# Scutellarin ameliorates ischemia/reperfusion injury-induced cardiomyocyte apoptosis and cardiac dysfunction via inhibition of the cGAS-STING pathway

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**Abstract.** Ischemic heart disease is a common cardiovascular disease. Scutellarin (SCU) exhibits protective effects in ischemic cardiomyocytes; however, to the best of our knowledge, the protective mechanism of SCU remains unclear. The present study was performed to investigate the protective effect of SCU on cardiomyocytes after ischemia/reperfusion (I/R) injury and the underlying mechanism. Mice were intraperitoneally injected with SCU (20 mg/kg) for 7 days before establishing the heart I/R injury model. Cardiac function was detected using small animal echocardiography, apoptotic cells were visualized using TUNEL staining, the myocardial infarct area was assessed by 2,3,5-triphenyltetrazolium chloride staining, and the protein levels of cyclic GMP-AMP synthase (cGAS), stimulator of interferon genes (STING), Bcl-2, Bax and cleaved Caspase-3 were detected by western blotting. In *in vitro* experiments, H9c2 cells were pretreated with SCU, RU.521 (cGAS inhibitor) and H-151 (STING inhibitor), before cell hypoxia/reoxygenation (H/R) injury. The viability of H9c2 cells was detected using a Cell Counting Kit-8 assay, the rate of apoptosis was determined by flow cytometry, and the protein expression levels of cGAS, STING, Bcl-2, Bax and cleaved Caspase-3 were detected by western blotting. It was revealed that SCU ameliorated cardiac dysfunction and apoptosis, and inhibited the activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways in I/R-injured mice. It was also observed that SCU significantly increased cell viability and decreased apoptosis in H/R-induced H9c2 cells. Furthermore, H/R increased the expression levels of cGAS, STING and cleaved Caspase-3, and decreased the ratio of Bcl-2/Bax, which could be reversed by treatment with SCU, RU.521 and H-151. The present study demonstrated that the cGAS-STING signaling pathway may be involved in the regulation of the activation of the Bcl-2/Bax/Caspase-3 signaling pathway to mediate I/R-induced cardiomyocyte apoptosis and cardiac dysfunction, which could be ameliorated by SCU treatment.

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*Key words:* scutellarin, ischemia/reperfusion injury, apoptosis, cyclic GMP-AMP synthase, stimulator of interferon genes

## Introduction

Ischemic heart disease (IHD) is a common cardiovascular disease. In 2016, the World Health Organization estimated that there are currently ~126.5 million cases of IHD, with >9 million deaths of patients with IHD per year worldwide, making IHD a leading cause of morbidity and mortality worldwide (1), and therefore it is a great burden on society and the economy, and has become a major global public health problem (2,3). For patients with acute myocardial infarction, immediate and successful myocardial reperfusion is the most effective strategy to reduce the size of myocardial infarction and improve clinical efficacy (4). Surgical treatment, such as coronary artery bypass grafting and percutaneous coronary intervention, are performed immediately to restore blood supply in ischemic cardiomyocytes (5,6). However, reperfusion itself can lead to further cardiomyocyte death and systolic dysfunction, which is known as ischemia/reperfusion (I/R) injury, thereby eliminating the benefits of reperfusion therapy in patients with IHD and leading to secondary myocardial injury (7,8). Apoptosis is considered to be the main form of cardiomyocyte death, and cardiomyocyte apoptosis is usually observed in myocardial I/R injury (9). Therefore, inhibiting I/R injury-induced apoptosis of cardiomyocytes may be an effective approach in the treatment of IHD.

Myocardial I/R injury is involved in the activation of the inflammatory cascade, which serves a role in the acute expansion of injury and myocardial repair (10-12). This inflammatory response is associated with the massive production of a series of mediators that determine the outcome of reperfusion injury, and these mediators, such as proteases, chemokines and interleukins, may contribute to cardiomyocyte apoptosis after I/R injury (13). Certain inflammatory molecules or pathways may be involved in mediating apoptosis. Cyclic GMP-AMP synthase (cGAS) is a nuclease with the function of recognizing cytoplasmic DNA and stimulating the transcription of

the stimulator of interferon genes (STING), as well as regulating the secretion of type I IFN and other cytokines, which contribute to the activation of immune responses (14). In innate immune cells, the presence of cytosolic DNA is sensed by the cGAS-STING signaling pathway, which initiates cytokine production and apoptosis induction (15). It has been reported that cGAS can mediate the activation of Caspase-3-dependent apoptosis by decreasing Bcl-2 expression and increasing Bax expression (16). It has also been demonstrated that the cGAS-STING signaling pathway is involved in the development of I/R-induced neuroinflammation (17). However, to the best of our knowledge, the role of the cGAS-STING signaling pathway in I/R injury-induced cardiomyocyte apoptosis is unclear. Therefore, it was hypothesized that cGAS-STING signaling may be an important target for preventing myocardial I/R injury.

Scutellarin (SCU), a natural bioactive flavonoid, is extracted from *Erigeron breviscapus* (18). It has been demonstrated to have anti-inflammatory and anti-apoptotic effects (19,20). It has been reported that SCU can alleviate hypoxia-induced cerebral injury (21,22). SCU has been demonstrated to protect rat cortical neurons from oxygen-glucose deprivation-induced apoptosis (21). SCU treatment has also been demonstrated to suppress apoptosis after brain I/R injury, as evidenced by reduced DNA fragmentation, NAD depletion and mitochondrial dysfunction (22). Wang *et al* (23) reported that SCU exerted anti-apoptotic and anti-oxidative stress activity to protect cardiomyocytes from I/R injury. Xu *et al* (24) reported that SCU inhibited activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome to attenuate myocardial I/R injury. However, the mechanisms underlying the protective effect of SCU are not well understood.

