Effects of Shenfu Qiangxin Drink on H_2O_2-induced oxidative stress, inflammation and apoptosis in neonatal rat cardiomyocytes and possible underlying mechanisms

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Abstract. The aim of the present study was to investigate the effects of Shenfu Qiangxin Drink (SFQXD) on acute myocardial infarction (AMI) and identify the possible underlying mechanisms. Levels of reactive oxygen species (ROS) and inflammatory factors, including interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) in the blood samples of patients with AMI were measured using commercially available kits by visible spectrophotometry after SFQXD administration. The contents of phosphorylated (p-) forkhead box O3a (FOXO3a) was examined using an ELISA kit. In addition, a hydrogen peroxide (H_2O_2)-induced myocardial injury model was established *in vitro* using neonatal rat cardiomyocytes. Following treatment with SFQXD, the levels of intracellular ROS, cell apoptosis, oxidative stress- and inflammation-related markers were measured using commercially available kits by visible spectrophotometry. Additionally, western blot analysis was used to measure the expression of sirtuin-4 (SIRT4), p-FOXO3a, acetylated FOXO3a (ace-FOXO3a) and apoptosis-related genes (Bcl-2, Bax, BIM and cleaved caspase-3). Subsequently, to investigate the possible underlying regulatory mechanisms, SIRT4 expression was silenced by transfection with small hairpin RNA against SIRT4, following which changes in the extent of oxidative stress, inflammation and apoptosis were assessed. The levels of ROS and interleukin (IL)-1β were found to be significantly reduced, whilst FOXO3a phosphorylation was markedly increased following administration with SFQXD. *In vitro*, SFQXD dose-dependently inhibited H_2O_2-induced oxidative stress, inflammation and apoptosis in neonatal rat cardiomyocytes. In addition, FOXO3a phosphorylation was markedly upregulated whilst FOXO3a acetylation was downregulated following treatment of H_2O_2-induced primary neonatal cardiomyocytes with SFQXD. SIRT4 knockdown also markedly reversed the effects of SFQXD on oxidative stress, inflammation and apoptosis in neonatal rat cardiomyocytes. In conclusion, these findings demonstrated that SFQXD may alleviate oxidative stress-induced myocardial injury by potentially regulating SIRT4/FOXO3a signaling, suggesting that SFQXD may be of clinical value for the treatment of AMI.

Introduction

Acute myocardial infarction (AMI) is caused by the sudden occlusion of coronary blood flow and is a major cause of morbidity and mortality in developed countries (1,2). AMI leads to >4 million deaths in North Asia and Europe, and >1/3 of all deaths in developed countries every year (3,4). It poses a major threat to human health (5). To date, significant advances have been made in developing efficient treatment strategies, including thrombolysis and direct coronary intervention (6,7). However, myocardial injury as a result of ischemia followed by restoration of blood flow remains to be a major cause of cardiovascular disease and contributes to mortality associated with cardiovascular events (8). Therefore, identification and development of novel and effective therapeutic methods for the treatment of myocardial injury remain to be in demand.

Myocardial ischemia-reperfusion (I/R) injury is an inherent response to the recovery of blood flow following ischemia during myocardial infarction (MI) (9). This is a complex process that involves numerous mechanisms, the most well studied of which is that mediated by reactive oxygen species (ROS) (10). Excessive production of ROS leads to oxidative stress, which in turn mediates the pathological process of almost all cardiovascular diseases, including myocardial injury post-MI (3,4). Hydrogen peroxide (H_2O_2) is an exogenous form of ROS that is produced as a by-product of I/R and

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a direct free radical donor during oxidative stress injury (11). H_2O_2-induced myocardial cell injury has been applied widely for the investigation of AMI *in vitro* (12,13). A growing body of evidence has demonstrated that the overload of ROS, such as H_2O_2, during oxidative stress may impair cell viability and trigger myocardial cell apoptosis by inducing DNA, protein and lipid damage (14,15). In addition, tt has been previously documented that cardiomyocyte cell death is a prominent pathological change post-MI, which results in irreversible cardiac dysfunction (16).

Forkhead box O3a (FOXO3a) is a member of the O subclass of the Forkhead family of transcription factors that has previously been investigated as a key protein involved in the regulation of oxidative stress, inflammation and apoptosis (17,18). Accumulating evidence has demonstrated that FOXO3a is closely associated with the pathogenesis of MI (19,20). It was previously documented that the activity of FOXO3a may be regulated by the Sirtuin (SIRT) family of proteins. In particular, *trans*-sodium crocetinate, a derivative compound of the carotenoid crocetin, has been reported to attenuate myocardial I/R injury through SIRT3/FOXO3a signaling (21). SIRT4 is a deacetylase that is normally localized to the mitochondrial matrix and is highly expressed in cardiomyocytes (22). SIRT4 has been previously found to alleviate oxidative stress and apoptotic damage during myocardial I/R (23).

Traditional Chinese Medicine (TCM) has long been used for the treatment of a number of diseases. Shenfu Qiangxin Drink (SFQXD) was created by adding and subtracting ingredients based on the Shenfu Qiangxin Decoction as described in Good Remedies For Women, which has been reported to effectively relieve the clinical symptoms of patients with heart failure (24,25). SFQXD is mainly composed of a mixture of seven herbal Chinese medicine, including *Codonopsis codonopsis* (30 g), *Aconitum carmichaeli Debx* (4 g), *Ophiopogon japonicus* (10 g), *Schisandra fructus* (10 g), *Polygonatum odoratum* (20 g), *Semen lepidii* (20 g), *Semen plantaginis* (20 g) and *Radix paeoniae rubra* (15 g) in specific proportions (25). To date, SFQXD has been used to treat a variety of cardiac diseases, including AMI and chronic heart failure (26). In addition, Shenfu injection has been developed based on this recipe and has become an important procedure for AMI complicated by cardiac shock (27). However, the effect of SFQXD on SIRT4/FOXO3a signaling for the treatment of MI remains to be fully elucidated.

In the present study, the levels of ROS and inflammatory factors were investigated in patients with MI before and after the administration of SFQXD. In addition, an *in vitro* model of H_2O_2-induced myocardial injury was established in neonatal rat cardiomyocytes to explore the effects of SFQXD on myocardial damage and possible underlying regulatory mechanism. The ultimate aim was to elucidate the mechanisms underlying the effect of SFQXD on myocardial injury during MI.

