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Cystatin C alleviates H_2O_2-induced H9c2 cell injury

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Abstract. – OBJECTIVE: At present, the incidence of acute myocardial infarction is increasing year by year, and it has become one of the diseases with the highest mortality rate in humans. Myocardial ischemia-reperfusion injury (MIRI) is a major problem in the treatment of myocardial infarction, but clinically there is no effective way to treat MIRI. This study used Cystatin C (Cys C) to treat cardiomyocytes and rats to investigate the effect of Cys C on MIRI.

MATERIALS AND METHODS: We used H_2O_2 to induce rat cardiomyocytes (H9c2 cells) injury and stimulated the cells with Cys C. Cell counting kit 8 (CCK8) assay was used to determine the optimal concentration of H_2O_2 and Cys C to stimulate H9c2 cells. We determined the effects of Cys C on oxidative stress and apoptosis levels in H9c2 cells by measuring the activity of dehydrogenase (LDH), superoxide dismutase (SOD) and malondialdehyde (MDA), and the expression of apoptosis-related molecules (caspase3/8/9, Bax and Bcl-2). Changes in the activity of the NF-κB signaling pathway in H9c2 cells were also detected. In addition, we made rat MIRI models by ligating the coronary arteries and used Cys C to treat rats to verify the effect of Cys C on MIRI.

RESULTS: According to the results of the CCK8 assay, 1000 μM of H_2O_2 and 15 μM of Cys C were used to stimulate H9c2 cells. Cys C alleviated H_2O_2-induced H9c2 cell injury, manifested as a decrease in LDH and MDA activity and an increase in SOD activity. Cys C also reduced the apoptosis level in H9c2 cells. The activity of NF-κB signaling pathway in injured H9c2 cells was increased, and stimulation of Cys C could inhibit the NF-κB signaling pathway in H9c2 cells. The application of Cys C in MIRI rats also verified its therapeutic effect on MIRI.

CONCLUSIONS: Cys C reduced the oxidative stress and apoptosis levels of cardiomyocytes by inhibiting the activity of NF-κB signaling pathway in cardiomyocytes, thereby reducing cardiomyocyte injury and treating MIRI.

Key Words:
Cystatin C, H9c2, Myocardial ischemia-reperfusion injury, Apoptosis.

Introduction

Acute myocardial infarction (AMI) is one of the main causes of death worldwide[1]. Its main cause is the acute obstruction of coronary blood flow, which causes acute ischemia and hypoxia in the cardiomyocytes of the corresponding blood supply area. Timely reperfusion to restore coronary blood flow is the most effective method to save surviving ischemic cardiomyocytes and reduce mortality in patients with myocardial infarction. Reperfusion terminates further ischemic injury, but it may cause fatal cardiomyocyte injury, called myocardial ischemia-reperfusion injury (MIRI), which together with ischemia-induced injury determines the final area of myocardial infarction and the process of ventricular remodeling and eventually progresses to heart failure[2]. The mechanism of MIRI involved increased oxygen free radical production, calcium overload, and inflammatory reactions caused by the activation of various immune cells[3]. MIRI reduces the therapeutic effect of reperfusion therapy in patients with AMI, so exploring the mechanism of MIRI is an urgent problem in the prevention and treatment of ischemic diseases[4]. The adjustment of endogenous protective mechanism can reduce reperfusion injury and improve the prognosis of AMI patients. The current methods of mobilizing endogenous protective mechanisms to reduce MIRI include ischemic preconditioning, ischemic postconditioning and pharmacological postconditioning[5]. The myocardial protective effect of

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ischemic preconditioning was first discovered by Murry et al[6] in 1986. This measure needs to be performed before ischemia. Due to the unpredictability of myocardial infarction in patients, the clinical application of ischemic preconditioning is very limited[6]. The myocardial protective effect of ischemic postconditioning was first discovered by Zhao et al[7]. It is used after myocardial ischemia before reperfusion, but the balloon needs to be expanded repeatedly in the implementation process. There may be a risk of vessel wall damage and plaque falling off to form microthrombus, so its application is also limited. Pharmacological postconditioning refers to a class of myocardial protection measures that administer drugs within a few minutes before or after reperfusion to regulate the endogenous protective mechanism *in vivo*[8]. Its advantage is to avoid mechanical damage to the vascular system caused by ischemic postconditioning. Pharmacological postconditioning occurs after ischemia. It has the characteristics of predictability, controllability, and convenient operation, so it has a good clinical application prospect in the treatment of MIRI[9]. At present, some animal experiments have found that the use of various substances can reduce MIRI, such as bradykinin, adenoids, opioids, adrenomedullin, natriuretic peptide, urotensin, urothelin, leptin, Glucagon-like skin-1, certain endogenous lipid molecules (such as sphingosine-1-phosphate) and cytokines (such as erythropoietin)[10]. However, few effective drugs have been used clinically to treat MIRI. Therefore, it is of great clinical significance to find an effective MIRI drug.

Cystatin is a class of natural cysteine protease inhibitors that are widely distributed in organisms and participate in various physiological and pathological processes, such as protein catabolism, infection and immunity, tumor invasion and metastasis, and so on[11]. Serum cystatin C (Cys C) is a member of the endogenous Cystatin superfamily. Cys C has a variety of biological functions, such as inhibiting cysteine peptidase, controlling extracellular proteolysis, regulating the immune system, and antibacterial and antiviral activity[12]. It plays an important role in the metabolism of peptides and proteins in cells. Studies have shown that the level of Cys C in serum increases with the severity of heart failure[13]. In addition, the level of Cys C in patients with heart failure who had adverse cardiovascular events was significantly higher than those who did not, indicating that the level of Cys C is closely related to the severity of heart disease and the prognosis of patients[14]. However, the effects of Cys C on cardiomyocytes have not been studied. Therefore, in this study, we used Cys C to treat rat cardiomyocytes (H9c2 cells) to study the effect of Cys C on cardiomyocytes, and used rat MIRI models to verify the effect of Cys C on MIRI.

