

Original Article

Sanguis draconis flavones promotes ischemic tolerance by targeting Prx-6 in myocardial ischemia and reperfusion injury

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Abstract: To observe whether *sanguis draconis* flavonoids (SDF) could protect the rat myocardial ischemia reperfusion and the corresponding mechanism of action through adopting the extracted SDF to treat MIRI rats. The TTC staining was used to detect the size of infarction zone. The TUNEL staining was used to detect the apoptosis rate of cardiac muscle cells. H9C2 cells were used for the *in vitro* experimental, the commercialized siRNA was used for the targeted inhibition on the Peroxiredoxin-6 (Prx-6) gene expression; after the cells were processed using the SDF, their SOD and MDA were detected; the CCK8 was used to test the impact of SDF on H9C2 cells; the flow cytometer was used to detect the apoptosis of H9C2 cells, and the WB was used to detect the protein expression level. The experimental results indicated that, SDF could significantly reduce the myocardial infarction regions of MIRI rats, and lower the oxidative stress and apoptosis of myocardial tissues. The results of cell experiment showed that, SDF could significantly inhibit the apoptosis of cardiac muscle cells and induce the proliferation of H9C2 cells, and the knock-out of the Prx-6 gene could reverse this trend. In the MIRI rat myocardial tissues and H9C2 cells, SDF could significantly down-regulate the expression of Bax, Caspase-9 and NF- κ -B2, and this phenomenon could be reversed by the knock-out of Prx-6 gene. Our experimental results indicated that, SDF could inhibit the oxidative stress and apoptosis of myocardial cells and improve the tolerance to myocardial ischemia through the mediation of Prx-6.

Keywords: *Sanguis draconis* flavones, Prx-6, MIRI, oxidative stress

Introduction

Myocardial ischemia reperfusion injury (MIRI) is a serious problem which often arises during the clinical practice. Early clinical observation found that, generally speaking, no reperfusion injury would occur if human heart recovered myocardial blood supply within 1.5 to 2 hours after acute myocardial infarction (AMI) [1, 2]; however, the recovery of blood supply after 2 to 4 hours would result in relatively obvious reperfusion injuries; the recovery of blood supply over 6 hours after the occurrence of AMI would be of no significance, but also potentially cause complications such as infarction, myocardial bleeding and myocardial rupture [3-7]. However, currently there is no report on Chinese medicine with special efficacy in prophylaxis and treatment of the myocardial reperfusion injury,

so it is very necessary to seek safe and effective anti-MIRI drugs and investigate their mechanism of action.

Oxidative stress is one of the main pathological mechanisms that result in the occurrence of MIRI [8, 9]. Due to the enhancement in the generating system activity, in case of the recovery of tissue blood supply and oxygen supply, OFRs will be massively generate, causing an acute accumulation and subsequently damaging the myocardium [10-12]. Peroxiredoxin 6 (Prx6) is expressed in multiple tissues, and it is mainly expressed in heart and brain. It is shown in studies that Prx6 has the biological activity of glutathione peroxidase; it is mainly responsible for the reduction of H₂O₂ and phosphatide peroxide [13, 14]. Therefore, we make a hypothesis that Prx6 may be involved in the regulation of

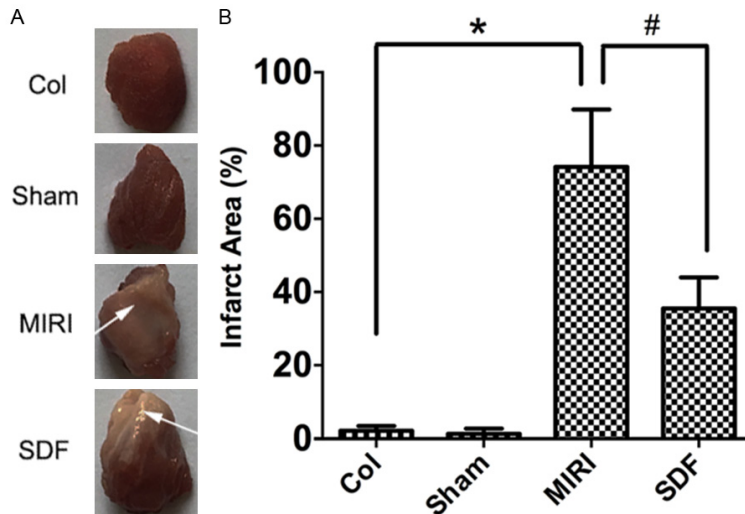


Figure 1. SDF can improve the ischemic tolerance of rats which receive MIRI surgery. A: The infarct size of rats in five groups, the arrows represent the infarct size that is negative in TTC staining. B: Percentage of infarct size. Col, control group; Sham, Sham group; MIRI, myocardial ischemia reperfusion injury group; SDF, MIRI plus SDF treatment group. Compared with control group, $*P<0.05$; compared with MIRI group, $\#P<0.05$.

