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Research Article
Role of Nrf2/ARE Pathway in Morphine Preconditioning to Alleviate Rat Myocardial Ischemia-Reperfusion Injury

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Abstract

Background and Objective: Myocardial Ischemia-Reperfusion Injury (MIRI) results in damage to heart structure and dysfunction, which seriously threatens life safety. To explore the role of nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway in morphine preconditioning to alleviate MIRI in rats with doxorubicin hydrochloride-induced heart failure (HF). **Materials and Methods:** HF model was established by tail vein injection of doxorubicin hydrochloride. Then model rats were randomly divided into sham operation (sham), ischemia-reperfusion (model), morphine preconditioning (MFC), oleanolic acid derivative 85 (OAD-85) +morphine preconditioning (MOA) and OAD-85 control (OAD) groups. Myocardial infarction, changes in histopathology and ultrastructure, reactive oxygen species (ROS) level, oxidative stress, apoptosis rate and protein expressions of Nrf2 and Heme Oxygenase-1 (HO-1) were detected by triphenyl tetrazolium chloride staining, hematoxylin-eosin staining, transmission electron microscopy, 2',7'-dichlorodihydrofluorescein diacetate staining, TUNEL staining and western blotting, respectively. **Results:** Compared with the model group, myocardial infarction proportion, ROS, malondialdehyde (MDA) and apoptosis rate significantly reduced in the MFC group, whereas superoxide dismutase (SOD) activity and expressions of Nrf2 and HO-1 increased. Compared with the MFC group, the MOA group exhibited increased myocardial infarction proportion, ROS, MDA and apoptosis rate as well as decreased activity of SOD and expressions of Nrf2 and HO-1. Compared with the model, MFC and MOA groups, myocardial infarction proportion, ROS, MDA and apoptosis rate rose significantly, while SOD activity and expressions of Nrf2 and HO-1 decreased significantly in the OAD group. **Conclusion:** The Nrf2/ARE pathway is involved in morphine preconditioning to alleviate MIRI in rats with HF induced by doxorubicin hydrochloride. Morphine can markedly activate the Nrf2/ARE pathway to play an antioxidant role.

Key words: Doxorubicin hydrochloride, heart failure, morphine, myocardial ischemia-reperfusion injury, Nrf2/ARE, reactive oxygen species

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Heart failure (HF) is the terminal stage of primary and secondary cardiovascular diseases, with a relatively high incidence rate[1]. When patients with HF caused by coronary heart disease and heart valves undergo cardiac or non-cardiac surgery, myocardial ischemia-reperfusion injury (MIRI) easily occurs because of their sensitivity to ischemia and other stimuli, resulting in damage to heart structure and dysfunction, which seriously threatens the life safety of patients and becomes an intractable challenge faced by clinical anesthesiologists[2]. Opioids are the first-line analgesics in cardiac anaesthesia surgery. Opioids, such as sufentanil and morphine, can reduce myocardial cell apoptosis and the probability of HF during cardiac surgery[3]. Besides, opioid preconditioning inhibits the abnormal apoptosis of myocardial cells in MIRI, suppresses myocardial infarction and plays a role in cardiac protection[4]. Chen *et al.*[5] confirmed that morphine preconditioning relieved MIRI in rats. However, the effect of morphine preconditioning on HF animal models suffering from MIRI has been rarely reported. The nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signalling pathway is the most important endogenous antioxidant pathway in the body. The Nrf2/ARE pathway eliminates reactive oxygen species (ROS) in cells and suppresses oxidative stress to alleviate MIRI by modulating the expression of antioxidant enzymes such as Heme Oxygenase-1 (HO-1) and the transporter Glutathione (GSH)[6].

In this study, the HF rat model of MIRI was established to explore the protective effect of Nrf2/ARE against MIRI induced by doxorubicin hydrochloride in morphine preconditioning in HF rats, aiming to provide theoretical support for its clinical application.

MATERIALS AND METHODS

Study area

Experimental animals: A total of 72 SPF-grade SD male rats aged 8-10 weeks old, weighing 220-250 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Production License No. SCXK (Beijing) 2016-0011). The experimental operation of this study was approved by the Animal Ethics Committee of the hospital. During the experiment, the animals were given humanitarian concern under the guidance of the 3R principle. This study was conducted in the experimental animal centre of Taizhou Central Hospital from January-August, 2020.

Main reagents and apparatus: Reagents included morphine (Approval No. 2013071022F, Fresenius Kabi, Germany), doxorubicin hydrochloride for injection (Shanxi Pude Pharmaceutical Co., Ltd., Approval No. NMPN H14023879), Nrf2/ARE pathway inhibitor oleanolic acid derivative 85 (OAD-85, Jilin Jinheng Pharmaceutical Co., Ltd., Approval No. NMPN BHX011634), paraformaldehyde (Sigma, USA), hematoxylin-eosin (HE) staining kits, triphenyl tetrazolium chloride (TTC) staining kits, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining kits (Shanghai Beyotime Biotechnology Co., Ltd.), Nrf2 and HO-1 antibodies (Abcam, USA) and TUNEL staining kit and western blotting kit (Rebstock, Germany).

