Original Research

Leonurine pretreatment protects the heart from myocardial ischemia-reperfusion injury

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Impact Statement

We aimed to investigate the effect of Leonurine on myocardial I/R injury *in vitro* and *in vivo*, respectively, as well as to decipher the mechanism involved. Leonurine reduced the release of infarction-related enzymes and restored cardiac functions, as indicated by the hemodynamic and echocardiography measurements, the findings suggesting that Leonurine could significantly reduce infarct size, maintaining cardiomyocyte morphology, and significantly reduce cardiomyocytes apoptosis. Moreover, Leonurine could exert a cardioprotective effect by reducing reactive oxygen species (ROS) level and restoring Bcl-2 expression and Akt phosphorylation while reducing the expression of Bax and the phosphorylation of p38 and JNK. In addition, Leonurine-induced the expression of p-Akt Bcl-2 and Bax were blocked by PI3K inhibitor LY294002, and the total protein level of Akt was not affected by Leonurine pretreatment. Our experimental results indicated Leonurine could have a cardioprotective effect on I/R injury, which confirmed the utility of Leonurine preconditioning as a strategy to prevent I/R injury.

Abstract

Myocardial ischemia-reperfusion (I/R), an important complication of reperfusion therapy for myocardial infarction, is characterized by hyperactive oxidative stress and inflammatory response. Leonurine (4-guanidino-n-butyl syringate, SCM-198), an alkaloid extracted from *Herbaleonuri*, was previously found to be highly cardioprotective both *in vitro* and *in vivo*. Our current study aimed to investigate the effect of SCM-198 preconditioning on myocardial I/R injury *in vitro* and *in vivo*, respectively, as well as to decipher the mechanism involved. Rats were pretreated with SCM-198 before subjected to 45 min of myocardial ischemia, which was followed by 24 h of reperfusion. Primary neonatal rat cardiac ventricular myocytes (NRCMs) were exposed to hypoxia (95% N_2 + 5% CO_2) for 12 h, and then to 12 h reoxygenation so as to mimic I/R. The enzymatic measurements demonstrated that SCM-198 reduced the release of infarction-related enzymes, and the hemodynamic and echocardiography measurements showed that SCM-198 restored cardiac functions, which suggested that SCM-198 could significantly reduce infarct size, maintaining cardiomyocyte morphology, and that SCM-198 pretreatment could significantly reduce cardiomyocytes apoptosis. Moreover, we demonstrated that SCM-198 could exert a cardioprotective effect by reducing reactive oxygen species (ROS) level and Akt phosphorylation while reducing the phosphorylation of p38 and JNK. In addition, the upregulation of p-Akt, Bcl-2/Bax induced by SCM-198 treatment were blocked by PI3K inhibitor LY294002, and the total protein level of Akt was not affected by SCM-198 pretreatment. Our experimental results indicated that SCM-198 could have a cardioprotective effect on I/R injury, which confirmed the utility of SCM-198 preconditioning as a strategy to prevent I/R injury.

Keywords: Leonurine, ischemia-reperfusion injury, cardioprotective, apoptosis, anti-oxidation

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Introduction

Globally, cardiovascular disease (CVD) still remains a huge health problem and exerts a social and economic burden, despite considerable improvement in its treatment. At present, CVD have taken an estimated 17.9 million lives annually calculated from the World Health Organization (WHO).[1,2] Coronary heart disease, one of the leading causes of morbidity and mortality worldwide, resulting in more than seven million deaths each year, is becoming a pyramidally significant problem in the developing countries.[1,3] Myocardial ischemia-reperfusion (I/R), an important complication of reperfusion therapy for myocardial infarction, is characterized by hyperactive oxidative stress and inflammatory response. Currently, there is no clinically effective therapy for I/R injury, and the conventional pharmacologic agents cannot reverse the damage to the ischemic-reperfused myocardium. Thus, it is of great clinical significance to develop

cardioprotective agents to limit the extent of infarction in the ischemic tissues induced by I/R injury.

Leonurine (4-guanidino-n-butyl syringate), also named SCM-198, is a unique single compound extracted from *Herba Leonuri* (Chinese motherwort), which is widely used in China to treat menoxenia, abnormal vaginal bleeding, and other gynecological disorders.[4,5] Studies have recently reported that SCM-198 improves the symptoms of myocardial infarction (MI),[6] including blood hyperviscosity, coronary flow, and microcirculation,[7] and alleviates cerebral ischemia.[8] SCM-198 has also been reported to be capable of improving the antioxidative capacity of myocardium,[9,10] promoting angiogenesis in ischemic myocardium, and ameliorating endothelial dysfunction caused by hyperlipidemia.[11] Recently, SCM-198 has been demonstrated to be effective for the treatment of stroke, and in Parkinson's disease models, to have the capacity of modulating mitochondrial function and the redox state of the brain, respectively.[8,12] SCM-198 can be a potential drug candidate to treat CVD, for its promising cardioprotective effects, low toxicity, and appropriate pharmacokinetic characteristics, as previously reported.[13] Our recent promising pharmacological results of SCM-198 secured the approval of its clinical trials in China as a cardioprotective agent for patients with atherosclerosis.[8]

However, the role of SCM-198 in the conditions of ischemia and reperfusion remains to be elucidated. Our study aimed to investigate the effect of SCM-198 preconditioning on myocardial I/R injury and investigate its underlying mechanism involved.

Materials and methods

Experimental animals

Adult male Sprague-Dawley (SD) rats weighing 250–280 g, purchased from Sippr-bk Experimental Animal Center (Shanghai, China), were housed under the standard conditions (temperature: 25°C; humidity: 55–60%; light/dark:12 h/12 h). All animal procedures, approved by the Ethics Committee of Experimental Research, Shanghai Pudong Hospital of Fudan University, were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Experiment design and drug administration

A total number of 134 adult male SD rats aged eight weeks were randomly divided to four groups: the sham-operated control group treated with vehicle (saline solution; $n = 30$); the I/R group treated with vehicle (saline solution; $n = 36$); and the I/R groups treated intraperitoneally with SCM-198 solution at the dose of 7.5 and 15 mg/kg, respectively, 5 min before I/R ($n = 32$ & 35).

For the induction of I/R injury, the rats were anesthetized with 30 mg/kg pentobarbital sodium, intubated, and ventilated using a rodent ventilator (Chengdu Instrument Factory, Sichuan, China). Their core body temperature was maintained at 37°C during surgery by continuously monitoring a rectal *probe and using an automatic heating blanket. A parasternal incision was performed between the left third and fourth rib and intercostal muscles using* surgical scissors. Myocardial ischemia was induced with a passing 5-0 silk suture beneath the left anterior descending artery at a point 1–2 mm inferior to the left auricle.[14,15] All the rats were subjected to 45 min of myocardial ischemia, followed by 24 h of reperfusion. The death rates were recorded.

