparation, is composed of 11 kinds of Chinese herbal medicine extracts, and is safe and effective for the treatment of CHF. A multicenter, randomized, double-blind, parallel-group, placebo-controlled study has shown that QLQX can be used in the treatment of CHF. It has also been demonstrated that QLQX can inhibit myocardial remodeling and protect cardiac function via down-regulating inflammation, attenuating myocardial apoptosis and fibrosis, and promoting angiogenesis in rats with CHF. However, whether QLQX can improve oxidative stress damage and the related mitochondria-dependent apoptosis in cardiomyocytes is still unclear. In this study, we focused on whether and how QLQX attenuates oxidative stress-induced mitochondrion-dependent apoptosis in cardiomyocytes, both in vivo and in vitro.

**MATERIALS AND METHODS**

**Drugs and Reagents.** QLQX, in ultrafine powder form, was supplied by Shijiazhuang Yiling Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China). According to the National Pharmacopoeia Committee of China (2005), the herbs were identified and standardized. The fingerprint of QLQX was performed by ultrahigh performance liquid chromatography as reported previously. In vivo, QLQX was weighed and dissolved in sterile saline at specified concentrations. In vitro, based on the suggestion from Shijiazhuang...
Yiling Pharmaceutical Co., Ltd and the previous study\textsuperscript{17}, QLQX was weighed and dissolved in Dulbecco’s Modified Eagle’s Medium (DMEM), sonicated for 30 min, and then centrifuged at 10,000 rpm/min for 10 min. The supernatant was obtained and filtered with a 0.22μm filter. The final concentration was calculated, and the stock solution was stored at -20°C for future use.

Benazepril was manufactured by Beijing Novartis Pharmaceutical Co., Ltd. (Beijing, China); H\textsubscript{2}O\textsubscript{2} was purchased from Tianjin Yongda Chemical Reagent Co., Ltd. (Tianjin, China). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], the Eastep\textsuperscript{®} Super Total RNA Extraction Kit and the Reverse Transcription System were obtained from Promega (Wisconsin, WI, USA); The Fast SYBR Green Master Mix was purchased from Thermo Fisher Scientific (Waltham, MA, USA); The primers of heme oxygenase-1 (HO-1), catalase (CAT) and GAPDH were bought from Sangon Biotech CO., Ltd (Shanghai, China). An in situ cell death detection kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany); The lactate dehydrogenase (LDH) Assay Kit, ROS/superoxide assay kit, SOD Activity Assay Kit, JC-1- MMP Assay Kit, and primary antibodies against cytochrome c, apoptotic protease activating factor-1 (Apaf-1), cleaved-caspase9, cleaved-caspase3, Drp1, and phosphorylated Drp1 (p-Drp1) at serine 637 were purchased from Abcam (Cambridge, England, UK). An Annexin V-FITC apoptosis detection kit was bought from BD Biosciences (Franklin Lakes, NJ, USA); A bicinchoninic acid (BCA) kit was purchased from Beyotime (Shanghai, China). The MPTP detection kit was obtained from BestBio (Shanghai, China); Mito-Tracker Red CMXRos was from Invitrogen (Carlsbad, CA, USA), and radio immunoprecipitation assay (RIPA) and phenylmethylsulfonyl fluoride (PMSF) were bought from Solarbio (Beijing, China). LY294002 and primary antibodies against Bcl2, Bax, AKT, phosphorylated AKT (p-AKT), GSK3β, and phosphorylated GSK3β (p-GSK3β) at serine 9 were obtained from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated secondary antibody was bought from LI-COR (Lincoln, NE, USA).

**Animal Model and Administration.** Male Sprague-Dawley rats, weighing 200-220g, were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China), and housed at five/cage with a standard diet and water and a 12 h light/12 h dark cycle. The model of CHF post-AMI was constructed by ligating the left anterior descending coronary artery (LAD) as previously described\textsuperscript{16, 18}. The animals were anesthetized with 10% chloral hydrate (0.35g/kg) and artificially ventilated after tracheotomy. The left thoracotomy was performed and the LAD was ligated at the point below the left atrial appendage by a single 6-0 nylon suture. Then the thoracic cavity was closed and stitched with sterility. The sham group underwent the same procedure but without ligation. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996) and approved by the Care and Use Committee of Hebei Medical University. All possible steps were taken to avoid animal suffering at each stage of the experiment.

Four weeks later, rats with left ventricular ejection fraction (LVEF) < 50% examined by echocardiography were diagnosed with CHF for subsequent studies. The rats were randomly

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assigned to six groups (n=10): sham group (Sham), model group (Model), QLQX low-dose group (QLQX-L, 0.25g/kg/d), QLQX middle-dose group (QLQX-M, 0.5g/kg/d), QLQX high-dose group (QLQX-H, 1.0g/kg/d), and Benazepril group (Benazepril, 10mg/kg/d). The sham group and model group were given equal volumes of normal saline by gavage. Angiotensin-converting enzyme inhibitor Benazepril, inhibiting ventricular remodeling and treating heart failure, was used as a positive control in the present study. All groups were administered drugs or normal saline for 4 weeks.