oxidative stress during the MIRI process. *Sanguis draxonis* (SD) is a resin extracted from the resiniferous woods of *Dracaena draco*. During the recent years, the extraction methods of its active components and its mechanism of action are increasingly revealed, with the constant deepening in the SD chemical components, pharmacology and clinical studies [15]. The investigators of this study carried out an in-depth investigation on whether SDF had the effects of relieving ischemia reperfusion injuries in myocardial tissues and cells, as well as the cellular molecular mechanism through the preparation of in vivo ischemia reperfusion injury models and in vitro cultured myocardial cell hypoxia-reoxygenation injury models, for the purpose of providing experimental basis to seek safe and effective anti-myocardial-ischemia-reperfusion traditional Chinese medicines.

Materials and methods

Reagents and devices

SDF, purchased from the Pharmaceutical Factory of Guangxi Institute of Chinese Medicine & Pharmaceutical Science, was prepared by using the normal saline to the SDF suspension with a mass concentration of 3.5 mg/mL, for the subsequent experimental use.

The superoxide dismutase (SOD) kit and malondialdehyde (MDA) kit were purchased from Nanjing Jiancheng Bioengineering Institute. The in situ cell apoptosis (TUNEL) kit was purchased from US Roche. DAB color developer and hematoxylin were purchased from Beijing Chemical Reagent Company. Flow cytometer was purchased from US BD.

Processing of experimental animals

SD rats were purchased from Experimental Animal Center of Guangxi Medical University, 40 in total, weighing from 180 to 220 g. The rats were grouped into different groups and cages at the environment of alternating illumination between light and shade for 12 hours, at the temperature of 18 to 21°C and the relative humidity of (50±5)%, and they were fed with standard feeds, and they were free to eat and drink. In one week after the feeding, MIRI surgery was performed on rats by referring to the methods provided in the literatures [16]. Briefly, SD rats were anesthetized with 2% isoflurane and mechanical ventilation, and then the chest was opened at the 4th intercostal space. The left anterior descending coronary artery was ligated at the location of the 1-2 mm under the boundary of pulmonary conus and left auricle. The left anterior descending coronary artery was occluded for 30 minutes for ischemia, and then released for 120 minutes for reperfusion. In one day after the MIRI surgery, the rats were sacrificed, the rat myocardial tissue samples were collected and divided into two portions: one portion was first rapidly cryopreserved in liquid nitrogen and then stored in a -80°C refrigerator for future use; the other portion was stored in a 4°C refrigerator for future use.

Evaluation of infarct area

As for the samples stored in a 4°C refrigerator, one portion was taken and cut into 1.5 mm sections using a microtome. According to the operation instructions provided by the reagent sup-