Materials and Methods

Cell Culture

H9c2 cells were used. Fetal bovine serum (FBS; Gibco, Rockville, MD, USA) was added to Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) to configure the complete medium with 10% FBS. H9c2 cells were cultured in cell culture dishes and we changed the DMEM medium once every two days. All cell experiments were performed in a sterile ultra-clean bench, and cell culture dishes were placed in an incubator with 37°C and 5% CO_2. H_2O_2 is used to stimulate H9c2 cells to induce cardiomyocyte injury.

Cell Counting Kit 8 (CCK8) Assay

Water-soluble tetrazolium salt-WST-8 is reduced to orange-yellow formazan under the action of 1-methoxy PMS. The amount of formazan is directly proportional to the number of living cells. We first seeded H9c2 cells in 96-well plates, and added 100 μL of medium to each well. After processing the cells, we added 10 μL of CCK8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) to each well and placed the 96-well plate in the incubator for 2 hours. The blank group had only medium and no cells; the control group had cells but no treatment. Finally, we used a microplate reader (Molecular Devices, Santa Clara Valley, MD, USA) to detect the absorbance (OD) of each well at a wavelength of 450 nm. Cell viability = $(OD_{\text{intervention group}}-OD_{\text{blank group}}) / (OD_{\text{control group}}-OD_{\text{blank group}})$.

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

H9c2 cells were digested and collected using trypsin. After discarding the supernatant by centrifugation (1500 rpm, 5 min), we added phosphate-buffered saline (PBS) to the centrifuge tube to wash the cells and centrifuge again (1500 rpm, 5 min). 1 mL of TRIzol (Sigma-Aldrich, St. Louis, MO, USA) was added to the centrifuge tube for full cell lysis. After centrifugation

(12000 rpm, 5 min, 4°C), we collected the supernatant and transferred them to new Eppendorf (EP) tubes. 200 μL of chloroform was added to each EP tube. After thorough mixing, we collected the supernatant by centrifugation (12000 rpm, 5 min, 4°C) and added 500 μL of isopropanol. After full oscillation, precipitation is collected by centrifugation (12000 rpm, 5 min, 4°C). Finally, we washed the RNA precipitation with 75% ethanol and lysed the RNA with RNase-free water (Sigma-Aldrich, St. Louis, MO, USA). The RNA solution was stored in a refrigerator at -80°C.

We used a NanoDrop spectrophotometer to measure the RNA concentration, and then took 1 μg of RNA based on the RNA concentration for reverse transcription. 5 × HiScript II qRT SuperMix (Invitrogen, Carlsbad, CA, USA) was used to reverse mRNA into complementary deoxyribose nucleic acid (cDNA). The reverse system was 1 μg RNA + 4 μL 5 × HiScript II qRT SuperMix + RNase-free ddH_2O (to 20 μL). The cDNA was stored at -20°C. 2 × SYBR Green qPCR Mix (Invitrogen, Carlsbad, CA, USA) was used to amplify cDNA. The system is 25 μL 2 × SYBR Green qPCR Mix + 2 μL DNA Template + 1 μL Forward Primer + 1 μL Reserve Primer + 21 μL ddH_2O. The primer sequences are shown in Table I. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as control. $2^{-\Delta\Delta Ct}$ is used to represent the relative concentration of RNA.

Immunocytofluorescence (IF) Staining

We placed cell slides in 24-well plates and seeded H9c2 cells into 24-well plates. After processing the cells, we took the 24-well plate and discarded the medium. We washed the cells with cold phosphate-buffered saline (PBS) and fixed them with 4% paraformaldehyde for 15 minutes. We then treated the cells with 0.1% TritonX-100 for 20 minutes. After washing the cells with PBS, we blocked the cells with 10% goat serum (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour. We then discarded the blocking solution and incubated the cells at 4°C with primary antibody dilution (caspase3, ab13847; caspase9, ab32539; p65, ab16502; IKKα, ab178870; IκBα, ab109300; Abcam, Cambridge, MA, USA) overnight. After washing the cells with PBS, we incubated the cells with fluorescent secondary antibody dilution (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour in the dark. We then washed the cells with PBS and incubated the cells with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes. Finally, we used Antifade Mounting Medium to fix the slide on a glass slide and observed the staining results with a fluorescence microscope (LEICA, Koln, Germany) in the dark.

Animals and MIRI Model

This investigation was approved by the Animal Ethics Committee of Guangxi Medical University Animal Center. A total of 20 6-week-old male Sprague Dawley (SD) rats were used in this study. Rats were purchased and bred at the Guangxi Medical University Experimental Animal Center. The rearing conditions included the room temperature of 22-24°C, the relative humidity of 40-75% and alternating light for 12 hours. We selected littermates and divided them into Sham group, MIRI group, MIRI + Cys C (10 μg/kg) (Abcam, Cambridge, MA, USA) group and MIRI + Cys C (20 μg/kg) group. Rats in the Sham group were only opened the thorax and ligated the coronary arteries. Rats in the MIRI group, MIRI + Cys C (10 μg/kg) group and MIRI + Cys C (20 μg/kg) group were ligated with the left anterior descending coronary artery to make MIRI models. Rats in the MIRI + Cys C (10 μg/kg) group and MIRI + Cys C (20 μg/kg) group were injected subcutaneously with Cys C daily one week before the modeling.

Table I. RT-PCR primer sequences.

Name	Sense sequences (5′-3′)	Anti-sense sequences (5′-3′)
caspase3	GGAACGCGAAGAAAAGTG	ATTTTGAATCCACGGAGGT
caspase8	CACATCCCGCAGAAGAAG	GATCCCGCCGACTGATA
caspase9	GGAGTTGACTGAGGTGGGA	GGAGTTGACTGAGGTGGGA
Bax	GAGGTCTTCTTCCGTGTGG	GATCAGCTCGGGCACTTT
Bcl-2	AGGAACTCTTCAGGGATGG	GCGATGTTGTCCACCAG
p65	GGGGAAGGGAAGGAAGA	CGACTAAAAGGACCGCAA
IKKα	GCAAATGAGGACCAGAGC	CGCAGGAAAGATGACCAC
IκBα	CACCAACTACAACGGCCACA	TTCAACAGGAGCGAGACCAG
GAPDH	ATGGCTACAGCAACAGGGT	TTATGGGGTCTGGGATGG