Materials and methods

Clinical sample collection. The present study involved 30 patients with acute non-ST segment elevation MI who fulfilled the diagnostic criteria for patients with AMI. The inclusion criteria were as follows: i) Electrocardiogram with characteristic alterations including the emergence of Q, the spread of ST segment elevation and the dynamic evolution of ST-T; ii) elevated serum biomarkers for myocardial necrosis; myocardial necrosis detected by serum biomarkers; and iii) an intracoronary thrombus identified by angiography (28). The following exclusion criteria applied: i) Previous history of myocardial infarction; ii) having received percutaneous coronary intervention treatment; iii) having acute heart failure upon admission; iv) having myocardial disease, infectious pericarditis or pericardial disease; v) having an infectious disease, severe diabetes mellitus, malignant tumor, liver or kidney disease, pulmonary fibrosis, bone metabolic disorder, systemic immune disease or complications caused by malignant tumors; and vi) having cardiac shock. The patients (male, 17; female, 13; mean age, 45.8±10.2 years) were recruited from The Jiangsu Provincial Hospital of Integrated Chinese and Western Medicine (Nanjing) between February 2019 and October 2019. Compositions of SFQXD (Beijing Tongrentang Pharma Co., Ltd.) were mixed and boiled in ddH_2O twice, with the first boiling processing (100˚C) lasting for 1.5 h and the second lasting for 30 min, before being finally concentrated using ddH_2O into a decoction at a concentration of 0.43 g/ml.

Blood samples (5 ml of each patient) were obtained from the patients prior to any treatment. Subsequently, the patients received SFQXD administration (300 ml) every day for 2 weeks. SFQXD was consistently prepared in the preparation room of Jiangsu Provincial Hospital of Integrated Chinese and Western Medicine. Blood samples (5 ml of each patient) were obtained from the patients after 2 weeks of treatment. Samples were immediately frozen in liquid nitrogen at -80˚C for storage. The protocol of the present study was approved by the Ethics Committee of Jiangsu Provincial Hospital of Integrated Traditional Chinese and Western Medicine (approval no. 2018LW012; Nanjing, China). All study participants were informed on the purpose of the study and provided written informed consent.

Primary cultures of cardiomyocytes. A total of 10 Sprague-Dawley (SD) rats aged 1-3 days (weight, 8-10 g; 5 males and 5 females) were provided by the Model Animal Research Center of Nanjing University (Nanjing, China; license number: 20181221-63). All animal procedures were performed according to the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health (29) and experimental protocols were approved by the Jiangsu Provincial Hospital of Integrated Chinese and Western Medicine (Nanjing, China). Neonatal rat cardiomyocytes were prepared and cultured as described previously (30,31). Briefly, neonatal SD rats were euthanized using carbon dioxide (CO_2) with the flow rate displacing 20% of the chamber volume/min. Rats were exposed to 50% CO_2 until they were euthanized, which was subsequently confirmed by decapitation. Subsequently, hearts of the neonatal rats were removed and placed in pre-cooled (4˚C) D-Hanks' Balanced Salt Solution (Sigma-Aldrich; Merck KGaA) under sterile conditions. The ventricles were first excised and cut into small pieces, which were then digested three times with 0.08% trypsin solution for 8 min at 37˚C each. After centrifugation at 1,200 x g at 4˚C for 6 min, the supernatants were discarded. The pellet was then re-suspended and cultured in

DMEM/F12 (1:1; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) with 5-Bromo-2'-deoxyuridine (0.1 mM; Sigma-Aldrich, Merck KGaA) to prevent fibroblast proliferation in 60-mm culture dishes. The cells were cultured at 37˚C in a 95% O_2 and 5% CO_2 incubator and the medium was replaced daily. After 72-96 h, rhythmic contractions could be observed and cells were deemed ready for subsequent experiments.

Experimental groups and treatments. Cardiomyocytes were treated with a series of H_2O_2 concentrations (25, 50, 100, 200, 400 and 600 μM; Sigma-Aldrich, Merck KGaA) to determine the optimal dose of H_2O_2 for mimicking oxidative stress-induced injury in cardiomyocytes. Compositions in SFQXD were concentrated into the decoction to a concentration of 0.43 g/ml as the aforementioned. For SFQXD treatment, SFQXD was diluted using DMEM/F12 (1:1). Cardiomyocytes were pretreated with 25, 50, 100, 200, 400 and 800 μl/ml SFQXD for 12 h at 37˚C and then incubated in medium containing 100 μM H_2O_2 for another 2 h at 37˚C. Cells in the control group (con) were cultured in complete DMEM/F12 (1:1) for 14 h at 37˚C. Doses of 25, 50 and 100 μl/ml SFQXD were selected for subsequent experimentation, which were designated as model + low dose (L), model + medium dose (M) and model + high dose (H) groups, respectively.

Cell viability assay. Cell Counting Kit-8 (CCK-8) kit (Sigma-Aldrich; Merck KGaA) was performed to measure cell viability. The cells were plated into 96-well plates (3,000 cells/100 μl). After treatment with H_2O_2 and/or SFQXD (mentioned in the previous section) for 14 h at 37˚C, 10 μl CCK-8 solution was added to each well and the cells were incubated for an additional 4 h at 37˚C. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Test for inflammatory factors and phosphorylated (p-)-FOXO3a. Levels of inflammatory cytokines, specifically interleukin (IL)-6 (cat. no. F01310), IL-1β (cat. no. F01220) and tumor necrosis factor-α (TNF-α; cat. no. F02810) in serum from patients and culture media of the cultured myocytes ($3x10^5$ cells/well) were measured using ELISA in accordance with the manufacturer's protocols (Shanghai Xitang Biotechnology Co., Ltd.; http://westang.bioon.com.cn/). The levels of p-FOXO3a (cat. no. JL49691-96T) in the serum samples of patients before and after SFQXD treatment was determined using an ELISA kit obtained from Shanghai Jianglai Industrial Co., Ltd. according to the manufacturer's protocol.

Detection of intracellular ROS. Generation of intracellular ROS was examined using 2,7-dichlorofluorescein diacetate (DCFH-DA) assay. Primary cardiomyocytes ($1x10^6$/well) were first collected and washed with PBS three times, followed by incubation in DMEM containing 10 μM DCFH-DA (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 20 min in a dark chamber. After centrifugation at 800 x g at 4˚C for 5 min, fluorescence was measured using a flow cytometer (BD Biosciences; Becton, Dickinson and Company) at 488 nm excitation and 525 nm emission wavelength. The data analysis was performed using BD CellQuest™ Pro Software (version 5.1; BD Biosciences; Becton, Dickinson and Company). The quadrant(s) from Q3 reflected the ROS levels.

Measurement of oxidative stress markers. Primary cardiomyocytes were seeded in 6-well plates at a density of $5x10^5$ cells/well l and treated as mentioned above. The culture media or cell lysate supernatants were first collected. Levels of oxidative stress-related markers, namely malondialdehyde (MDA; cat. no. A003-4-1), catalase (CAT; cat. no. A007-1-1) and total-superoxide dismutase (T-SOD; cat. no. A001-1-2), were assessed using their respective commercial kits (Nanjing Jiancheng Bioengineering Institute), according to colorimetric methods.

Cell transfection. Small hairpin RNA (shRNA) specific against SIRT4 (50 nM; shRNA-SIRT4-1, shRNA-SIRT4-2 and shRNA-SIRT4-3) and a non-targeting sequence serving as a negative control (shRNA-NC) were synthesized by Guangzhou RiboBio Co., Ltd. For transfection, primary neonatal rat cardiomyocytes were placed in the wells of six-well plates at $2x10^5$ cells/well and cultured at 37˚C until reaching 70% confluence. Lipofectamine® 200 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized to perform the transfection experiments. At 48 h post-transfection, transfection efficacy was assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Subsequently, transfected cells were treated with H_2O_2 and/or SFQXD mentioned in the previous section.

