INTRODUCTION

Cardiovascular disease is the leading cause of morbidity and mortality in humans. Research has shown that ischaemic heart disease was one of the leading causes of death and disability-adjusted life-years (DALYs) in China.1 In children, congestive heart failure is mainly caused by different forms of cardiomyopathy or congenital heart disease. The cause of myocardial infarction in most paediatric patients is still unclear and the mortality rate is still high; neonatal myocardial infarction is a rare disease compared with adults. However, the treatment options

Diosmetin exerts cardioprotective effect on myocardial ischaemia injury in neonatal rats by decreasing oxidative stress and myocardial apoptosis

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Abstract
Myocardial injury caused by the myocardial ischaemia (MI) is still a troublesome condition in the clinic, including apoptosis, oxidative stress and inflammation. Diosmetin inhibits the cellular apoptosis and inflammatory response and enhances antioxidant activity. So, this study was designed to investigate the cardioprotective effects of diosmetin on MI model neonatal rats. Forty Sprague Dawley (SD) rats 7 days old were randomly divided into five groups. Four groups of rats received diosmetin (50, 100, and 200 mg/kg) or vehicle (MI group) after ischaemia. Another group received vehicle without ischaemia to serve as a control group. Rats were pretreated with diosmetin intraperitoneally for 7 days and intoxicated with isoproterenol (ISO, 85 mg/kg, sc) on the last 2 days. The expression of apoptotic molecules, myocardial systolic function index, antioxidant enzymes and myocardial enzyme was analyzed. Compared with the control group, the proliferation marker proteins of Ki67 were increased significantly (P < .05), the MI group significantly increased the cardiac apoptosis, oxidative stress and myocardial enzymes, and weakened myocardial contractility. The levels of p-P65/P65 were increased significantly (P < .05) with decreased p-AKT/AKT and p-Nrf2/Nrf2 (P < .05). Nevertheless, pretreatment with diosmetin reversed these changes, especially high-dose group. In summary, diosmetin has significant potential as a therapeutic intervention to ameliorate myocardial injury after MI and provides the rationale for further clinical studies.

KEYWORDS
diosmetin, myocardial apoptosis, myocardial ischaemia, oxidative stress
in this case are limited. In myocardial ischaemia and hypoxia of the newborn, the body has a compensation reaction that could lead to hypoxic-ischaemic injury to multiple organs, especially to the brain and heart. Restoring the blood perfusion and oxygen supply of the tissue and organs as soon as possible is one of the best treatment methods. However, reperfusion injury and reoxygenation injury would aggravate the injury after regaining the blood perfusion and oxygen supply.

Myocardial ischaemia (MI) is the leading cause of myocardial infarction, and studies have shown that apoptosis plays a role in cell death of cardiomyocytes for patients with acute myocardial infarction. These findings provide evidence that oxidative stress and myocardial apoptosis are exacerbated in a rat model of myocardial ischaemia. There are many complex factors contributing to its pathogenesis, in which apoptosis has been identified as the main factor contributing to myocardial dysfunction and is vital process in the occurrence and development of myocardial ischaemia reperfusion injury (MIRI). Studies have shown that MIRI is an indispensable factor leading to inflammation and oxidative stress. For newborns, it could have serious consequences due to MI. The neonatal rat MI model is established in this study to evaluate the injury caused by myocardial ischaemia.

Studies have shown that left ventricular dysfunction is inextricably linked to myocardial infarction, and myocardial cells in the left ventricle are killed by oxygen, and eventually the ventricular wall becomes thinner. Cardiac systolic function can be reflected by left ventricular wall thickness (LVWT), left ventricular end-systolic volume (LVEDV), left ventricular ejection fraction (LVEF), shortened fraction (FS) and heart rate (HR). Myocardial enzymes are released from the injury of myocardial cells and are an important marker of myocardial injury. The detection of cTnl, AST, LDH, α-hydroxybutyrate dehydrogenase (α-HBDH), CK-MB and Mb are the most used as biomarkers of myocardial injury.

Diosmetin is a flavonoid isolated from the leaves of Olea europaea L. Diosmetin has been studied to demonstrate anti-proliferative and pro-apoptotic activities in cancer, and antiinociceptive properties in different pain models in mice and suppressed inflammation under conditions of pneumococcal meningitis infection. It has also been demonstrated that diosmetin could inhibit osteoclast formation and activation, and could serve as a potential therapeutic drug for osteolytic bone disease. Evidence has been provided that Isovitexin and 6-C-glycoside-Diosmetin could exert concomitant cognitive-enhancement and anxiolytic-like effects via GABAA receptor modulation in a mice model. However, the therapeutic activity of diosmetin in myocardial ischaemia is still unknown.