After weighing the rats with an electronic balance, we anesthetized the rats by intraperitoneal injection using 2% sodium pentobarbital (0.25 ml/100 g) and fixed the rats on the operating table. We used scissors to cut fur off the neck and chest of rats, and then disinfected the skin with iodophors. We used scissors to cut the neck skin down from the thyroid cartilage along the midline of the rat neck, and then used a hemostat to bluntly separate the subcutaneous tissue and muscle. Then, we cut the cartilage ring under the thyroid cartilage and insert the endotracheal tube. Then, we connected the ECG lead to the limbs of the rat and set the tidal volume of the ventilator to 7 ml, the respiration ratio to 3:2, and the frequency to 75/min. We use scissors to cut the skin and muscles of the left chest and cut the left three or four ribs. After exposing the heart, we opened the pericardium and found the left coronary artery between the left atrial appendage and the arterial cone. We then used sutures to ligate the left descending coronary artery. Graying of the left ventricular anterior wall and ST-segment elevation in ECG lead II indicated successful myocardial ischemia. After 40 minutes, we loosened the suture. The disappearance of cyanosis and the decrease of ST segment in the ischemic area of the left ventricle indicated successful reperfusion. We then took rat serum and collected heart tissue.

Histology and Hematoxylin-Eosin (HE) Staining

After obtaining rat myocardial tissues, we placed them in 4% paraformaldehyde solution for 24 hours. Myocardial tissues were then dehydrated in gradient alcohol and then placed in turn in xylene and paraffin. We use an embedder to make paraffin blocks and a microtome (LEICA RM2235, Koln, Germany) to make paraffin sections. Before HE staining, paraffin sections were baked in a 55°C incubator for 1 hour. We put paraffin sections into xylene and gradient alcohol in order to dewax and hydrate. After rinsing the sections with running water, we soaked the sections in hematoxylin solution for 1 minute. We then rinsed the sections with running water and differentiated them with hydrochloric acid alcohol. The sections were then placed in eosin solution for 1 minute and continued to dehydrate in gradient alcohol. Finally, we used a high power light microscope to observe the results.

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

We collected rat hearts and immersed them in 1% heparinized saline at 37°C and cleaned them. We then perfused the hearts with 1% Evans Blue. Myocardial tissue in the non-ischemic area will gradually turn blue. The myocardial tissues were frozen in a refrigerator at -20°C for 20 minutes. We cut the myocardial tissue into 2mm thickness tissue pieces along the direction perpendicular to the longitudinal axis. We then placed the tissue pieces in 2% TTC solution (Sigma-Aldrich, St. Louis, MO, USA) and incubated them at 37°C for 15 minutes. The blue area means the non-ischemic myocardial tissue; the red area means the ischemic and non-infarcted myocardial tissue; the gray-white area means the infarcted myocardial tissue. The sum of the red and gray-white areas is the danger zone. Ischemic myocardial area = area of the dangerous / area of the left ventricle × 100%; infarct range = area of the infarct area / area of the dangerous area × 100%.

Detection of Lactic Dehydrogenase (LDH), Troponin T (TnT), Superoxide Dismutase (SOD) and Malondialdehyde (MDA)

LDH and TnT are markers of myocardial injury. We detected the activity of LDH and TnT in rat serum and the activity of LDH, MDA and SOD in H9c2 cell culture fluid. We collected rat blood and left it at room temperature for 30 minutes. After centrifugation (8000 rpm, 5 min, 4°C), we collected the serum and used the LDH detection kit (R&D Systems, Emeryville, CA, USA) and TnT detection kit (R&D Systems, Emeryville, CA, USA) to detect the activity of LDH and TnT in the serum according to the manufacturer's instructions. In addition, we collected cell culture fluid and collected the supernatant by centrifugation (12000 rpm, 15 min, 4°C). We then used the LDH, MDA and SOD detection kit to detect activity of LDH, MDA and SOD.

Statistical Analysis

Measurement data are expressed as mean ± SD (standard deviation). We used Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (La Jolla, CA, USA) statistical software for statistical analysis. Comparison between multiple groups was done using One-way ANOVA test followed

by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant. All experiments were repeated 3 times.

Results

Cys C Increased Cardiomyocyte Viability and Reduced H_2O_2-Induced Cardiomyocyte Injury

To determine the effect of Cys C on H9c2 cells, we stimulated the cells with Cys C and detected the cell viability by CCK8 assay (Figure 1A). To find the optimal concentration of Cys C for cardiomyocyte stimulation, we used different concentrations of Cys C (1 μM, 5 μM, 10 μM, 15 μM, 20 μM) to stimulate H9c2 cells. 15 μM was considered to be the optimum concentration for Cys C to stimulate H9c2 cells in subsequent experiments. H_2O_2 is used to induce H9c2 cell injury. We detected the effects of different concentrations of H_2O_2 on H9c2 cells through CCK8 assay (Figure 1B). 1000 μM and 2000 μM H_2O_2 can significantly reduce the activity of H9c2 cells, so we use 1000 μM H_2O_2 to stimulate H9c2 cells in subsequent experiments. The cells were divided into control group, Cys C group, H_2O_2 group and H_2O_2 + Cys C group. We detected the activity of LDH, MDA and SOD in H9c2 cells. After H_2O_2 was used to stimulate cells, the activity of LDH (Figure 1C) and MDA (Figure 1D) in the cells increased and the activity of SOD (Figure 1E) decreased. However, stimulation of Cys C could lead to the opposite result. On the basis of H_2O_2 stimulation, Cys C intervention can reduce the damaging effect of H_2O_2 on H9c2 cells.