Flow cytometry analysis. Cardiomyocytes ($1x10^6$/well) were harvested and double-stained with Annexin V-FITC (5 μl) and propidium iodide (PI; 10 μl). The mixture was placed in the dark for 15 min at room temperature. Cardiomyocytes were then subjected to apoptosis assay (Nanjing KeyGen Biotech Co., Ltd.) using flow cytometry (BD Biosciences; Becton, Dickinson and Company) and analyzed using the CellQuest™ Pro Software (version 5.1; BD Biosciences; Becton, Dickinson and Company).

RT-qPCR analysis. After treatment, total RNA was extracted from primary neonatal rat cardiomyocytes using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Complementary DNA (cDNA) was synthesized using PrimeScript™ RT reagent (Takara Bio, Inc.; 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min). iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) was employed to conduct qPCR according to the manufacturer's protocol in the ABI 7500 PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: Initial denaturation at 95˚C for 7 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec; and a final extension at 72˚C for 30 sec. Sequences of the gene-specific primers used in this study were as follows: SIRT4 forward, 5'-ACCCTGAGAAGGTCAAAG AGTTAC-3' and reverse, 5'-TTCCCCACAATCCAAGCAC-3' and GAPDH forward, 5'-ACCACAGTCCATGAA ATCAC-3' and reverse, 5'-AGGTTTCTCCAGGCGGCATG-3'. All primers used in the present study were synthesized by Sangon Biotech Co., Ltd. GAPDH served as the endogenous

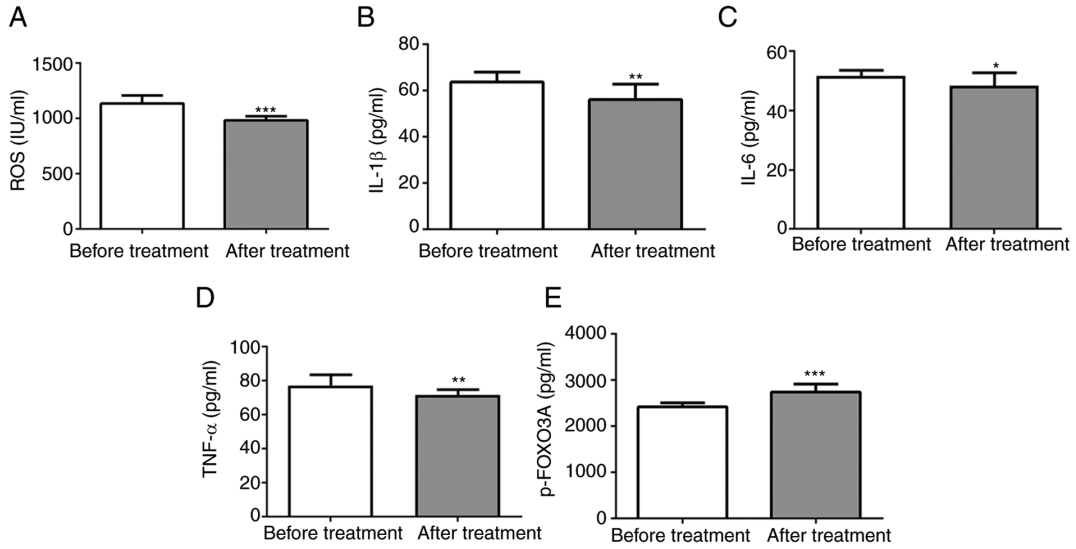

Figure 1. SFQXD treatment significantly reduces the levels of ROS, inflammatory cytokines and FOXO3a phosphorylation in blood samples of patients with acute myocardial infarction. The levels of (A) ROS, (B) IL-1β (C) IL-6, (D) TNF-α and (E) phosphorylated FOXO3a were measured using the corresponding commercially available kits. Experimental results were generated from three independent experimental repeats. *P<0.05, **P<0.01 and ***P<0.001 vs. Before treatment. SFQXD, Shenfu Qiangxin Drink; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL, interleukin; FOXO3a, forkhead box O3a; p-, phosphorylated.

control. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (32).

Western blot analysis. Total proteins were extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) in the presence of a protease inhibitor cocktail (Beyotime Institute of Biotechnology). Bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) was used to measure protein concentration. Subsequently, 40 μg protein per lane was separated via 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were then blocked with 5% non-fat milk for 1.5 h at room temperature and incubated with primary antibodies against the target proteins at 4˚C overnight. Following incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074S; 1:5,000; Cell Signaling Technology, Inc.) for 1.5 h at room temperature, the bands were visualized using an enhanced chemiluminescence assay (EMD Millipore) and analyzed using ImageJ software (version 1.52r; National Institutes of Health). Anti-Bcl-2 (1:1,000; cat. no. sc-7382), anti-Bax (1:1,000; cat. no. sc-7480), anti-Bcl-2-like protein 11 (BIM; 1:1,000; cat. no. sc-374358) and anti-SIRT4 (1:1,000; cat. no. sc-135797) were purchased from Santa Cruz Biotechnology, Inc. Anti-cleaved caspase-3 (1:1,000; cat. no. 9664T), anti-FOXO3a (1:1,000; cat. no. 12829S), anti-p-FOXO3a (1:1,000; cat. no. 5538S), anti-acetyl lysine (1:1,000; cat. no. 9441S) and anti-β-actin (1:1,000; cat. no. 4970S) antibodies were obtained from Cell Signaling Technology, Inc. β-actin was considered as the internal control.

Statistical analysis. All experiments were repeated independently in triplicate. All data were represented as mean values ± SD and statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc.). Comparisons between two groups in the clinical sample analysis was evaluated using paired student's t-test. Comparisons involving two groups and multiple samples in the cell experiments were analyzed by unpaired t-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, respectively. P<0.05 was considered to indicate a significantly different difference.

Results

SFQXD treatment reduces the levels of ROS, IL-1β and p-FOXO3a in the blood of patients with AMI. To investigate the effects of SFQXD in AMI, the levels of ROS, IL-6 and TNF-α and IL-1β in the blood samples from patients were measured before and after SFQXD administration. SFQXD notably reduced ROS, IL-1β, IL-6 and TNF-α levels (Fig. 1A-D). Furthermore, a significant increase in the level of phosphorylated FOXO3a was observed in the SFQXD after treatment group (Fig. 1E).