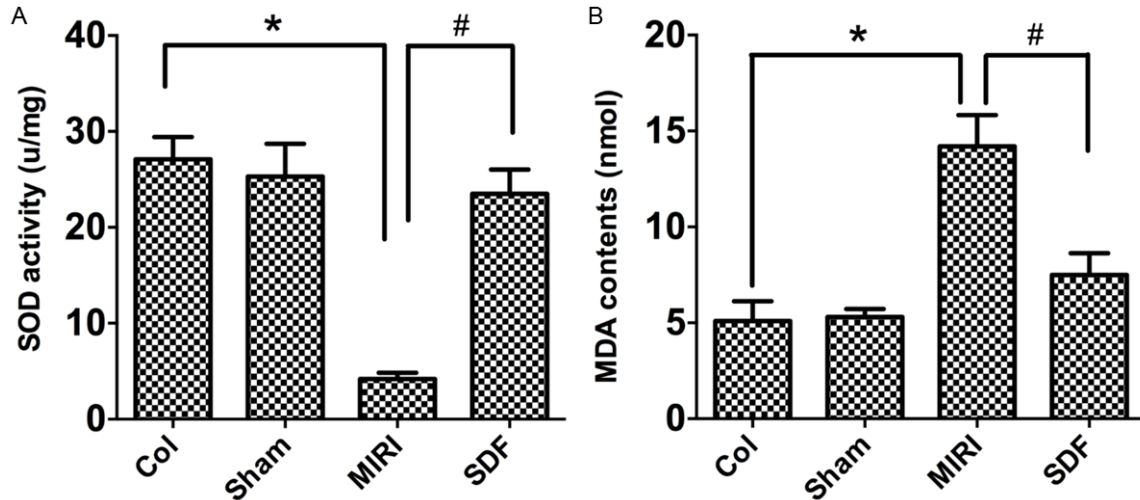


Figure 2. SDF lowered the hypoxia stress of myocardial tissues of Rats that received MIRI surgery. A: SOD activity of rat myocardial tissues. B: Content of MDA of rat myocardial tissues. Col, control group; Sham, Sham group; MIRI, myocardial ischemia reperfusion injury group; SDF, MIRI plus SDF treatment group. Compared with control group, * $P < 0.05$; compared with MIRI group, # $P < 0.05$.

plier, the sections were stained by using the 1% TTC solution at 37°C in the dark places. The tissues with a good blood supply were stained into red by TTC, and the staining of infarcted tissues was relatively superficial and whitish. 20 minutes thereafter, after the liquid on the slice surface was sucked dry, the ImageJ software was used to analyze the infarct size after the pictures were taken.

The TUNEL test was carried out to detect the apoptosis of myocardial histiocyte

Frozen sectioning was performed on the rat myocardial tissues. The TUNEL reaction solution was mixed to generate reactions in the dark for 1 hour according to the operation instructions of TUNEL kit. After DAB color development for three minutes, the hematoxylin was used to the counterstain of the histological slices. Under the 400-fold magnification high power field, 10 different regions were chosen from each slice for analysis, and the number of TUNEL positive cells per unit area of the field (567 pixels * 567 pixels, 72 dpi as the unit area) was measured, then the number was averaged. The cell apoptosis rate was expressed by the ratio between the number of apoptotic myocardial cells and the total number of muscle cells at the center of the field.

Expression of WB test related proteins

60 mg of myocardial tissues were weighed, the total protein was extracted, the BCA method

was used to measure the protein concentration, and the WB method was used to detect the Prx-6, Bax, Caspase-9 and the protein level of NF- κ -B2 of the myocardial tissue of rats in each group. With the sample loading volume of 50 μ g, 6% SDS-PAGE electrophoresis was performed, the membrane was traversed under the cold conditions and then sealed, and the primary antibody concentration of antibody incubation was: Prx-6 (1:200), GAPDH (1:800), Bax (1:500), Caspase-9 (1:200), and NF- κ -B2 (1:400). The chemical substrates were luminescent, the images were developed, and photographic fixing was performed. The Quantity One 4.6 software was used to analyze the optical density values of all bands.

Cell culture and siRNA interference

The rat myocardial cells H9C2 were provided by ATCC (USA). 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin were added to the cell culture and DMEM culture medium. Then they were placed into a 5% CO₂ incubator, the temperature was adjusted to 37°C, and the culture medium was replaced every other day. After the cell growth was stable, the cells were transferred to the six-hole cell culture plates for culture, and the cell concentration was adjusted to 5 × 10⁶ per hole. The cultured H9C2 cells were divided into four groups, and the culture was repeated five times per group: control group (Col), hypoxia group (Hypoxic), hypoxia + SDF group (HS), siRNA +