Instruments included E800 fluorescence microscope (Nikon, Japan), Universal Hood Bio-Rad gel imaging system (Bio-Rad, USA), -80°C cryogenic refrigerator (Wiggens, Germany), a JEM-ACE200F electron microscope (JEOL, Japan) and Leica RM2135 tissue slicer (Leica, Germany).

Establishment of HF mouse model: Before the experiment, doxorubicin hydrochloride for injection was prepared into a 2 mg L^{-1} solution using standard medical normal saline. A total of 60 healthy adult rats with weight maintained at 220-250 g were injected with 2 mg kg^{-1} doxorubicin hydrochloride solution through the tail vein once a week for 8 consecutive weeks according to the methods described in a previous literature[7], after which a rat model of HF was constructed. The rats in the normal group (n = 12) were injected with an equal volume of medical normal saline through the tail vein. 8 weeks later, cardiac colour doppler ultrasound was applied to measure Left Ventricular Ejection Fraction (LVEF) and Left Ventricular Fractional Shortening (LVFS). The rats with LVEF>58% and LVFS>30% indicated successful modelling of rat HF.

Establishment of MIRI mouse model and grouping: The 60 HF model rats were randomly divided into 5 groups (n = 12), namely, sham operation group (sham group), ischemia-reperfusion group (model group), morphine preconditioning group (0.1 mg kg^{-1} morphine, MFC group), Nrf2/ARE signalling pathway inhibitor OAD-85+morphine preconditioning group (0.1 mg kg^{-1} OAD-85+0.1 mg kg^{-1} morphine, MOA group) and OAD-85 control group (0.1 mg kg^{-1} OAD-85, OAD group). The rats in the MFC group and MOA group were intravenously infused with 0.1 mg kg^{-1} morphine for 5 min for 3 times, with 5 mL/time, at 1 hr before the modelling and those in other groups were infused with the same volume of normal saline.

After ischemic preconditioning, the rats in each group were anaesthetized by intraperitoneal injection of pentobarbital sodium and fixed on a sterile operating table in the supine position. Then the left upper limb, right upper limb and left lower limb were connected to needle-shaped

electrodes to record the Electrocardiogram (ECG) and heart rate (HR) of the rats. Subsequently, a longitudinal incision was made at the trachea and the intubation tube was inserted to connect an animal ventilator. Meanwhile, the left anterior descending coronary artery was ligated using a 6-0 thread and after stabilization for 10 min, the left coronary artery occlusion caused by tightening the thread knot resulted in myocardial ischemia in rats[8]. After successful ischemia in rats, i.e., there was local myocardial cyanosis dominated by the left coronary artery, the limited local beat of the heart, decreased blood pressure and great fluctuation (either increase or decrease) of ST-segment and myocardial infarction changes shown on ECG, reperfusion was realized by loosening the thread knot. In this experiment, the heart of the rats in each group was treated with ligation for 30 min and reperfusion for 120 min. In the sham group and normal group, the suture was not ligated only at the left anterior descending coronary artery. During the surgery, heat should be preserved. In this experiment, the rats should be regularly ligated for 30 min and reperfused for 120 min every day for one week and all the rats were fed with standard feed and had free access to water.

Observation of myocardial infarction by TTC staining: The rats were anaesthetized by intraperitoneal injection of pentobarbital sodium and killed by bleeding from the abdominal aorta. On the sterile operating table, the heart was taken out by opening the chest and water was absorbed with sterile filter paper. After treatment, the heart tissues were sealed in liquid nitrogen and placed in the -80°C cryogenic refrigerator for later use. Next, 10% rat heart specimens were taken out from each group and prepared into coronary slices, which were then stained with 2% TTC staining solution at room temperature for 20 min and fixed with 10% formalin solution. Later, the slices were placed in glacial acetic acid at 4°C for 24 hrs, followed by photographing using a digital camera. Normal myocardial tissues were bright red and the myocardial infarction tissues were white. Finally, the images were preserved and statistical analysis of myocardial infarction was carried out in the meantime.

Observation of pathological changes of myocardial tissues by light microscopy: A total of 10% rat myocardial tissues were taken out from the refrigerator, subjected to HE staining and cut into slices. Then the pathological changes in rat myocardial tissues in each group were observed under a light microscope.

Observation of ultra-microstructure by transmission electron microscopy: A total of 10% of rat myocardial tissues were taken out from the refrigerator and cut into ultra-thin tissue slices as follows: The tissues were fixed with 30% polyoxymethylene and 0.05% osmic acid for 1 hr and subjected to sterile dehydration for 5 min and pre-cooling at 4°C ethanol for 3 min. After soaking in epoxy resin 812 for 5 min, the tissues were embedded in paraffin, followed by uranyl acetate and lead citrate double staining. Ultimately, the tissues were prepared into 50 nm slices and the slices were put on a copper net, whose ultramicroscopic pathological structure was observed on a transmission electron microscope.