SFQXD ameliorates oxidative stress and inflammation in H_2O_2-treated neonatal rat cardiomyocytes. To study the mechanism of action of SFQXD in the treatment of AMI, neonatal rat cardiomyocytes were isolated (Fig. 2A) and a H_2O_2-induced primary cardiomyocyte damage model was established *in vitro*. Viability of cardiomyocytes was reduced after stimulation with H_2O_2 in a dose-dependent manner (Fig. 2B). At 100 μM H_2O_2, cell viability was reduced by ~50% (Fig. 2B). Therefore, this concentration was selected for subsequent experiments. By contrast, SFQXD at a concentration of ≥200 μl/ml exerted significant inhibitory effects on the viability of primary cardiomyocytes (Fig. 2C). Therefore, 25, 50 and 100 μl/ml SFQXD were selected for the following experiments.

The potential effects of SFQXD on H_2O_2-induced oxidative stress was then tested. Generation of ROS was markedly increased after H_2O_2 induction, which was reversed with SFQXD treatment in a dose-dependent manner (Fig. 3A).

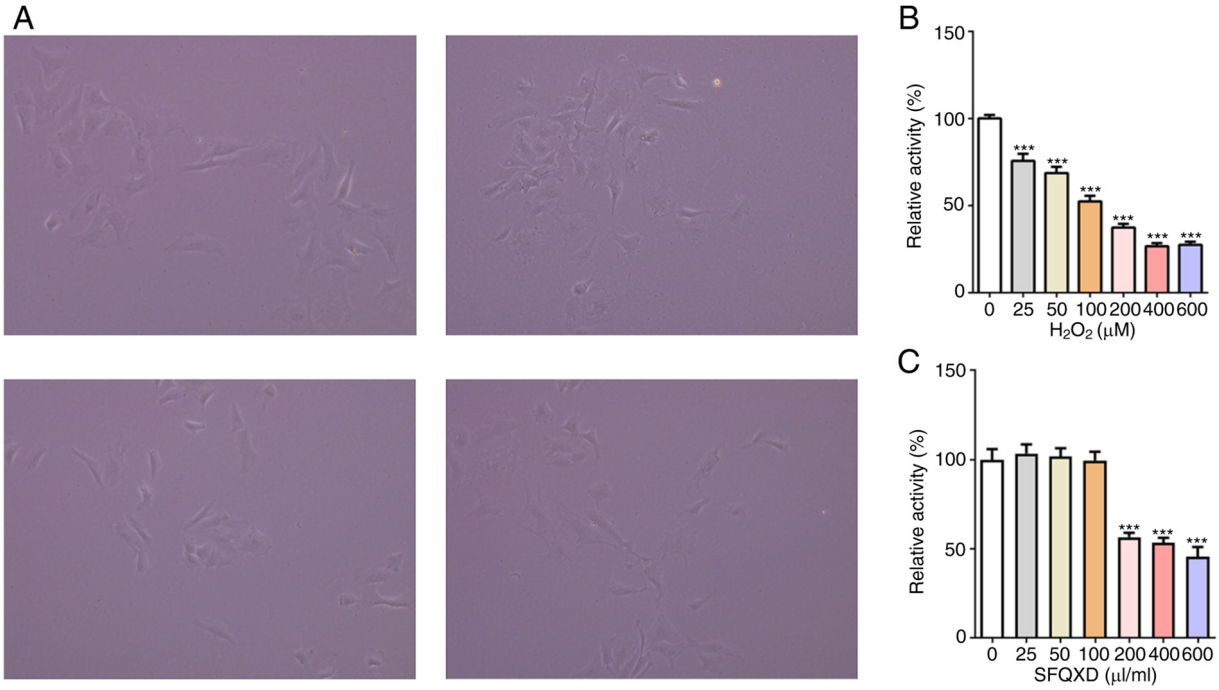

Figure 2. Establishment of the H_2O_2-induced primary cardiomyocyte injury model *in vitro*. (A) Cell morphology of neonatal rat cardiomyocytes. Magnification, x200. (B) Cell viability was tested using CCK-8 assay after treatment with different concentrations of H_2O_2. (C) Cell viability was examined using CCK-8 assay after treatment with a series of SFQXD doses. Results were generated from three independent experimental repeats. ***P<0.001 vs. 0. SFQXD, Shenfu Qiangxin Drink; CCK-8, Cell Counting Kit-8; H_2O_2, hydrogen peroxide.

Consistently, SFQXD pre-treatment reduced the concentration of MDA, whilst significantly increasing the activities of T-SOD and CAT in a dose-dependent manner in H_2O_2-induced primary cardiomyocytes compared with those in the model group (Fig. 3B-D). Concurrently, medium and high dose SFQXD pre-treatment group exhibited significantly lower levels of TNF-α, IL-1β and IL-6 compared with those in the model group (Fig. 3E-G). In summary, these data suggest that SFQXD was able to ameliorate oxidative stress and inflammation induced by H_2O_2 in neonatal rat cardiomyocytes.

SFQXD alleviates apoptosis in H_2O_2-treated neonatal rat cardiomyocytes. Subsequently, cell apoptosis was measured using flow cytometry analysis. The number of apoptotic cells was markedly increased in the model group compared with that the control group, whilst SFQXD pre-treatment markedly prevented this in a dose-dependent manner (Fig. 4A and B). Compared with those in the control group, the expression levels of Bcl-2 was significantly downregulated, whilst those of Bax, BIM and cleaved caspase-3 were significantly upregulated after the primary cardiomyocytes were treated with H_2O_2 (Fig. 4C). There effects aforementioned were significantly prevented by all three doses of SFQXD pre-treatment (Fig. 4C). These observations suggest that SFQXD suppressed H_2O_2-induced apoptosis in primary cardiomyocytes.

SFQXD attenuates oxidative stress and inflammation in H_2O_2-treated neonatal rat cardiomyocytes via regulation of SIRT4/FOXO3a signaling. To investigate the potential regulatory mechanisms by which SFQXD exerts its effects on H_2O_2-treated neonatal rat cardiomyocytes, the levels of SIRT4 expression, FOXO3a phosphorylation and acetylation were measured using western blot analysis. H_2O_2 treatment significantly downregulated the levels of SIRT4 and p-FOXO3a, but significantly upregulated in ace-FOXO3a levels compared with those in the control group (Fig. 5). After the primary cardiomyocytes were pretreated with SFQXD, reductions in the levels of SIRT4 expression and FOXO3a phosphorylation and increments in FOXO3a acetylation induced by H_2O_2 were significantly prevented (Fig. 5).

Subsequently, SIRT4 expression was silenced by transfection with shRNA-SIRT4. Cardiomyocytes that were transfected with shRNA-SIRT4-1 were selected for the following experiments, since they exhibited the lowest expression levels of SIRT4 compared with those transfected with shRNA-NC (Fig. 6A). Among all H_2O_2- and SFQXD-treated cells, it was subsequently observed that SIRT4 silencing markedly enhanced the levels of intracellular ROS and MDA (Fig. 6B and C), whilst significantly reducing the activities of T-SOD and CAT (Fig. 6D and E), compared with those in the model + H + shRNA-NC group. Similarly, the levels of the inflammatory factors TNF-α, IL-1β and IL-6 exhibited similar trends with ROS, all of which were significantly increased after SIRT4 knockdown compared with those after shRNA-NC transfection (Fig. 6F-H). The aforementioned findings suggest that SFQXD was able to alleviate oxidative stress and inflammation in H_2O_2-treated neonatal rat cardiomyocytes through regulation of SIRT4/FOXO3a signaling.

