

# Stanniocalcin 1 alleviates myocardial ischemia-reperfusion injury through inhibiting inflammation and apoptosis of myocardial cells

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**Abstract.** – **OBJECTIVE:** Myocardial ischemia-reperfusion injury (MIRI) is the main cause of death from ischemic heart diseases. Stanniocalcin 1 (STC1) has a potential therapeutic effect on MIRI. The purpose of this study is to investigate the effect of STC1 on inflammation and apoptosis of myocardium in MIRI.

**MATERIALS AND METHODS:** We used rats to make ischemia-reperfusion (I/R) models and determined the efficiency of modeling by 2, 3, 5-triphenyl tetrazolium chloride staining, echocardiography, and lactate dehydrogenase detection. We injected subcutaneously recombinant human STC1 (2.5 µg/kg, 5 µg/kg) into rats daily one week before modeling to detect the effect of STC1 pretreatment on inflammation and apoptosis of rat myocardial cells. In addition, we cultured rat myocardial cell lines (H9c2 cells) to investigate the effect of STC1 on myocardial cells.

**RESULTS:** The cardiac function and structure of I/R rats were obviously destroyed. After treating rats with STC1, we found that the cardiac function and structure of the rats were significantly improved. In addition, STC1 reduced the expression of inflammatory factors and apoptosis levels in rat myocardium. Stimulation of STC1 also improved the viability of H9c2 cells *in vitro*.

**CONCLUSIONS:** Therefore, STC1 can alleviate MIRI by inhibiting inflammation and apoptosis. It indicated that STC1 may have a potential therapeutic effect on MIRI.

*Key Words:*

Myocardial ischemia-reperfusion injury, Stanniocalcin 1, Inflammation, Apoptosis.

## Introduction

Ischemic heart disease (IHD) is the leading cause of death from cardiovascular disease<sup>1</sup>.

The basis of IHD is the reduction of coronary blood flow, which leads to insufficient blood supply to the myocardium<sup>2</sup>. The pathological characteristics include coronary artery lumen stenosis and even occlusion. It is commonly found in the coronary atherosclerotic cardiopathy and coronary spasm<sup>3</sup>. Myocardial ischemia can induce angina and myocardial infarction, leading to a sharp decrease in cardiac output and blood supply. Reperfusion therapy can restore the coronary blood supply to the ischemic area of the heart, and is considered to be the most effective treatment for myocardial ischemia<sup>4</sup>. However, although the restored coronary blood flow relieves the myocardial ischemia and hypoxia, irreversible damage to the myocardium may be exacerbated during the reperfusion period, resulting in deterioration of cardiac function. This is called myocardial ischemia-reperfusion (I/R) injury (MIRI)<sup>5</sup>. Factors such as the generation of large amounts of oxygen free radicals during the reperfusion period, the release of inflammatory cytokines, calcium overload, and dysfunction of mitochondrial energy utilization of myocardial cells are important causes of myocardial cells death<sup>6</sup>. MIRI can lead to myocardial metabolic disorders, myocardial ultrastructure changes, arrhythmia, and systolic dysfunction<sup>7</sup>. Therefore, alleviating MIRI and improving the biological function of ischemic myocardium is the key to improving the prognosis of IHD patients.

Stanniocalcin (STC) is a glycoprotein hormone commonly expressed in mammals. The human STC family consists of STC1 and STC2. STC1 participates in various physiological func-

tions in the body in an autocrine or paracrine manner<sup>8</sup>. STC1 was found to prevent damage to brain neurons caused by cerebral ischemia. Bonfante et al<sup>9</sup> used recombinant human STC1 to treat cerebral I/R rats and found that STC1 can alleviate cerebral I/R injury by reducing oxidative stress and blood-brain barrier permeability. In addition, STC1 has also been found to relieve renal I/R injury by regulating the level of reactive oxygen species (ROS)<sup>10</sup>. However, the effect of STC1 on MIRI was still unclear. Therefore, we used rats to make I/R models and treated I/R rats with recombinant STC1 to study the effect of STC1 on MIRI.

## Materials and Methods

### Animals

Forty-eight 8-week-old healthy male Sprague Dawley rats were used in this study. Rats are housed in SPF-class animal houses and fed with clean food and water. This study has been approved by the Ningxia Medical University Experimental Animal Ethics Committee.