Cys C Reduced H_2O_2-Induced Cardiomyocyte Apoptosis

To further determine the effect of Cys C on cardiomyocyte injury, we examined changes in the apoptosis level of H9c2 cells. First, we detected the expression of the pro-apoptotic molecules caspase3 (Figure 2A) and caspase9 (Figure 2B) by IF staining. The results showed that Cys C reduced the expression of caspase3 and caspase9 in H9c2 cells, while H_2O_2 increased their expression. After using Cys C to treat H9c2 cells on the basis of H_2O_2, the promotion effect of H_2O_2 on the expression of caspase3 and caspase9 was sig-

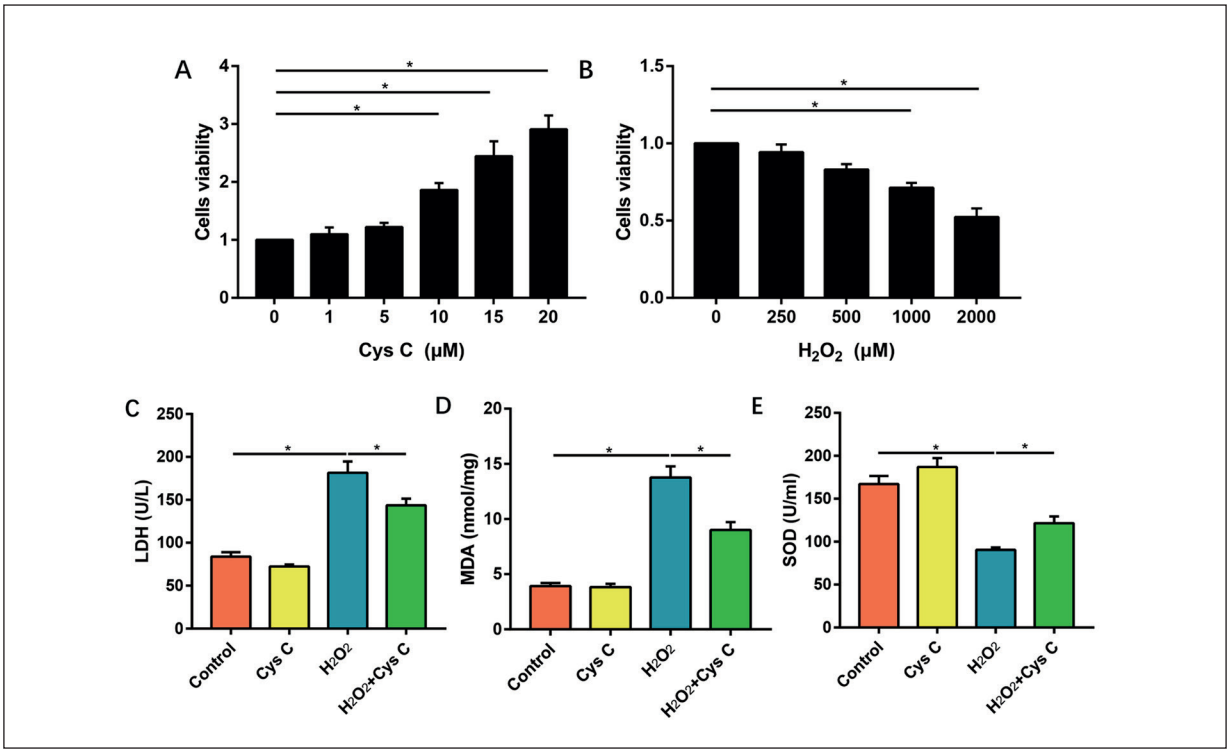

Figure 1. Cys C increased cardiomyocyte viability and reduced H_2O_2-induced cardiomyocyte injury. **A-B,** Optimum concentration of Cys C and H_2O_2 was determined by CCK8 assay; **C,** LDH activity in H9c2 cells; **D,** MDA activity in H9c2 cells; **E,** SOD activity in H9c2 cells. ("*" means the difference is statistically significant, $p<0.05$).