SFQXD inhibits apoptosis in H_2O_2-treated neonatal rat cardiomyocytes by regulating SIRT4/FOXO3a signaling. Next, cell apoptosis was examined by flow cytometry analysis after SIRT4 knockdown in H_2O_2-treated primary cardiomyocytes. After H_2O_2 and SFQXD treatment, the apoptotic ratio in

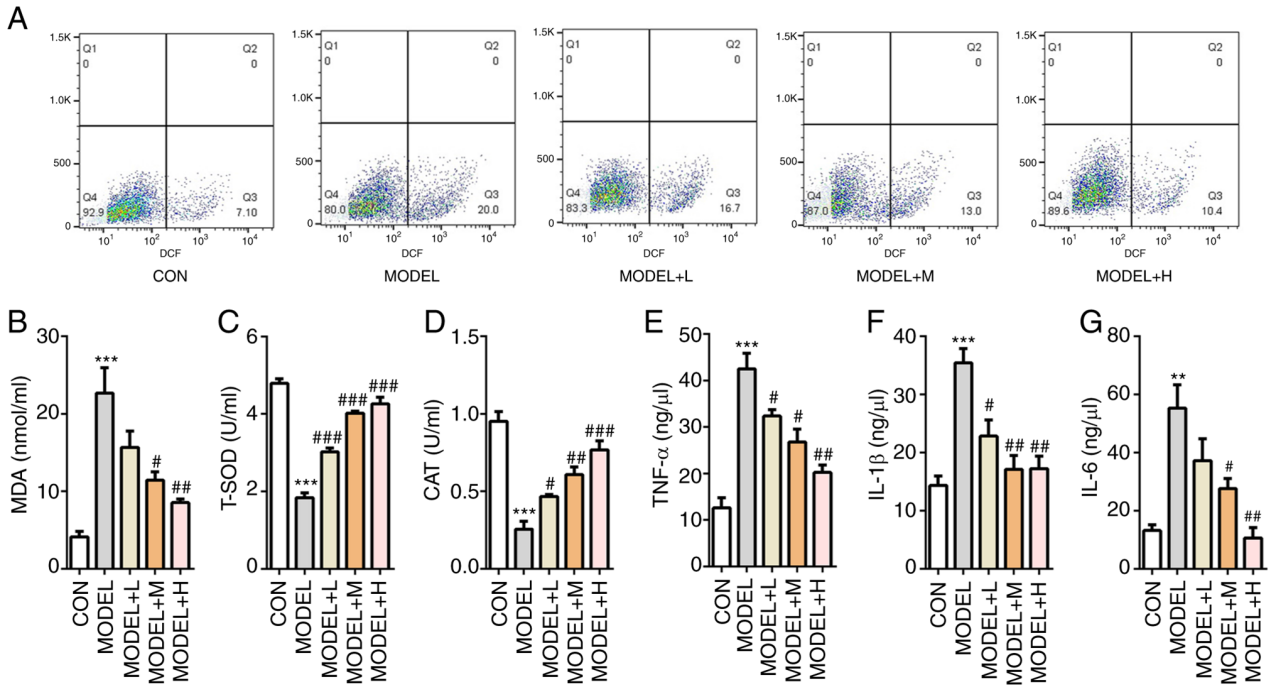

Figure 3. SFQXD ameliorates H_2O_2-induced oxidative stress and inflammation in primary cardiomyocytes. (A) Generation of intracellular ROS was tested using 2,7-dichlorofluorescein diacetate staining followed by flow cytometry. The levels of (B) MDA and activities of (C) T-SOD and (D) CAT were measured using commercially available kits by visible spectrophotometry. Concentrations of (E) TNF-α, (F) IL-1β and (G) IL-6 were determined using ELISA. The experimental results were generated from three experimental independent repeats. **P<0.01 and ***P<0.001 vs. CON; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MODEL. SFQXD, Shenfu Qiangxin Drink; MDA, malondialdehyde; T-SOD, total superoxide dismutase; CAT, catalase; ROS, reactive oxygen species; TNF, tumor necrosis factor; IL, interleukin; TNF, tumor necrosis factor; CON, control; L, low dose; M, medium dose; H, high dose.

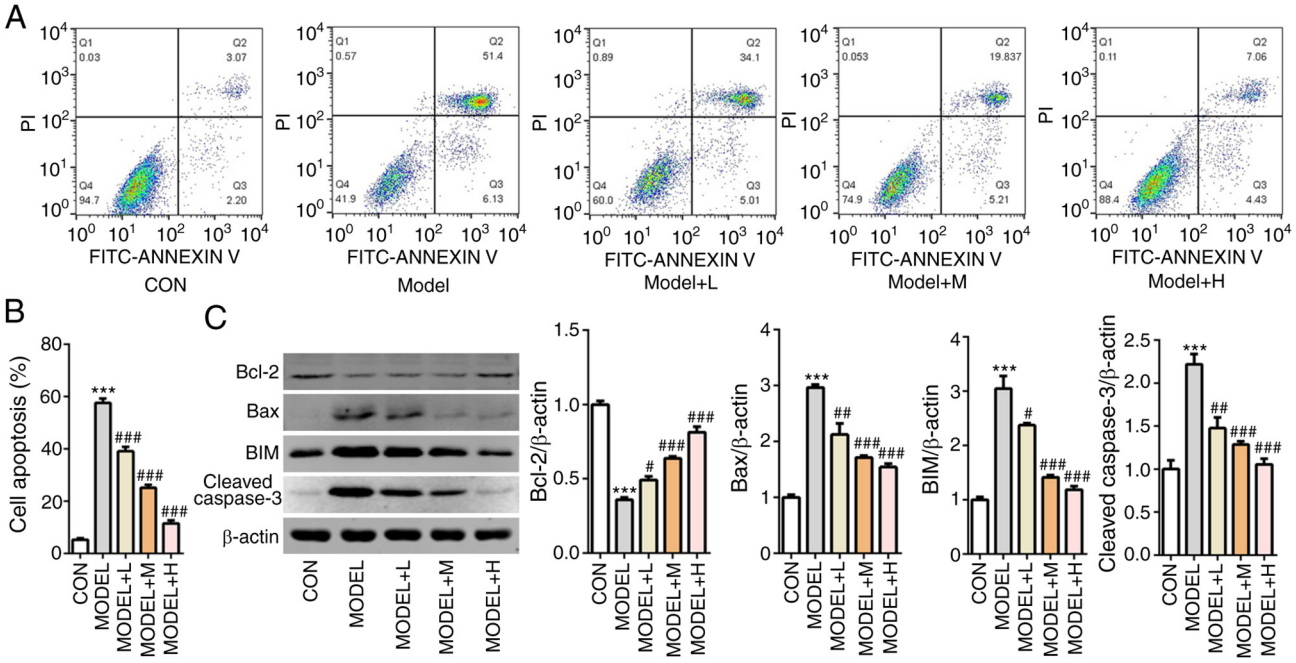

Figure 4. Shenfu Qiangxin Drink alleviates apoptosis in H_2O_2-treated neonatal rat cardiomyocytes. (A) Cell apoptosis was measured using flow cytometry. (B) Quantification of apoptotic rate. (C) Representative images of western blot analysis, which was used to quantify the expression levels of Bcl-2, Bax, BIM and cleaved caspase-3. The experimental results were generated from three independent experimental repeats. ***P<0.001 vs. CON; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MODEL. BIM, Bcl-2-like protein 11; CON, control; L, low dose; M, medium dose; H, high dose.