### Procedure of I/R Model

We anesthetized the rats by intraperitoneal injection with 2% sodium pentobarbital (40 mg/kg) and connected the rats' limbs to the ECG monitor. Then, we used a small animal ventilator (CWE SAR-830, Orange, CA, USA) to keep the rats breathing. The parameters of the small animal ventilator were set as the heart rate of 90 times/min, the breathing rate of 60 times/min and the breathing ratio of 1: 2. We, then, used sterile scissors to open the left thoracic cavity of the rats and sutured the left anterior descending coronary artery of the rats with sterile sutures. The elevated ST segment on the electrocardiogram indicated that the coronary artery was successfully blocked. Then, we loosened the suture after 30 min. A decrease in the ST segment of the ECG indicated coronary recanalization. After 180 min of reperfusion, we detected the rat cardiac function and collect serum and heart tissue. The rats were divided into sham group, I/R group, I/R + low-dose STC1 (LSTC1) group and I/R + high-dose STC1 (HSTC1) group. The rats in the sham group were only opened the chest cavity without ligating the coronary arteries. Rats in the I/R group, the I/R + LSTC1 group and the I/R + HSTC1 group were made I/R models. Rats in

the I/R + LSTC1 group and the I/R + HSTC1 group were injected with recombinant human STC1 (2.5 µg/kg, 5 µg/kg) (Sigma-Aldrich, St. Louis, MO, USA) daily subcutaneously one week before the model was made<sup>11</sup>.

### 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) Staining

After collecting the rat heart, we washed the heart with normal saline. Then, we put the rat heart in the -20°C refrigerator for 20 min. The rat heart tissue was cut into slices with thickness of 2 mm along the vertical direction of the longitudinal axis. Then, we put the heart slices into 1% TTC staining solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Normal tissue appeared red, while ischemic tissue appeared pale. Myocardial ischemic area was represented as the percentage of ischemic area in the left ventricle.

### Echocardiography

After 180 min of reperfusion in rats, we used Acuson Sequoia 512 high-frequency ultrasound diagnostic equipment (Acuson, Malvern, PA, USA) to detect rat cardiac function. We determined the left ventricular end-systolic diameter (LVSD), left ventricular end-diastolic diameter (LVDD), left ventricular end-systolic volume (LVSV), and left ventricular end-diastolic volume (LVDV) by continuously detecting 3 cardiac cycles. Left ventricular ejection fraction (LVEF) = (LVDV-LVSV) / LVDV ×%. Left ventricular shortening fraction (LVFS) = (LVDD-LVSD) / LVDD × 100%.

### Hematoxylin-Eosin (HE) Staining

After collecting the rat heart, we put the rat myocardial tissue in 4% paraformaldehyde for 24 h and made paraffin blocks. Then, we used a microtome to cut the paraffin blocks into paraffin sections with 5µm. Subsequently, we placed the paraffin slices in a 37°C oven for 3 d. The slices were put in turn in xylene and gradient alcohol (100%, 95%, 80% and 75%). Then, we stained slices with hematoxylin (Beyotime, Shanghai, China) for 1 min and rinsed the slices with running water. We also stained the cytoplasm with eosin (Beyotime, Shanghai, China) for 1 min and dehydrated them with gradient alcohol. Finally, we used neutral gum to seal the slides and observe the staining results using an optical microscope.

**Immunohistochemical (IHC) Staining**

After dewaxing and hydrating the slices, we used citrate buffer to repair the antigens in myocardial tissue. Then we used 3% H<sub>2</sub>O<sub>2</sub> to incubate myocardial tissue for 30 min. 10% goat serum was used to block myocardial tissue for 1 h. Then, we used primary antibody dilution (caspase3, ab13847; caspase9, ab202068. Abcam, Cambridge, MA, USA) to incubate myocardial tissue at 4°C overnight. After washing the slices with phosphate-buffered saline (PBS), we used secondary antibody dilution (GeneTech, Shanghai, China) to incubate the myocardial tissue for 1 h and developed the color. Then, we stained the cell nucleus with hematoxylin and dehydrated myocardial tissue with gradient alcohol. Finally, we used neutral gum to seal the slides and observe the staining results using an optical microscope.

**Cell Culture and Hypoxia Reoxygenation (H/R) Model**

The rat myocardial cells line (H9c2) was used to study the effect of STC1 on myocardial cells. Dulbecco's modified eagle medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% double antibody was used to culture H9c2 cells. We induced I/R *in vitro* through the H/R model. We firstly used sugar-free DMEM instead of normal DMEM to culture H9c2 cells and placed cells in an anaerobic chamber for 6 h. Then, we used normal DMEM to continue culturing H9c2 cells for 24 h.