Echocardiography

In each group, the animals were anesthetized with pentobarbital sodium (30 mg/kg). Their cardiac function was dynamically evaluated by echocardiography using the Vevo770 (VisualSonics Inc., Toronto, Canada) with a 716 probe. The transducers with frequency of 17.5 MHz for ventricular structures provided spatial resolutions. Ejection fraction (EF) and fractional shortening (FS) were automatically derived using the High-Resolution Electrocardiograph system. All measurements were averaged for five consecutive cardiac cycles.[16,17]

Hemodynamic measurements

When they were anesthetized with pentobarbital sodium (30 mg/kg), the rats received a subcutaneous injection of heparin (2000 U/kg) to have blood coagulation prevented. After 10 min electrocardiogram (ECG) recording, the neck was incised longitudinally so that the right common carotid artery (CCA) was exposed, which was distally ligated with the sutures placed proximal to the artery. A small opening was then made in the artery with mini-scissors for a fluid-filled polyethylene catheter (P50), which was gently inserted into the left ventricular (LV) cavity to record intracavitary pressure. Hemodynamic parameters were analyzed using the MFlab 200 (AMP 20130830) connecting to a pressure transducer (TRI 21; Letica Scientific Instruments). The hemodynamic assessment of LV contractility was measured by an analysis of pressure volume, including the heart rate (HR), the maximum pressure of the left ventricular (peak), maximum rate of rise of left ventricular pressure ($+dp/dt_{max}$), and minimum rate of rise of left ventricular pressure ($-dp/dt_{max}$). A calculation was made of maximum speed of myocardial contractility shortening (physiology, cm/s; Vpm), L0 (the total area of the heart strength loop, CFU), and contractility index (a major determinant of cardiac output and an important factor in cardiac compensation). Afterward, the animals were euthanized with an overdose of anesthetic after hemodynamic recordings, the hearts of which were rapidly removed to be stored at −80°C for Western blot and histological analysis.[14,18]

Biochemical analyses

At the end of the experiment, the rats were fasted for 12 h before euthanized with 30 mg/kg pentobarbital sodium, from which blood samples were collected to be centrifuged at 3500 rpm for 15 min so that plasma was separated. The plasma levels of lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase isoenzyme-MB (CK-MB), and aspartate aminotransferase (AST) were determined using the automatic biochemical analyzer (Cobas 6000; Roche).[14]

Measurement of plasma lipid peroxidation levels and SOD activity

The lipid peroxidation in plasma was determined with thiobarbituric acid-reactive substances. To 0.5 mL plasma sample was added 1.0 mL of 0.67% TBA and 2.5 mL of 20% trichloroacetic acid (TCA) was added to 0.5 mL plasma sample, which was then heated in a boiling water bath (100°C) for 30 min. After 15 min cooling in a water bath at 2°C, the mixture received an addition of 4 mL of n-butanol. Following 2 min vortex, the mixture was centrifuged at 3000 rpm for 10 min, the upper n-butanol layer separated and the absorbance read at 535 nm. Serial dilution curves were plotted based on a series of standard solutions (1,1,3,3-tetramethoxypropane), and the sample values were derived from the standard curve. The results were expressed as nmol/mL. The superoxide dismutase (SOD) levels were measured using assay kits (Ransel and Ransod test kits, Laboratories Ltd, UK).

Determination of infarct size

Intraperitoneally treated with SCM-198 or vehicle, the animals were subjected to 45-min myocardial ischemia and subsequently to 24 h reperfusion. With the sutures tightened again, they were intravenously injected with 1% Evans blue (Sigma-Aldrich, St. Louis, MO, USA) so that their hearts were immediately removed. The hearts were washed twice in phosphate-buffered saline (PBS) (pH 7.4), before cut into 1- to 2-mm-thick slices parallel to the atrioventricular groove. The slices were dipped in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) solution at 37°C for 30 min, after which they were flushed with saline and fixed in 10% paraformaldehyde in PBS (pH 7.4) for 2 h. Subsequently, the slices were placed on a glass slide to be photographed under a digital camera. Image J software (NIH, Boston, MA, USA) used in a blinded fashion to analyze the images, and the infarct size was expressed as a ratio of the infarct area and the area at risk.

TUNEL staining for detecting myocardium apoptosis

TdT-mediated dUTP nick-end labeling (TUNEL) staining was used to detect the apoptotic cardiomyocytes in the border zone of the infarct area. To determine cardiomyocyte apoptosis in a quantitative manner, eight rats in each group were examined in an independent experiment using TUNEL. The detection of apoptotic cardiomyocytes was performed with TUNEL staining using an apoptosis detection kit (Roche Applied Science) according to the manufacturer's instructions. Heart samples were fixed in 10% formalin and then embedded with paraffin, which were to be cut into 5 μm sections. TUNEL staining carried out as described previously,[16] the apoptotic cells were shown as green, and the nucleus was counterstained to be blue.

Cell culture (hypoxia/reoxygenation) and treatment

Neonatal rat cardiac ventricular myocytes (NRCMs) were prepared as previously described.[16–18] To establish the hypoxia-reoxygenation model, the cells were cultured in DMEM/F-12 without glucose and serum. The cells were exposed to hypoxia (95% N_2 + 5% CO_2) for 12 h, and then to reoxygenation for 12 h. The cells were pretreated with different concentrations of SCM-198 solution for 0.5 h before the hypoxia-reoxygenation procedure; the untreated ones were cultured in DMEM/F-12 with low glucose (1000 mg/L) and 2% serum under standard conditions to be treated with vehicle.

Cell viability assays

The viability of NRCMs cultured in 96-well plates was measured using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies), according to the manufacturer's instructions, and the absorbance of CCK-8, using a microplate reader at 450 nm. The values were normalized to those of the untreated cells.

Measurement of intracellular ROS levels

ROS levels in NRCMs were determined using dihydroethidium (DHE, Sigma-Aldrich) fluorescence of a confocal microscopy. After 24 h treatment, the cells were washed twice with PBS before incubated with DHE (10 μmol/L) at 37°C for 30 min in the dark. Washed with PBS, DHE was removed. Fluorescent signals were measured at excitation of 488 nm and emission of 610 nm under a laser confocal microscope (Zeiss).

Western blot

The infarcted myocardial tissues were visually identified under a microscope, the samples of which were lysed in 250 μL of lysis buffer and zirconium oxide beads (Bertin Technologies), before processed for 2 × 30 s using a tissue homogenizer (Precellys 24; Bertin Technologies) and kept on ice for 30 min. The samples were centrifuged for 2 × 10 min at 12,000 g, with the supernatant to be stored at −80°C in tissue lysis buffer (cOmplete Lysis-M; Roche). The cultured NRCMs were lysed with ice-cold RIPA lysis buffer (Pierce, Pittsburgh, PA, USA) so that the protein was extracted to be quantified using the BCA reagent (Shen Neng Bo Cai Corp).

The protein samples (60 μg) were separated using a 10% SDS-PAGE gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore-Upstate). The blots were blocked with 5% non-fat milk before incubated with the following primary antibodies: phospho-Akt, Akt, phospho-JNK, JNK, Bcl-2 and Bax (Santa Cruz Biotechnology Company), and phospho-p38 and p38 (Abcam Company) at 4°C overnight. Washed with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Specific bands were detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific-Pierce).

Statistical analysis

Data were expressed as mean ± SEM. Differences between multiple groups were analyzed using one-way analysis of variance (ANOVA) based on the SPSS software version 25 (SPSS Inc., Chicago, IL, USA). ANOVA was performed with a Bonferroni test for post hoc analysis. The results were considered to be statistically significant at $P < 0.05$.