The protein kinase B (AKT) signalling pathway work on a survival signal during myocardial ischaemia, and activation of this signal act on the mitochondrion, thereby exerts anti-apoptotic effects via upstream and downstream signalling. Studies have shown that P13K and AKT are involved in the regulation of NF-κB signalling. AKT could up-regulate the expression of Bcl-2, while NF-κB signalling plays an important role in regulating the release of cytokines and chemokines. Nrf2, belongs to the CNC family of transcription factors and is a key transcription factor against oxidative stress, controlling various antioxidant genes and enzymes.

However, it has not been reported that diosmetin has a therapeutic effect on the injury caused by myocardial ischaemia. To evaluate the effects of diosmetin on anti-oxidative stress, cardiomyocyte apoptosis, myocardial enzymes and left ventricular function in neonatal MI rats, the neonatal rat MI model is established by subcutaneous injection of isoproteonol (ISO) in this work.

2 | RESULTS

2.1 | Diosmetin enhanced the proliferation marker proteins of Ki67 and the ratio of Bcl-2/Bax in MI model rats

The effects of diosmetin treatment on the proliferation marker proteins of Ki67 and the ratio Bcl-2/Bax are shown in Figure 1. The percentage of Ki67-positive cells of MI model rats was decreased significantly compared with the control group \( (P < .05) \), and the ratio of Bcl-2/Bax of relative mRNA level and relative protein level in the MI model rats also declined significantly contrasted with those in the control group \( (P < .05, P < .05) \). However, in MI model rats treated with diosmetin at the doses of 50 mg/kg, 100 mg/kg and 200 mg/kg, we found the percentage of Ki67-positive cells was increased notably in different doses of treated rats compared to the MI model rats group \( (P < .05, P < .05, P < .05) \), and the ratio of Bcl-2/Bax of relative mRNA level and relative protein level in the MI model rats was also elevated notably contrasted with those in the MI model rats group \( (P < .05, P < .05, P < .05) \).

2.2 | Diosmetin enhanced anti-cardiomyocyte apoptosis in MI model rats

From the above results, it can be found that diosmetin might inhibit cardiomyocyte apoptosis. To further verify the results, the protein expression levels of caspase-3, MDM2 and p53 were detected. As shown in Figure 2, the protein expression level of caspase-3 and p53 was enhanced markedly compared with the control group \( (P < .05, P < .05) \); moreover, the protein expression level of MDM2 was inhibited markedly when compared with the control group \( (P < .05) \). However, there was a significant decrease for the protein expression level of caspase-3 and p53 contrasted with those in the MI model group \( (P < .05, P < .05) \), while the protein expression level of MDM2 was up-regulated markedly when contrasted with the MI model group \( (P < .05) \). These results shown diosmetin could inhibit the myocardial apoptosis caused by myocardial ischaemia.

2.3 | Change of cardiac pathological and systolic function index after diosmetin treatment

The previous study indicated that myocardial cell apoptosis could cause cardiac dysfunction in mice. Change of cardiac pathological and systolic function index after diosmetin treatment is shown...
in Figure 3, haematoxylin and eosin (HE) staining showed that the cardiomyocytes were disordered, the cells were swollen, and some cells were dissolved in the MI model of the neonatal rat. From the results of echocardiography, it can be seen that LVWT, LVEF, FS and HR were significantly lower than the control group (\(P < .05\), \(P < .05\), \(P < .05\), \(P < .05\), respectively), while LVESV was significantly higher than the control group (\(P < .05\)). However, after treatment with diosmetin, it can be seen that LVWT, LVEF, FS and HR were significantly increased compared with the MI model group (\(P < .05\), \(P < .05\), \(P < .05\), \(P < .05\), respectively), while LVESV was significantly decreased in the MI model group (\(P < .05\)). These results shown diosmetin would restore the function of heart contraction caused by myocardial ischaemia.