**RNA Isolation and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

The TRIzol (Invitrogen, Carlsbad, CA, USA) method was used to extract total RNA from rat myocardium and H9c2 cells. Then, we used a spectrophotometer to detect the concentration of total RNA. The reverse transcription system was configured according to the concentration of RNA. After reversing the mRNA into complementary deoxyribose nucleic acid (cDNA) using a PCR machine, we amplified the corresponding sequence using SYBR Green Master Mix (Vazyme, Nanjing, Jiangsu, China) and primers. Primer sequences were shown in Table I. The expression of endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to calculate the expression of different targets. The relative expression level of RNA was represented as  $2^{-\Delta\Delta Ct}$ .

**Cell Counting Kit 8 (CCK8) Assay**

After H9c2 cells were passaged to 96-well plates and treated differently. We added 10  $\mu$ L of CCK8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) to each well and placed the 96-well plate in an incubator for 2 h. Then, we used a microplate reader to measure the absorbance (OD) of each well at 450 nm. We set up blank and control wells. The blank wells contained only medium, while the control wells contained cells but were not treated. Cell viability =  $(OD_{\text{sample}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}})$ .

**Table I.** RT-PCR primers.

Name	Sense/anti-sense (S/AS)	Sequence (5'-3')
IL-1 $\beta$	S	CCCTTGACTTGGGCTGT
	AS	CGAGATGCTGCTGTGAGA
IL-6	S	CTCACGCACCGATGTCT
	AS	AGGCTGTGGGCTCAATC
IL-8	S	CTCTTGCCGCTGTTCAT
	AS	GCGTTTCTGTCTCATGTGG
TNF- $\alpha$	S	CAGCCAGGAGGGAGAAC
	AS	GTATGAGAGGGACGGAACC
Bax	S	CGGCTGCTTGTCTGGAT
	AS	TGGTGAGTGAGGCAGTGAG
Bcl2	S	GTCACAGAGGGGCTACGA
	AS	GTCCGGTTGCTCTCAGG
caspase3	S	TGGACAACAACGAAACCTC
	AS	ACACAAGCCCATTTTCAGG
caspase9	S	TCGCCAATGCTGTTTCTG
	AS	TCGAAATCCTTGCTTCACG
GAPDH	S	GTTGTGGCTCTGACATGCT
	AS	CCCAGGATGCCCTTTAGT

### Enzyme Linked Immunosorbent Assay (ELISA)

We detected the levels of lactate dehydrogenase (LDH) and inflammatory factors (including IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ ) in rat serum by ELISA kits (R&D Systems, Emeryville, CA, USA). We collected myocardial tissue lysate and collected the supernatant by centrifugation (13000 rpm, 15 min, 4°C). Then we used the standard in ELISA kits to make a standard curve. Subsequently, we calculated the concentration of LDH and inflammatory factors based on the absorbance of the sample and the standard curve. The LDH concentration in H9c2 cells was also detected.

### Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick End Labeling (TUNEL) Assay

We fixed the cells with 4% paraformaldehyde for 30 min and soaked the cells with 0.1% Triton X-100 for 15 min. After washing the cells with PBS, we added 50  $\mu$ L of TUNEL detection solution (Sigma-Aldrich, St. Louis, MO, USA) and incubated cells for 60 min in the dark. Then, we stained the cell nucleus with DAPI and washed cells three times with PBS. Finally, we sealed the slides with anti-fluorescence quencher and observe the staining results using a fluorescence microscope.