the SIRT4-knockdown group was significantly increased compared with that in the model + H + shRNA-NC group (Fig. 7A and B). Additionally, silencing of SIRT4 combined with SFQXD treatment in H_2O_2-stimulated primary cardiomyocytes significantly inhibited the expression of Bcl-2 whilst significantly promoting the expression of Bax, BIM and cleaved caspase-3 compared with those in the shRNA-NC group (Fig. 7C). The levels of SIRT4 expression and FOXO3a phosphorylation were

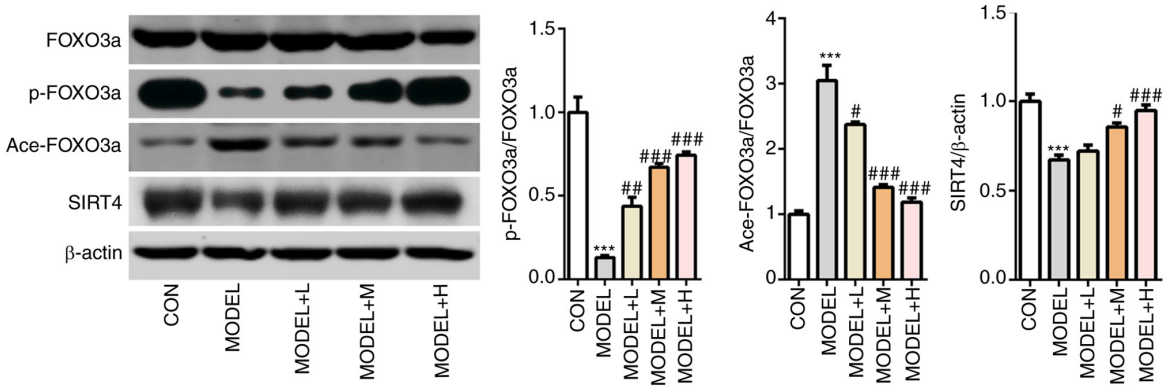

Figure 5. Shenfu Qiangxin Drink can regulate SIRT4/FOXO3a signaling. Expression levels of SIRT4, FOXO3a phosphorylation and FOXO3a acetylation were measured using western blot analysis. Experimental results were obtained from three independent experimental repeats. ***P<0.001 vs. CON; #P<0.05, ##P<0.01, ###P<0.001 vs. MODEL. SIRT4, sirtuin-4; FOXO3a, Forkhead box O3; p-, phosphorylated; ace-, acetylated; CON, control; L, low dose; M, medium dose; H, high dose.