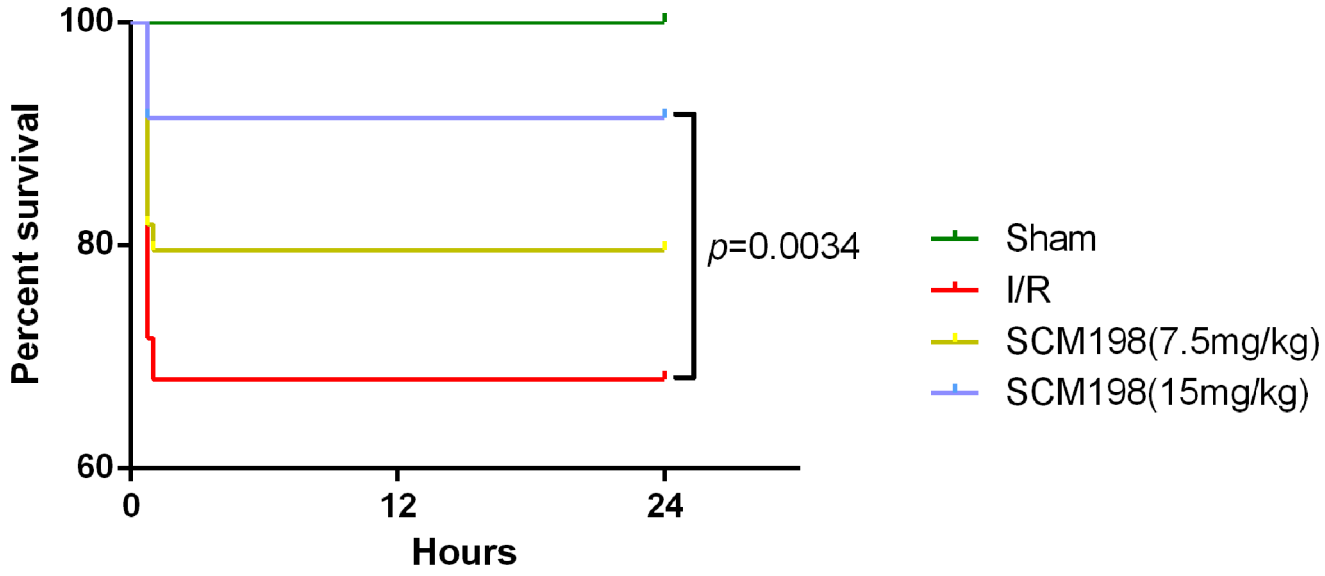


Figure 1. Effect of SCM-198 on survival rate of I/R rats.
Kaplan-Meier survival curve; $P = 0.0034$; SCM-198 (15 mg/kg) group versus I/R group ($n = 30$–53 in each group).

Results

SCM-198 increased survival rate of I/R injury rats

In the different groups, the rats were subjected to I/R or sham surgery, and over the following 24 h the survival rate was determined. In the I/R group, 36 of 53 rats (67.92%) survived up to 24 h, while in the SCM-198 (7.5 or 15 mg/kg) groups, 35 of 44 and 32 of 35 (79.55%, 91.43%) rats survived, respectively. Those which were intraperitoneal injected with SCM-198 (7.5 mg/kg) had an increased survival rate when compared with those in the I/R group (35/44; $P < 0.05$; Figure 1). There was a significantly higher mortality in the I/R group (36/53) than in the SCM-198 15 mg/kg) group (32/35) ($P < 0.001$; Figure 1).

SCM-198 mitigated cardiac functional deterioration and oxidative stress following I/R

To examine the effect of SCM-198 on myocardial I/R, the male SD rats were divided into four groups: Sham, I/R, I/R + SCM-198 (7.5 mg/kg and 15 mg/kg, respectively). An intraperitoneal injection of SCM-198 (15 mg/kg) was observed to result in a significant dose-dependent reduction in plasma myocardial enzymes 24 h after I/R, as indicated in LDH ($P = 0.019$; Figure 2(A)), CK ($P = 0.024$; Figure 2(B)), CK-MB ($P = 0.032$; Figure 2(C)), AST ($P < 0.001$; Figure 2(D)), and HBDH ($P = 0.037$; Figure 2(E)) ($n = 30$ in each group). The effect of SCM-198 was dose-dependent, significantly decreasing MDA level at 15 mg/kg ($P = 0.029$; Figure 2(F)) and significantly increasing the activity of total SOD in plasma 24 h after I/R (Figure 2(G)).

Furthermore, SCM-198 was observed to ameliorate cardiac morphology through transmission electron microscopy (TEM) 24 h after I/R. In the I/R group, the TEM results revealed rupture of muscle fibers, mitochondrial swelling and intracellular edema, the shape of nucleus irregular, with the presence of mitochondrial overflow after cell death. In the I/R + SCM-198 group, less rupture of muscle fibers was observed, with mild mitochondria swelling, mild intercellular edema, and less cell death. In the sham group, we observed no rupture of muscle fibers, no mitochondrial or intracellular edema, and no dead cells (Figure 2(J)).

To confirm the protective effect of SCM-198, we measured the myocardial infarct size using TTC and Evans Blue staining for all four groups. The infarct size was reduced in the group treated with SCM-198 (7.5 mg/kg) 24 h after I/R, when compared with that in the vehicle-treated group ($35.5 \pm 3.5\%$ versus $26.5 \pm 2.7\%$; $P = 0.02$; Figure 2(H)). Similarly, the infarct size was significantly reduced in the group treated with SCM-198 (15 mg/kg) when compared with that in the vehicle-treated group ($35.5 \pm 3.5\%$ versus $20.7 \pm 2.4\%$; $P < 0.001$; Figure 2(H) and (I)).

SCM-198 restored hemodynamics and cardiac functions after I/R

To evaluate the effect of the SCM-198 on LV function, a series of hemodynamic measurements were conducted 5 h after I/R, the results of which demonstrated that the value of HR, peak, $\pm dP/dt_{max}$, Vpm, and L0 declined, respectively, in the I/R group, but was higher in the SCM-198-treated group 5 h after reperfusion (Figure 3(A) to (F)), as indicated by the echocardiographs in Figure 3(G) to (I). The statistical analysis showed that FS and EF were significantly decreased in the I/R group in comparison with the sham group; that SCM-198 treatment significantly increased FS and EF; and that FS and EF in the SCM-198 (15 mg/kg) group were similar to those in the sham group.

SCM-198 reduced cardiac apoptosis in I/R injury rats

As indicated by the results of TUNEL assays to determine whether the beneficial effect of SCM-198 on myocardial I/R injury was associated with reduced apoptosis, the I/R group showed elevated apoptosis when compared with