In the present study, C57BL/6 mice were used to establish an I/R-induced heart injury model and H9c2 cells were used to construct a hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury model to explore the effects of SCU on I/R or H/R injury-induced cardiomyocyte apoptosis and cardiac dysfunction, and its potential mechanisms.

## Materials and methods

*Animals and treatment*. The animal experiment was approved by the Animal Ethics Committee of The People's Hospital of Yue Chi County (Guang'an, China). A total of 20 male C57BL/6 mice (age, 6-8 weeks; weight, 18-25 g) purchased from Western Biotechnology, Inc. were randomly divided into four groups: Sham, I/R, SCU and I/R + SCU group (n=5/group). The mice were treated as follows: In the Sham group, PBS was injected intraperitoneally 1 h before sham surgery; in the SCU group, SCU (20 mg/kg) was injected intraperitoneally each day for a total of 7 days to achieve a stable blood concentration of the drug (25) before sham surgery; in the I/R group, PBS was injected intraperitoneally 1 h before ligation of the left anterior descending coronary artery; and in the I/R + SCU group, SCU (20 mg/kg) was injected intraperitoneally for 7 days before ligation of the left anterior descending coronary artery. The mice had free access to food and water and were housed in an environment at 22±2˚C with a humidity between 40 and 60% and a light-dark cycle of 12 h.

*Ischemia/reperfusion (I/R) model*. The mice were anesthetized with isoflurane gas (dose for both induction and maintenance, 2%) and the thorax was opened. Subsequently, the heart was compressed and quickly positioned in the third and fourth intercostal spaces. In the sham and SCU groups, only the chest was opened without proceeding with ligation, while mice in the I/R and I/R + SCU groups underwent ligation of the left anterior descending coronary artery for 30 min to mimic the ischemia phase, and then the ligation line of the left anterior descending coronary artery was removed for 24 h to mimic the reperfusion phase, to establish the I/R model.

*Echocardiography*. Left ventricular function in mice was assessed using echocardiography (Philips TIS 0.8; Koninklijke Philips N.V.) and an RMV 707B transducer (frequency, 30 MHz; Siemens AG). The mice were anesthetized with isoflurane (dose for both induction and maintenance, 2%) before echocardiography. Images were obtained by identifying the interventricular septum and the left ventricular posterior wall. The left ventricular fractional shortening (LVFS; %) and left ventricular ejection fraction (LVEF; %) were automatically calculated by echocardiography. Each parameter was evaluated by calculating the average of four cardiac cycles. All mice in the present study were sacrificed by cervical dislocation at the end of this experiment.

*TUNEL staining*. Mouse heart tissues were fixed with 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin and cut into 5-$\mu$m-thick paraffin-embedded sections. The *In Situ* Cell Death Fluorescein Kit (Roche Diagnostics) was used to detect the free 3'-OH chain breaks induced by DNA degradation. The detailed steps were as follows: The paraffin sections were soaked in xylene twice (5 min each) and immersed in gradient ethanol (100, 95, 90, 80 and 70%) for 3 min each. Subsequently, the sections were soaked in steaming water once for 3 min, 3% $H_2O_2$ once for 10 min and rinsed with PBS twice (5 min each). The sections were placed in a wet box, treated with 100 $\mu$l proteinase K working solution (20 $\mu$g/ml) at 37˚C for 15 min and rinsed with PBS twice (5 min each). Subsequently, 50 $\mu$l TUNEL reaction solution (10% TdT + 90% fluorescein labeled dUTP) was added to the tissue sections on the glass slides, and the glass slides were covered and incubated at 37˚C for 1.5 h, and subsequently rinsed with PBS three times (5 min each). DAPI staining solution (5 $\mu$g/ml) was added and incubated at room temperature for 5 min, and subsequently rinsed with PBS three times (5 min each). Anti-Fade Mounting Medium (Sangon Biotech Co., Ltd.) was added. The slides were observed under a fluorescence microscope with an excitation wavelength of 450-500 nm and a detection wavelength of 515-565 nm. Five random visual fields were selected from each slide under the microscope. The TUNEL-positive-stained cells were counted using ImageJ software (version 1.46; National Institutes of Health).

*Staining with 2,3,5-triphenyltetrazolium chloride (TTC)*. The myocardial infarct area was assessed by TTC staining. After 24 h of reperfusion, the hearts were excised, and the 2-mm-thick tissue slices were incubated for 20 min in 1% TTC

(MilliporeSigma) at 37˚C, followed by fixation in 4% paraformaldehyde at room temperature overnight. The images were captured using a digital camera and the infarct area appeared white. The ratio of the infarct size (white area) was calculated using ImageJ software (version 1.46; National Institutes of Health).

*Cell culture and processing*. The H9c2 cell line was purchased from American Type Culture Collection. The cells were cultured in DMEM (low glucose, 5 Mm; Thermo Fisher Scientific, Inc.) with 10% FBS (Thermo Fisher Scientific, Inc.) at 37˚C, 5% $CO_2$ and saturated humidity. H9c2 cells were passaged at a ratio of 1:4 in culture plates. Plates with H9c2 ($4x10^5$ cells per well) cardiomyocytes were then placed in a sealed chamber (Modular Incubator Chamber MIC1; Billups-Rothenberg, Inc.) filled with 95% $N_2$ and 5% $CO_2$ to achieve an oxygen-deficient environment. Ventilation at 5 l/min for 15 min was used to achieve a 1% oxygen concentration in the chamber at 37˚C. Cells were incubated with PBS at 37˚C for 3 h, then PBS was removed and replaced with fresh medium containing 10% FBS. Further incubation for 3 h in 95% air and 5% $CO_2$ at 37˚C was performed for reoxygenation. The cells in the control and DMSO groups were kept in fresh medium containing 10% FBS in 95% air and 5% $CO_2$ at 37˚C. The cells in the RU.521 (10 mM in 1 ml DMSO; MedChemExpress) group were treated with 1 mmol/l RU.521 for 48 h at 37˚C. The cells in the H-151 (10 mM in 1 ml DMSO; MedChemExpress) group were treated with 2 $\mu$mol/l H-151 for 48 h at 37˚C. The cells in the SCU (10 mM in 1 ml DMSO; MedChemExpress) group were treated with 100 $\mu$mol/l SCU for 48 h at 37˚C. For the H/R + RU.521 group, the H/R-injured cells were treated with 1 mmol/l RU.521 for 48 h at 37˚C. For the H/R + H-151 group, the H/R-injured cells were treated with 2 $\mu$mol/l H-151 for 48 h at 37˚C. For the H/R + SCU group, the H/R-injured cells were treated with 100 $\mu$mol/l SCU for 48 h at 37˚C.