Detection of myocardial cell apoptosis by TUNEL assay: After 10% of rat myocardial tissues were taken out from the refrigerator, they were fixed using conventional methods, frozen and sliced. Following washing with xylene twice, the slices were rinsed by gradient concentrations of ethanol solutions for 5 min, soaked in 3% hydrogen peroxide-methanol for 10 min after air drying and rinsed in Phosphate-Buffered Saline (PBS) 3 times (3 min/time). Subsequently, the slices underwent the following operations on precooled ethanol at 4°C: The slices were treated with 0.1% Triton X-100 and 0.1% buffer solution for 2 min, washed with PBS three times (3 min/time) and added with TUNEL reaction mixture. Later, the slices reacted with the sealing film in a dark wet box for 1 hr at 37°C, rinsed with PBS, dehydrated with gradient concentrations of ethanol solutions, transparentized using xylene and sealed by neutral resins. Ultimately, the slices were observed under the fluorescence microscope.

Evaluation of myocardial oxidative stress based on ROS level detected by DCFH-DA staining: A total of 10% rat myocardial tissues were taken from the refrigerator, fixed with 10% paraformaldehyde for 2 hrs, embedded in paraffin and sliced. Then they were stained by 10 μmol L^{-1} DCFH-DA for 2 hrs and photographed under the fluorescence microscope. Later, 5 images of non-repeating areas were randomly selected under the fluorescence microscope, the average intensity of green fluorescence in 5 fields was assessed using image analysis software ImageJ 1.41 and the specimens in each group were statistically analyzed in turn. According to the instructions of each kit, malondialdehyde (MDA) detection kits and superoxide dismutase (SOD) detection kits were used to detect the change level of substrates related to oxidation reaction in myocardial tissues.

Detection of Nrf2 and HO-1 protein expressions in myocardial tissues by a western blot: A total of 10% rat myocardial tissues were taken out from the refrigerator in

each group, respectively. Then the skull was quickly peeled off to take ischemic hemisphere tissues and the cerebral cortex was separated under ice bath conditions. Afterwards, the tissues were added with an appropriate amount of RIPA tissue lysis buffer, broken in the constant-temperature homogenizer, lysed on ice for 30 min and centrifuged. Next, the supernatant was preserved and the concentration of each protein was measured using conventional BCA kits. Following the dilution of the buffer, the proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and quickly transferred onto a PVDF membrane. Later, the membrane was sealed by 5% Tris-buffered saline-Tween 20 (TBST) at room temperature for 2 hrs and primary antibodies (1: 1000) were added for incubation overnight at 4°C. The next day, the proteins were washed by TBST three times (10 min/time) and added with secondary antibodies (1: 10000). After dilution, the proteins were incubated at constant temperature for 2 hrs and the colour development agent DAB was added in the dark. Finally, the greyscale of the proteins was recorded under the Bio-Rad gel imaging system and the relative expression of the target proteins was displayed, respectively, with GAPDH as a reference.

Statistical analysis: SPSS 16.0 software was used for statistical analysis and GraphPad 5.01 was applied for plotting. Intergroup comparison was conducted by t-test. $p<0.05$ represented statistical significance.

RESULTS

Proportions of myocardial infarction: There was no myocardial infarction in the sham group and normal group. Compared with that in the sham group and normal group, the myocardial infarction proportion was increased significantly in the model group ($p<0.05$). Compared with the model group, the MFC group showed a significantly decreased myocardial infarction proportion ($p<0.05$) and compared with the MFC group, the MOA group displayed a significantly increased myocardial infarction proportion ($p<0.05$). Besides, in contrast to that in the model group, MFC group and MOA group, the myocardial infarction proportion rose significantly in the OAD group ($p<0.05$) (Fig. 1).

Pathological and ultra-microstructural changes of myocardial tissues: HE staining results revealed that in the normal group, the structure of myocardial cells was complete, clear and distinguishable, the cells were arranged orderly, there was no oedema in the intercellular matrix and myocardial cross striations were clear and regular (Fig. 2a). In the sham group, myocardial cells were swollen and arranged irregularly, interstitial oedema was obvious, the extracellular matrix was increased, inflammatory cells infiltrated and fibroblasts were increased (Fig. 2b). In the model group, the arrangement of myocardial cells became more disordered, some nuclear membranes were broken and disappeared, some cell membranes were missing, cells showed apoptosis, interstitial congestion of myocardial cells was serious, myocardial cross striations disappeared and inflammatory cells infiltrated (Fig. 2c). Compared with those in the model group, the myocardial cells were arranged regularly, the infiltration of inflammatory cells was reduced, there was no bleeding point, the symptoms of myocardial cell injury were markedly alleviated and the apoptosis was notably improved in the MFC group (Fig. 2d). In contrast to those in the MFC group, the myocardial cells were arranged disorderly, inflammatory cell infiltration was increased and the number of apoptotic cells in visual fields was remarkably increased in the MOA group (Fig. 2e). Compared with those in the model group, MFC group and MOA group, the cytoplasm and nucleus boundaries of cardiac cells in the OAD group overlapped significantly, boundaries were unclear and cell apoptosis symptoms were more evident (Fig. 2f).