**Echocardiography.** Echocardiography was conducted in a standard setting using a 15 MHz ultrahigh frequency scan head (MyLabFive, Esaote, Italy) in rats anesthetized with 10% chloral hydrate, 4 weeks after surgery and at the end of medical interventions. Several indicators were measured blinded by experienced researchers, including left ventricular end-diastolic internal diameter (LVEDd), left ventricular end-systolic internal diameter (LVESd), left ventricular ejection fraction (EF), and left ventricular fractional shortening (FS). All parameters were measured over 5 consecutive cardiac cycles.

**Cell Culture and Treatment.** Rat H9c2 cardiomyocytes were purchased from Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 1.5 g/L NaHCO3, and 1% penicillin/streptomycin in the incubator at 37°C with 5% CO2. The cells were treated in subsequent experiments as follows. Normal group: H9c2 cells were treated with equal volume of DMEM for 24h; QLQX group: H9c2 cells were treated with QLQX for 24h; H2O2 group: H9c2 cells were exposed to H2O2 for 24h; H2O2+QLQX group: H9c2 cells were pretreated with QLQX for 4h, and then co-incubated with H2O2 for 24h. H2O2+QLQX+LY294002 group: H9c2 cells were pretreated with QLQX for 4h, then 50μM LY294002 for 0.5h. The cells were then co-incubated with H2O2 for 24h. H2O2+LY294002 group: H9c2 cells were pretreated with LY294002 for 0.5h, and then co-incubated with H2O2 for 24h.

**Cell Viability Assay.** Cell viability was determined by MTS assay. H9c2 cells were seeded at 1×10^4 cells/well in 96-well plates for 24h and then respectively exposed to serial concentrations of H2O2 and QLQX for another 24h. After treatment, the cells were incubated with 1mg/mL MTS working solution (100μl/well) for 2h at 37°C. The absorbance values at 490 nm were measured using a microplate spectrophotometer. Based on the viability of H9c2 cells, 80μM was determined as the appropriate concentration of H2O2 for subsequent experiments. H9c2 cells were pretreated with increasing concentrations of QLQX for 4h, followed by exposure to 80μM H2O2 and QLQX in different concentrations for further 24h. The cell viability was assessed using a microplate spectrophotometer.

**Measurement of Oxidative Stress.** Oxidative stress injury both in vivo and in vitro was confirmed by measuring LDH release level, ROS level and SOD activity. In vivo, the blood was collected from the abdominal aorta and centrifuged for 10min at 3500rpm. Then the serum was loaded into Eppendorf tubes for further use. In vitro, H9c2 cardiomyocytes were seeded at 1×10^4 cells/well in the 96-well plate for 24h. After treatment, the culture supernatant and cells were obtained. According to the manufacturer’s instructions, the ROS level in serum and H9c2 cells was performed by ROS/superoxide detection assay kit using a fluorescent plate reader with excitation/emission rates of 488 nm/520 nm, and the ROS level was calculated according to the fluorescence intensity. The LDH release level and SOD...
inhibition rate in serum and supernatant were detected using kits according to the manufacturer’s instructions with a microplate reader at 450 nm. The values were calculated by the standard curve.

In addition, HO-1 and CAT mRNA expressions were measured by quantitative real time polymerase chain reaction (RT-PCR) to detect the antioxidant effects of QLQX. Total RNA was extracted from H9c2 cardiomyocytes using Eastep® Super Total RNA Extraction Kit, and reversely transcribed into cDNA using Reverse Transcription System according to the manufacturer’s instructions. All reactions were performed in a reaction volume of 20 µl. Quantitative RT-PCR analysis was performed using Fast SYBR Green Master Mix on an applied biosystems 7500 FAST RT-PCR System. PCR thermal cycling involved 95°C for 20sec denature, followed by 40 cycles each containing 95°C for 3 sec and 60°C for 30sec. HO-1, CAT and GAPDH were amplified by using their specific primers as follows. HO-1 forward, 5'‐ CGA AAC AAG CAG AAC CCA -3' and reverse, 5'- CAC CAG CAG CTC AGG ATG -3' (product, 192bp); CAT forward, 5'- CCT CGT TCA AGA TGT GGT -3' and reverse, 5'- CAC TTT TGC CTT GGA GTA -3' (product, 148bp); GAPDH forward, 5'- GCA AGT TCA ACG GCA CAG -3' and reverse, 5'- GCC AGT AGA CTC CAC GAC AT -3' (product, 140bp). GAPDH was used for normalization. The relative mRNA expressions were calculated using the 2^ΔΔCt method.