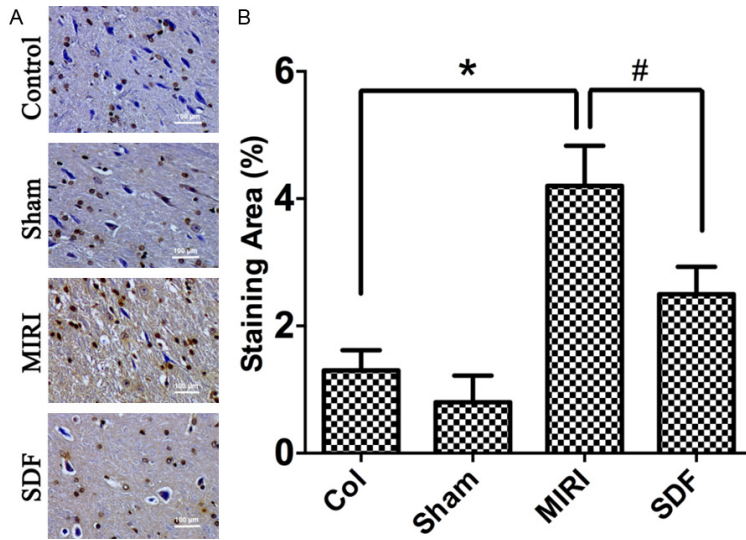


Figure 3. SDF lowered the apoptosis rate of myocardial tissues of Rats that received MIRI surgery. A: The TUNEL staining of myocardial tissues of rats in five groups, the blue granules are karyons and the brownish-black regions are the apoptotic cells. B: Percentage of cell apoptosis rate. Col, control group; Sham, Sham group; MIRI, myocardial ischemia reperfusion injury group; SDF, MIRI plus SDF treatment group. Compared with control group, * $P < 0.05$; compared with MIRI group, # $P < 0.05$.

hypoxia + SDF group (SHS). Before accepting the processing by other drugs, the cells to be used to the Prx-6 gene expression down-regulation test were first processed according to the user's manual provided by reagent manufacturer (Guangzhou RiboBio Co., Ltd.); the cells that should be hypoxia-processed were hypoxia-cultured for one day at the environment of 1% O_2 , 5% CO_2 , and 94% N_2 after other processing.

CCK8 was used to detect the cell proliferation

The Cell Counting Kit was used to detect the cell proliferation; the processed cells in each group were transferred to 96-hole plates. The culture plates were pre-cultured in the incubators under the conditions of 37°C and 5% CO_2 for 24 hours. 10 ml of CCK-8 solution was added to each hole. The culture plate was placed into the incubator for 2-hour incubation. The microplate reader was used to determine the absorbance at 450 nm.

The flow cytometer was used to detect the cell apoptosis

5×10^6 processed cells were collected, centrifugation was performed at the rate of 500 to 1000 r/min for 5 minutes, and the culture fluid was abandoned. After the cells were washed

with 3 ml PBS, centrifugation was performed to have the PBS removed, and then the ice precooling 70% ethanol was added for fixation at 4°C for 1 hour. Centrifugation was performed to abandon the fixation fluid, and then they were resuspended with 3 ml PBS for 5 minutes. The solution was filtered once with the 400-mesh sieve, and centrifugation was performed at 500 to 1000 r/min for 5 minutes. After the PBS was abandoned, 1 ml PI staining solution was used, the cells were placed in the dark place at 4°C for 30 minutes, and finally they were loaded in the flow cytometer for detection.

Detection of cell SOD and MDA

After the processing, the cells were cultured for 24 hours, and centrifugation was performed at 1000 r/min for 5 minutes to aspirate the supernatant. According to the user's manual provided by reagent supplier, the superoxide dismutase (SOD) kit was used to detect the SOD concentration, and the malondialdehyde (MDA) kit was used to detect the MDA concentration. Each processing was performed for three times repeatedly, the detection results were averaged, and the test results were analyzed.

Statistical analysis

The SPSS 19.0 software was used for data processing and analysis, an intergroup comparison was made using the single factor analysis of variance, all the measurement data were expressed by $V \pm s$, and $P < 0.05$ meant the difference was significant.

Results

SDF decreased infarct area of MIRI rats

We performed the transient central muscular arterial blocking surgery to simulate the rat ischemia myocardial injury and detected the impact of SDF on the ischemic tolerance of rats which received MIRI surgery. After the rats received the sham-operation, no ischemia was