*Cell viability detection using the CCK-8 assay*. In brief, H9c2 cells ($5x10^3$ per well) were seeded into 96-well microplates, then placed in a sealed chamber filled with 95% $N_2$ and 5% $CO_2$ at 37˚C and treated with 0, 25, 50 and 100 $\mu$mol/l SCU (10 mM in 1 ml DMSO; MedChemExpress) for 48 h or treated with 100 $\mu$mol/l SCU for 0, 12, 24 and 48 h. A total of 10 $\mu$l CCK-8 (Beyotime Institute of Biotechnology) was added, followed by incubation at 37˚C for 1 h. The absorbance value of each well was detected at 490 nm using a microplate reader (Agilent Technologies, Inc.).

*Annexin V/PI staining*. Flow cytometry was used to detect the apoptosis rate of H9c2 cells. In brief, the H/R-injured cells were treated with 0, 25, 50 and 100 $\mu$mol/l SCU for 24 h. Subsequently, $\geq 1x10^5$ cells were resuspended in 100 $\mu$l binding buffer containing Annexin V-FITC and PI (Beyotime Institute of Biotechnology) and incubated at room temperature for 15 min. The BD FACScan™ system (BD Biosciences) was used to quantify Annexin V-FITC and PI binding using the channels FL-1 (Annexin V-FITC) and FL-3 (PI), and analysis was performed using BD CellQuest Pro™ software (version 5.1; BD Biosciences). Apoptosis was calculated as the sum of early and late apoptosis.

*Western blot analysis*. Pre-cooled H9c2 cells at 4˚C and left ventricular tissue from mice were lysed on ice for 30 min using RIPA Lysis Buffer (MedChemExpress), which contained 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1% Triton X-100 and 1% Phosphatase Inhibitor Cocktail I and III (MedChemExpress). Next, the cell lysis products were centrifuged for 10 min at 12,700 x g in a 4˚C refrigerated centrifuge and the supernatants were collected. The protein concentration was measured using a BCA protein kit (Thermo Fisher Scientific, Inc.) and the samples were boiled at 100˚C for 5 min. A total of 30 $\mu$g cellular protein/lane were electrophoresed using 10% SDS-PAGE, and transferred onto a PVDF membrane. The membrane was blocked for 2 h at room temperature with 5% BSA (Beyotime Institute of Biotechnology), incubated overnight at 4˚C with primary antibodies of GAPDH (dilution, 1:10,000; ab181602; Abcam), cGAS (dilution, 1:1,000; ab252416; Abcam), STING (dilution, 1:1,000; ab288157; Abcam), Bcl-2 (dilution, 1:1,000; ab196495; Abcam), Bax (dilution, 1:1,000; ab32503; Abcam) and cleaved Caspase-3 (dilution, 1:1,000; ab184787; Abcam), washed with TBS containing 0.1% Tween 20, and incubated for 2 h with HRP Anti-Rabbit IgG antibody (dilution, 1:10,000; ab184787; Abcam) at room temperature. The immunoreactivity of the proteins was visualized by chemiluminescence with immobilon western chemilum HRP substrate (MilliporeSigma). Signals were detected and analyzed with ChemiDoc™XRS + with Image Lab™ Software Gel Imaging System (version 2.0; Bio-Rad Laboratories, Inc.).

*Statistical analysis*. All experiments were performed in triplicate and the data are presented as the mean ± SD. Statistical significance was analyzed using one-way ANOVA followed by Dunnett's test for comparisons vs. a single control or Tukey's test for comparisons among multiple groups. GraphPad Prism (version 5.01; GraphPad Software; Dotmatics) was used to analyze the data. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*SCU ameliorates I/R injury-induced cardiac dysfunction and inhibits cardiomyocyte apoptosis*. Cardiac function (indicated by LVEF and LVFS) was measured by echocardiography to determine the effect of SCU treatment on I/R-injured mice. The results demonstrated that I/R injury induced a decrease in LVEF and LVFS in mice, which was ameliorated by SCU treatment (Fig. 1A and B). The effect of SCU treatment on I/R-induced cardiomyocyte apoptosis was detected using the TUNEL technique. The results revealed that I/R injury induced an increase in cardiomyocyte apoptosis in cardiac tissues, which was ameliorated by SCU treatment (Fig. 1C and D). The effect of SCU treatment on I/R-induced myocardial infarction (MI) was detected by TTC staining. The results demonstrated that the I/R-induced myocardial infarct area was decreased by SCU treatment compared with the I/R group (Fig. 1E and F).