**Assessment of Cardiomyocyte Apoptosis.** In vivo, myocardial apoptosis in rats was analyzed by TUNEL assay using a In Situ Cell Death Detection Kit according to the manufacturer's instructions. Briefly, the paraffin sections were pretreated with proteinase K (20µg/mL) after dewaxation and rehydration, incubated with TUNEL reaction mixture, and then converter-POD. After coloration with DAB and counterstaining with hematoxylin, cell nuclei labeled with brown were considered TUNEL-positive. Images were visualized under an optical microscope at 200 × magnification in at least three fields chosen randomly. The apoptotic index of cardiomyocytes was calculated from the ratio of TUNEL-positive nuclei to total number of cardiomyocytes per field. In vitro, H9c2 cells apoptosis was detected with flow cytometry. H9c2 cells were seeded at a density of 3 × 10^5 cells/well in 6-well plates for 24h. After cells were treated as above, they were collected and the apoptosis rate was measured with the Annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions. The cellular apoptosis was detected with flow cytometry.

**Western Blot Analysis.** Heart tissues in the infarct border region or cells were collected and lysed with RIPA buffer containing a cocktail of phosphatase inhibitor and PMSF for 30min on ice. The lysate was centrifuged at 12,000 rpm at 4°C for 25min. Protein concentration was determined with the BCA kit following manufacturer's instructions. The supernatant was added to the protein sample buffer and denatured at 100°C for 5min. Protein samples were separated with 4%–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto Nitrocellulose Blotting membrane (Life Sciences, Mexico). After blocking with the Odyssey blocking buffer (LI-COR, Lincoln, USA) for 1h, the membrane was incubated with primary antibodies at 4°C overnight, and then it was incubated with horseradish peroxidase-conjugated secondary antibody at 37°C for 1 h. Then the protein bands were scanned and analyzed with the Odyssey scanner.
Confocal Laser Scanning Microscopy Analysis. H9c2 cells were seeded in a glass bottom cell culture dish for 24h. After cell treatment, the cells were stained with 300nM Mito-Tracker Red CMXRos in the dark for 30 min. and then fixed with 4% paraformaldehyde. Images of mitochondrial morphology were evaluated using confocal laser scanning microscopy (Oberkochen, Germany) at least three fields randomly.

Assessment of MPTP Opening. The MPTP opening was measured with the MPTP detection kit according to the manufacturer’s instructions. After cell treatment, the cells were collected and re-suspended at the density of 1×10⁶ cells/ml. Subsequently, the cells were incubated with the BB cell ProbeTM M61 probe and quencher in the dark for 15 min. The fluorescence intensity in the mitochondria was detected using flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 515 nm.

Detection of MMP. The MMP was detected with the JC-1- MMP Assay Kit according to the manufacturer’s instructions. H9c2 cardiomyocytes were seeded at 1×10⁴ cells/well in the 96-well black-bottomed plate for 24h. After treatment, the cells were incubated with JC-1 in the dark at 37°C for 10 min. Then the fluorescence intensity was detected using a fluorescent plate reader with excitation/ emission rates of 485nm/535nm (Green) and 535nm/595nm (Red). The MMP was determined based on the ratio of red/green fluorescence intensity.

Statistical Analysis. Data were expressed as mean ± SD from three or more independent experiments. Comparison between groups was determined by one-way ANOVA. P <0.05 and P <0.01 were considered statistically significant. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

QLQX Rescued Cardiac Function in Rats with HF Post-AMI. Cardiac function was measured with echocardiography 4 weeks after treatment. The results showed that, compared with the sham group, cardiac function was significantly deteriorated in the model group, including decreasing the levels of ejection fraction (EF) and fractional shortening (FS) but increasing left ventricular end-diastolic diameter (LVEDd) and left ventricular end-systolic diameter (LVESd) in the model group (P < 0.01). After treatment with QLQX and benazepril, the values of EF and FS were remarkably up-regulated (P < 0.01), whereas LVEDd and LVESd were significantly down-regulated (P < 0.01) (Fig. 1a and 1b).

QLQX Attenuated Oxidative Stress Injury in Rats with HF Post-AMI. The results showed that the levels of LDH and ROS in serum significantly increased (P < 0.01), whereas SOD activity was decreased in the model group more than in those in the sham group (P < 0.01). Besides, QLQX and benazepril could down-regulate the levels of LDH and ROS, but up-regulate SOD activity compared with those of the model group (P < 0.05) (Fig. 1c).