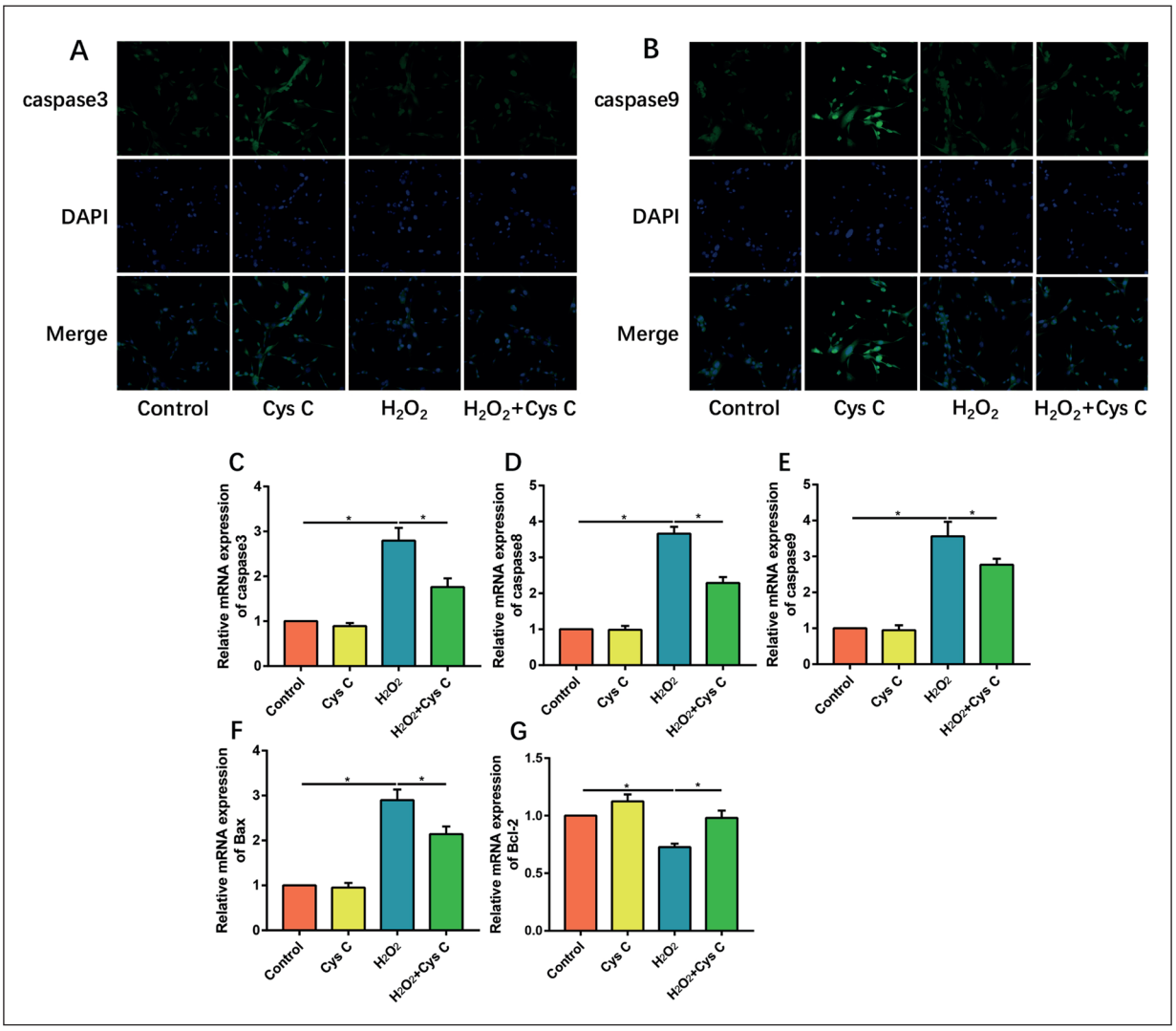

Figure 2. Cys C reduced H_2O_2-induced cardiomyocyte apoptosis. **A-B,** IF staining detected the expression of caspase3 and caspase9 (magnification: 200×); **C-G,** RT-PCR detected the expression of caspase3, caspase8, caspase9, Bax and Bcl-2 mRNA. ("*" means the difference is statistically significant, $p<0.05$).

nificantly weakened. In addition, we detected the expressions of pro-apoptotic molecules caspase3 (Figure 2C), caspase8 (Figure 2D), caspase9 (Figure 2E) and Bax (Figure 2F) mRNA, and anti-apoptotic molecules Bcl-2 (Figure 2G) mRNA by RT-PCR. Cys C was found to reduce caspase3, caspase8, caspase9 and Bax at the mRNA level and promote Bcl-2 mRNA expression.

Cys C Inhibited NF-κB Signaling In Cardiomyocytes

NF-κB signaling pathway is an important pathway for cardiomyocyte injury. We examined the expression of p65 (Figure 3A), IKKα (Figure 3B) and IκBα (Figure 3C) by IF staining. The results showed that stimulation of H_2O_2 increased the expression of p65 and IKKα, the key signaling molecules of the NF-κB signaling pathway in H9c2 cells, and reduced IκBα. After using Cys C to treat H9c2 cells, the activity of NF-κB signaling pathway was significantly reduced. The results of RT-PCR also proved that Cys C can inhibit the activity of NF-κB signaling pathway at mRNA level (Figure 3D-3F).

Cys C Relieved MIRI In Rats

To verify the effect of Cys C on rat MIRI, we made rat MIRI models and treated rats with