*SCU inhibits activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways in cardiac tissues of I/R-injured mice*. The effect of SCU on the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways in I/R-injured

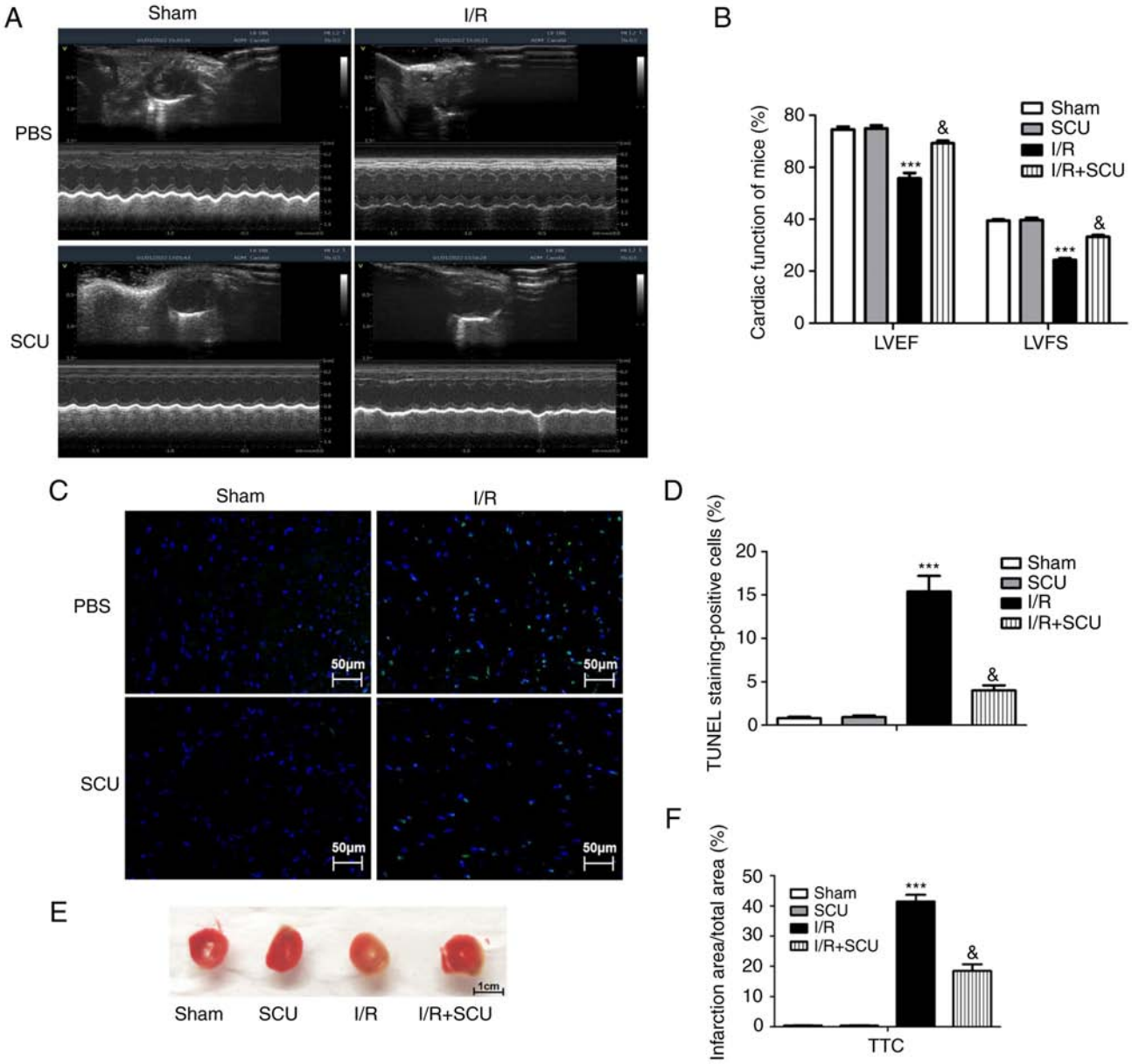


Figure 1. Effect of SCU on cardiac dysfunction and cardiomyocyte apoptosis in cardiac tissues of I/R-injured mice. (A) Cardiac function (LVEF and LVFS) of mice in the sham, SCU, I/R and I/R + SCU groups was detected by echocardiography. (B) LVEF and LVFS data were analyzed using GraphPad Prism. Data are presented as the mean ± SD. $^{***}$P<0.001 compared with the sham group; $^{\&}$P<0.001 compared with the I/R group. (C) Apoptotic cells were detected using a TUNEL assay, and (D) the TUNEL-positive cell percentage was determined using ImageJ and analyzed using GraphPad Prism. Scale bar, 50 $\mu$m. Data are presented as the mean ± SD. $^{***}$P<0.001 compared with the sham group; $^{\&}$P<0.001 compared with the I/R group. (E) Myocardial infarction was detected by TTC staining. (F) Infarct size (white area) was calculated using ImageJ software and analyzed using GraphPad Prism. Scale bar, 1 cm. Data are presented as the mean ± SD. $^{***}$P<0.001 compared with the sham group; $^{\&}$P<0.001 compared with the I/R group. SCU, scutellarin; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; I/R, ischemia/reperfusion; TTC, 2,3,5-triphenyltetrazolium chloride; ADM, add/drop multiplexer.

mice was assessed. The results revealed that SCU treatment reversed the I/R injury-induced increase in cGAS, STING and cleaved Caspase-3 expression, as well as the decrease in the Bcl-2/Bax ratio, in mouse cardiac tissues (Fig. 2).

*SCU inhibits cell apoptosis and activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways in H9c2 cells after H/R injury.* H/R-injured H9c2 cells were treated with SCU (0, 25, 50 and 100 $\mu$mol/l) for 48 h and cell viability was detected using a CCK-8 assay. The cell viability was significantly increased by 50 and 100 $\mu$mol/l SCU treatment compared with the H/R group (Fig. 3A). H/R-injured H9c2 cells were treated with SCU (100 $\mu$mol/l) and a CCK-8 assay was used to detect cell viability at 0, 12, 24 and 48 h. A significant increase in cell viability from 0 to 48 h was observed in H/R-injured cells treated with 100 $\mu$mol/l SCU compared with that at 0 h (Fig. 3B). The rate of apoptosis in H/R-injured H9c2 cells was detected by flow cytometry. SCU treatment significantly decreased the high apoptotic rate of HR-injured H9c2 cells (Fig. 3C and D). Since 100 $\mu$mol/l SCU had the highest anti-apoptotic effect in H/R-injured cells, 100 $\mu$mol/l SCU was selected for subsequent experiments.