2.4 | Changes of myocardial enzymes and oxidative stress index after diosmetin treatment

The main indicators of myocardial ischaemia injury include cTnl, CK-MB, LDH and Mb. As shown in Figure 4, the expression levels of cTnl, CK-MB, LDH and Mb declined observably compared with the control group (\(P < .05\), \(P < .05\), \(P < .05\), \(P < .05\), respectively). After diosmetin treatment, the expression levels of cTnl, CK-MB, LDH and Mb declined observably compared with the MI model group (\(P < .05\), \(P < .05\), \(P < .05\), \(P < .05\), respectively). The main indicator of oxidative stress includes MDA and ROS. The levels of MDA and ROS were elevated observably compared with the control group (\(P < .05\), \(P < .05\)). After diosmetin treatment, they were observably repressed for MDA and ROS compared with the MI model group (\(P < .05\), \(P < .05\)). These results indicated that diosmetin might alleviate the damage caused by myocardial ischaemia and inhibit oxidative stress.

2.5 | Diosmetin affects the level of AKT, P65 and Nrf2 in the MI model

Previous results have demonstrated that diosmetin could attenuate cardiomyocyte apoptosis and inhibit oxidative stress. To further verify the results, the phosphorylation level of AKT, P65 and Nrf2 in the MI model was analyzed and is shown in Figure 5. The ratio of p-AKT/AKT and p-P65/P65 was increased significantly (\(P < .05\)) while the ratio of p-Nrf2/Nrf2 was decreased significantly (\(P < .05\)) in
the MI model compared with the control group. However, there was significant reduction in the ratio of p-P65/P65 ($P < .05$) while the ratios of p-AKT/AKT and p-Nrf2/Nrf2 were up-regulated significantly ($P < .05$) after diosmetin treatment compared with the MI model. These results shown diosmetin could restrain oxidative stress and secretion of cytokines and chemokines, cardiomyocyte apoptosis, thereby playing a role in protecting against myocardial ischaemic injury.

3 | DISCUSSION

Myocardial ischaemia is one of the causes of a series of heart diseases that endangers child health. Generally, the heart and kidneys have complex interactions physiologically and endocrinologically, in other words, dysfunction of the heart affects the kidneys and vice versa. Cardiac tissues in rats given isoproterenol treatment showed universal accumulation of fibrous tissue, myocardial hypertrophy, injured myocardial structure, leukocyte infiltration and vacuolization. At the same time, stained myocardial and renal sections of rats given isoproterenol treatment showed more blue fibrous tissues and hydropic degeneration damage in tubular epithelial cells, protein casts and haemorrhage. Renal sympathetic denervation alleviates isoproterenol-induced left ventricle remodelling potentially via down-regulation of TGF-β/CTGF and up-regulation of miR-29b, miR-30c and miR-133a. Interestingly, diosmetin can protect mice against renal I/R injury by suppressing inflammation and apoptosis and enhancing antioxidant capabilities. In the present study, myocardial ischaemia mainly leads to accelerated cardiomyocytes apoptosis, increased oxidative stress, and weakened myocardial contraction.

Myocardial enzymes are released from the damage of myocardial cells and are an important marker of myocardial injury. The biomarkers cTnl, CK-MB, Mb and LDH are the most used for detection of myocardial injury. The results of the present study found that diosmetin administration caused effective ameliorative effects on the experimental isoproterenol-induced MI in neonatal rats. In addition, a significant increase in serum cTnl, CK-MB, Mb and LDH in the samples confirmed the development of MI in the rats. One of the main features of myocardial ischaemic injury is myocardial systolic and diastolic dysfunction; LVWT, LVEFS, LVEF, FS and HR are commonly used to assess myocardial systolic and diastolic function.
The present result showed that diosmetin treatment improved LVWT, LVEF, FS and HR, while reduced LVESV in neonatal rat with MI model. Feng et al. showed that the cardiac EF and FS of rats with myocardial ischaemia was significantly lower, which is consistent with our findings. Furthermore, HE staining showed that diosmetin treatment attenuated pathological damage from MI such as necrosis in heart tissue. Therefore, it can be seen from the results that diosmetin could restore the contractile function of cardiac myocytes in neonatal rats.

Ki67 is an indicator of cell proliferation and has been proven to be a strong prognostic marker for several cancer types. In this study, the expression of proliferation marker proteins of Ki67 was significantly increased in neonatal MI model treatment with diosmetin. Research has shown that Ki67 is necessary for normal G2/M transition through E2F1 activation in HaCaT cells. Studies have shown that the expression of Ki67 in cardiomyocytes is associated with cell activation. These findings suggested that Ki67 might promote cardiomyocyte proliferation.