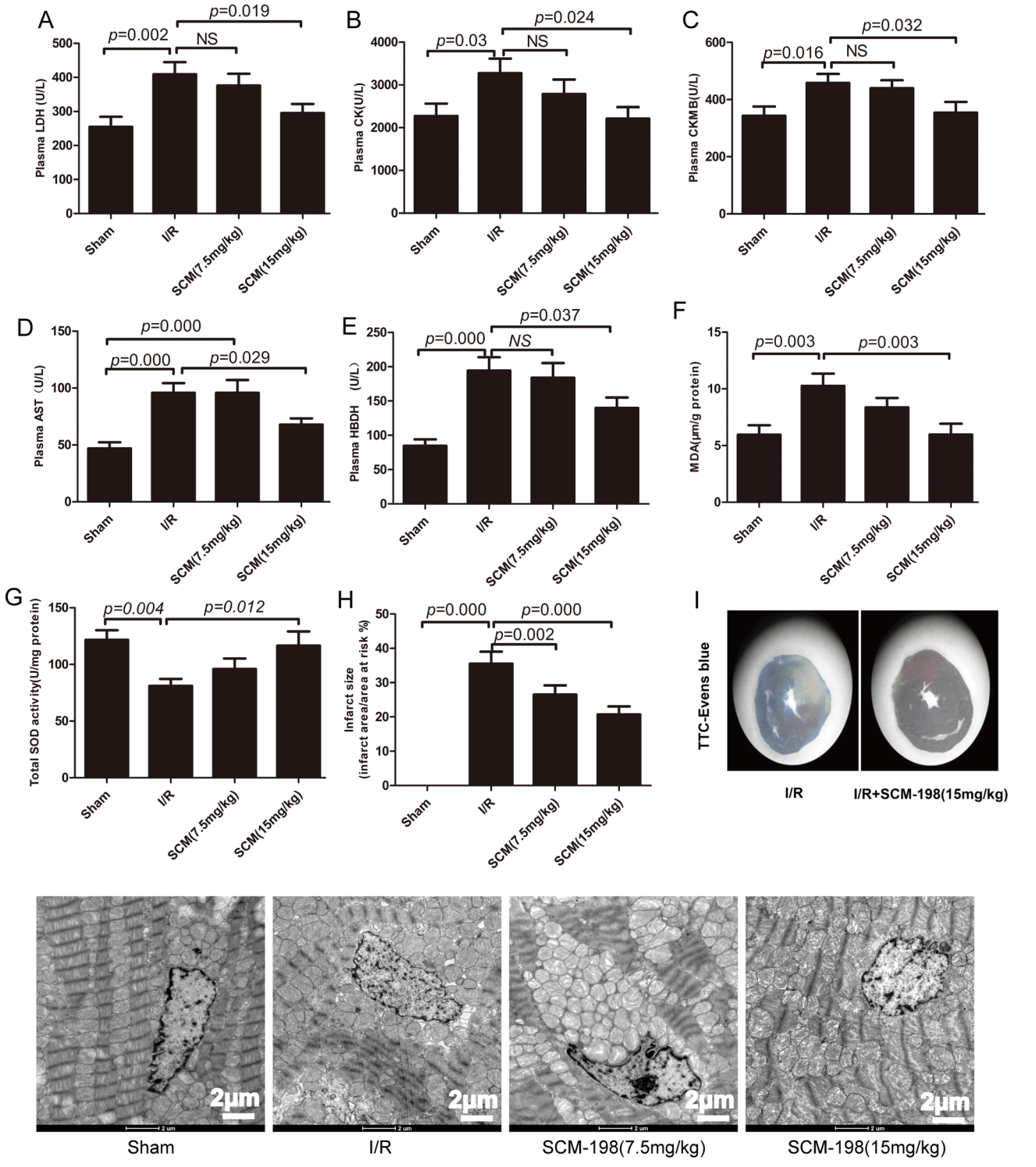


Figure 2. Effect of SCM-198 on the activities of myocardium zymogram, oxidative stress, infarct size, and morphological changes in cardiac cells 24 h after I/R. Plasma myocardial enzymes: (A) LDH ($n=28$); (B) CK ($n=28$); (C) CKMB ($n=28$); (D) AST ($n=28$); (E) HBDH ($n=28$); (F) myocardial malondialdehyde (MDA) content ($n=28$); (G) myocardial superoxide dismutase (SOD) activity ($n=28$); (H) statistical analysis; and (I) representative images demonstrating the role of SCM-198 in reducing infarct size in a rat model of I/R; infarct size expressed as a ratio of the infarct area and the area at risk ($n=19$); (J) morphological changes in cardiac cells assessed by TEM. Scale bar, 2 μm ($n=6$); $P<0.05$ considered statistically significant.

the sham-treated group, and SCM-198 pretreatment significantly decreased the cell apoptosis in myocardial infarct tissues (Figure 4(A) and (B)). Furthermore, the measurement of the anti-apoptotic protein expressions, Bcl-2 and Bax, indicated that I/R led to a substantial decrease and increase in Bcl-2 and Bax level, respectively, in the SCM-198 (15 mg/kg) group compared with the sham-treated group (Figure 4(C) to (E)). Bcl-2 was almost restored to the normal level, while Bax level was significantly reduced because of SCM-198 pretreatment.