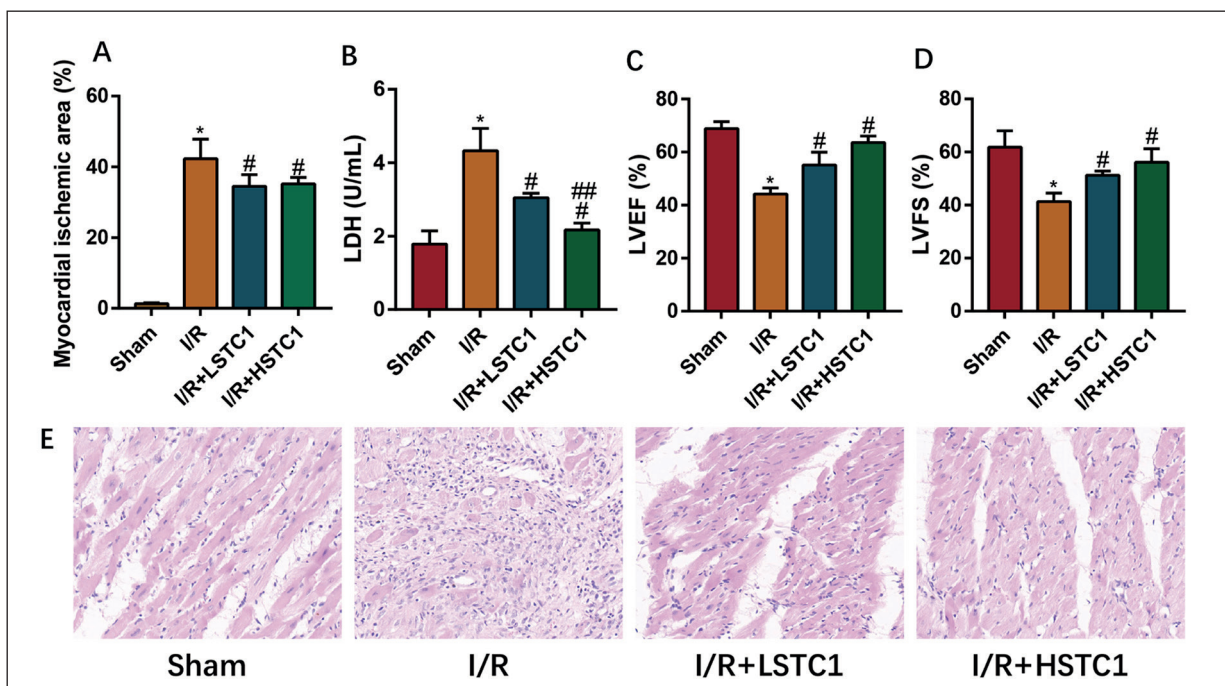
### Statistical Analysis

Statistical Product and Service Solutions 21.0 statistical software (IBM, Armonk, NY, USA) was used to analyze the results of this study. GraphPad Prism 7.0 (La Jolla, CA, USA) was used to draw column graphs. The *t*-test was used to analyze the difference between the two sets of data. For the differences between multiple groups, we use one-way analysis of variance for analysis.  $p < 0.05$  was considered statistically significant. All experiments were repeated three times.

## Results

### STC1 Improved the Structure and Function of Myocardium in I/R Rats

To determine the effect of STC1 on MIRI, we made the I/R rat model and treated the rats with recombinant human STC1. TTC staining detected the myocardial ischemic area in rats. The myocardium of I/R rats showed a large area of ischemia, while the myocardial ischemia area of STC1-treated rats was significantly reduced (Figure 1A). In addition, we detected the level of myocardial injury marker LDH in rat serum. STC1 significantly reduces the level of LDH in



**Figure 1.** STC1 improved the structure and function of myocardium in I/R rats. **A**, Myocardial ischemic area of rats; **B**, LDH level in rat serum; **C**, **D**, LVEF and LVFS of rats; **E**, HE staining of rat myocardium (200 $\times$ ). (“\*”) means  $p < 0.05$  vs. sham group; (“#”) means  $p < 0.05$  vs. I/R group; (“##”) means  $p < 0.05$  vs. I/R+LSTC1 group).

the serum of I/R rats and high concentrations of STC1 had a better effect (Figure 1B). Echocardiography detected changes in rat cardiac function. LVEF (Figure 1C) and LVFS (Figure 1D) of I/R rats were significantly reduced, while STC1 increased LVEF and LVFS of rats. HE staining detected the structure of rat myocardium. The myocardial tissue structure of I/R rats was disordered, and inflammatory cells infiltrate in the interstitium, while the myocardial structure of rats treated with STC1 was significantly improved (Figure 1E).