Apoptosis and activation of the cGAS-STING signaling pathway were observed after I/R and/or H/R injury; therefore, the protein levels of cGAS, STING, Bcl-2, Bax and cleaved

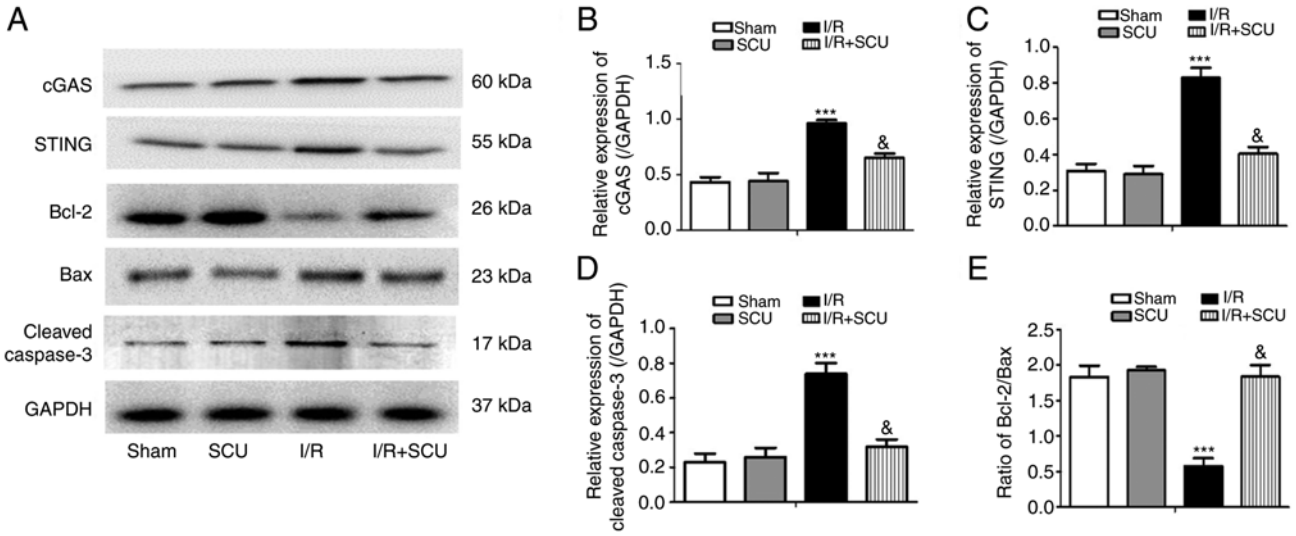


Figure 2. SCU inhibits the activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 pathways in cardiac tissues of I/R-injured mice. (A) Western blotting was performed to detect the expression levels of cGAS, STING, Bcl-2, Bax, cleaved Caspase-3 and GAPDH in mouse cardiac tissues. Relative expression levels of (B) cGAS, (C) STING and (D) cleaved Caspase-3, and (E) the Bcl-2/Bax ratio were calculated from the gray-scan value and analyzed using GraphPad Prism. Data are presented as the mean ± SD. $^{***}$P<0.001 compared with the sham group; $^{\&}$P<0.001 compared with the I/R group. SCU, scutellarin; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; I/R, ischemia/reperfusion.

Caspase-3 in H/R-injured H9c2 cells were detected. It was found that 100 $\mu$mol/l SCU significantly decreased the expression levels of cGAS, STING and cleaved Caspase-3, and increased the ratio of Bcl-2/Bax in H9c2 cells after H/R injury (Fig. 3E-I).

*Inhibition of cGAS inhibits activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways induced by H/R injury, similarly to SCU treatment.* To explore whether cGAS serves a role in the protective effective of SCU in cardiomyocytes from H/R injury-induced apoptosis, the activity of cGAS was inhibited using RU.521 (a cGAS inhibitor) and the effect was compared with that of SCU in H9c2 cells after H/R injury. The expression levels of cGAS, STING, Bcl-2, Bax and cleaved Caspase-3 were detected. H/R injury increased the expression levels of cGAS, STING and cleaved Caspase-3 and decreased the ratio of Bcl-2/Bax, and these effects were significantly reversed by RU.521 treatment, which was similar to the effect of SCU treatment (Fig. 4).

*Inhibition of STING inhibits activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways induced by H/R injury, similarly to SCU treatment.* STING is the downstream target of and can be activated by cGAS (14). However, it is unclear whether the cGAS-mediated apoptosis of H9c2 cells after H/R injury is dependent or independent of STING. Therefore, the STING inhibitor H-151 was used to inhibit STING, and the effect on H9c2 cells after H/R injury was compared with the effect of SCU. H/R injury induced upregulation of the expression levels of STING and cleaved Caspase-3, and reduced the ratio of Bcl-2/Bax, and these effects were significantly reversed by H-151 treatment, which was similar to the effect of SCU treatment (Fig. 5). However, H-151 treatment exhibited no effect on cGAS expression in response to H/R injury.

## Discussion

The present study revealed that SCU ameliorated I/R injury-induced cardiac dysfunction, and inhibited cardiomyocyte apoptosis and activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways in I/R-injured mice. *In vitro* experiments demonstrated that SCU significantly increased cell viability and decreased the apoptosis of H/R-induced H9c2 cells. Furthermore, it was observed that H/R injury increased the expression levels of cGAS, STING and cleaved Caspase-3, and decreased the ratio of Bcl-2/Bax, which was reversed by treatment with SCU and both cGAS and STING inhibitors.