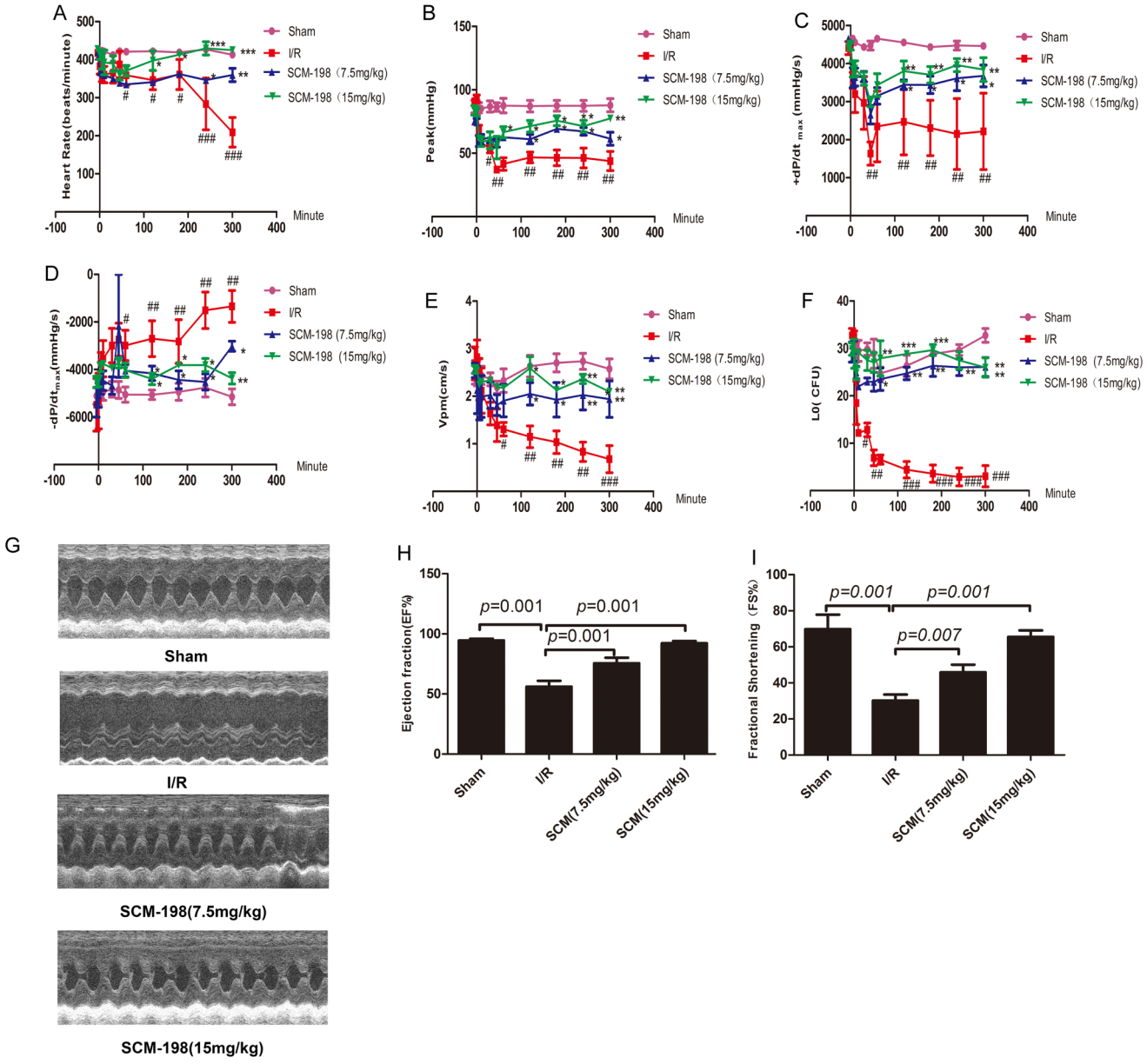


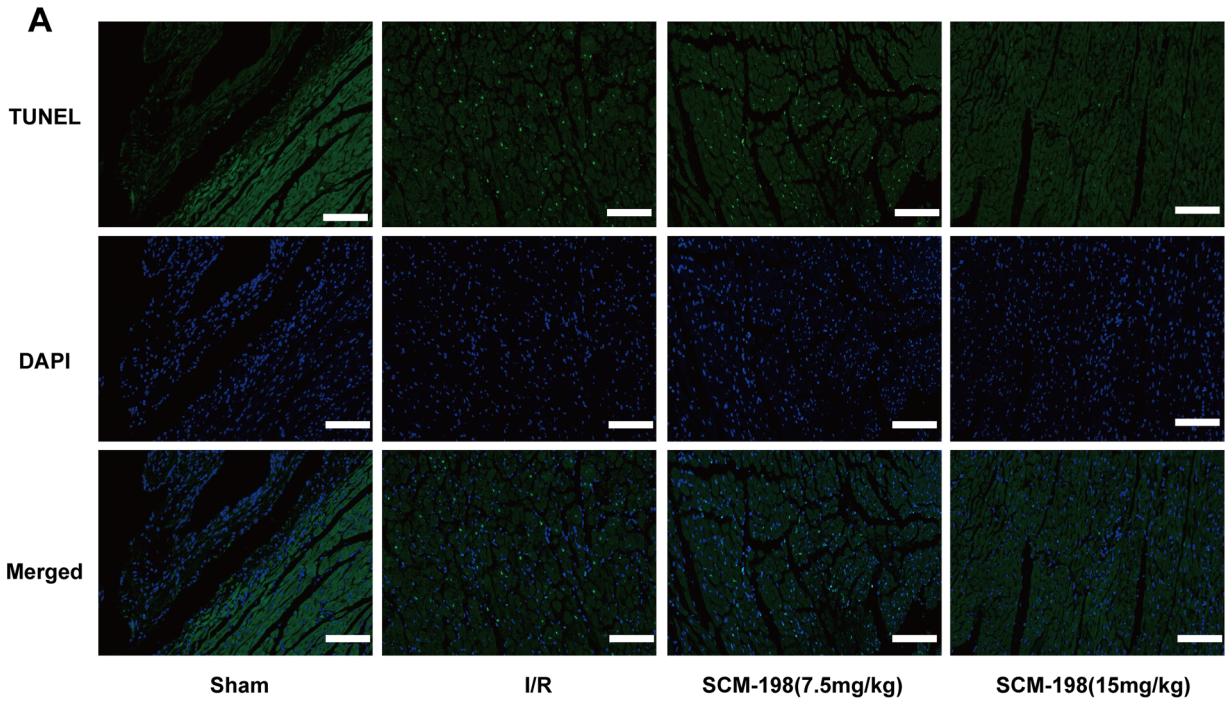
Figure 3. Hemodynamic assessments and echocardiogram of the left ventricular after I/R. Cardiac function assessed by intraventricular pressure measurement: (A) heart rate (HR); (B) Peak; (C) and (D) the maximum positive and negative value of dP/dt; (D) and (E) maximum speed of myocardial contractility shortening (Vpm) and total area of the heart strength loop (L0); SCM-198 treatment showing improved cardiac HR in the group of 7.5 mg/kg and 15 mg/kg, respectively, compared with the I/R group showing increased +dp/dt, –dp/dt and Peak and L0, respectively; these parameters increased in the group of 7.5 mg/kg although lower than those in the group of 15 mg/kg; values being mean ± SEM, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Tukey's test versus I/R group and $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, Tukey's test versus Sham group ($n=12$ in each group); (G) representative echocardiographic records obtained from mid-ventricular short-axis views of the rat hearts ($n=12$–16 in each group); (H) and (I) statistical analysis of the data from the original echocardiographic records; data presented as mean ± SEM ($n=12$–16 in each group).
EF: ejection fraction; FS: fractional shortening

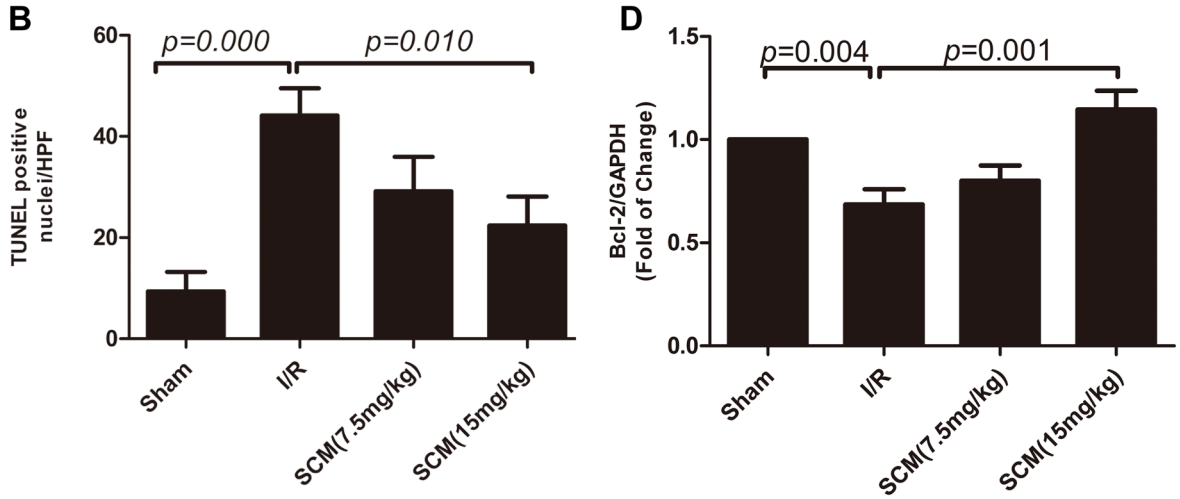
SCM-198 reduced intracellular ROS level and apoptosis and enhanced the viability of primary cardiomyocytes induced by H/R in NRCMs

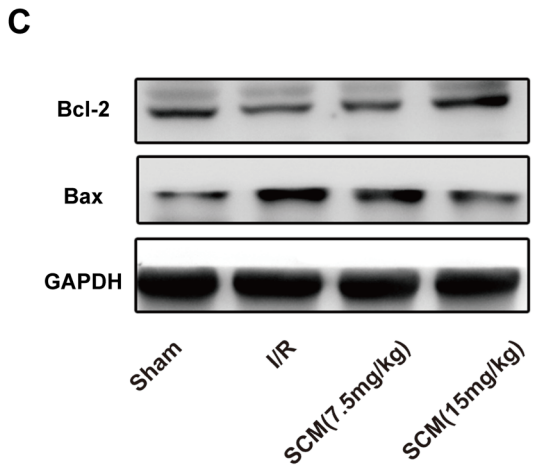
Since the reoxygenation of cardiomyocytes contributes to cell death, we set up the hypoxia/reoxygenation (H/R) injury *in vitro* model using primary NRCMs. As shown in Figure 5(A) and (B), ROS level increased significantly 24 h after NRCMs were subjected to H/R in the H/R group, while it decreased significantly in the SCM-198 pretreatment group. The effect of SCM-198 was detected to be on myocardial apoptosis, as indicated by TUNEL staining. In addition, the cell apoptosis increased significantly in the H/R group, while SCM-198 pretreatment decreased significantly the apoptosis of myocardial cells after H/R (Figure 5(D) and (E). To investigate whether SCM-198 had the same effect *in vitro*, we incubated NRCMs to hypoxia using an incubator to mimic myocardial I/R injury, as described in the section of methods.[16,19] We found that SCM-198 treatment significantly improved cell viability, when compared with the H/R group (Figure 5(C)).

The effect of SCM-198 on the Akt/JNK/p38 pathway and expression of Bcl-2/Bax in NRCMs

With the cultured NRCMs treated with SCM-198 in a dose-dependent manner, the cell lysates were collected to find







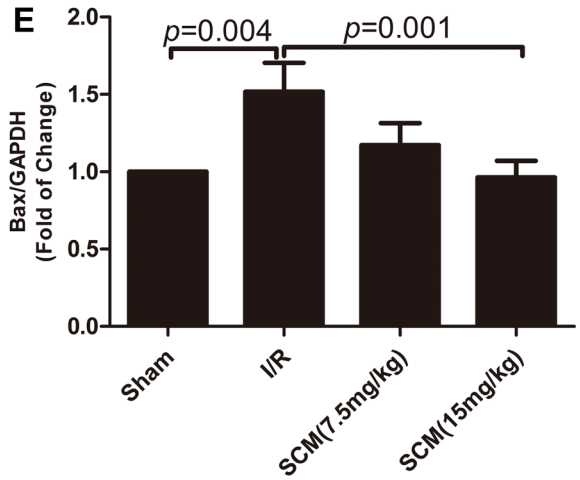


Figure 4. Reduction of myocardial apoptosis in SCM-198-administered I/R rats. Cardiomyocyte sections harvested 24 h after I/R was stained with TUNEL; green = apoptotic cardiomyocyte; blue = TUNEL-negative cells; (A) representative photomicrographs of TUNEL staining taken at ×200 magnification. Scale bar, 100 μm; (B) quantitative analysis of the number of apoptotic cells per field; (C) apoptosis-related protein Bcl-2 and Bax level in the different groups, measured by Western blot analysis (D) to (E) ($n = 8$ in each group); data represented as mean ± SEM.