Cardiomyocyte apoptosis plays a necessary role in the development process of myocardial ischaemic diseases. The Bcl-2 family proteins are key regulators of apoptosis, including anti-apoptotic proteins and pro-apoptotic proteins, and slight changes in the homeostasis of these proteins may lead to inhibition or promotion of cell death. The expression of the two regulatory proteins, Bcl-2 and Bax, has been studied. Cardiomyocyte apoptosis was inhibited by the expression protein of Bcl-2, while cardiomyocyte apoptosis was promoted by the over-expression protein of Bax in the hearts of patients with myocardial infarction. It has been found that the ratio of Bcl-2/Bax determines the survival or apoptosis of cells. If Bcl-2 expression is higher than Bax, the cells survive, Bcl-2 expression is lower than Bax, then apoptosis. Our results indicated that the ratio of Bcl-2/Bax is significantly increased after treatment with diosmetin. It is indicated that anti-apoptosis plays a dominant function in neonatal MI model rats after treatment with diosmetin. Caspases are the initiators and executors of cell apoptosis, caspase-3 is the most critical apoptotic protease, including regulation of cardiomyocytes, which plays an important role in the downstream of cascade junctions and has a decisive role in the process of cell death. The result indicated that the expression protein level of caspase-3 is observably down-regulated in neonatal MI model rats. Studies have shown that Bcl-2 family pro-apoptotic members lead to loss of integrity of the outer mitochondrial membrane, promoting the release of intermembrane space proteins, such as cytochrome c, to stimulate caspase activation accelerating apoptosis. These results indicated that diosmetin could inhibit the apoptosis of cardiomyocytes.

The tumour suppressor p53 is a master regulator of cellular stress response, primarily through regulation of apoptosis, cell cycle arrest, senescence, DNA repair and genetic stability. The negative feedback regulation is mainly strictly controlled by MDM2. Current research shows MDM2 controls cellular p53 levels and
FIGURE 4 Changes of myocardial index and oxidative stress index after diosmetin treatment. Effect of diosmetin in sham group, MI model group, low-dose group, medium-dose group and high-dose group. A, cTnl concentration in serum was measured by enzyme-linked immunosorbent assay (ELISA). B, CK-MB concentration in serum was measured by ELISA. C, Mb concentration in serum was measured by ELISA. D, MDA concentration in supernatant with tissue was detected by the thiobarbituric acid chromogenic method. E, LDH concentration in serum was measured by ELISA. F, ROS production in serum was measured by ELISA. The results were presented as mean ± SD and represent three individual experiments. (*P < .05 vs sham group, #P < .05 vs MI model group, ##P < .01 vs MI model group)

FIGURE 5 Diosmetin effect the level of AKT, P65 and Nrf2 in the MI model. A, A representative result for western blot analysis ATk, p-AKT, P65, p-P65, Nrf2 and p-Nrf2 in sham group, MI model group, low-dose group, medium-dose group and high-dose group. B, Semi-quantitative analysis of the relative ratio of p-AKT/ATK, p-P65/P65 and p-Nrf2/Nrf2 in each group of neonatal rats. The results were presented as mean ± SD and represent three individual experiments. (*P < .05 vs sham group, #P < .05 vs MI model group)
activity through at least three mechanisms. Our results shown that the levels of MDM2 were markedly enhanced at the expression of mRNA and proteins in neonatal MI model rats after treatment with diosmetin, whereas the levels of p53 were markedly reduced at the expression of mRNA and proteins. This means that diosmetin could activate the expression of MDM2, thereby inhibiting p53 activation in neonatal MI model rats. There is evidence to demonstrate that p53 interacts with Bcl-xL and Bcl-2 and neutralizes their inhibitory effects on pro-apoptotic Bax and Bak, which are the only members of the Bcl-2 family able to oligomerize and form lipid pores on the mitochondrial outer membrane. The protein kinase B (AKT) signalling pathway exhibits a crucial role in cytophysiological processes including cell survival, growth and migration. It may also work on a survival signal during myocardial ischaemia, and activation of this signal act on the mitochondrion, thereby exerts anti-apoptotic effects via upstream and downstream signalling. Recent studies indicate that activation of the AKT signalling pathway leads to phosphorylation and nuclear entry of MDM2, as well as degradation of p53 thus inhibiting apoptosis. The result show that diosmetin promotes AKT phosphorylation and prevents activation of the AKT signalling pathway in neonatal MI model rats. It was further illustrated that diosmetin could inhibit apoptosis and thereby exerts a cardioprotective effect.