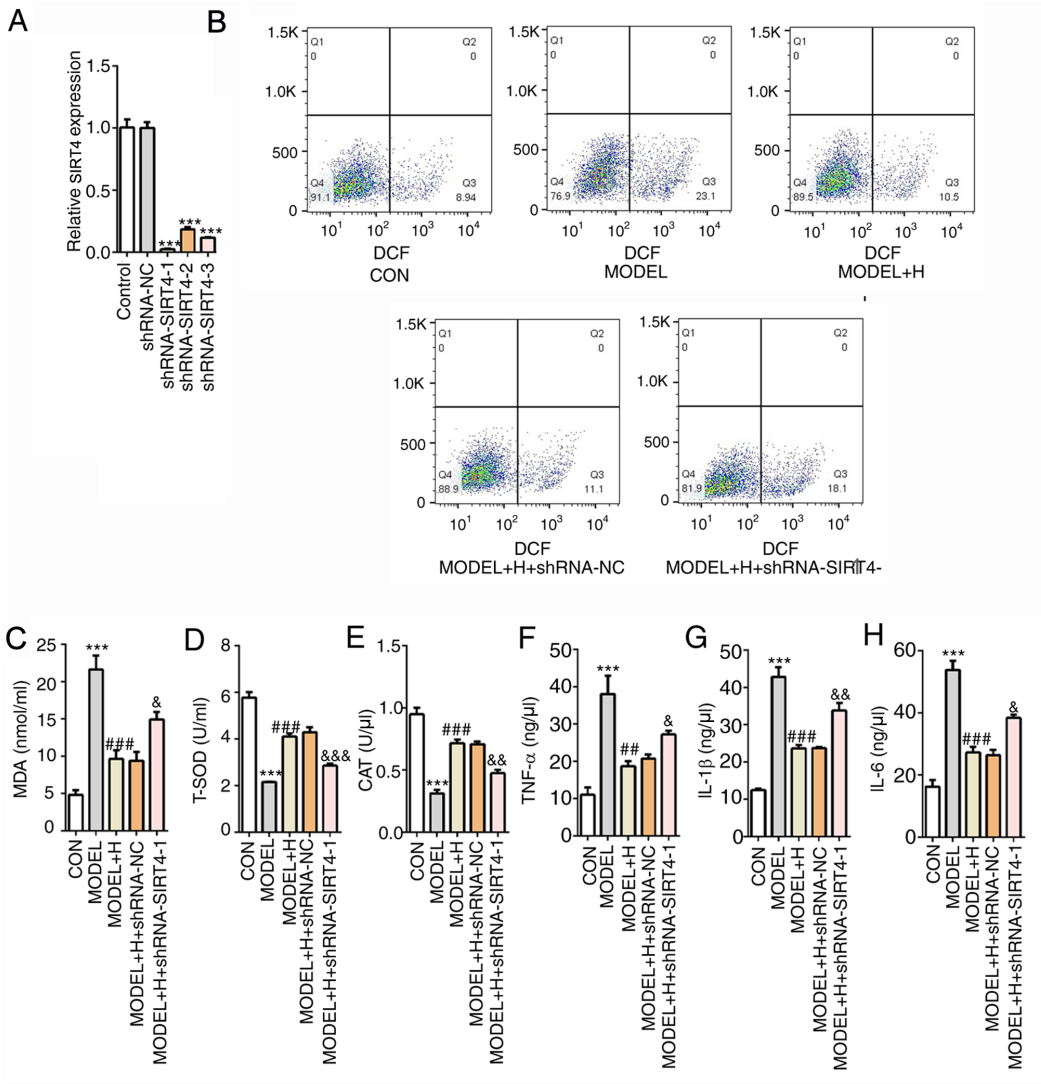

Figure 6. Shenfu Qiangxin Drink reduces H_2O_2-induced oxidative stress and inflammation in neonatal rat cardiomyocytes by regulating SIRT4/FOXO3a signaling. (A) Expression of SIRT4 was measured using reverse transcription-quantitative PCR after transfection with shRNA-SIRT4. ***P<0.001 vs. shRNA-NC. (B) Generation of intracellular reactive oxygen species was tested by staining with 2,7-dichlorofluorescein diacetate follow by flow cytometry. The levels of (C) MDA and activities of (D) T-SOD and (E) CAT were determined using commercially available kits by visible spectrophotometry, respectively. Concentrations of (F) TNF-α, (G) IL-1β and (H) IL-6 were determined using ELISA. The experimental results were generated from three independent experimental repeats. ***P<0.001 vs. CON; ##P<0.01 and ###P<0.001 vs. MODEL; &P<0.05, &&P<0.01 and &&&P<0.001 vs. MODEL + H + shRNA-NC. SIRT4, sirtuin-4; FOXO3a, Forkhead box O3; shRNA, short hairpin RNA; IL, interleukin; TNF, tumor necrosis factor; MDA, malondialdehyde; T-SOD, total superoxide dismutase; CAT, catalase; CON, control; H, high dose; NC, negative control.

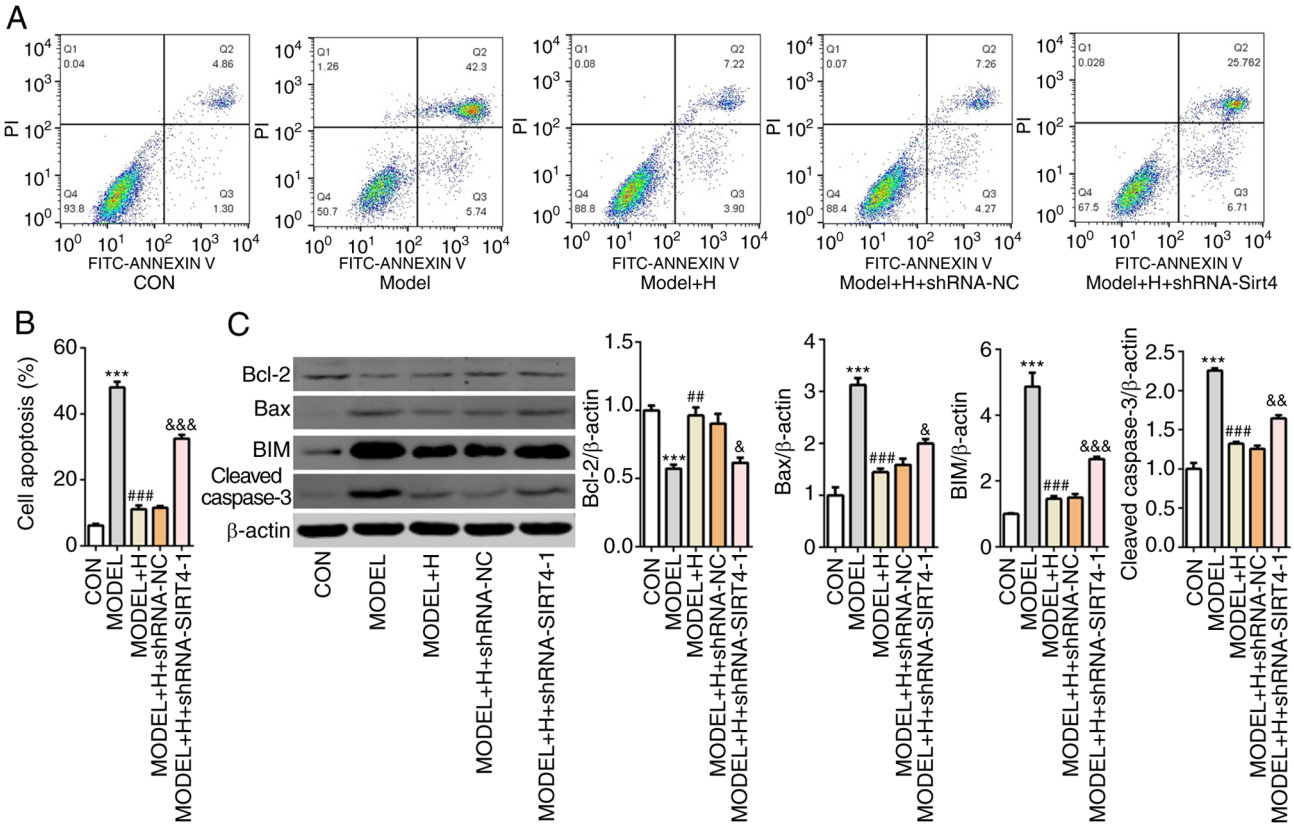

Figure 7. Shenfu Qiangxin Drink inhibits apoptosis in H_2O_2-treated neonatal rat cardiomyocytes by regulating SIRT4/FOXO3a signaling. (A) Cell apoptosis was assessed using flow cytometry. (B) Quantification of apoptotic rate. (C) Western blot analysis was used to measure the expression of Bcl-2, Bax, BIM and cleaved caspase-3. Experimental results were obtained from three independent experimental repeats. ***P<0.001 vs. CON; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MODEL; $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. MODEL + H + shRNA-NC. SIRT4, sirtuin-4; FOXO3a, Forkhead box O3; shRNA, short hairpin RNA; BIM, Bcl-2-like protein 11; CON, control; H, high dose; NC, negative control.