The ultrastructural changes of myocardial tissues in each group were observed under the electron microscope. In the normal group, myocardial sarcomeres were in alignment, with clear cross striations, regular shape of nucleus located in the centre of cells, clear structure of myocardial mitochondria, clear double membranes, the regular and continuous structure of mitochondrial cristae and no swelling, endoplasmic reticulum folded around the cells and its surroundings and bright and dark bands of myocardial cells

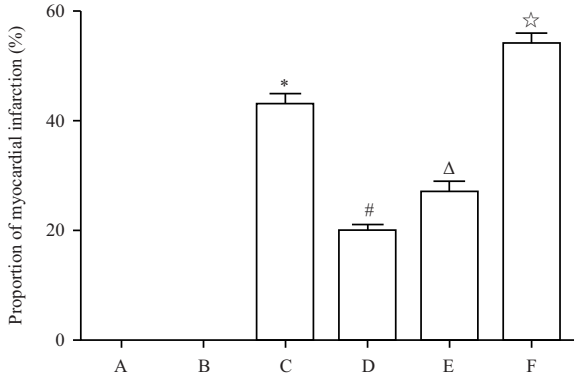

Fig. 1: TTC staining results of rat myocardial tissues

A: Normal group, B: Sham group, C: Model Group, D: MFC group, E: MOA group, F: OAD group. *: $p<0.05$ vs. sham group, #: $p<0.05$ vs. model group, Δ: $p<0.05$ vs. MFC group and ☆: $p<0.05$ vs. MOA group

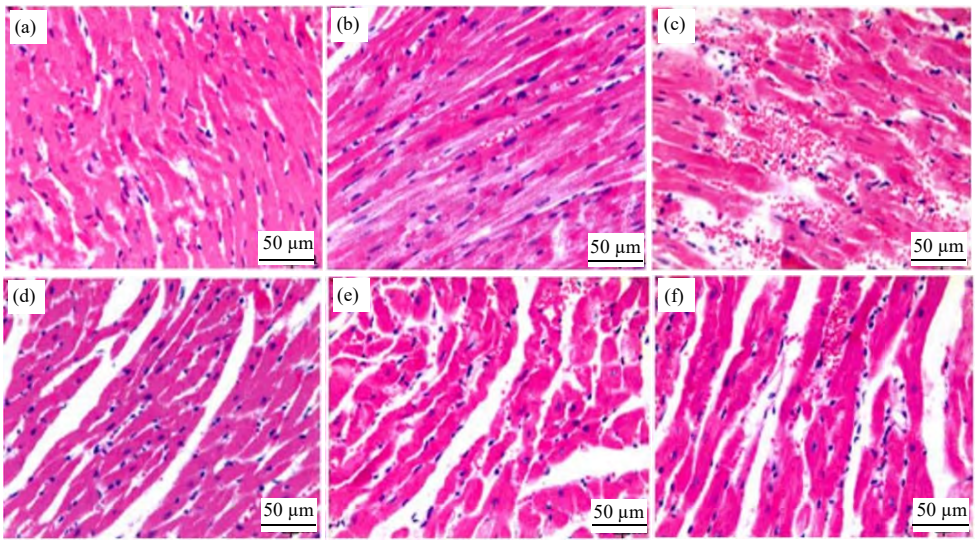

Fig. 2(a-f): HE staining results of rat myocardial tissues
A: Normal group, B: Sham group, C: Model group, D: MFC group, E: MOA group and F: OAD group

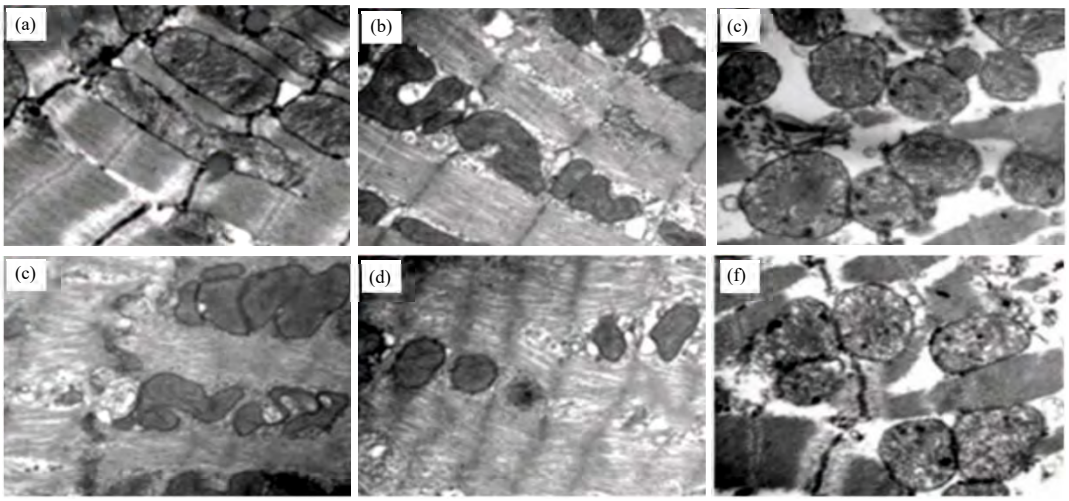

Fig. 3(a-f): Ultrastructure of rat myocardial tissues
A: Normal group, B: Sham group, C: Model group, D: MFC group, E: MOA group and F: OAD group