QLQX Inhibited Cardiomyocyte Apoptosis via the PI3K/AKT/GSK3β Signaling Pathway in HF Post-AMI Rats. In the present study, TUNEL staining showed that the apoptosis rate in the infarct border region, compared with the sham group, was significantly elevated in the model group (P<0.01). However, QLQX and benazepril significantly decreased the apoptosis rate compared with the model group (P<0.01). In addition, we observed a dose dependent effect among QLQX groups (Fig. 2a). Meanwhile, we
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investigated apoptosis-related protein expressions in the infarct border area. Our results showed that, compared with the sham group, the Bcl-2 expression was remarkably down-regulated ($P < 0.01$), whereas the expressions of Bax, cytochrome c, Apaf-1, cleaved-caspase9 and cleaved-caspase3 were significantly up-regulated in the model group ($P < 0.01$). However, QLQX and benazepril reversed the trends of the above indicators ($P < 0.01$) (Fig. 2b). We further detected the protein expressions of AKT, p-AKT, GSK3β and p-GSK3β to investigate the PI3K/AKT/GSK3β signaling pathway involved in the anti-apoptosis effect of QLQX in rats with CHF. Our results demonstrated that, compared with the sham group, the ratios of p-AKT/AKT and p-GSK3β/GSK3β were significantly decreased in the model group ($P<0.01$). However, QLQX and benazepril treatment reversed the trend of the ratios ($P<0.05$) (Fig. 2c). Taken together, the results suggested that QLQX inhibited myocardial apoptosis through the PI3K/AKT/GSK3β pathway in HF post-AMI rats.

**Effects of QLQX on Cell Viability in H2O2-Induced H9c2 Cardiomyocytes.** After H9c2 cell treatment with H2O2 at 60–100μM for 24h, the results showed that, compared with the normal group, H2O2 impaired H9c2 cell viability in a dose-dependent state ($P <0.01$). In particular, compared with the normal group, cell viability exposed to H2O2 at 80μM was reduced to 70.44%±3.81% ($P<0.01$). Thus, 80μM was determined to be the appropriate concentration of H2O2 for subsequent experiments (Fig. 3a). Similarly, after H9c2 cell treatment with QLQX at 25–400μg/ml for 24h, the results showed that there was no significant toxic effect on H9c2 cells from QLQX of different concentrations (25-200μg/ml) compared with the control group ($p > 0.05$) (Fig. 3b). In addition, H9c2 cardiomyocytes were pretreated with QLQX at 30–150μg/ml for 4h and then co-incubated with 80μM H2O2 for 24h. Compared with the H2O2 group, cell viability increased with QLQX at 90μg/ml ($P <0.01$). Therefore QLQX at 90μg/ml was used for further experiments (Fig. 3c).

**Inhibition of QLQX on Oxidative Stress Injury.** in H9c2 Cells Induced by H2O2. We investigated the effects of QLQX on LDH release level, ROS level, SOD activity, HO-1 and CAT mRNA expressions in H9c2 cardiomyocytes impaired with H2O2. Compared to the normal group, our results showed that LDH release level and ROS level were significantly elevated, but SOD activity, HO-1 and CAT mRNA expressions were inhibited in the H2O2 group ($P<0.01$). However, these trends were reversed after QLQX treatment ($P<0.01$). Meanwhile, we did not observe any effects of QLQX on the cells without the stress of H2O2 in the levels of LDH release, ROS expression, SOD activity, HO-1 and catalase mRNA expressions. Therefore, we could presume that QLQX do not disturb the normal physiological function of cells. According to the results above, QLQX prevented oxidative stress injury in H9c2 cells (Fig. 3d-3h).

**QLQX Inhibits H2O2-Induced Apoptosis in H9c2 Cells.** To investigate the effects of QLQX on H2O2-induced apoptosis in H9c2 cardiomyocytes, the apoptosis rate was determined using flow cytometry. Our results showed that, compared to the normal group, the apoptosis rate significantly increased after H2O2 exposure ($P<0.01$). However, compared to the H2O2 group, QLQX treatment decreased the apoptosis rate in H9c2 cells induced by H2O2 ($P<0.01$) (Fig. 4a). To further investigate the role of QLQX in inhibiting cellular apoptosis, apoptosis-related protein expressions were detected using western blot analysis. The results demonstrated that, compared to the normal group, H2O2 exposure significantly
down-regulated Bcl-2 expression, but up-regulated expressions of Bax, cleaved-caspase9, and cleaved-caspase3 in H9c2 cells ($P<0.01$). However, QLQX treatment reversed these trends of the above proteins ($P<0.01$) (Fig. 4b). These results suggested that QLQX treatment could inhibit H2O2-induced apoptosis in H9c2 cardiomyocytes.

**Involvement of PI3K/AKT/GSK3β Signaling Pathway in QLQX Prevented H2O2-Induced Mitochondria-Dependent Apoptosis in H9c2 Cardiomyocytes.** To determine the regulatory mechanisms of QLQX involved in mitochondria-dependent apoptosis in H9c2 cardiomyocytes, the PI3K inhibitor, LY294002, was added to cultures 0.5h before QLQX and H2O2 treatments. Then we investigated the effects of QLQX on the apoptosis rate, expressions of apoptosis-related protein expressions, mitochondrial fission, MPTP opening, and MMP decline in H9c2 cells induced by H2O2.