SCU is a flavone isolated from *Stipa barbata* and *Erigeron breviscapus*, which has been reported to exert a broad range of cardiovascular pharmacological effects, including vasodilative, anti-inflammatory, anticoagulative and antithrombotic, myocardial protection and protection against I/R injury effects (26). It has been reported that SCU has an anti-apoptotic effect in cerebral I/R injury (21,22,25,27). SCU also inhibits apoptosis and oxidative stress in hepatocytes after H/R injury (28). Furthermore, SCU has protective effects in cardiovascular ischemia in rats (29). Xu *et al* (24) reported that SCU suppressed activation of the NLRP3 inflammasome to protect against myocardial I/R injury. Wang *et al* (23) found that SCU treatment protected cardiomyocytes from I/R injury-induced oxidative stress and apoptosis. In the present study, it was observed that SCU protected against cardiomyocyte I/R injury by increasing cell viability and decreasing apoptosis, and also improved the cardiac function of I/R-injured mice.

Different cell death forms, such as autophagy, ferroptosis and apoptosis, contribute to I/R injury. Deng *et al* (30) provided evidence that ferroptosis aggravated intestinal I/R injury. Huang *et al* (31) reported that inhibition of autophagy suppressed I/R injury-induced inflammation and apoptosis. Apoptosis is a gene-regulated programmed cell death process,