Reactive oxygen plays an integral role in both myocardial injury and repair. All biological molecules are destroyed by the strong oxidative activity of ROS, especially cells damaged by lipid peroxidation. The results of the test indicate that myocardial ischaemia leads to the production of a large amount of ROS and MDA, indicating that rats in the model group were in a state of peroxidative stress. However, ROS and MDA were significantly reduced in neonatal MI model rats after treatment with diosmetin, indicating that diosmetin had a protective effect on oxidative stress injury of myocardial ischaemia induced by isoproterenol. Nrf2, belongs to the CNC family of transcription factors and is a key transcription factor against oxidative stress, controlling various antioxidant genes and enzymes. In the present study, the ratio of p-Nrf2/Nrf2 was significantly increased in neonatal MI model rats after treatment with diosmetin, indicating that diosmetin activates the Nrf2 pathway and promotes antioxidant activity. This means that the oxidative stress caused by myocardial ischaemia is attenuated.

Studies have shown that when AKT phosphorylation is induced and the NF-κB signalling pathway is subsequently activated, NF-κB located in the cytoplasm induces phosphorylation of NF-κBp65. In addition, phosphorylated NF-κBpP65 can be transferred to the nucleus to regulate transcription of pro-inflammatory mediators. Our results showed that the levels of P65 phosphorylation were observably down-regulated at the expression of proteins in neonatal MI model rats after treatment with diosmetin. The research has exhibited that activation of the p13/Akt pathway inhibits MIRI injury, inhibits cardiomyocyte apoptosis and inflammation, but inhibits NF-κB-P65 signalling, consistent with our results.

Cardiomyocyte apoptosis and inflammation are associated with inhibition of pathway. These results indicate that the inflammatory response caused by myocardial ischaemia was attenuated.

Taken together, the neonatal rat model of myocardial ischaemia was established by subcutaneous injection of ISO for two consecutive days. The result found that diosmetin could restore myocardial contractile function, prevent myocardial injury, and protect against oxidative stress injury in myocardial ischaemia. Diosmetin could promote cardiomyocyte proliferation and inhibit cardiomyocyte apoptosis. However, our study was conducted in animal models with limited sample size and clinical significance. Further research is needed to confirm the protective effect of diosmetin on patients with clinical myocardial ischaemia.

### 4 MATERIAL AND METHODS

#### 4.1 MI model and diosmetin treatment

All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by Affiliated Hospital of North Sichuan Medical College. Specific-pathogen-free (SPF) neonatal Sprague Dawley (SD) rats of 7 days old, were purchased from the Chengdu Dashuo Biotechnology Co. Experimental rats began the experiment after one week of adaptation. Rats were randomly divided into five groups: control group, MI model group, low-dose group (50 mg/kg diosmetin), medium-dose group (100 mg/kg diosmetin) and high-dose group (200 mg/kg diosmetin). Rats were pretreated with diosmetin intraperitoneally for 7 days and intoxicated with ISO (85 mg/kg, sc) on the last 2 days. After the end of the experiment, the neonatal rats were returned to the cage and breastfed by the female rats, and were housed in a controlled environment at 25 ± 3°C, humidity 60%, in a 12-h light/dark cycle with free access to water.

Diosmetin was obtained from Mansite (Chengdu, China) with the purity > 97% and molecular weight of 300.26 g/mol, which prepared at a stock concentration of 100 mmol/L in dimethyl sulfoxide. As the negative control, equal amounts of saline were injected in the same manner.

Control rats were subcutaneously injected with saline for 2 days and intraperitoneally injected with saline for 7 days. MI model rats were subcutaneously injected with isoproterenol (85 mg/kg/day) for 2 days and intraperitoneally injected with saline for 7 days. MI + diosmetin rats were subcutaneously given isoproterenol for two consecutive days and intraperitoneally injected with diosmetin (50 mg/kg, 100 mg/kg, 200 mg/kg) for 7 days. The blood samples were collected on the third day 24 hours after the second injection of ISO for the measurement of CK-MB and cTnI markers. Before killing the rats, blood samples were collected on the 10th day 24 hours after the final injection of diosmetin for the measurement of myocardial enzymes and oxides index.
4.2 | Histology

The neonatal rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (40–60 mg/kg) and then, the heart tissue was removed. The myocardium of neonatal rats was fixed with 4% paraformaldehyde for 24 hours, embedded in paraffin, and sectioned to a thickness of about 4 μm. The samples were performed with HE, and then histopathological morphology was observed under a light microscope.