**Inhibition of QLQX on Mitochondrial Apoptosis in H2O2-Induced H9c2 Cells via the PI3K/AKT/GSK3β Pathway.** We first investigated the effects of QLQX on mitochondrial apoptosis in H9c2 cells induced by H2O2 via the PI3K/AKT/GSK3β pathway. The results showed that the apoptosis rate, compared with the normal group, was significantly elevated in the H2O2 group ($P<0.01$). Compared to the H2O2 group, QLQX noticeably decreased the apoptosis rate in H9c2 cells impaired by H2O2 ($P<0.01$). However, LY294002 treatment partly reversed the effect of QLQX on H2O2-induced apoptosis in H9c2 cells ($P<0.01$). It is interesting to note that no remarkable difference was observed in the apoptosis rate between the H2O2+ LY294002 group and the H2O2 group ($P>0.05$) (Fig. 5a).

Similar trends were found in the detection of apoptosis-related protein expressions using western blot analysis. Compared to the normal group, after H2O2 treatment, Bcl-2 expression was significantly down-regulated ($P<0.01$), while the expression levels of Bax, cytochrome c, Apaf-1, cleaved-caspase9, and cleaved-caspase3 were significantly up-regulated in H9c2 cells ($P<0.01$). However, QLQX treatment reversed the trends of these apoptosis-related protein expressions ($P<0.01$). Interestingly, LY294002 treatment partly abolished the anti-apoptosis effects of QLQX in H2O2-treated H9c2 cells ($P<0.05$) (Fig. 5b).

We further detected the protein expressions of AKT, p-AKT, GSK3β, and p-GSK3β to investigate the PI3K/AKT/GSK3β signaling pathway involved in the effects of QLQX in H2O2-induced H9c2 cells. Our results showed no remarkable changes in the expressions of AKT and GSK3β, while the expressions of p-AKT and p-GSK3β changed significantly. Compared to the normal group, the ratios of p-AKT/AKT and p-GSK3β/GSK3β appreciably decreased in the H2O2 group ($P<0.01$). However, QLQX treatment reversed the trends of the above indicators ($P<0.01$). Furthermore, LY294002 partially blocked the influence of QLQX on the ratios of p-AKT/AKT and p-GSK3β/GSK3β in H9c2 cells exposed to H2O2 ($P<0.01$). Meanwhile, we observed no significant difference in the ratios of p-AKT/AKT and p-GSK3β/GSK3β between the H2O2+LY294002 group and the H2O2 group (Fig. 5c). Taken together, these results suggested that QLQX inhibited mitochondrial-dependent apoptosis in H9c2 cardiomyocytes induced by H2O2 via PI3K/AKT/GSK3β pathway.

**The Effect of QLQX on Mitochondrial Fission in H2O2-Induced H9c2 Cells through the PI3K/AKT/GSK3β Pathway.** The cells were stained with the Mito-Tracker Red CMXRos, and the mitochondrial morphology was evaluated under ZEISS confocal microscopy. Our results showed that, compared with the normal group, more obvious
fragmented mitochondria were observed in H_2O_2-treated H9c2 cells. However, QLQX alleviated mitochondrial fission in H9c2 cells following oxidative stress injury, but this effect was reversed in cells pretreated with LY294002 (Fig. 6a). Moreover, protein expressions of Drp1 and p-Drp1 (Ser637) were further detected using western blot analysis. Similarly, compared to the normal control, the ratio of p-Drp1 (Ser637)/Drp1 was markedly decreased in H9c2 cells exposed to H_2O_2 (P<0.01). QLQX treatment markedly increased the ratio in H9c2 cells induced by H_2O_2 (P<0.01). However, LY294002 partly blocked the effect of QLQX, and there was no significant difference in the ratio between the H_2O_2+LY294002 group and the H_2O_2 group (Fig. 6b). Therefore, our results suggested that QLQX attenuated H_2O_2-induced mitochondrial fission in H9c2 cardiomyocytes via the PI3K/AKT/GSK3β pathway.

**Effects of QLQX on MPTP Opening and MMP in H9c2 Cells Induced by H_2O_2 via the PI3K/AKT/GSK3β Pathway.** To determine the effect of QLQX on the MPTP opening in H9c2 cells exposed to H_2O_2, cells were treated with the BB cell ProbeTM M61 probe and quencher, and fluorescence intensity was detected with flow cytometry. Our results showed that, compared to the normal group, the relative fluorescence intensity of the BB cell ProbeTM M61 probe in mitochondria significantly declined (P<0.01). Furthermore, QLQX noticeably elevated fluorescence intensity in H_2O_2-induced H9c2 cells (P<0.01), but the effect of QLQX was reversed in H_2O_2-induced cells pretreated with LY294002 (P<0.01).