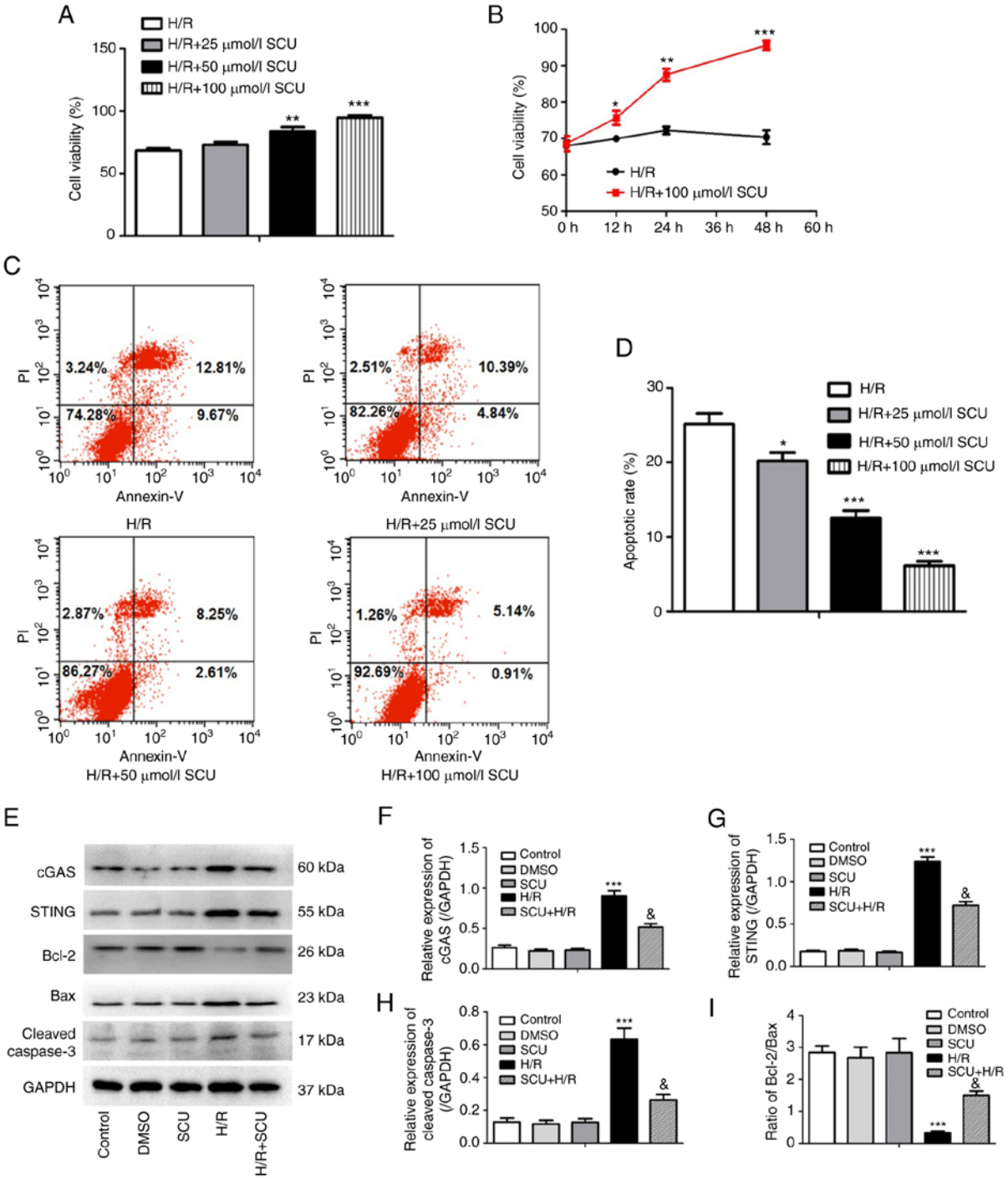


Figure 3. SCU inhibits cell apoptosis and activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways in H9c2 cells after H/R injury. (A) After H/R injury, H9c2 cells were exposed to SCU at concentrations of 0, 25, 50 and 100 $\mu$mol/l for 48 h, and then a CCK-8 assay was performed to detect cell viability. $^{**}$P<0.01 and $^{***}$P<0.001 compared with the H/R group. (B) After H/R injury, H9c2 cells were either exposed or not exposed to SCU (100 $\mu$mol/l), and then a CCK-8 assay was performed to detect viability at 0, 12, 24 and 48 h. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 compared with the 0 h group. (C) After H/R injury, H9c2 cells were treated with 0, 25, 50 and 100 $\mu$mol/l SCU for 24 h, and then the apoptotic rate of H9c2 cells was determined by flow cytometry. (D) Apoptosis rate was calculated as the sum of early and late apoptosis and analyzed using GraphPad Prism. Each graph represents the results of three independent experiments (data are presented as the mean ± SD). $^{*}$P<0.05 and $^{***}$P<0.001 compared with the H/R only group. (E) After H/R injury, H9c2 cells were exposed to 100 $\mu$mol/l SCU. The expression levels of cGAS, STING, Bcl-2, Bax and cleaved Caspase-3 were determined by western blotting, and the relative expression levels of (F) cGAS, (G) STING and (H) cleaved Caspase-3, and (I) the Bcl-2/Bax ratio were calculated from the gray-scan value and analyzed using GraphPad Prism. Each graph represents the densitometry results of three independent experiments (data are presented as the mean ± SD). $^{***}$P<0.001 compared with the control group; $^{\&}$P<0.001 compared with the H/R group. SCU, scutellarin; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; H/R, hypoxia/reoxygenation; CCK-8, Cell Counting Kit-8.

and abnormal regulation of this process has been associated with a variety of human diseases, including immune and developmental disorders, neurodegeneration and cancer (32). Members of the Bcl-2 protein family mainly regulate apoptosis via the mitochondrial pathway (33). The family contains pro-apoptotic and anti-apoptotic proteins that share

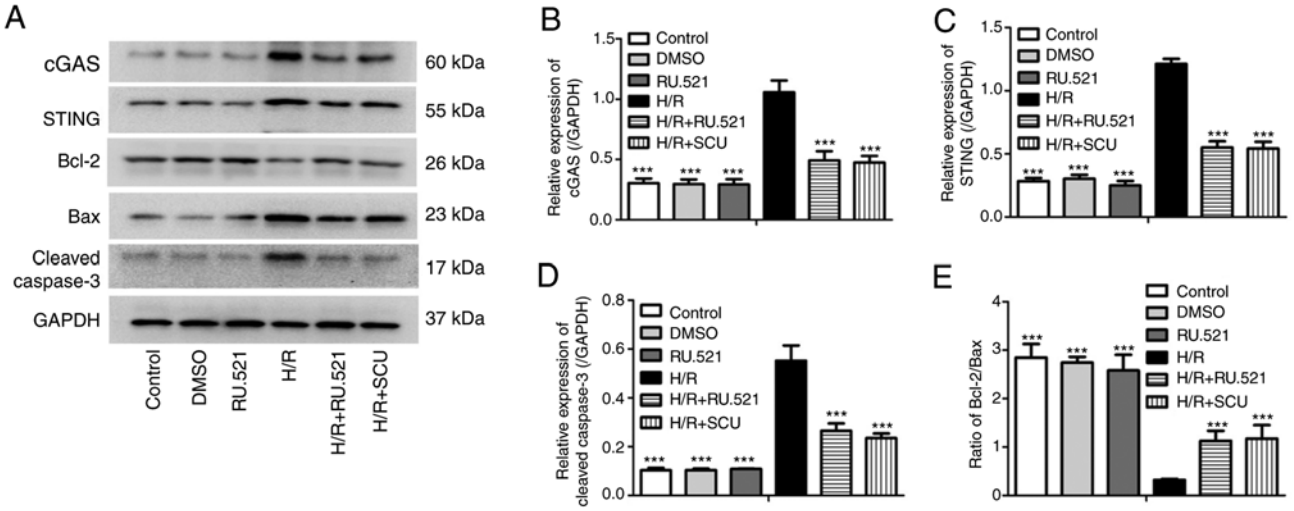


Figure 4. Inhibition of cGAS inhibits activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways induced by H/R injury, similarly to SCU treatment. After H/R injury, H9c2 cells were treated with 100 $\mu$mol/l SCU or 1 mmol/l RU.521 for 48 h. (A) Expression levels of cGAS, STING, Bcl-2, Bax and cleaved Caspase-3 were examined by western blotting. and the relative expression levels of (B) cGAS, (C) STING and (D) cleaved Caspase-3, and (E) the Bcl-2/Bax ratio were calculated from the gray-scan value and analyzed using GraphPad Prism. Each graph represents the densitometry results of three independent experiments (data are presented as the mean ± SD). $^{***}$P<0.001 compared with the H/R group. cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; H/R, hypoxia/reoxygenation; SCU, scutellarin.