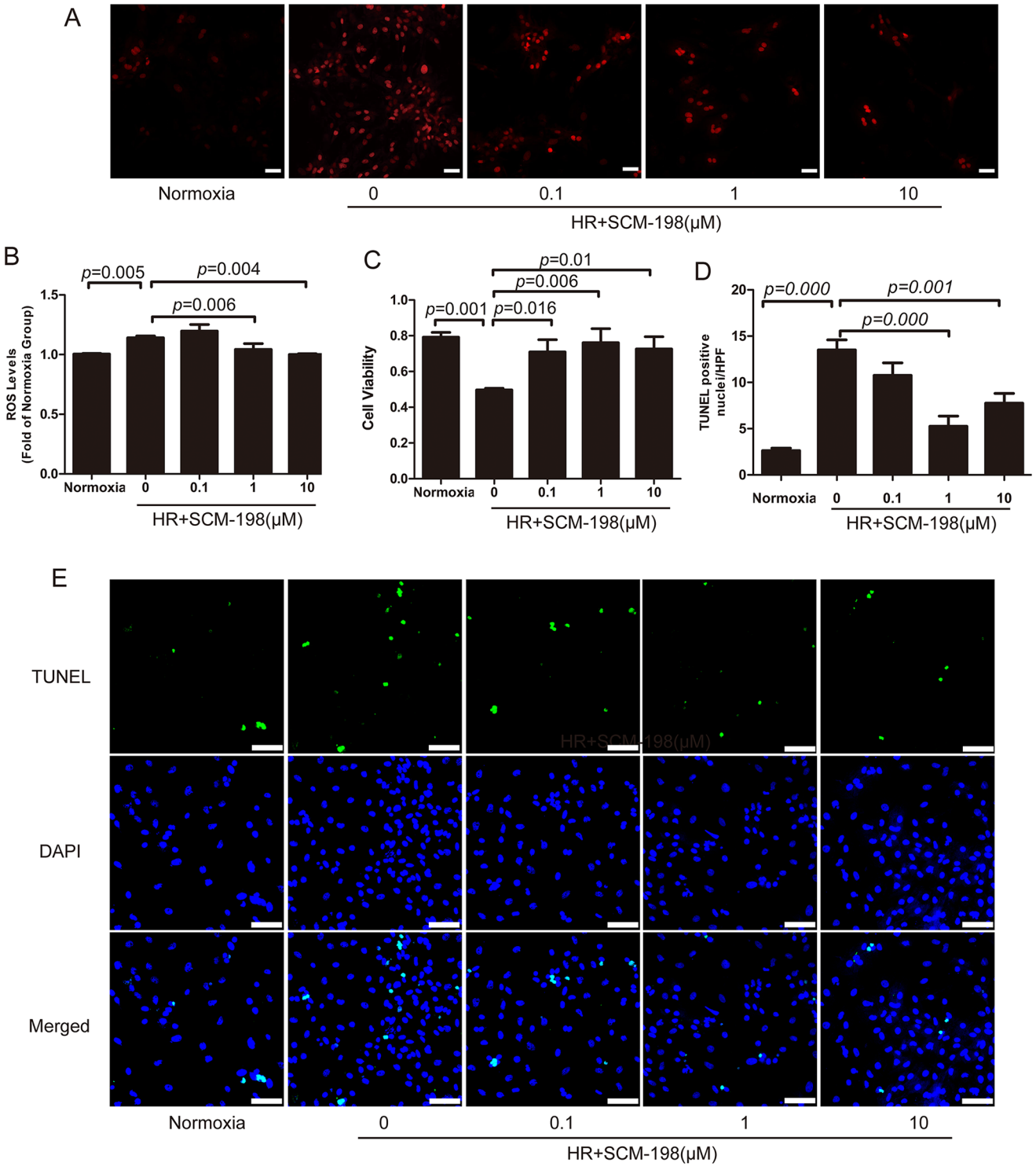


Figure 5. NRCMs protected from H/R-induced apoptosis by decreasing ROS level. (A) representative images of ROS level in NRCMs detected by confocal microscope. Scale bar, 100 μm; (B) quantitative analysis for ROS level in NRCMs detected using dihydroethidium in a microplate reader; (C) enhanced viability of NRCMs measured by CCK-8 assay at the end of 72 h treatment; (D) TUNEL-positive nuclei quantification represented as number per high-power field (HPF); (E) representative images of cardiomyocyte apoptosis from NRCMs detected by confocal microscope-TUNEL (green), apoptotic nuclei, DAPI (blue), and total nuclei. Scale bar, 50 μm; data represented as the mean ± SEM ($n = 6$ in each group).

that the level of Bcl-2 and phosphorylation of Akt increased, respectively, while the level of Bax, p-P38, and p-JNK was downregulated, respectively (Figures 6 and 7). The cell lysates were collected from the culture NRCMs treated with SCM-198, 1 μM for 0.5 h and HR for 24 h, respectively, the findings of which showed that SCM-198 increased the level of p-Akt and Bcl-2 and downregulated the level of Bax *in vitro*. Importantly, SCM-198-induced the expression