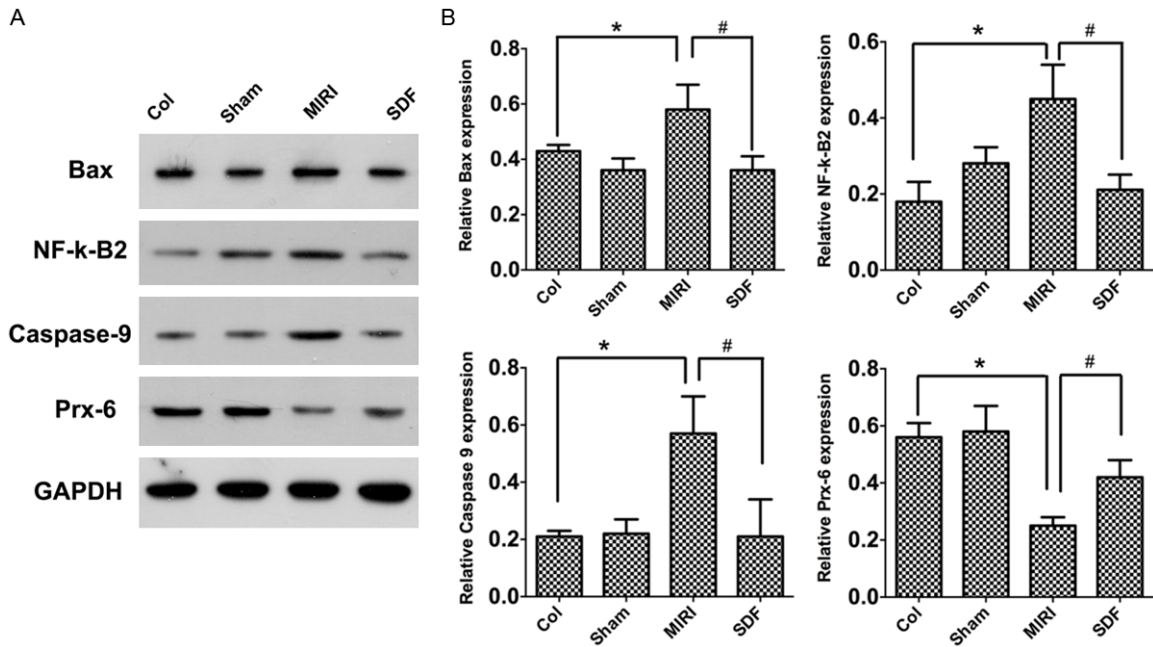


Figure 4. SDF could regulate the expression of Prx-6, Bax, Caspase-9 and NF-κ-B2 proteins. A: SDS-PAGE image of myocardial tissue protein of all rats in five groups. B: Levels of expression of Prx-6, Bax, Caspase-9 and NF-κ-B2 proteins. Col, control group; Sham, Sham group; MIRI, myocardial ischemia reperfusion injury group; SDF, MIRI plus SDF treatment group. Compared with control group, * $P < 0.05$; compared with MIRI group, # $P < 0.05$.

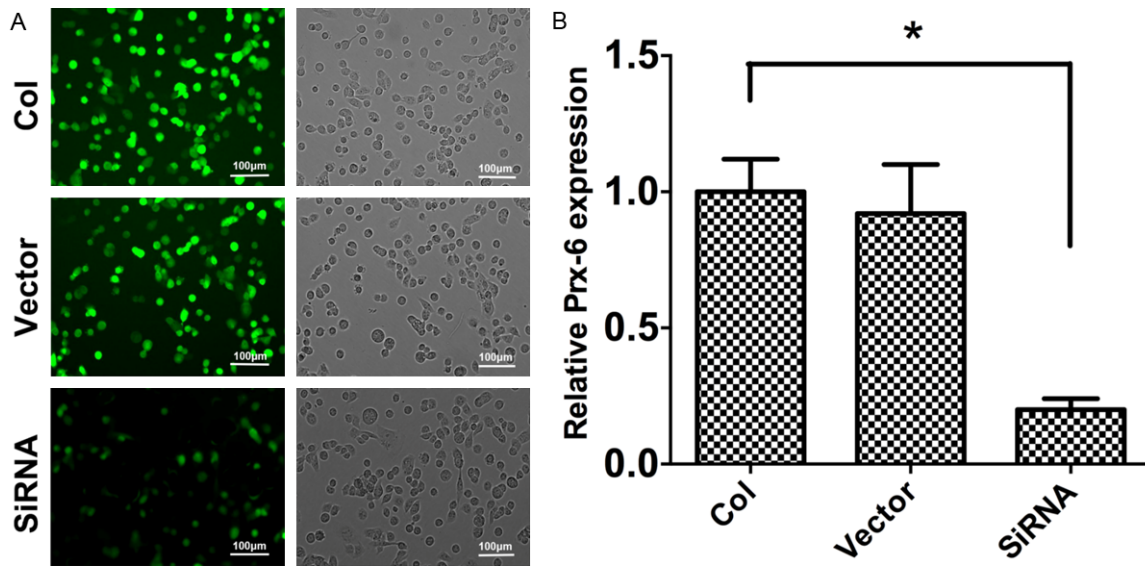


Figure 5. Establishment of H9C2 cells with a low Prx-6 expression. A: The fluorescence microscopy indicated that, siRNA inhibited the Prx-6 gene expression; B: After the siRNA processing, the expression of Prx-6 gene was significantly inhibited. Col, control group; Vector, H9C2 cells with vector; SiRNA, H9C2 cells with siRNA treatment. Compared with control group, * $P < 0.05$.

detected in the myocardial issues, and after the MIRI surgery, no obvious infarction occurred. However, after the SDF-processed rats received the MIRI surgery, their infarct size

was smaller than those who received simple MIRI surgery (Figure 1A, 1B). The experimental results indicated that, SDF could lower the infarct size of MIRI receiving rats.