Meanwhile, there was no statistical difference in fluorescence intensity between the LY294002+ H_2O_2 group and the H_2O_2 group (P>0.05) (Fig. 6c). To further investigate the effect of QLQX on MMP in H_2O_2-induced H9c2 cardiomyocytes, the cells were stained with JC-1, and fluorescence intensity was detected using a fluorescent plate reader. Compared to the normal group, MMP significantly declined after H_2O_2 exposure (P<0.01). QLQX treatment significantly elevated MMP in the H_2O_2-treated H9c2 cells (P<0.01), whereas the effect of QLQX was partly eliminated with LY294002 treatment (P<0.05). Interestingly, there were no significant changes in MMP levels between the LY294002 group + H_2O_2 and the H_2O_2 group (P>0.05) (Fig. 6d). Taken together, these results suggested that QLQX inhibited MPTP opening, and MMP decline in H9c2 cardiomyocytes was impaired by H_2O_2 through the PI3K/AKT/GSK3β pathway.

**DISCUSSION**

QLQX consists of 11 herbs, including ginseng, astragalus, aconite, semen lepidii, salvia, safflower, alisma orientale, cassia twig, polygonatum, cortex periplocae, and tangerine peel, which represents a traditional Chinese formula widely used to treat CHF in China. Previous study demonstrated that QLQX was effective in inhibiting apoptosis and cardiac remodeling via activation of the Neuregulin-1/Akt signaling pathway and suppression of p53 pathway in rats with heart failure post AMI 16). Another study indicated that QLQX protected against cardiac dysfunction and ventricular remodeling by attenuating apoptosis and cardiac fibrosis via up-regulation of Akt phosphorylation in heart failure rats post AMI19). Recent studies also
showed that QLQX could decrease myocardial apoptosis and fibrosis via activation of PPARγ in male mice and bilateral ovariectomized female mice post AMI. However, few studies have been conducted to explore the role of QLQX in mitochondrion-dependent apoptosis induced by oxidative stress in cardiomyocytes. In this study, we demonstrated for the first time that QL protected cardiomyocytes from oxidative stress-induced mitochondrion-dependent apoptosis in vivo and in vitro, which was associated with the PI3K/AKT/GSK3β signaling pathway.

In myocardium failure, the imbalance of ROS and antioxidant system resulting from ischemia or hypoxia causes oxidative stress damage and triggers mitochondria-dependent apoptosis, which is an important mechanism to cause or aggravate CHF. ROS is a remarkable biomarker of oxidative stress, as “the redox messenger,” which plays a vital role in intracellular signaling and regulation. Additionally, LDH, SOD, HO-1 and CAT are adequate biomarkers to evaluate oxidative stress injury and antioxidant effects. Previous studies have shown that oxidative stress injury causes up-regulation of LDH level and a large accumulation of ROS against SOD activity in cardiomyocytes. Other studies have also indicated that luteolin and shengmai injection have antioxidant effects on the oxidative stress injury by up-regulating the HO-1 or CAT mRNA expression level in rat cardiomyocytes. These are consistent with the results of the present study. In vivo, QLQX decreased myocardial LDH release and relative expression of ROS but increased myocardial SOD activity in rats with CHF post-AMI. The effect of QLQX was, to some extent, in a dose-dependent manner. In vitro, QLQX promoted a proliferation of H9c2 cardiomyocytes after oxidative stress injury and improved the above indicators to attenuate oxidative stress. In addition, QLQX had antioxidant effects by up-regulating the HO-1 and CAT mRNA relative expressions. The results suggested that QLQX exerted a protective effect on oxidative stress injury in cardiomyocytes, which provided a theoretical basis for QLQX in treating CHF.

Oxidative stress is closely related to activation of the mitochondrion-dependent apoptosis pathway in chronic heart failure (CHF). It has been shown that inhibiting oxidative stress damage and apoptosis could improve cardiac function in rats with CHF. The imbalance between pro-apoptosis proteins (Bax, cytochrome c, cleaved caspase-9, and cleaved caspase-3) and the anti-apoptosis protein (Bcl-2) is a major determinant in the mitochondrial apoptosis pathway in cardiomyocytes. Over-expression of Bax would transfer from the cytoplasm into mitochondria to disrupt the membrane permeability, delivering excessive cytochrome c from the mitochondria into the cytoplasm. Cytochrome c, in combination with Apaf-1, forms apoptotic bodies which activate caspase-9 and consequently activate the downstream caspases-3, resulting in mitochondrial-mediated apoptosis in cardiomyocytes. Recent studies indicated that α-Bisabolol abrogated myocardial infarction induced by isoproterenol, attenuated dysfunction of myocardial mitochondria, and inhibited mitochondrion-mediated apoptosis in rats by up-regulating the expression of Bcl-2 and down-regulating the expressions of Bax, cytochrome c, Apaf-1, active caspase-9, and active caspase-3. Another previous study showed that rhamnetin ameliorated oxidative stress-induced H9c2 cardiomyocyte apoptosis by regulating the expressions of Bcl-2, Bax, and cleaved-caspase. Our in vivo and in vitro results both showed that QLQX inhibited the apoptosis rate of
cardiomyocytes post-oxidative stress injury, up-regulated Bcl-2 expression, and down-regulated the expressions of Bax, cytochrome c, Apaf-1, cleaved-caspase9, and cleaved-caspase3. Additionally, QLQX improved cardiac function in rats with HF post-AMI. These data indicate that QLQX could inhibit oxidative stress-induced myocardial apoptosis, thereby improving cardiac function in CHF. These findings are consistent with previous studies.