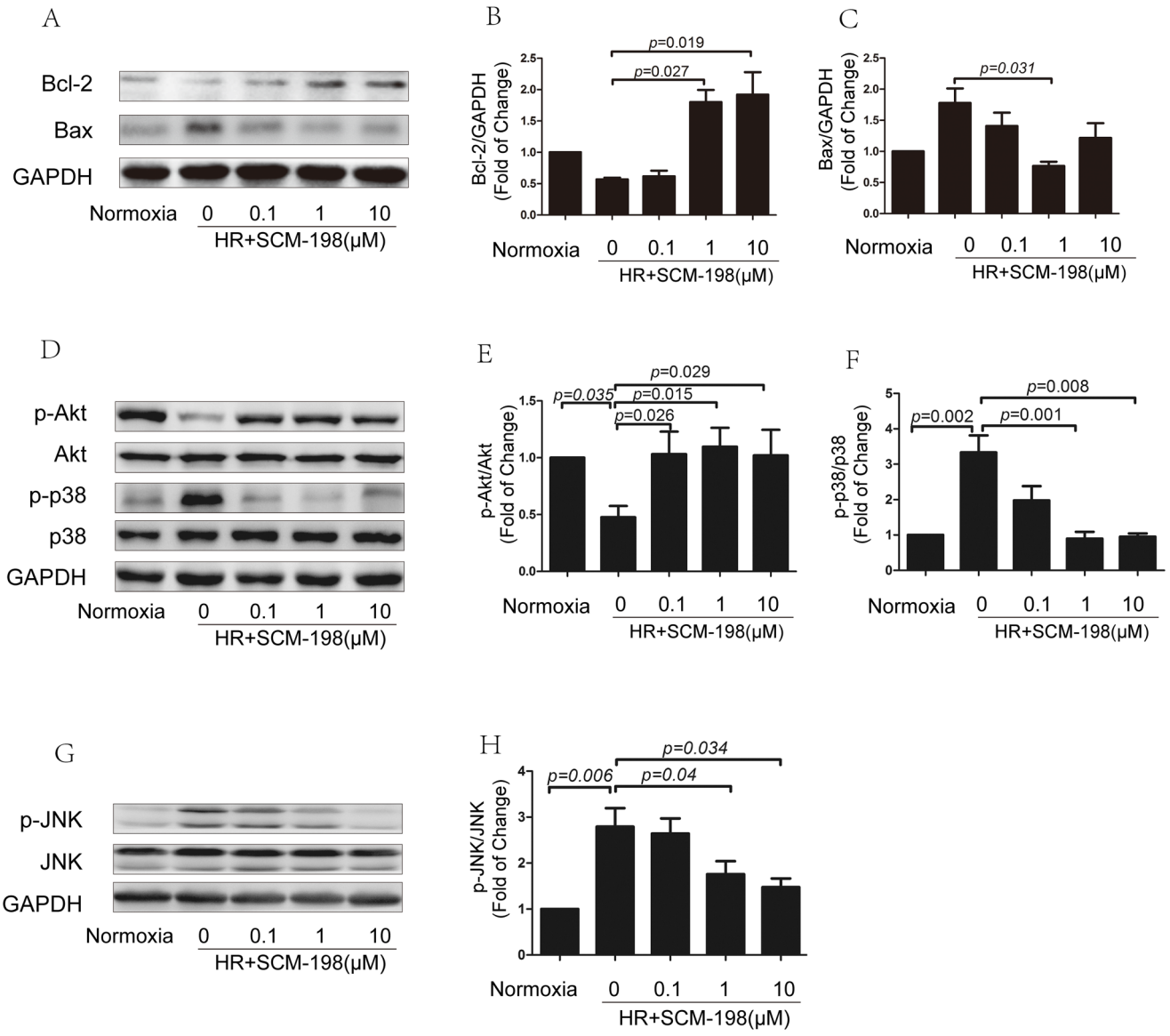


Figure 6. Modulation of apoptosis-related protein expression by SCM-198 after H/R. Representative Western blots (A), (D), (G) and quantitative analysis for (B) Bcl-2, (C) Bax, (E) p-Akt/Akt, (F) p-p38/p38, (H) p-JNK/JNK expression levels after H/R ($n = 6$ in each group); data represented as the mean ± SEM. The P of <0.05 was considered significant.

of p-Akt Bcl-2 and Bax were blocked by the PI3K inhibitor LY294002 (10 μM; Figure 8). The total protein level of Akt was not affected by SCM-198 pretreatment.

Discussion

It is well recognized that I/R is a major cause of cardiac dysfunction during cardiovascular surgery and heart transplantation, inducing cardiomyocyte injury.[14,19,20] Under the conditions of ischemia and hypoxia, the endogenous-free radical scavenger, glutathione, was reported to decrease, leading to the accumulation of reactive-free radicals to induce cellular damage.[21] In this study, we demonstrated that SCM-198 not only reduced infarct size but also improved the cardiac function and overall survival rate.

Moreover, LDH and CK are representative indexes of cardiac cellular damage during MI.[26] They are released from myocardial tissues into the plasma. In our experimental animal model, the occlusion of the left coronary resulted in a high level of a leakage of cardiac enzymes, including LDH, CK, CK-MB, AST, and HBDH.[27–29] These biomarkers are pivotal to clinical diagnosis and prognosis. As previously reported, HBDH could predict cardiac necrosis because it did not change its activity spatially.[30] However, our previous study demonstrated that SCM-198 could significantly decrease the levels of LDH and CK in the plasma of MI rats.[31] In this study, we investigated the changes in additional number of enzymes, finding similar changes in AST, CKMB, and HBDH after MI. These indexes were almost similar to the normal ranges in the high-dose SCM-198 group in comparison with the I/R group.

Previous studies have demonstrated that SCM-198 ameliorates atherosclerosis, inhibits osteoclastogenesis, and prevents osteoporosis.[32–34] Consistent with the previous

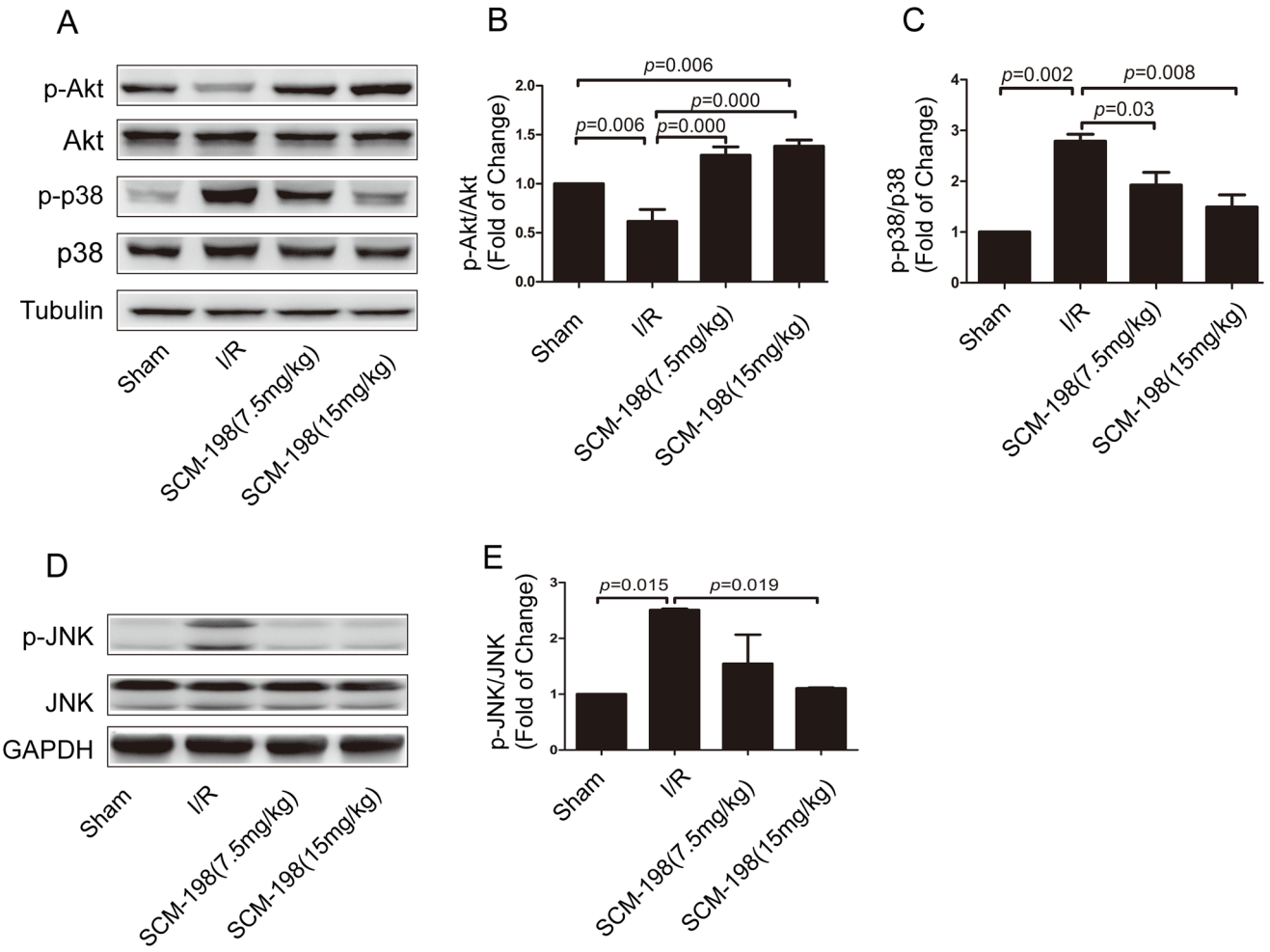


Figure 7. Akt and JNK protein expressions modulated of by SCM-198 after I/R. Representative Western blots (A), (D) and quantitative analysis for (B) p-Akt/Akt, (C) p-p38/p38, (E) p-JNK/JNK expression levels after myocardial I/R ($n=6$ in each group); data represented as the mean ± SEM. The P of <0.05 was considered significant.