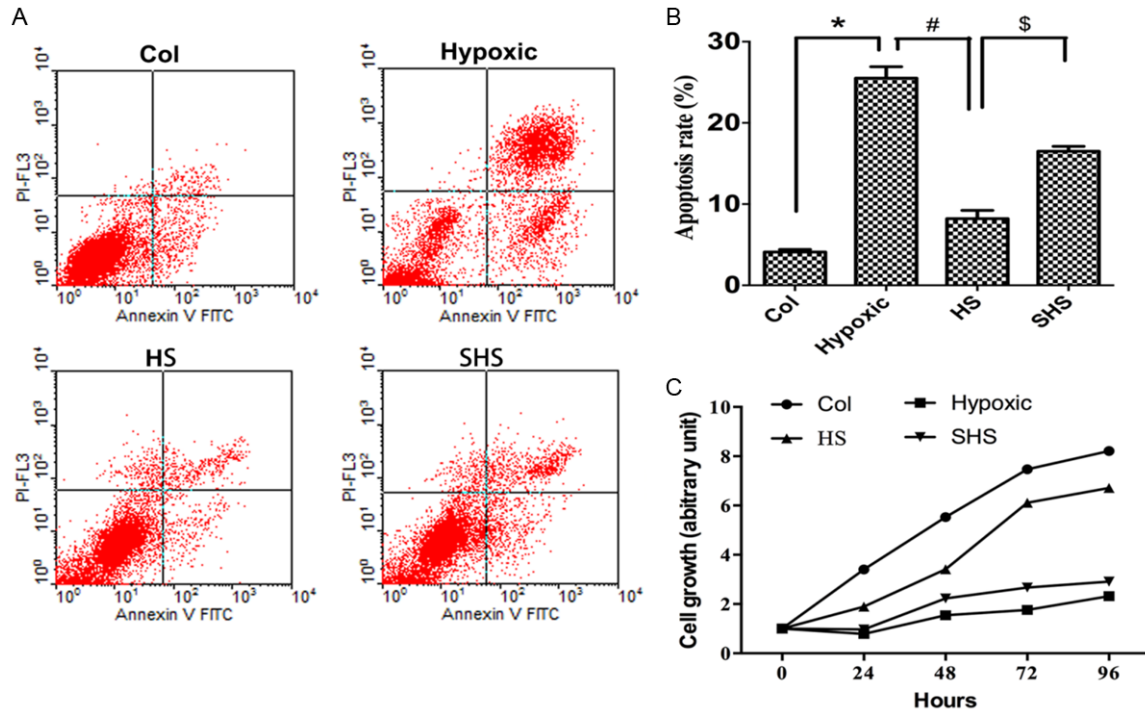


Figure 6. Impact of low Prx-6 expression on the apoptosis and proliferation of H9C2 cells. A, B: Apoptosis was detected in the cells in the four groups using the flow cytometer, and the down-regulation of Prx-6 gene expression significantly offset the SDF effects. C: CCK8 was used to detect the proliferation of all cells in the four groups. Col, control group; Hypoxic, hypoxic condition; HS, hypoxic condition plus SDF; SHS, hypoxic condition plus SDF after Prx-6 gene silencing. Compared with control group, * $P < 0.05$; compared with H group, # $P < 0.05$; compared with HC group, \$ $P < 0.05$.

SDF lower the oxidative stress of MIRI surgery

In order to detect the impact of SDF on the oxidative stress induced by the MIRI surgery, we detected the levels of MDA and SOD of rat myocardial tissues. The experimental results indicated that, after the SDF processing, the rats that received the MIRI surgery had a significantly decreased level of MDA in rat myocardial tissues and a significantly increased level of SOD (Figure 2A, 2B). The experimental results indicated that, SDF processing could inhibit the oxidative stress of Rats that received MIRI surgery.

SDF can inhibit the apoptosis of myocardial cells of rats that received MIRI surgery

We detected the apoptosis of myocardial cells of rats that received MIRI surgery and rats processed by SDF through the TUNEL test. The test results indicated that, after the rats received the MIRI surgery, the apoptosis rate of their myocardial tissues and cells was signifi-

cantly higher than that in the normal group. However, after the SDF preprocessing, the apoptosis rate of the myocardial tissues significantly decreased (Figure 3). The results showed that, SDF could lower the apoptosis rate of myocardial cells of Rats that received MIRI surgery.