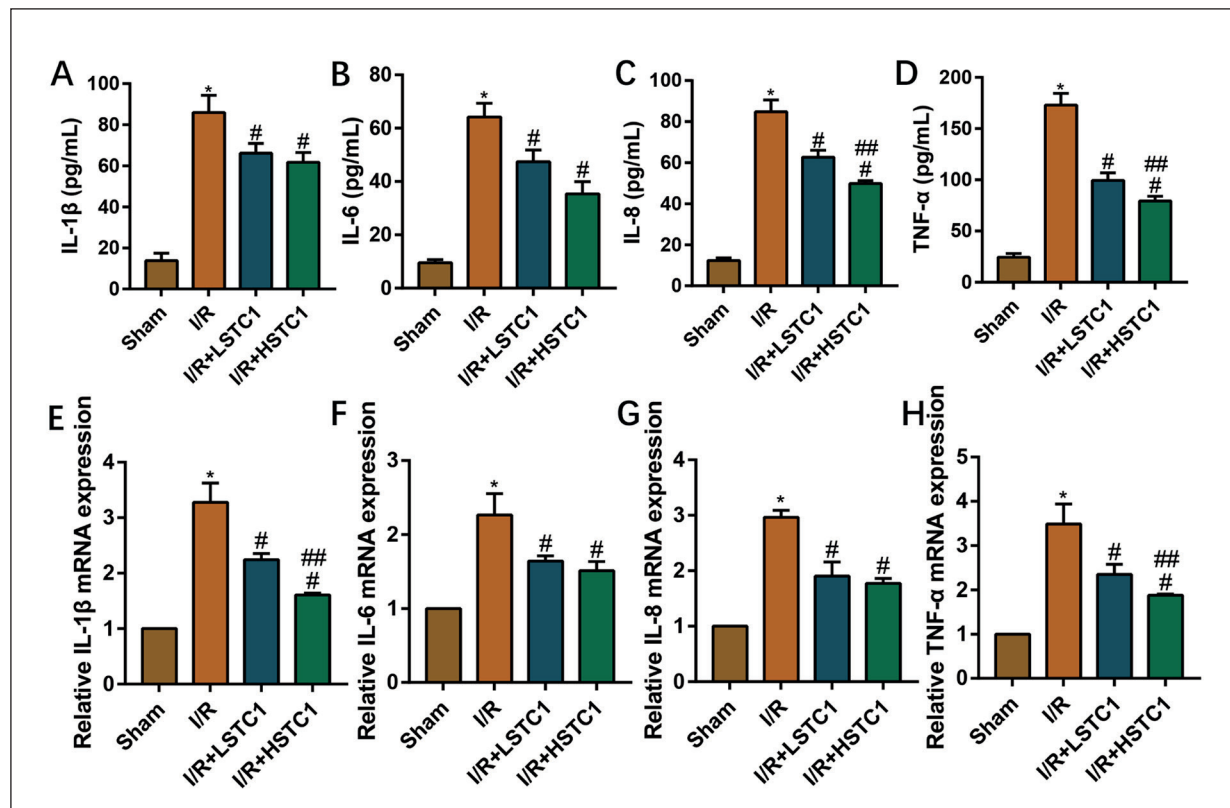
### STC1 Inhibited the Inflammation Level of I/R Rat Myocardium

In order to determine the effect of STC1 on MIRI-induced inflammation, we examined changes in the expression of inflammatory factors in rats. ELISA detected the concentration of IL-1 $\beta$  (Figure 2A), IL-6 (Figure 2B), IL-8 (Figure 2C) and TNF- $\alpha$  (Figure 2D) in rat serum. The expression of inflammatory factors in I/R rats increased significantly, and the treatment of STC1 reduced the concentration of these in-