were visible (Fig. 3a). In the sham group, the nucleus of myocardial cell nuclei was swollen obviously, the shape and structure deformed remarkably and the mitochondrial structure was damaged dramatically. The mitochondrial cristae structure partially overlapped and was relatively loose, but it still filled the whole mitochondrial cavity (Fig. 3b). Compared with those in the normal group, the number of mitochondria did not notably drop down and the endoplasmic reticulum was slightly loose and still surrounded the nucleus in the sham group. In the model group, the myocardial sarcomere was disordered and dissolved in a large area. Besides, the nucleus of the cells was markedly enlarged, the nuclear membrane was broken, the cytoplasm and nucleus boundaries were unclear, the nuclear chromatin was remarkably condensed, the mitochondria were seriously swollen, the cristae structure was irregular, shortened, sparse and blurred and obvious vacuolar degeneration could be seen. Moreover, some mitochondrial bilayer membranes were fused and broken, the endoplasmic reticulum was irregular in shape, severely expanded and filled the cytoplasm loosely. The myocardial collagen fibres were disordered, the connection between myocardial cells was severely broken and dissolved and the bands were difficult to distinguish (Fig. 3c). In comparison with those in the model group, the myocardial

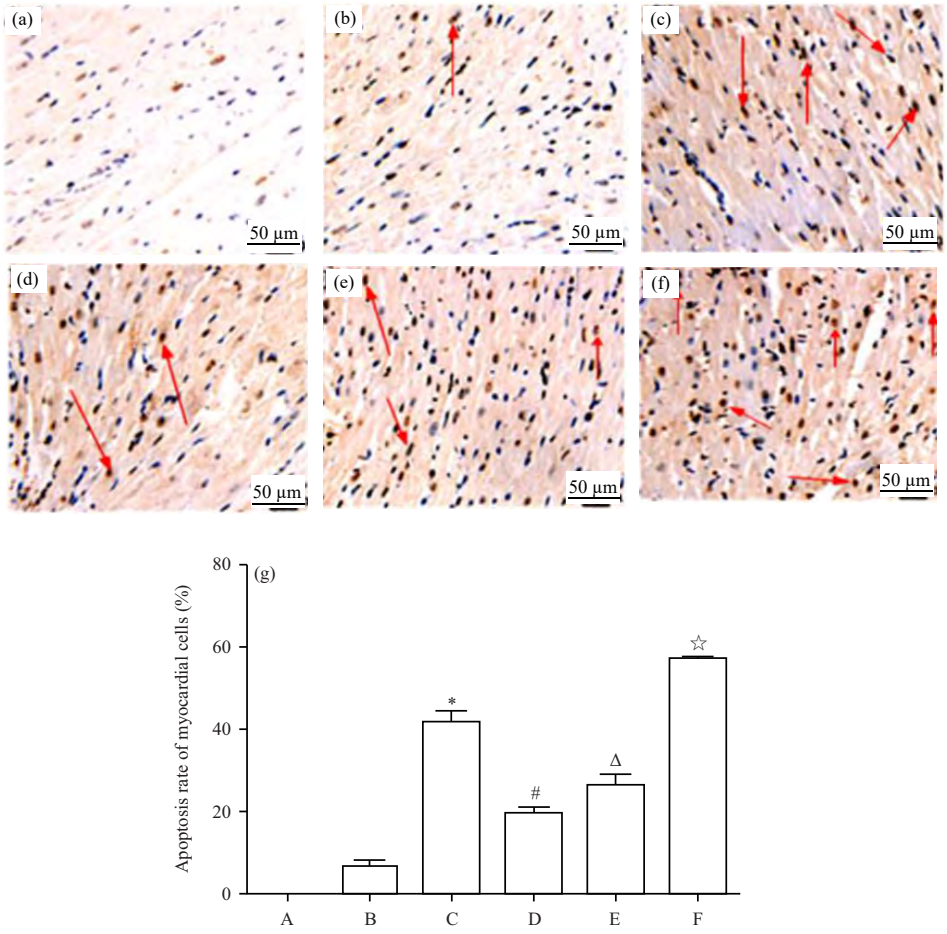

Fig. 4(a-g): TUNEL staining results of rat myocardial tissues

A: Normal group, B: Sham group, C: Model group, D: MFC group, E: MOA group, F: OAD group, *: p<0.05 vs. sham group, G: Histogram of mean fluorescence intensities of ROS, #: p<0.05 vs. model group, Δ: p<0.05 vs. MFC group and ☆: p<0.05 vs. MOA group

cells were arranged regularly, the oedema of myocardial mitochondria was significantly alleviated and some cristae were distinguishable in the MFC group. Although some of the cristae were broken or missing, the severity was lighter than that in the model group. In the meantime, the stable endoplasmic reticulum surrounded the cells and around them and the myocardial collagen fibres were relatively neatly arranged, showing relatively slight pathological changes (Fig. 3d). Furthermore, in contrast to those in the MFC group, the myocardial cells were disordered and dissolved and the damage of myocardial organelles in the MOA group was more serious than that in the MFC group but lighter than that in the model group (Fig. 3e). Compared with the model group, MFC group and MOA group, the OAD group displayed significantly aggravated myocardial cell injury (Fig. 3f).