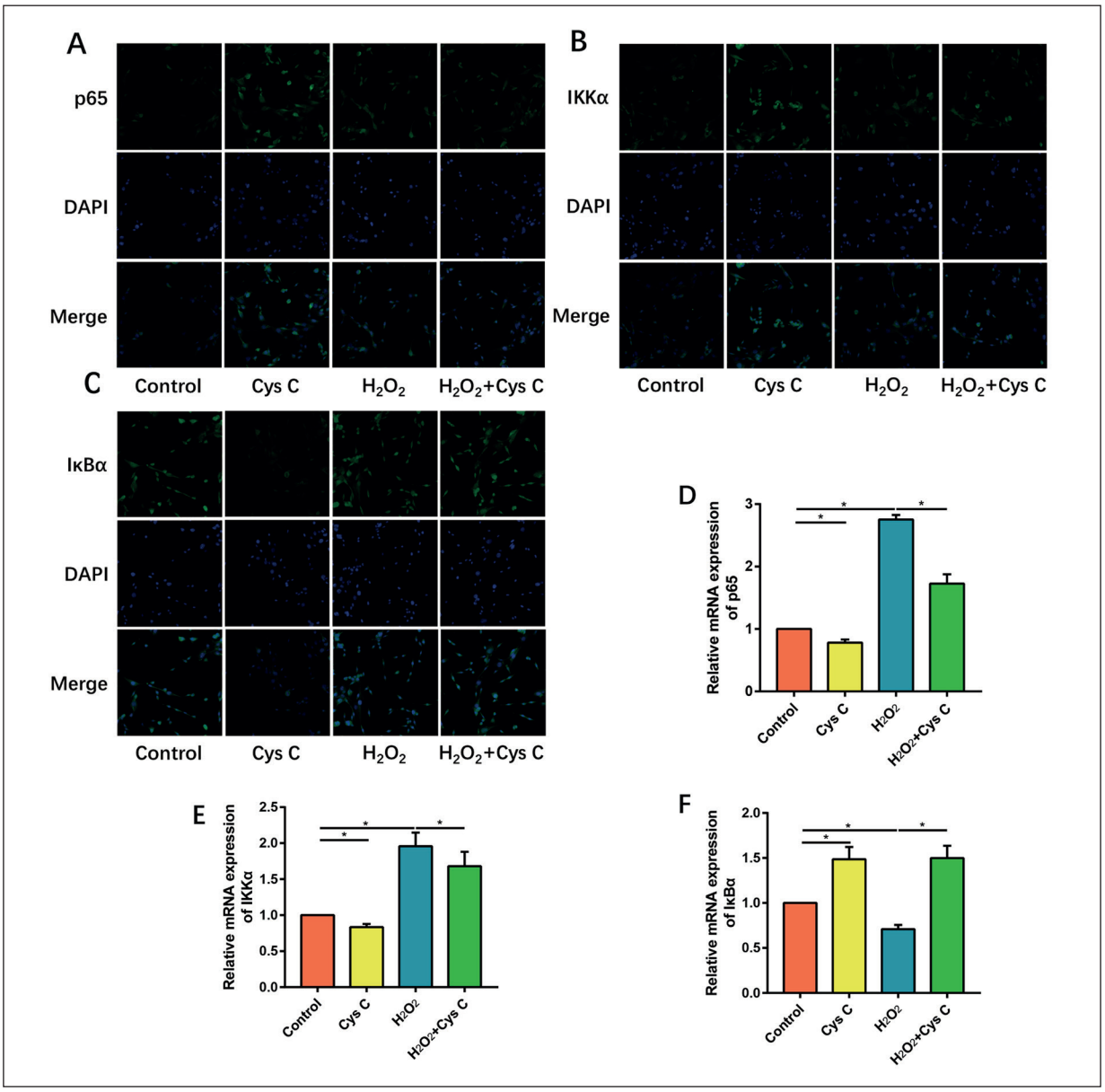

Figure 3. Cys C inhibited NF-κB signaling in cardiomyocytes. **A-C,** IF staining detected the expression of p65, IKKα and IκBα (magnification: 200×); **D-F,** RT-PCR detected the expression of p65, IKKα and IκBα mRNA. ("*" means the difference is statistically significant, $p<0.05$).

different concentrations of Cys C. HE staining revealed cardiomyocyte disorders in MIRI rats, indicating that the MIRI model was successfully made. After treatment of MIRI rats with Cys C, the morphology of cardiomyocytes was significantly improved (Figure 4A). TTC staining detected the range of myocardial infarction in rats. The area of myocardial ischemia (Figure 4B) and the range of myocardial infarction (Figure 4C) increased significantly in MIRI rats, and Cys C was found to reduce the area of myocardial ischemia and myocardial infarction. We also measured the activity of myocardial injury markers LDH (Figure 4D) and TnT (Figure 4E) in rat serum. Cys C was shown to effectively reduce LDH and TnT activity in rat serum. This again proved the protective effect of Cys C on rat myocardium.

Discussion

Fatal cell injury due to reperfusion reduces the therapeutic effect of coronary recanaliza-

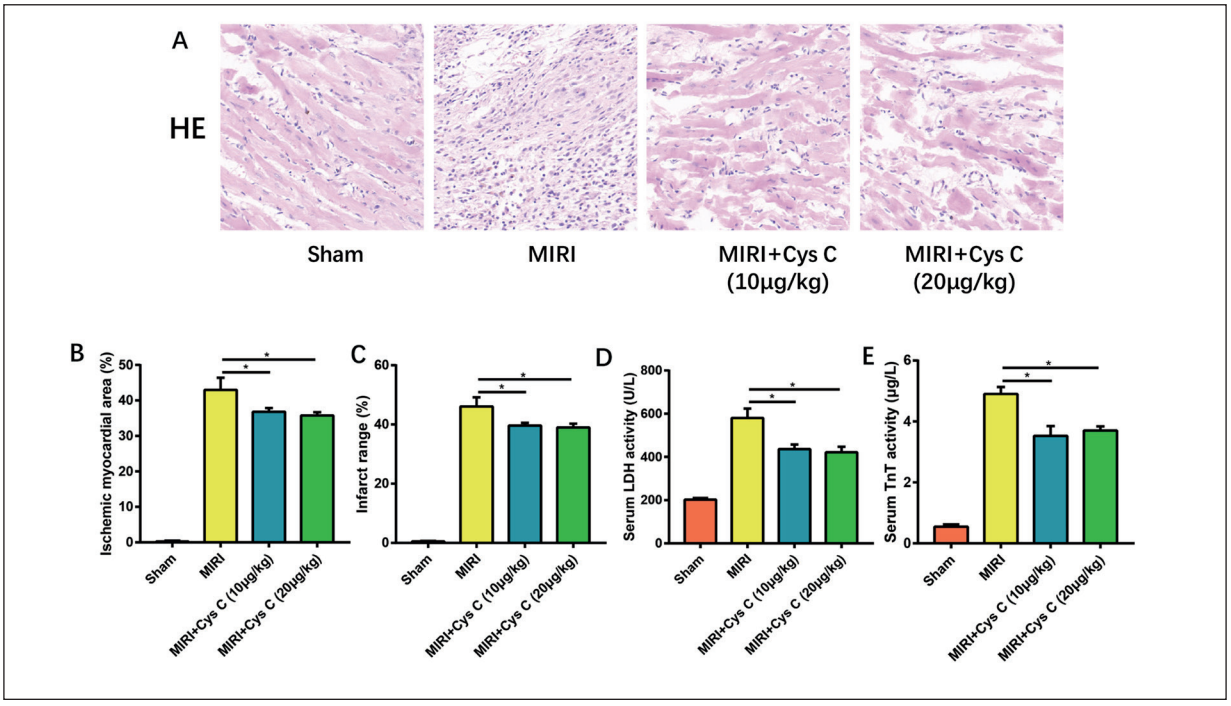

Figure 4. Cys C relieved MIRI in rats. **A,** HE staining of rat myocardium (magnification: 200×); **B-C,** TTC staining detected the ischemic myocardial area and infarct range; **D,** LDH activity in rat serum; **E,** TnT activity in rat serum. ("*" means the difference is statistically significant, $p<0.05$).

tion in patients with ischemic heart disease. It is of great clinical significance to further study the mechanism of MIRI and explore strategies to alleviate MIRI[15]. Pharmacological postconditioning is an effective cytoprotective mechanism, which has better clinical application prospects than preconditioning[8]. We used H9c2 cells and SD rats in this study. First, we successfully induced cardiomyocyte injury using H_2O_2. The intervention of Cys C increased the H9c2 cells viability, reduced the activity of LDH and MDA in cardiomyocytes and increased the activity of SOD, indicating that Cys C can reduce the cardiomyocytes injury induced by H_2O_2. Therefore, the apoptosis level of H9c2 cells also changed accordingly. The pro-apoptotic molecules (caspase3, caspase8, caspase9 and Bax) of H_2O_2 stimulated cardiomyocytes increased significantly, while the anti-apoptotic molecules (Bcl-2) decreased significantly. Cys C stimulated cardiomyocytes had a relatively low level of apoptosis and Cys C inhibited the pro-apoptotic effect of H_2O_2. The NF-κB signaling pathway is an important pathway leading to the occurrence and development of MIRI. The activity of NF-κB signaling pathway in H_2O_2 stimulated cardiomyocytes was also significantly increased, while the activity of NF-κB signaling pathway was significantly inhibited in Cys C stimulated cardiomyocytes, which may be an important mechanism for Cys C to protect cardiomyocytes. Finally, we used Cys C in a MIRI rat model to verify its therapeutic effect on MIRI. Histological and molecular biological experiments have verified the alleviating effect of Cys C on MIRI.