4.3 | Total RNA and quantitative real-time PCR

Approximately 100 mg of heart tissue was homogenized in 1 mL Trizol (Invitrogen) and processed for isolation according to the manufacturer's instructions. The concentration 1 μg of total RNA samples was measured using a Gene Quant Pro RNA/DNA Calculator (Amersham Pharmacia Biotech), and then, the RNA was reverse-transcribed into cDNA by using Prime Script RT Mix reagent kit (TakaRa); β-actin was amplified as a housekeeping gene. The quantitative real-time polymerase chain reactions (qRT-PCR) were assembled using the 2 SYBR Premix Ex Taq II (TakaRa) and subjected to the following protocol in Bio-Rad CFX-96 (Bio-Rad): 30 s at 95°C, 40 cycles of 10 s at 95°C, 30 s at 60°C and 30 s at 72°C. The melting curve was performed from 65°C to 95°C in 1°C/10 s increments. The qRT-PCR data were analyzed using $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of mRNA. All assays were performed in duplicate.

4.4 | Western blot

The neonatal rats’ heart tissues (0.1 g) were collected from each group and were homogenized in 1 mL protein extraction buffer. The supernatant was collected following centrifugation and the protein concentrations were detected using the BCA Protein Assay Kit (Boaoweixin Biotechnology Co. (Abcam)). The band densities were determined and analyzed with a automatic digital gel image analysis system Bio-Rad CFX-96 (Bio-Rad). These antibodies were purchased from Chengdu Boaoweixin Biotechnology Co. (Abcam) and Shanghai Youningwei Biotechnology Co. (Cell Signaling).

4.5 | Immunohistochemistry

The myocardium of neonatal rats was fixed with 4% paraformaldehyde for 24 hours, embedded in paraffin, and sectioned. Paraffin sections were separated in xylene and rehydrated in gradient ethanol. After the antigen was extracted in 10 mmol/L citric acid buffer, the tissue sections were incubated in 3% H$_2$O$_2$ for 10 minutes and sealed at room temperature for 1 hour. The heart tissue sections were then incubated overnight with rabbit anti-Ki67 antibody (1:25, ab833, Abcam) and rabbit anti-caspase-3 antibody (1:1000, ab44976, Abcam). The corresponding second antibody was incubated at room temperature for 1 hour. The images were observed under an Olympus DX51 fluorescence microscope (Olympus). The data were analyzed by image 6.0. These two antibodies were purchased from Chengdu Boaoweixin Biotechnology Co. (Abcam).

4.6 | Echocardiography

The neonatal rats were anaesthetized with 2% isoflurane. Then, a MyLab 30CV ultrasound system (Biosound Esaote Inc) with a 10-MHz linear array ultrasound transducer was used to perform the echocardiographic examination, and the cardiac structure and function parameters of each mouse were recorded, including the left ventricular wall thickness (LVWT), left ventricular end-systolic volume (LVESV), left ventricular ejection fraction (LVEF), fraction shortening (FS) and heart rate (HR).

4.7 | Determination of myocardial enzymes and oxides index

Arterial blood samples of neonatal rats were collected and placed in 1 mL heparinized centrifuge tubes, and the supernatant was collected by centrifugation at 4°C, 3500 rpm/min for 20 min and stored at −80°C. The cTnl, CK-MB, Mb, ROS and LDH were measured by enzyme-linked immunosorbent assay (ELISA). The tissue was fully ground in an ice bath with sterile PBS, and centrifuged at 4°C, 3500 rpm/min for 10 min. The supernatant was taken, and then, the ROS and the MDA content in the myocardial homogenate were measured. The content of MDA was detected by the thiobarbituric acid chromogenic method. These kits were purchased from Nanjing Institute of Bioengineering.
4.8 | Statistical analysis

All experiments were performed three times or more independently, and all experimental data were presented as the mean ± standard deviation (SD). The statistical analyses between the two groups were conducted by using Student's t tests and SPSS 22.0 software (SPSS Inc). A value of P < .05 was considered statistically significant. Differences were considered significant at P < .05.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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