Myocardial cell apoptosis: It was discovered from TUNEL staining results that compared with that in the normal group (Fig. 4a) and sham group (Fig. 4b), the apoptosis rate of myocardial cells in the model group was significantly elevated (Fig. 4c) and it was significantly decreased after morphine preconditioning in the MFC group in contrast to that in model group (Fig. 4d). In comparison with that in the MFC group, the apoptosis rate of myocardial cells significantly rose after adding the signalling pathway inhibitor in the MOA group (Fig. 4e) and the OAD group exhibited a significantly increased apoptosis rate compared with that in the model group, MFC group and MOA group ($p<0.05$) (Fig. 4f). The histogram of apoptosis rates is exhibited in Fig. 4g.

Oxidative stress of myocardial tissues: The content of ROS was detected by DCFH-DA staining to evaluate the oxidative stress of the heart in each group of rats. DCFH-DA itself shows no fluorescence, but it can be hydrolyzed freely through the cell membrane and will fluoresce after ROS in the cell oxidizes DCFH. Compared with those in the normal group (Fig. 5a) and

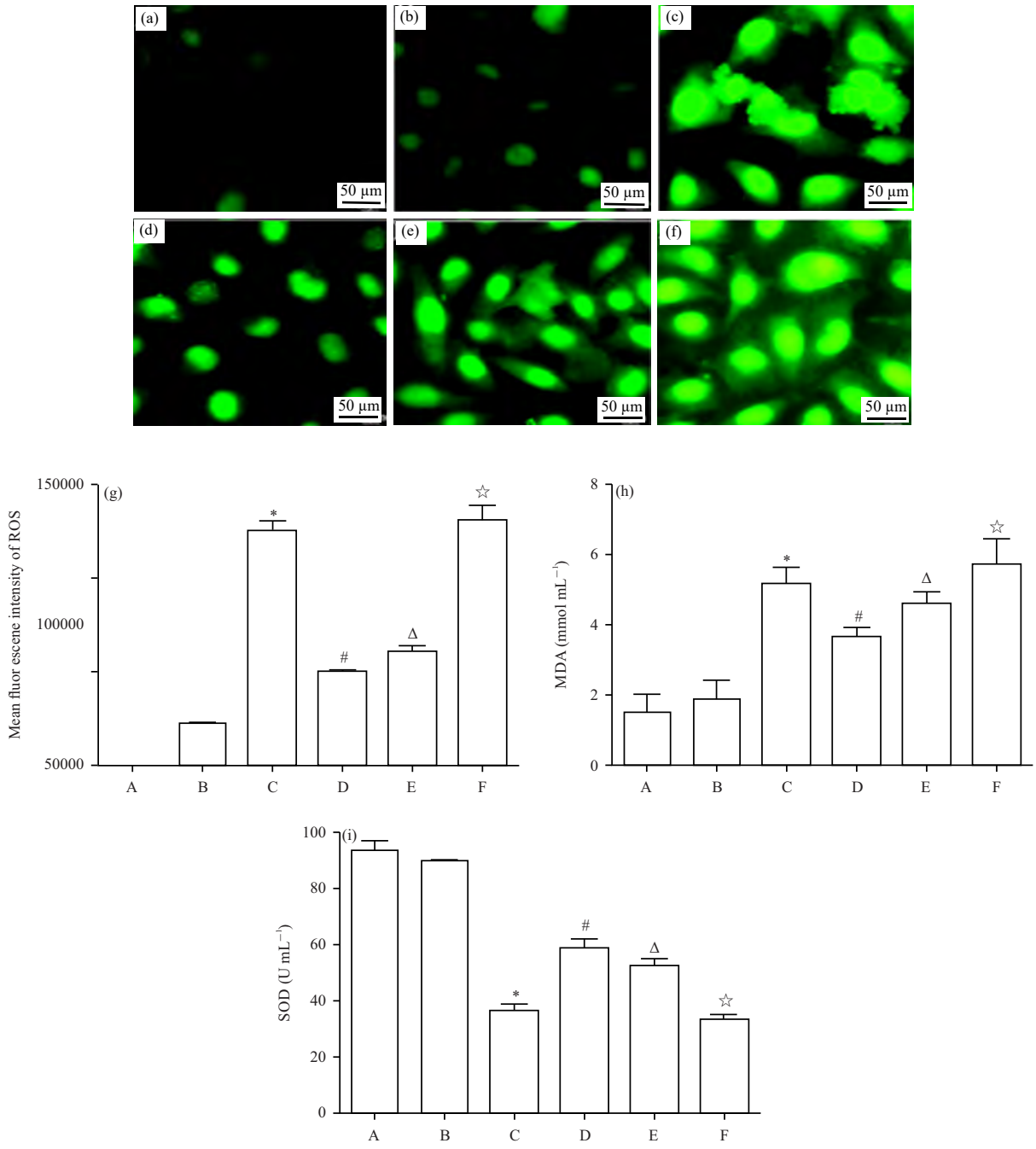

Fig. 5(a -i): Oxidative stress of myocardial tissues

A: Normal group, B: Sham group, C: Model group, D: MFC group, E: MOA group, F: OAD group, G: Histogram of mean fluorescence intensities of ROS, H: MDA contents, I: SOD activities. *: p<0.05 vs. sham group, #: p<0.05 vs. model group, Δ: p<0.05 vs. MFC group and ☆: p<0.05 vs. MOA group