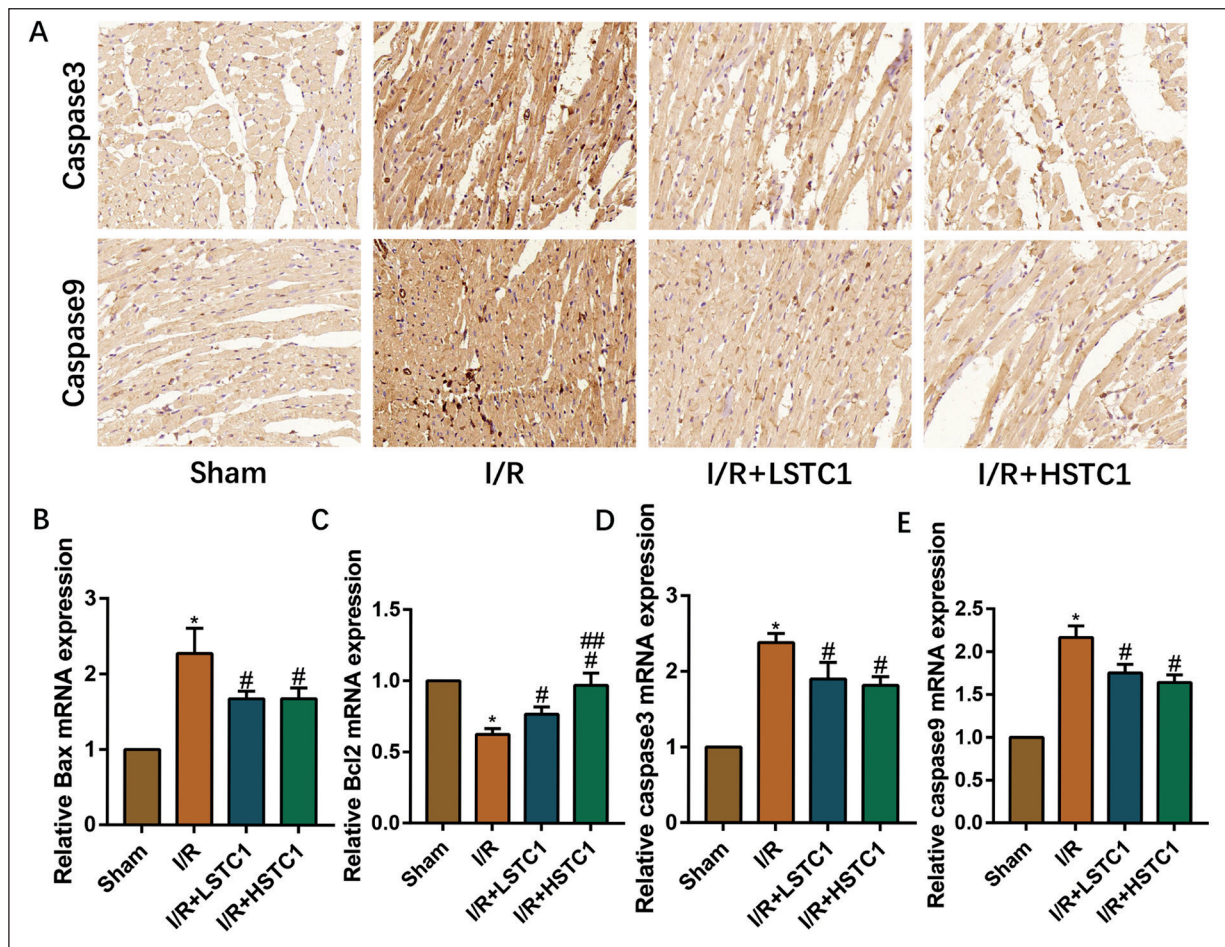
flammatory factors in serum. The treatment of high concentrations of STC1 got better results. RT-PCR detected the mRNA expression changes of inflammatory factors in rat myocardium (Figure 2E-2H). STC1 also significantly reduced the mRNA expression of inflammatory factors in myocardial cells.

### STC1 Reduced the Apoptosis of Myocardial Cells in I/R Rats

The occurrence of MIRI was accompanied by a large number of myocardial cells apoptosis, so we examined the effect of STC1 on rat myocardial cells apoptosis. IHC staining detected the expression of caspase3/9, the key signaling molecules in the cascade of apoptosis. The expression of caspase3/9 in myocardial cells of I/R rats was significantly increased, and treatment with STC1 can reduce their expression (Figure 3A). RT-PCR detected the mRNA expression of caspase3/9, pro-apoptotic molecule Bax and anti-apoptotic molecule Bcl2. STC1 was found to promote Bcl2 mRNA expression and inhibit caspase3/9 and Bax mRNA expression (Figure 3B-3E).



**Figure 2.** STC1 inhibited the inflammation level of I/R rat myocardium. **A-D**, ELISA results of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  in rat serum; **E-H**, mRNA expression of IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  in rat myocardium. (“\*” means  $p < 0.05$  vs. sham group; “#” means  $p < 0.05$  vs. I/R group; “##” means  $p < 0.05$  vs. I/R+LSTC1 group).