Myocardial apoptosis is one of the important causes of myocardial infarction and MIRI[16]. Cardiomyocytes are terminally differentiated cells and cannot be regenerated[17]. The decrease of cardiomyocytes caused by apoptosis is considered to be the main reason for the continuous decline of ventricular function during ischemia-reperfusion and the pathological basis for the development of the heart from compensatory hypertrophy to pathological hypertrophy[18]. The morphological characteristics of apoptosis are vesicularization, cell shrinkage, nuclear division, and chromatin condensation. Activation of caspase family members is its main biological characteristic. Signal transduction pathways of apoptotic cells

can occur through extrinsic (death receptor) and intrinsic (mitochondrial) pathways, and there are multiple biochemical and functional connections between the two pathways[19]. Extrinsic or death receptor pathways include death receptors composed of TNFR family members (TNFR1/2, Fas and DR3/4/5), and their related ligands (TNF-α, FasL, TRAIL and TWEAK). The ligands induce receptor activation, which in turn forms a death-inducing signal complex with the death domain-containing linker proteins FADD and TRADD, activating caspase8 and caspase3. Caspase3 can hydrolyze various cellular proteins and cause apoptosis[20]. Bcl-2 family proteins control intrinsic pathways of apoptosis. Anti-apoptotic proteins Bcl-2 and Bcl-xL are localized on the outer membrane of mitochondria and inhibit the release of pro-apoptotic proteins such as cytochrome C. The pro-apoptotic members of the Bcl-2 family, Bad, Bid, Bax and Bim, are located in the cytoplasm[21]. Various apoptosis-stimulating signals, such as hypoxia and radiation, cause Bax protein to shift to the outer membrane of the mitochondria and polymerize to form membrane channels, which stimulates the mitochondria to release cytochrome C and Smac. Cytochrome C and caspases9 can form apoptotic bodies, leading to the downstream cascade reaction. Smac regulates apoptosis by promoting this cascade reaction. The ratio of Bcl-2/Bax protein is one of the important factors determining the sensitivity of apoptosis. When Bcl-2 level is higher than Bax, cells are not sensitive to apoptosis, otherwise cells are prone to apoptosis. Pro-apoptotic Bcl-2 family members are up-regulated in ischemic cells. Bax activation, translocation, and integration into the mitochondrial membrane can also occur[22]. Cardiomyocyte apoptosis plays a dominant role in the pathogenesis of MIRI and is also the main cause of persistent myocardial dysfunction after reperfusion[23]. The increase of cardiomyocyte necrosis rate is related to the duration of myocardial ischemia, and continuous ischemia and reperfusion can induce cardiomyocyte apoptosis. Especially after reperfusion, the supply of oxygen and glucose is restored, which provides the energy required for apoptosis, and a large number of oxygen free radicals are generated, which accelerates the occurrence of apoptosis[24]. Inhibiting cardiomyocyte apoptosis can reduce the infarct area by 50-70% and reduce the incidence of cardiac dysfunction after ischemia-reperfusion[25]. In H9c2 cells, Cys C effectively inhibited the expression of the caspase family and increased Bcl-2/Bax. In addition, the increase in SOD activity and the decrease in MDA activity also confirmed the antioxidant effect of Cys C on H9c2 cells.

NF-κB signaling pathway plays a key role in the MIRI process. It has been observed on an *in vitro* heart model that simple short-term ischemia of the heart can cause a rapid increase in NF-κB activity, while short-term ischemia plus ischemia-reperfusion can further increase NF-κB activity. This showed that NF-κB is a fast-expressing gene for cardiac stress response[26]. Functional NF-κB binding sequences are widely found in promoters and enhancers of cytokines and adhesion molecules such as TNF-α and ICAM-1. The activation of NF-κB leads to the expression of the above genes, and the TNF-α and ICAM-1 produced by transcription can directly cause damage to vascular endothelial cells and cardiomyocytes[27]. Currently, TNF-α, in addition to having direct effects on local tissues and cells, also induces the expression of a variety of adhesion molecules in vascular endothelial cells and cardiomyocytes, and mediates the adhesion of leukocytes to vascular endothelial cells. Leukocytes migrate out of blood vessels, adhere to cardiomyocytes, release cytotoxicity, and damage myocardium. Reducing the secretion of TNF-α and ICAM-1 is beneficial to myocardial cells[27]. In short, the NF-κB signaling pathway indirectly mediates the binding of leukocytes and vascular endothelial cells, which is a key step in MIRI. We found a decrease in the activity of the NF-κB signaling pathway in H9c2 cells treated with Cys C. This is the key to the treatment of MIRI by Cys C. Through this study, we hope to clarify the protective effect of Cys C on cardiomyocytes and the therapeutic effect on MIRI, so as to provide a new method and theoretical basis for clinical MIRI treatment.

Conclusions

Cys C was found to reduce H_2O_2-induced cardiomyocyte injury. By inhibiting the activity of the NF-κB signaling pathway, Cys C reduced the oxidative stress and apoptosis levels in H9c2 cells, thereby alleviating H9c2 cell injury. In