sham group (Fig. 5b), the fluorescence intensity and ROS content of myocardial cells in the model group were significantly increased (Fig. 5c). Compared with those in the model group, the fluorescence intensity and ROS content of myocardial cells were significantly reduced after morphine preconditioning in the MFC group (Fig. 5d). Besides, the MOA group exhibited significantly increased fluorescence intensity and ROS content of myocardial cells after the addition of the pathway inhibitor in contrast to the MFC group but displayed significantly reduced fluorescence intensity and ROS content compared with the model group (Fig. 5e). Moreover, in comparison with those in the model group, MFC group and

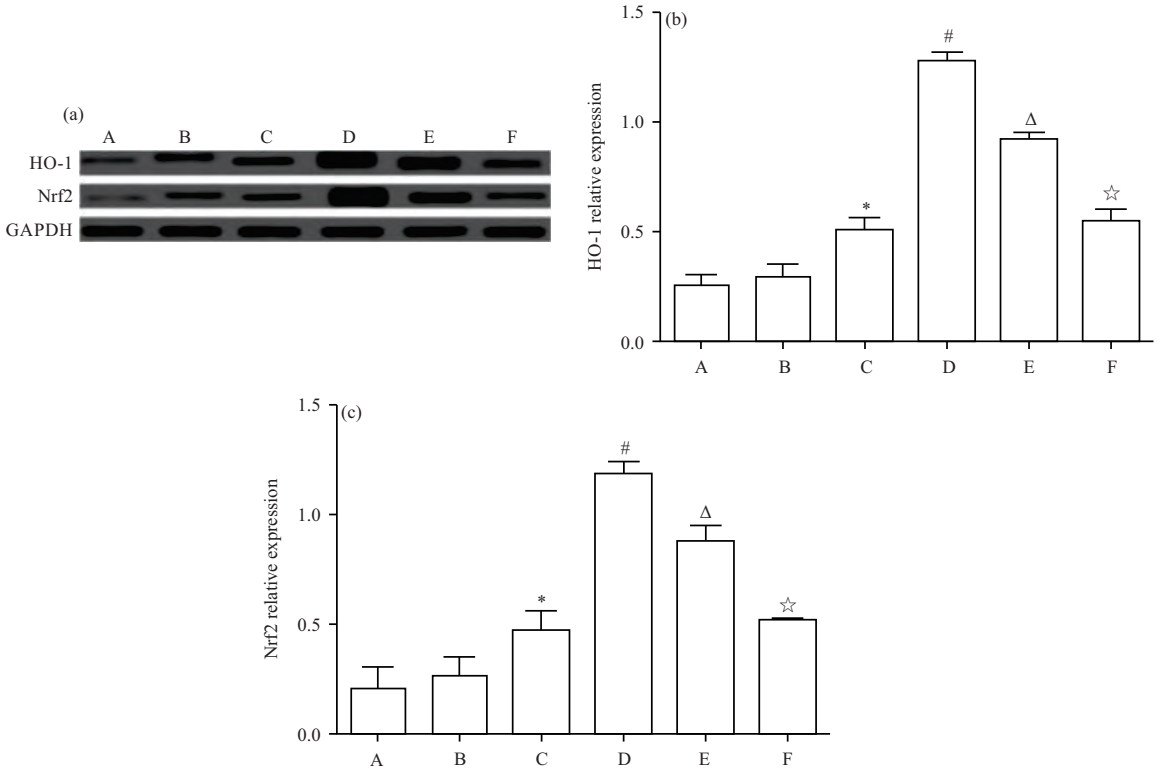

Fig. 6(a-c): Protein expressions of Nrf2 and HO-1 in rat myocardial tissues, (a) Western blotting bands, (b) HO-1 relative expression and (c) Nrf2 relative expression

A: Normal group, B: Sham group, C: Model group, D: MFC group, E: MOA group, F: OAD group, *: p<0.05 vs. sham group, #: p<0.05 vs. model group, Δ: p<0.05 vs. MFC group and ☆: p<0.05 vs. MOA group

MOA group, the fluorescence intensity and the ROS content of myocardial cells were significantly raised in the OAD group (Fig. 5f). The histogram of mean fluorescence intensities of ROS is presented in Fig. 5g.