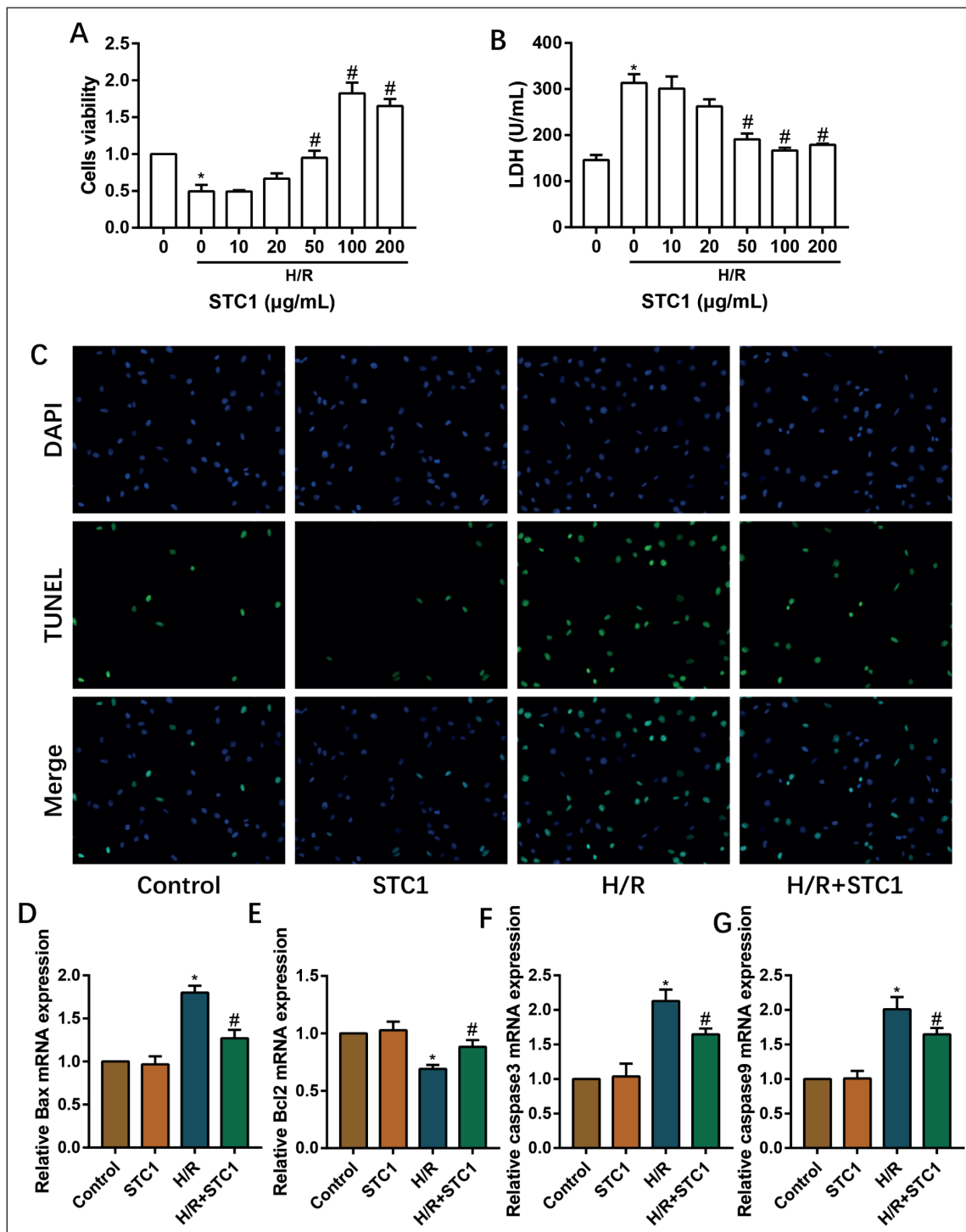
**Figure 3.** STC1 reduced the apoptosis of myocardial cells in I/R rats. **A**, IHC staining results of caspase3/9 in rat myocardium (200 $\times$ ); **B-E**, mRNA expression of Bax, Bcl2 and caspase3/9 in rat myocardium. (“\*” means  $p < 0.05$  vs. sham group; “#” means  $p < 0.05$  vs. I/R group; “##” means  $p < 0.05$  vs. I/R+LSTC1 group).

### STC1 Improves the Viability of H9c2 Cells

To clarify the effect of STC1 on myocardial cells, we induced H9c2 cells injury through H/R and stimulated H9c2 with STC1. CCK8 detected the effects of 10, 20, 50, 100 and 200  $\mu\text{g}/\text{mL}$  recombinant human STC1 on the viability of H9c2 cells. The viability of H9c2 cells in the H/R group decreased significantly, while STC1 dose-dependently increased the viability of H9c2 cells and 100  $\mu\text{g}/\text{mL}$  was considered as the optimal concentration (Figure 4A). In addition, STC1 also significantly reduced LDH levels in H9c2 cells (Figure 4B). The results of TUNEL staining showed that the stimulation of STC1 significantly inhibited H/R-induced apoptosis (Figure 4C). RT-PCR results also showed that STC1 decreased the expression of caspase3/9 and Bax, and increased the expression of Bcl2 (Figure 4D-4G).

### Discussion

Ischemic heart disease (IHD) causes myocardial ischemia and insufficient perfusion, which greatly threatens human health. However, reperfusion can lead to malignant arrhythmias, heart failure and even death<sup>12</sup>. Therefore, finding a way to prevent or alleviate MIRI is essential to improve the prognosis of IHD patients. As a glycoprotein hormone in the human body, STC1 has been found to have a good preventive effect on MIRI. After using STC1 to treat I/R rats, the cardiac function and structure of the rats were significantly improved. In addition, the levels of inflammation and apoptosis in rat myocardium were significantly reduced. STC1 also improves the viability of H9c2 cells *in vitro* and reduces the level of apoptosis. Therefore, STC1 has potential therapeutic prospects for MIRI.