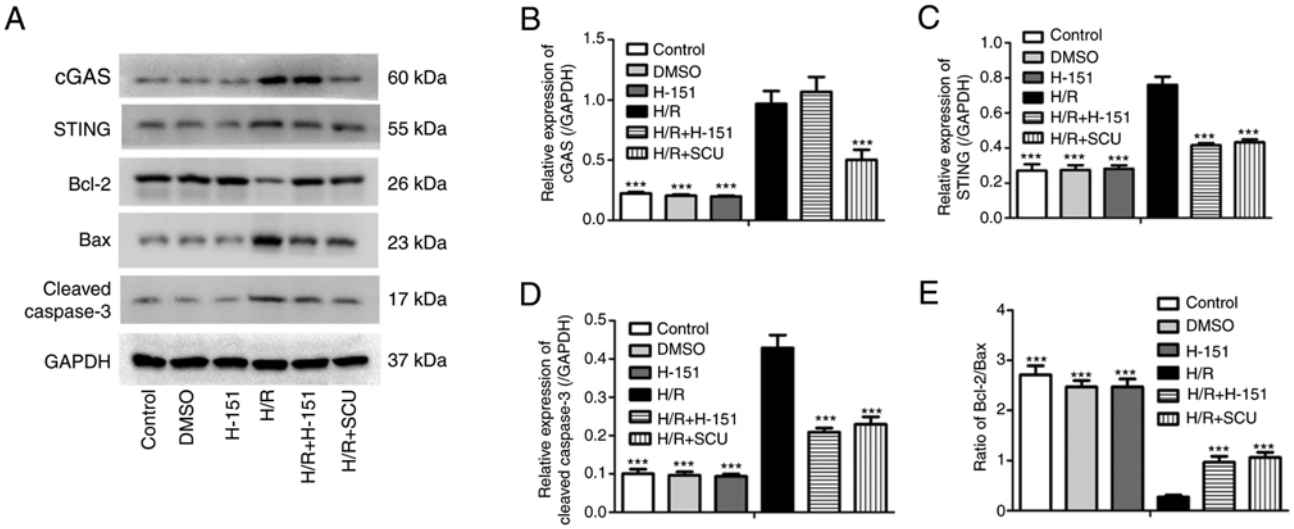


Figure 5. Inhibition of STING inhibits the activation of the cGAS-STING and Bcl-2/Bax/Caspase-3 signaling pathways induced by H/R injury, similarly to SCU treatment. After H/R injury, H9c2 cells were treated with 100 $\mu$mol/l SCU or 2 $\mu$mol/l H-151 for 48 h. (A) Expression levels of cGAS, STING, Bcl-2, Bax and cleaved Caspase-3 were examined by western blotting, and the relative expression levels of (B) cGAS, (C) STING and (D) cleaved Caspase-3, and (E) the Bcl-2/Bax ratio were calculated from the gray-scan value and analyzed using GraphPad Prism. Each graph represents the densitometry results of three independent experiments (data are presented as the mean ± SD). $^{***}$P<0.001 compared with the H/R group. STING, stimulator of interferon genes; cGAS, cyclic GMP-AMP synthase; H/R, hypoxia/reoxygenation; SCU, scutellarin.

four conserved Bcl-2 homology (BH) domains and form complexes by binding to the common BH3 domain (34). Bcl-2 itself is a key regulator that inhibits cell death by reducing cell permeability, cytochrome c release and calcium flow across endoplasmic reticulum membranes (35). The pro-apoptotic protein Bax of the Bcl-2 family is also an important regulator of apoptosis, mainly promoting apoptosis by inducing the release of Caspase-3 (36). Therefore, the Bcl-2/Bax ratio determines the sensitivity of cells to apoptotic stimuli (37). When the Bcl-2/Bax ratio is increased, apoptosis is inhibited, and when the Bcl-2/Bax ratio is decreased, apoptosis is promoted (38). The present study demonstrated that SCU reversed the I/R- or H/R-induced decrease in the Bcl-2/Bax ratio, which indicated that SCU had a protective role in apoptosis.

The inflammatory reaction is one of the most important elements in myocardial I/R injury (39). cGAS is a critical cytosolic DNA sensor with the function of generating cyclic GMP-AMP (40), which further binds and activates STING (41), inducing a strong innate immune response. Self-DNA leaked from the nucleus or mitochondria can also serve as a cGAS ligand to activate this pathway and trigger extensive inflammatory responses (42). The cGAS-STING signaling pathway is recognized as a main mediator

bridging innate and adaptive immunity (43,44), which is involved in local tissue inflammation (45). The cGAS response to cardiac ischemia is similar to that of a pattern recognition receptor in the sterile immune response (46). With regard to ischemic MI, cGAS can sense cytoplasmic DNA released from dying ruptured cells and can lead to fatal post-MI cardiac inflammation, which can be reversed by inhibition of the cGAS-STING signaling pathway (47). In the present study, it was revealed that H/R injury significantly increased the expression levels of cGAS, STING and cleaved Caspase-3, and decreased the Bcl-2/Bax ratio, which could be reversed by blocking the cGAS-STING signaling pathway using RU.521 and H-151. These results indicated that the cGAS-STING signaling pathway is involved in H/R injury-induced apoptosis of H9c2 cells.

SCU has been indicated to exhibit an anti-inflammatory effect in experimentally induced cerebral ischemia (48). Yuan *et al* (49) reported that SCU effectively suppressed the inflammatory responses in activated microglia, which were induced by cerebral ischemia, by decreasing TNF-α expression. It has also been reported that SCU exerts an anti-inflammatory effect to protect against myocardial I/R injury by suppressing NLRP3 inflammasome activation (24). Furthermore, it has been demonstrated that SCU exhibits strong anti-oxidative activity against ischemic injury (50). Zhang *et al* (51) reported that SCU alleviated cerebral I/R by suppressing oxidative stress and inflammatory responses via the MAPK/NF-κB signaling pathways in rats. Wu and Jia (28) reported that SCU attenuated H/R injury in hepatocytes by inhibiting apoptosis and oxidative stress. It has also been demonstrated that cellular oxidative stress activated the cGAS/STING/IFN-I signaling pathway via FOXO3-regulated lamin post-translational modification (52). However, to the best of our knowledge, the effect of SCU on the cGAS-STING signaling pathway has not been elucidated. The present study revealed that SCU treatment reversed the I/R injury-induced upregulation of cGAS and STING, which indicated that SCU can inhibit the cGAS-STING signaling pathway. Considering the anti-oxidative stress role of SCU (50) and the activation of cGAS-STING by oxidative stress (52), inhibition of the cGAS-STING pathway by SCU may be achieved by inhibiting oxidative stress, which should be confirmed in future studies.

In summary, to the best of our knowledge, the present study was the first to demonstrate that SCU ameliorated cardiac dysfunction and protected cardiomyocytes from I/R injury-induced apoptosis via deactivation of the cGAS-STING signaling pathway. Considering the protective effect of SCU on cardiomyocyte H/R injury, SCU may be a promising agent for the treatment of IHD in the future.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

JKL contributed to performing the experiments and data analysis. ZPS contributed to performing the experiments. XZH contributed to data analysis and study design. JKL and XZH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The animal experiment was approved by the Animal Ethics Committee of The People's Hospital of Yue Chi County (Guang'an, China; approval no. 21000104).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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