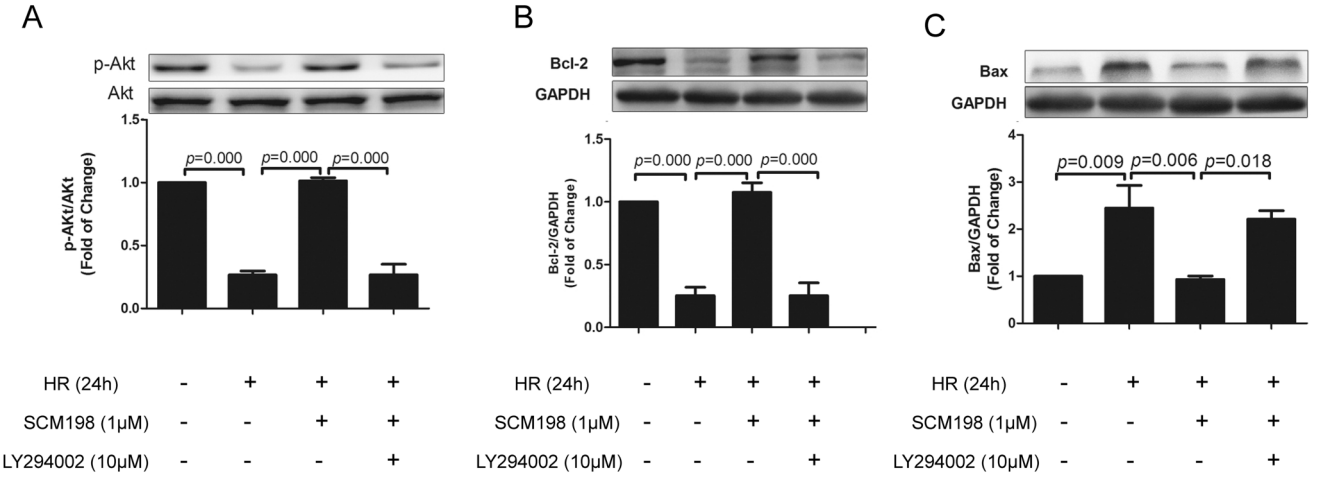


Figure 8. The anti-apoptotic effect of SCM-198 in NRCMs was blocked by the PI3K inhibitor LY294002 after H/R. Representative Western blots and quantitative analysis for p-Akt/Akt and Bcl-2/Bax expression after H/R (A) to (C) ($n=6$ in each group). GAPDH was used as the internal control; data represented as the mean ± SEM. The P of <0.05 was considered significant.

studies,[35,36] our findings demonstrated that SCM-198 could increase the heart rate, $\pm dp/dt_{max}$, Vpm, and L0. *In vivo*, we observed that SCM-198 reduced the infarct size, which was recognized to be one of the frequently used clinical end-points to predict clinical outcomes.[8] Importantly, the SCM-198 (15 mg/kg) group had the highest survival rate at the end of the experiment. To our knowledge, this was the first study to demonstrate that SCM-198 preconditioning

could not only reduce infarct size but also improve ventricular function and survival rate in a dose-dependent manner, thus alleviating cardiac dysfunction after I/R. This suggests that SCM-198 could be a novel promising therapeutic agent to prevent myocardial I/R-related death.

We also explored the mechanism by which SCM-198 can regulate cardiac function during I/R. Although several therapies have been developed over the recent years, acute myocardial infarction (AMI) is the leading cause of death in the world.[2,8] MI/R injury can lead to both necrosis and apoptosis of cardiomyocytes,[37–40] which could be ascribed to reperfusion,[41,42] as it does not completely reverse the ischemia-induced changes but causes further cardiomyocyte apoptosis.[14,43–46] Studies on experimental animal models and *in vitro* experiments have consistently demonstrated that cardiomyocyte apoptosis is responsible for MI/R injury and gets involved in post-infarction remodeling.[47,48] Cardiomyocyte apoptosis has been highly investigated for therapeutic applications in AMI since it was first described as the pathological progress.[14,38,46] In mammalian cells, ROS are known to act as second messengers to activate diverse redox-sensitive signaling transduction cascades, including the stress-activated MAP kinases p38 and the Jun-N Terminal kinase (JNK).[22–25] We demonstrated that SCM-198 inactivated the phosphorylation of p38 and JNK and that SCM-198 prevented cardiomyocyte apoptosis via the upregulation of Akt phosphorylation and Bcl-2, and downregulated Bax both *in vivo* and *in vitro* experiments. In our study, we found that cardiomyocyte apoptosis in the rats subjected to myocardial I/R as well in primary NRCMs exposed to H/R, which was in line with the previously reported findings.

Through *in vivo* and *in vitro* TUNEL tests, we found that MI/R significantly increased apoptosis in the rat heart, while SCM-198 significantly decreased the level of apoptotic myocytes. These findings suggest that apoptosis can be involved in I/R-induced cardiac infarction, and that SCM-198 can protect the heart from MI/R injury by inhibiting cardiac apoptosis. A number of studies have verified that SCM-198 can be a multi-target medical herb.[8,49,50] Although we made efforts to explore the mechanism of SCM-198 from various molecular pathways, the mechanism that underlies this apparent anti-apoptotic effect remains to be unknown. Bcl-2 family is known to be associated with the regulation of apoptotic response.[51] Recently, it was reported that impaired regulation of the Bcl-2 family could further aggravate ischemic damage, accompanied with increased cell death.[52] We observed that SCM-198 significantly increased Bcl-2 levels in the hearts of MI/R rats, while the expression of Bax was reduced in the rats treated with SCM-198.

From the experiments on NRCMs to prevent the myocardial interstitial cells influencing our conclusions, we found the same trend in expression level between Bcl-2 and Bax protein after H/R. SCM-198 treatment restored Bcl-2 expression, while it inhibited Bax expression. Therefore, it is reasonable for us to hypothesize that SCM-198 may exert an anti-apoptotic effect by inducing Bcl-2 and inhibiting Bax expression. This facilitates our conclusion that the cardioprotective effect of SCM-198 on cardiac function relies on its anti-apoptotic activity by upregulating Bcl-2 and downregulating Bax expression.

Pro-survival signaling pathways, such as the Akt pathway,[53,54] may play a functional role in preventing myocyte apoptosis under stress conditions.[55,56] In this study, we proved that there was a significant increase in Akt phosphorylation after SCM-198 treatment in the hearts of MI/R injury rats and in NRCMs subjected to H/R, as elucidated in some previous studies reporting that the Akt pathway was involved in the protection against myocardial dysfunction and failure induced by pressure overload.[57,58]

In summary, our experiment results suggested that SCM-198 could exert a cardioprotective effect on myocardial I/R rats *in vivo* and in NRCMs subjected to H/R *in vitro*; that SCM-198 could prevent cardiomyocyte apoptosis by elevating the protein expression of Bcl-2 and phosphorylation of Akt; and that SCM-198 could decrease Bax, phosphorylation of p38 and JNK. We can speculate that SCM-198 could have potential benefits of preconditioning in preventing ischemic heart disease so that it could serve as a novel promising candidate in preventing the progression of myocardial I/R.

AUTHORS' CONTRIBUTIONS

HL and JG designed and performed the experiments, analyzing data and writing the manuscript. TZ ZJ and WD performed some animal-model-based experiments and Western Blot. Corresponding Author: Dr. FM and JD proposed the idea and supervised the project.

DECLARATION OF CONFLICTING INTERESTS

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