**Figure 4.** STC1 improves the viability of H9c2 cells. **A**, CCK8 assay results of H9c2 cells; **B**, LDH level in DMEM of H9c2; **C**, TUNEL staining results in H9c2 cells (200×); **D-G**, mRNA expression of Bax, Bcl2 and caspase3/9 in H9c2 cells. (“\*” means  $p < 0.05$  vs. control group; “#” means  $p < 0.05$  vs. H/R group).

MIRI is a complex pathophysiological process involving myocardial cell apoptosis, inflammatory response, coronary endothelial injury and neutrophil infiltration etc<sup>13</sup>. Inflammation is closely

related to MIRI. When MIRI occurs, vascular endothelial cells can release a large number of inflammatory factors, such as IL-6 and TNF- $\alpha$ . Inflammatory factors can further promote the

cytokine cascade and induce the expression of intercellular adhesion molecules, thereby aggravating myocardial injury<sup>14</sup>. Birnbaum et al<sup>15</sup> found large amounts of inflammatory factors in rats in an I/R rat model, and they found that the anti-platelet drug ticagrelor can reduce the level of inflammation in I/R rats. Cain et al<sup>16</sup> found in a randomized controlled study that the inflammatory factors TNF- $\alpha$  and IL-1 $\beta$  can synergistically inhibit human cardiac function. Therefore, in the treatment of MIRI, it is necessary to suppress the destruction of myocardial cells by excessive inflammatory response. STC1 has been found to play an anti-inflammatory role in multiple diseases. A study found that up-regulation of STC1 can inhibit inflammation of fibroblast-like membrane cells and prevent the progression of osteoarthritis<sup>17</sup>. In addition, mesenchymal stem cells have also been found to inhibit the inflammatory response of endothelial cells by secreting STC1<sup>18</sup>. In our study, we detected the expression of inflammatory factors in rat serum and myocardial tissue by ELISA and RT-PCR. STC1 showed good anti-inflammatory effects and inhibited the expression of various inflammatory factors.

Apoptosis is one of the important links of MIRI pathogenesis and the degree of apoptosis determines the severity of MIRI injury<sup>19</sup>. Apoptosis genes can be divided into two categories. The first type is pro-apoptotic protein, which can induce apoptosis, such as Bax. The second type is anti-apoptotic protein, which has anti-apoptotic and protective cell survival effects, such as Bcl2. When Bcl2 is combined with Bax, it can inhibit the apoptosis of Bax. Under normal physiological conditions, the levels of Bcl2 and Bax are in dynamic equilibrium. When the balance between Bcl2 and Bax is broken by external influences, Bax is secreted in large amounts and Bcl2 cannot fully bind Bax in the body. Excessive Bax causes the caspase cascade reaction system to be activated, thereby promoting apoptosis<sup>20</sup>. STC1 has been found to be closely related to apoptosis in tumor diseases. In prostate cancer, monoclonal antibodies to STC1 have been found to reduce the proliferation capacity of tumor cells and promote their apoptosis<sup>21</sup>. In addition, Dai et al<sup>22</sup> found that klotho affects the growth and apoptosis of follicular thyroid cancer cells by regulating STC1. STC1 also showed a good anti-apoptotic effect in inflammatory diseases. Tang et al<sup>23</sup> found that STC1 inhibited lipopolysaccharide-induced inflammatory and apoptotic responses in mice. Therefore, we also studied the effect of STC1 on

myocardial cells apoptosis in MIRI. We found that STC1 reduced Bax/Bcl2 in myocardial cells *in vivo* and *in vitro*, and decreased the expression of caspase family. These results showed that STC1 effectively reduced myocardial cells apoptosis.

In short, this is the first study to investigate the effect of STC1 on MIRI. The results of this study indicated that STC1 has a potential effect on MIRI. We hoped that this research will provide new targets for the prevention and treatment of clinical MIRI.

## Conclusions

As a glycoprotein hormone in the human body, STC1 has a potential therapeutic effect on MIRI. Treatment with recombinant human STC1 reduced the expression of inflammatory factors in I/R rats. In addition, STC1 also reduced the expression of Bax and caspase3/9 in rat myocardial cells, and increased the expression of anti-apoptotic molecule Bcl2. Recombinant human STC1 also increased the viability of H9c2 cells *in vitro*.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

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