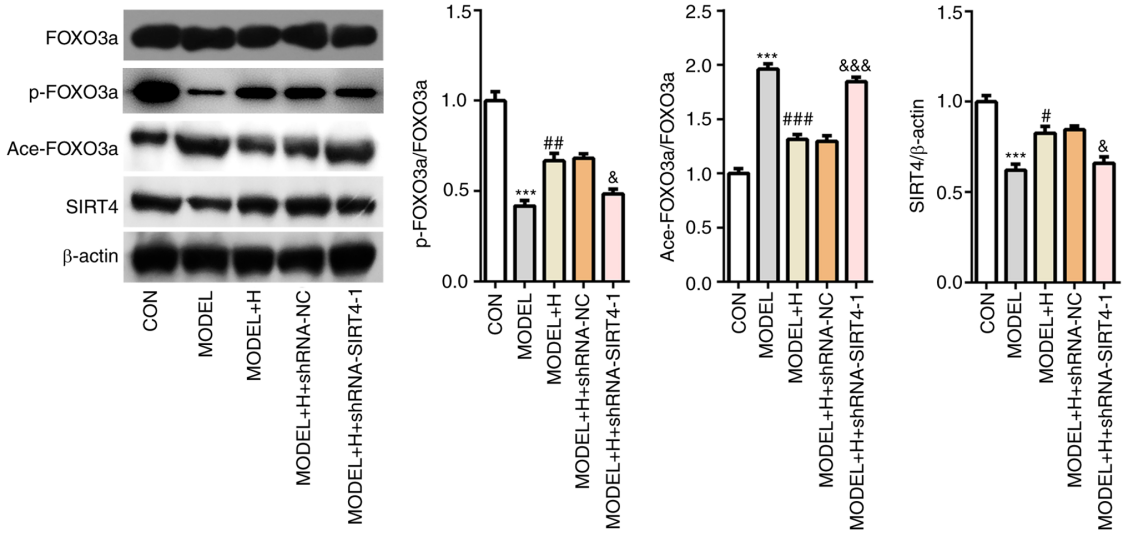

Figure 8. SIRT4 silencing reverses the effects of Shenfu Qiangxin Drink on SIRT4/FOXO3a signaling in H_2O_2-treated neonatal rat cardiomyocytes. The expression of SIRT4, FOXO3a phosphorylation and FOXO3a acetylation was detected using western blot analysis. The experimental results were generated from three independent experimental repeats. ***P<0.001 vs. CON; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MODEL; $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 vs. MODEL + H + shRNA-NC. SIRT4, sirtuin-4; FOXO3a, Forkhead box O3; shRNA, short hairpin RNA; BIM, Bcl-2-like protein 11; CON, control; H, high dose; NC, negative control.

subsequently found to be significantly decreased whilst FOXO3a acetylation was significantly increased in the SIRT4-knockdown group compared with those transfected with shRNA-NC (Fig. 8). Collectively, these results suggest that SFQXD attenuates apoptosis of H_2O_2-treated primary cardiomyocytes by regulating SIRT4/FOXO3a signaling.

Discussion

Cardiovascular diseases, particularly AMI, have become the leading cause of death worldwide (33). At present, TCM has been attracting increasing attention due to its clinical application potential for the treatment of various diseases, such as AMI. A previous study demonstrated that the Baoyuan decoction can improve oxidative stress-induced myocardial cell apoptosis in heart failure post-AMI (34). In addition, Qili qiangxin has been demonstrated to suppress the apoptosis of rat cardiomyocytes following MI (35). In the present study, it was demonstrated that SFQXD can alleviate oxidative stress-induced myocardial damage, potentially through regulation of the SIRT4/FOXO3a signaling pathway. This provides a theoretical basis and partly elucidates the underlying mechanism supporting the clinical application of SFQXD for the treatment of AMI.

Oxidative stress results from the excessive production of ROS, including O_2, ·OH and H_2O_2, which is implicated in the pathogenesis of myocardial damage post-AMI (36). In particular, MDA is the end-product of lipid peroxidation and serves a key role in the process of oxidative stress. By contrast, T-SOD and CAT are vital antioxidant enzymes that act against oxidative stress by removing free ROS (37). In addition, excessive oxidative stress can induce the overproduction of inflammatory cytokines, including TNF-α, IL-6 and IL-1β (38). TNF-α has been reported to serve a key role in inducing the production of free radicals, which in turn further damages the myocardium (39). IL-6 and IL-1β are pivotal regulatory markers that participate in the post-MI inflammatory response (40). The present study revealed that SFQXD alleviated the generation of ROS, MDA, TNF-α, IL-6 and IL-1β, whilst enhancing the activities of T-SOD and CAT in a dose-dependent manner, which was in agreement with a previous finding which reported that SFQXD could effectively improve the heart function of rats with heart failure (25). These results indicated the potential protective effects of SFQXD against MI.

Accumulated evidence has demonstrated that the imbalance between ROS production and clearance leads to myocardial cell apoptosis by activating caspase-3 and subsequently the mitochondria-dependent pathway (41,42). Caspases are a family of cysteine-aspartic proteases serve a key pro-apoptotic role (43). The Bcl-2 protein family serves as a crucial regulator of this apoptotic pathway (44). Among these proteins, Bax is a typical pro-apoptotic factor that can trigger apoptosis by inducing the release of mitochondrial apoptosis-inducing factors, such as cytochrome *c*, into the cytoplasm under stress conditions, which further results in the activation of the caspase cascade and cell apoptosis (45). Of note, the Shenfu Qiangxin decoction has been previously shown to improve the cardiac function of rats with heart failure (25). The present study revealed that SFQXD dose-dependently inhibited the apoptosis of H_2O_2-treated primary cardiomyocytes.

The serine 253 residue of FOXO3a can be phosphorylated, which subsequently results in its inactivation and translocation into the cytoplasm, leading to the inhibition of its transcriptional activity (46). Acetylation of FOXO3a interrupts its nuclear translocation to suppress its transactivation further (46). The activity of FOXO3a can be regulated by the proteins of the Sirtuin family (47,48). It has been reported that SIRT6 protects cardiomyocytes from I/R injury by augmenting FOXO3a-dependent antioxidant defense mechanisms (49). SIRT7 overexpression can reduce oxidative stress, prevent inflammatory injury and apoptosis of cardiomyocytes in a mouse model of cardiomyopathy (50). In addition, results from a previous study also supported the notion that SIRT4 can alleviate oxidative stress and apoptosis damage following myocardial I/R (23). In another study, SIRT4 was reported to ameliorate myocardial I/R injury by regulating mitochondria function and apoptosis (22). The present study revealed that FOXO3a phosphorylation was significantly lower whilst FOXO3a acetylation was notably enhanced in primary cardiomyocytes after H_2O_2 treatment, suggesting that the transcriptional activity of FOXO3a was inhibited. Additionally, the expression of SIRT4 was markedly reduced following H_2O_2 stimulation. Changes in SIRT4 expression and post-translational modification of FOXO3 were blocked by SFQXD treatment, suggested that the transcriptional activity of FOXO3a was protected. SIRT4 expression was subsequently silenced to investigate the regulatory mechanism between SIRT4 and FOXO3a. The results indicated that the inhibitory effects of SFQXD on H_2O_2-induced neonatal rat cardiomyocytes were reversed following SIRT4 silencing. Taken together, these findings provide evidence that SFQXD alleviates H_2O_2-induced oxidative stress, inflammation and apoptosis in neonatal rat cardiomyocytes by regulating SIRT4/FOXO3a signaling.

In summary, to the best of our knowledge, the present study was the first to investigate the mechanistic role of SFQXD in H_2O_2-induced myocardial damage. It was concluded that SFQXD was able to ameliorate H_2O_2-induced oxidative stress, inflammation and apoptosis in neonatal rat cardiomyocytes by regulating SIRT4/FOXO3a signaling. However, whether this herbal mixture can modify other signal pathways during myocardial damage remain a topic that require further study. In addition, the lack of *in vivo* animal experiments should be investigated in any future investigations, which serve as limitations of the present study. The findings of the present study may provide experimental data to support the clinical application of SFQXD in restoring myocardial function post-MI in the future.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SZ, YZ and XW searched the literature, designed the experiments and conducted the experiments. LW, JS, MG and ZF

analyzed and interpreted the data. MG and ZF wrote the manuscript. ZF revised the manuscript. SZ and ZF confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Jiangsu Province Hospital of Integrated Traditional Chinese and Western Medicine (Nanjing, China). Written informed consent was obtained from each patient or their legal guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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