addition, the results of *in vivo* experiments also verified the therapeutic effect of Cys C on MIRI rats.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Toldo S, Abbate A. The NLRP3 inflammasome in acute myocardial infarction. Nat Rev Cardiol 2018; 15: 203-214.
2) Herr DJ, Singh T, Dhammu T, Menick DR. Regulation of metabolism by mitochondrial enzyme acetylation in cardiac ischemia-reperfusion injury. Biochim Biophys Acta Mol Basis Dis 2020; 1866: 165728.
3) Mokhtari-Zaer A, Marefati N, Atkin SL, Butler AE, Sahebkar A. The protective role of curcumin in myocardial ischemia-reperfusion injury. J Cell Physiol 2018; 234: 214-222.
4) Boag SE, Andreano E, Spyridopoulos I. Lymphocyte communication in myocardial ischemia/reperfusion injury. Antioxid Redox Signal 2017; 26: 660-675.
5) Wu MY, Yiang GT, Liao WT, Tsai AP, Cheng YL, Cheng PW, Li CY, Li CJ. Current mechanistic concepts in ischemia and reperfusion injury. Cell Physiol Biochem 2018; 46: 1650-1667.
6) Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136.
7) Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003; 285: H579-H588.
8) Arnold M, Mendez-Carmona N, Gulac P, Wyss RK, Rutishauser N, Segiser A, Carrel T, Longnus S. Mechanical postconditioning promotes glucose metabolism and AMPK activity in parallel with improved post-ischemic recovery in an isolated rat heart model of donation after circulatory death. Int J Mol Sci 2020; 21: 964.
9) Aslan G, Atessahin A, Sahna E. The inhibition of apoptosis through myocardial postconditioning by affecting Fas/FasIg signaling through miR139-3p and miR181a-1. J Card Surg 2020; 35: 564-570.
10) Heusch G. Treatment of myocardial ischemia/reperfusion injury by ischemic and pharmacological postconditioning. Compr Physiol 2015; 5: 1123-1145.
11) Kos J, Nanut MP, Prunk M, Sabotic J, Dautovic E, Jewett A. Cystatin F as a regulator of immune cell cytotoxicity. Cancer Immunol Immunother 2018; 67: 1931-1938.
12) Tabara Y, Kohara K, Okada Y, Ohyagi Y, Igase M. Creatinine to Cystatin C ratio as a marker of bone property in older adults: the J-SHIPP study. J Nutr Health Aging 2020; 24: 277-281.
13) Pinsino A, Mondellini GM, Royzman EA, Hoffman KL, D'Angelo D, Mabasa M, Gaudig A, Zuver AM, Masoumi A, Garan AR, Mohan S, Husain SA, Toma K, Faillace RT, Giles JT, Takeda K, Takayama H, Naka Y, Topkara VK, Demmer RT, Radhakrishnan J, Colombo PC, Yuzefpolskaya M. Cystatin C- versus creatinine-based assessment of renal function and prediction of early outcomes among patients with a left ventricular assist device. Circ Heart Fail 2020; 13: e6326.
14) Zhi H, Zhang M, Cui X, Li Y. [Renal echography and cystatin C for prediction of acute kidney injury: very different in patients with cardiac failure or sepsis]. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 2019; 31: 1258-1263.
15) Caccioppo A, Franchin L, Grosso A, Angelini F, D'Ascenzo F, Brizzi MF. Ischemia reperfusion injury: mechanisms of damage/protection and novel strategies for cardiac recovery/regeneration. Int J Mol Sci 2019; 20: 5024.
16) Yao B, Wan X, Zheng X, Zhong T, Hu J, Zhou Y, Qin A, Ma Y, Yin D. Critical roles of microRNA-141-3p and CHD8 in hypoxia/reoxygenation-induced cardiomyocyte apoptosis. Cell Biosci 2020; 10: 20.
17) Zhang M, Zhu J, Qin X, Zhou M, Zhang X, Gao Y, Zhang T, Xiao D, Cui W, Cai X. Cardioprotection of Tetrahedral DNA nanostructures in myocardial ischemia-reperfusion injury. ACS Appl Mater Interfaces 2019; 11: 30631-30639.
18) Pei YH, Chen J, Wu X, He Y, Qin W, He SY, Chang N, Jiang H, Zhou J, Yu P, Shi HB, Chen XH. LncRNA PEAMIR inhibits apoptosis and inflammatory response in PM2.5 exposure aggravated myocardial ischemia/reperfusion injury as a competing endogenous RNA of miR-29b-3p. Nanotoxicology 2020: 1-16.
19) Dong Y, Chen H, Gao J, Liu Y, Li J, Wang J. Molecular machinery and interplay of apoptosis and autophagy in coronary heart disease. J Mol Cell Cardiol 2019; 136: 27-41.
20) Xu ZY, Zheng MX, Zhang L, Gong X, Xi R, Cui XZ, Bai R. Dynamic expression of death receptor adapter proteins tradd and fadd in Eimeria tenella-induced host cell apoptosis. Poult Sci 2017; 96: 1438-1444.
21) Pekarsky Y, Balatti V, Croce CM. BCL2 and miR-15/16: from gene discovery to treatment. Cell Death Differ 2018; 25: 21-26.
22) Vogler M, Walter HS, Dyer M. Targeting anti-apoptotic BCL2 family proteins in haematological malignancies - from pathogenesis to treatment. Br J Haematol 2017; 178: 364-379.
23) Zhao D, Feng P, Sun Y, Qin Z, Zhang Z, Tan Y, Gao E, Lau WB, Ma X, Yang J, Yu S, Xu X, Yi D, Yi W. Cardiac-derived CTRP9 protects against myocardial ischemia/reperfusion injury via calreticu-

lin-dependent inhibition of apoptosis. Cell Death Dis 2018; 9: 723.

24) Wei L, Zhou Q, Tian H, Su Y, Fu GH, Sun T. Integrin beta3 promotes cardiomyocyte proliferation and attenuates hypoxia-induced apoptosis via regulating the PTEN/Akt/mTOR and ERK1/2 pathways. Int J Biol Sci 2020; 16: 644-654.

25) Cai Y, Ying F, Liu H, Ge L, Song E, Wang L, Zhang D, Hoi CTE, Xia Z, Irwin MG. Deletion of Rap1 protects against myocardial ischemia/reperfusion injury through suppressing cell apoptosis via activation of STAT3 signaling. FASEB J 2020; 34: 4482-4496.

26) Gordon JW, Shaw JA, Kirshenbaum LA. Multiple facets of NF-kappaB in the heart: to be or not to NF-kappaB. Circ Res 2011; 108: 1122-1132.

27) Gutierrez SH, Kuri MR, Del CE. Cardiac role of the transcription factor NF-kappaB. Cardiovasc Hematol Disord Drug Targets 2008; 8: 153-160.