SDF can regulate the expression of Prx-6, Bax, Caspase-9 and NF- κ -B2 proteins in the myocardial tissues of Rats that received MIRI surgery

It was molecularly proven that, SDF could impact the infarct size, oxidative stress and apoptosis rate of the myocardial tissues of Rats that received MIRI surgery; we adopted the Western blot test to detect the expression of Prx-6, Bax, Caspase-9 and NF- κ -B2 proteins of rat myocardial tissues of rats. The results indicated that, the MIRI surgery could significantly up-regulate the expression of Prx-6, Bax, Caspase-9 and NF- κ -B2 proteins, and the SDF preprocessing could reverse this trend (Figure

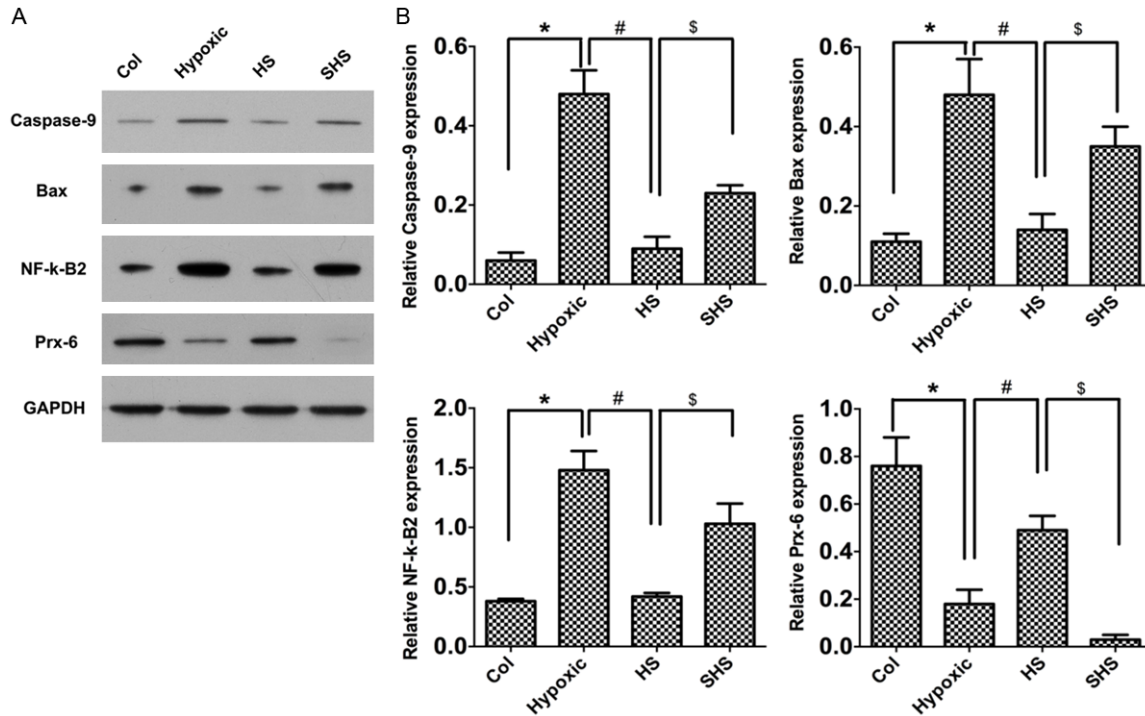


Figure 7. Impact of the low Prx-6 expression on the expression level of Prx-6, Bax, Caspase-9 and NF- κ -B2 proteins. A: SDS-PAGE images of proteins of all cells in the four groups. B: The expression level of Prx-6, Bax, Caspase-9 and NF- κ -B2 in H9C2 cells. Col, control group; Hypoxic, hypoxic condition; HS, hypoxic condition plus SDF; SHS, hypoxic condition plus SDF after Prx-6 gene silencing. Compared with control group, * $P < 0.05$; compared with H group, # $P < 0.05$; compared with HC group, $^sP < 0.05$.

4A, 4B). The results indicated that, the Prx-6, Bax, Caspase-9 and NF- κ -B2 proteins might be involved in the ischemic tolerance of Rats that received MIRI surgery to ischemia.