We further investigated the mechanism by which QLQX inhibits oxidative stress-induced myocardial apoptosis. The PI3K/AKT signaling pathway plays an important role in cellular apoptosis and survival. Previous research has shown that activating the PI3K/AKT signaling pathway inhibits doxorubicin-induced myocardial apoptosis and improves cardiac function in mice. Another study has demonstrated that activation of PI3K/AKT signaling antagonizes oxidative stress injury and inflammatory responses in rats with diabetic cardiomyopathy, inhibiting myocardial apoptosis and improving myocardial structure. GSK3β, a major downstream substrate of the PI3K/AKT signaling pathway, could be phosphorylated by activating PI3K/AKT signaling and inactivating GSK3β, thereby mediating apoptosis. Like previous studies, our results showed that compared with the control group, QLQX more often activated the PI3K/AKT signaling and up-regulated p-GSK3β in vivo and in vitro. Meanwhile, in vitro LY294002, a PI3K inhibitor, partially reversed the effects of QLQX on the apoptosis rate, apoptosis-related protein expression, and the signaling receptors in H9c2 cardiomyocytes induced by H2O2. These results suggest that QLQX partly inhibits oxidative stress-induced myocardial apoptosis via the PI3K/AKT/GSK3β signaling pathway.

Mitochondria are dynamic and continually undergo fission and fusion to maintain their function. DRP1, a dynamic GTPase, would over-activate and migrate from the cytoplasm into mitochondria after an oxidative stress injury, which aggravates mitochondrial fission, leading to cytochrome c release and caspase activation. Meanwhile, previous studies have shown that p-Drp1 can block the migration of Drp1 into mitochondria, thereby inhibiting mitochondrial fission and apoptosis. Additionally, oxidative stress would damage mitochondria and trigger MPTP opening and MMP decline, causing mitochondrial-dependent apoptosis. One study has shown that down-regulation of Drp1 by inactivation of the GSK3β receptor could inhibit mitochondrial fission. Previous studies have also demonstrated that activation of the PI3K/AKT signaling pathway prompts phosphorylation of GSK3β and inhibits mitochondrial MPTP opening induced by bupivacaine, attenuating the MMP decline induced by oxidative stress and hypoxia, and thereby inhibiting apoptosis. Therefore, mitochondrial fission, MPTP opening, and MMP decline could mediate initial apoptosis via the PI3K/AKT/GSK3β signaling pathway. Consistently with previous studies, the present study showed that QLQX could inhibit mitochondrial fission, MPTP opening, and MMP decline, but its effects could be partly reversed by LY294002. Hence, the results suggest that QLQX partly attenuates early mitochondrial-related apoptosis in H9c2 cardiomyocytes induced by H2O2 via the PI3K/AKT/ GSK3β signaling pathway.

In conclusion, the study clearly reveals that QLQX significantly attenuates mitochondria-dependent apoptosis in cardiomyocytes induced by oxidative stress. In vivo,
our results show that QLQX could attenuate oxidative stress injury and inhibit mitochondria-dependent myocardial apoptosis, thereby improving cardiac function in rats with HF post-AMI. In vitro, the present study demonstrates that QLQX protected H9c2 cardiomyocytes against H$_2$O$_2$-induced mitochondria-related apoptosis. Meanwhile, our results suggest that the PI3K/AKT/GSK3β signaling pathway could represent a new target pathway for QLQX to protect cardiomyocyte apoptosis after oxidative stress, both in vivo and in vitro. These findings may provide new insights to further understand the molecular mechanism of QLQX and alternative strategies for HF post-AMI treatment. Meanwhile, we will further investigate the underlying mechanism of antioxidant activities and the therapeutic benefits of QLQX.

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Conflicts of Interest The authors declare no conflict of interest.
References


Figure 1: QLQX improved cardiac function and attenuated oxidative stress injury in rats with HF post-AMI. (a) Representative images of echocardiography in all groups. (b) Echocardiography results showed that QLQX improved EF and FS but decreased LVEDd and LVESd. (c) QLQX down-regulated the LDH release level and the ROS relative expression level, but up-regulated the SOD inhibition rate, suggesting that QLQX could attenuate oxidative stress injury in rats with HF post-AMI. Data are expressed as mean ± SD. Compared with Sham, ** P<0.01; compared with Model, ## P<0.01, # P<0.05; compared with Benazepril, ▲▲ P < 0.01. n = 10 per group.

(Color figure can be accessed in the online version.)
Figure 2: QLQX inhibited cardiomyocyte apoptosis via the PI3K/AKT/GSK3β signaling pathway in HF post-AMI rats. (a) TUNEL results showed that QLQX attenuated the apoptosis rate of cardiomyocytes in rats with HF post-AMI. (b) Effects of QLQX on the
expressions of apoptosis-related proteins. Western blot analysis showed that QLQX increased Bcl-2 expression but decreased the expressions of Bax, cytochrome c, Apaf-1, cleaved-caspase9 and cleaved-caspase3 in rats with HF post-AMI. (c) Western blot analysis results showed that QLQX up-regulated the ratios of p-AKT/AKT and p-GSK3β/GSK3β in rats with HF post-AMI. Data are expressed as mean ± SD. Compared with Sham, ** P < 0.01; compared with Model, ‖‖ P < 0.01, ‖ P < 0.05; compared with Benazepril, ▲▲ P < 0.01, ▲ P < 0.05. n = 3 per group.

(Color figure can be accessed in the online version.)
Figure 3: Effects of QLQX on cell viability and oxidative stress injury in H9c2 cardiomyocytes induced by H2O2. (a) Cell viability of H9c2 cardiomyocytes exposed to H2O2 at different concentrations for 24 h. (n=6 per group) (b) Cell viability of H9c2 cardiomyocytes treated with QLQX at different concentrations for 24 h. (n=6 per group) (c) The result of cell viability showed that QLQX (90μg/ml) promoted proliferation of H9c2 cardiomyocytes exposed to H2O2. (n=6 per group) (d) QLQX down-regulated the LDH release level in H2O2-induced H9c2 cardiomyocytes. (n=6 per group) (e) QLQX decreased the ROS level in H2O2-induced H9c2 cardiomyocytes. (n=6 per group) (f) QLQX enhanced the SOD activity in H2O2-induced H9c2 cardiomyocytes. (n=6 per group) (g) QLQX up-regulated the HO-1 mRNA relative expression in H9c2 cardiomyocytes induced by H2O2. (n=3 per group) (h) QLQX up-regulated the CAT mRNA relative expression in H9c2 cardiomyocytes induced by H2O2. (n=3 per group) Data are expressed as mean ± SD. Compared with the normal group, ** P<0.01, * P<0.05; compared with the H2O2 group, ## P<0.01.

(Color figure can be accessed in the online version.)
Figure 4: QLQX inhibited H2O2-induced apoptosis in H9c2 cardiomyocytes. (a) The effect of QLQX on the apoptosis rate of H9c2 cardiomyocytes induced by H2O2. (b) Effects of QLQX on the protein expressions of Bcl-2, Bax, cleaved-caspase9, and cleaved-caspase3 in H9c2 cardiomyocytes induced by H2O2. Data are expressed as mean ± SD. Compared with the normal group, ** P<0.01; compared with the H2O2 group, ### P<0.01. n = 3 per group. (Color figure can be accessed in the online version.)
Figure 5: QLQX inhibited H$_2$O$_2$-induced mitochondrion-dependent apoptosis in H9c2 cardiomyocytes via the PI3K/AKT/GSK3β pathway. (a) The effect of QLQX on the apoptosis rate of H9c2 cardiomyocytes induced by H$_2$O$_2$. (b) Effects of QLQX on the expressions of apoptosis-related proteins in H9c2 cardiomyocytes induced by H$_2$O$_2$. (c) Effects of QLQX on the ratios of p-AKT/AKT and p-GSK3β/GSK3β. Data are expressed as mean ± SD. Compared with the normal group, ** $P<0.01$; compared with the H$_2$O$_2$ group, ## $P<0.01$; compared with the H$_2$O$_2$+ QLQX group, ▲ $P < 0.05$, ▲▲ $P < 0.01$. n = 3 per group.

(Color figure can be accessed in the online version.)
Figure 6: QLQX inhibited H$_2$O$_2$-induced mitochondrial fission, MPTP opening, and MMP decline in H9c2 cardiomyocytes via the PI3K/AKT/GSK3β signaling pathway. (a) Mitochondrial morphology was observed with LCSM. Scale bar, 20μm. (b) The effect of QLQX on the p-Drp1/Drp1 ratio. (c) The effect of QLQX on H$_2$O$_2$-induced MPTP opening in H9c2 cardiomyocytes. (d) The effect of QLQX on H$_2$O$_2$-induced MMP decline in H9c2 cardiomyocytes. Data are expressed as mean ± SD. Compared with the normal group, ** $P$<0.01; compared with the H$_2$O$_2$ group, ### $P$<0.01; compared with the H$_2$O$_2$+QLQX group, ▲ $P$<0.05, ▲▲ $P$<0.01. n = 3 per group.

(Color figure can be accessed in the online version.)