SDF can inhibit the myocardial apoptosis through Prx-6

In order to investigate the relation of Prx-6 and SDF in the myocardial ischemia reperfusion injury, we adopted the siRNA to inhibit the Prx-6 gene expression in the H9C2 cells (**Figure 5A, 5B**), then used SDF to process the hypoxia-cultured H9C2 cells and detected the cell apoptosis rate through the flow cytometry. The experimental data indicated that, SDF processing could significantly lower the apoptosis rate of hypoxia-processed H9C2 cells; then the down-regulation of Prx-6 gene expression lowered the SDF effects (**Figure 6A, 6B**).

SDF can regulate the myocardial cell proliferation via Prx-6

After the use of siRNA to inhibit the expression of Prx-6 gene expression, SDF was used to pro-

cess the hypoxia-cultured H9C2 cells; the test by using CCK8 indicated that, SDF processing could significantly induce the proliferation of hypoxia-processed H9C2 cells; then the down-regulation of Prx-6 gene expression lowered the SDF effects (**Figure 6C**).

SDF can regulate the expression of Bax, Caspase-9 and NF-κ-B2 through Prx-6

The results of early experiment indicated that, SDF could regulate the apoptosis and proliferation of the hypoxia-cultured myocardial cells through Prx-6. Therefore, we make a hypothesis that SDF has a protective effect on hypoxia stress, which is very likely achieved through using the Prx-6 to regulate the expression of Bax, Caspase-9 and NF- κ -B2 (**Figure 7A, 7B**). The results of western blot test indicated, after the H9C2 cells were hypoxia-cultured, the SDF processing could significantly inhibit the expression of Bax, Caspase-9 and NF- κ -B2 proteins; however, the SDF effect was significantly lowered after the Prx-6 gene expression of H9C2 cells was inhibited. Therefore, we further validated from the molecular perspective that, SDF

had protective effects on the myocardial ischemia reperfusion injury, which could be achieved through the regulation of Prx-6.

Discussion

During the early stage, investigators studied the protecting effect of SDF on suckling rat myocardial cells through the determination of myocardial cell activity and the concentration of LDH in the culture fluid [19]. In this study, the TTC staining was performed on the myocardial tissues to detect the infarct size of rat myocardial tissues through the construction of post-MIRI-surgery rat models. We preliminarily found that, SDF could significantly inhibit the oxidative stress induced by MIRI surgery, and TUNEL staining was performed on the myocardial tissues. The analysis results showed that, SDF could reverse the MIRI-induced cell apoptosis. At the level of tissues, we found through the experiment that, SDF had the protective effect on myocardial ischemia reperfusion injury, but the mechanism of action still remains unclear to us.

Under the normal growth conditions, SDF had no impact on the myocardial cell activity and the concentration of LDH in the culture fluid; in case of the hypoxia/reoxygenation ($\text{Na}_2\text{S}_2\text{O}_2$) models or peroxide (H_2O_2) injuries, SDF could significantly lower the concentration of LDH in the culture fluid, enhance the activity of impaired cells, and protect the impaired myocardial cells [20-22]. The results of these studies also indicate that, SDF has protective effects on myocardial ischemia reperfusion injury, and the mechanism may be the clearance or inhibition of free radicals on myocardial cells.

It's shown in some studies that SDF has the significant anti-oxidation effect, and its anti-inflammatory effect is related with the clearance of free radicals [23-25]. In this study, H9C2 cells were used as the experimental materials, the cell models were constructed through hypoxia induction to validate the impact of SDF on the hypoxia tolerance of myocardial cells, and then attempted to investigate the mechanism of action. Studies have indicated that, Bax and Caspase-9 are important indicators in the apoptosis signal pathway, NF- κ -B2 plays an important role in the cytotoxicity and proliferation process, and the abnormal expres-

sion of Prx-6 gene is closely associated with the cell hypoxia stress, so it may be a key factor in the process that SDF protects the myocardial ischemia reperfusion injury. Therefore, we adopted the siRNA to inhibit the expression of Prx-6 and observed the SDF effect. Like our hypothesis, the Prx-6 gene expression was inhibited, significantly impacting the protective effect of SDF on myocardial ischemia reperfusion injury. Therefore, we can judge that, SDF can improve the hypoxia tolerance of myocardial cells, and this protective effect can be achieved through the regulation of the Prx-6 gene expression. However, whether SDF can have a targeted regulation of the Prx-6 gene expression through other factors remains unclear and needs further exploration.

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Disclosure of conflict of interest

None.

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