Furthermore, the activities of related substrates and enzymes in oxidative stress were detected. Compared with the normal group and sham group, the model group exhibited significantly increased content of MDA in myocardial cells and significantly decreased SOD activity ($p<0.05$). In comparison with those in the model group, the content of MDA in myocardial cells dropped significantly, while the activity of SOD rose significantly after morphine preconditioning in the MFC group. In contrast to those in the MFC group, the content of MDA in myocardial cells was raised significantly, but the activity of SOD was reduced significantly after the addition of the signalling pathway inhibitor in the MOA group. In addition, compared with the model group, MFC group and MOA group, the OAD group showed significantly increased content of MDA in myocardial cells and significantly weakened SOD activity ($p<0.05$) (Fig. 5h-i).

A: normal group, B: sham group, C: model group, D: MFC group, E: MOA group and F: OAD group.

Nrf2 and HO-1 protein expressions in myocardial tissues: In this study, western blotting was adopted to examine the protein expressions of Nrf2 and HO-1 in the rat myocardial tissues in each group (Fig. 6a). It was discovered that compared with those in the normal group and sham group, the protein expressions of Nrf2 and HO-1 in myocardial cells in the model group were significantly elevated. In comparison with those in the model group, the protein expressions of Nrf2 and HO-1 in myocardial cells significantly rose after morphine preconditioning in the MFC group. In contrast to those in the MFC group, the protein expressions of Nrf2 and HO-1 in myocardial cells declined significantly after adding the signalling pathway inhibitor in MOA group. Besides, the OAD group displayed significantly reduced protein expressions of Nrf2 and HO-1 compared with those in the model group, MFC group and MOA group ($p<0.05$) (Fig. 6b-c).

DISCUSSION

MIRI is an inevitable organ function involvement faced by HF patients after diverse combined cardiac surgeries, which can result in abnormal apoptosis of myocardial cells,

ventricular remodelling and aggravation of myocardial fibrosis, thus leading to myocardial infarction and other serious consequences[8]. Therefore, it is of great significance to find an effective way to control MIRI, inhibit myocardial cell apoptosis and reduce myocardial infarction area in clinical practice for ensuring the life safely of patients.

Doxorubicin hydrochloride is a commonly used anti-tumour chemotherapy drug in clinics, which has a high affinity with heart receptors and shows evident cardiotoxicity. The changes in pathology, hormone level and hemodynamics in the animal model of HF induced by doxorubicin hydrochloride for injection are close to changes occurring in HF in patients[9]. Therefore, in this study, according to the method described in the literature[7], doxorubicin hydrochloride was injected into the tail vein to replicate the rat model of HF. The results of HE staining and the ultrastructural observation under the electron microscope illustrated that compared with those in the normal group, evident HF symptoms appeared, HF changes occurred in the rat heart structure and the animal model of HF was successfully established in the sham group. The left forelimb of the coronary artery is the most important blood supply vessel of the heart. In this study, the MIRI model was replicated by ligating the left forelimb of a coronary artery. In the research on MIRI, the proportion of myocardial infarction is the gold standard to measure the severity of the myocardial injury. TTC results in this study demonstrated that the proportion of myocardial infarction in the model group rose significantly ($p<0.05$). Morphine preconditioning can alleviate myocardial injury and reduce the proportion of myocardial infarction[10]. The results of this study revealed that compared with those in the model group, the apoptosis rate of myocardial cells and the proportion of myocardial infarction in the MFC group dropped significantly.

The increase of oxidative stress level during blood reperfusion is the most basic pathological change leading to MIRI[11]. In the reperfusion period of MIRI, the mitochondrial function in the heart vascular endothelial cells is damaged, catecholamine self-oxidation is enhanced and neutrophil respiration is suddenly increased, all of which increase ROS production, reduce the activity and expressions of intracellular antioxidant enzymes and related transporters and weaken the self-defence ability of myocardial cells. ROS is largely enriched in cells and tissues and oxidizes with intracellular components, which leads to the disordered myocardial cell structure and energy metabolism[12], thus triggering cell apoptosis. Kalogeris *et al.*[13] found that the increase of ROS level in mitochondria, the imbalance of energy metabolism in cells and the instability of cellular infrastructure were the major factors leading to abnormal apoptosis of MIRI myocardial cells. Herein, the levels of ROS, redox-related enzymes and substrates in the model group were elevated and the apoptosis rate of the myocardial cells was increased accordingly.

Nrf2/ARE is the most vital endogenous antioxidant signalling pathway in the body. Most downstream targets regulated by Nrf2/ARE are closely associated with cardiovascular diseases[14]. Xu *et al.*[15] reported that Nrf2 bound molecular chaperones and stably existed in the cytoplasm of cells under normal physiological conditions. When ischemia-reperfusion occurred in the heart, Nrf2 was translocated into the nucleus through direct stimulation by the enriched ROS and then bound ARE and activated the expressions of downstream target proteins such as HO-1, SOD, quinone oxidoreductase 1 and GSH to play an antioxidant role. However, this role is not strong enough to remove ROS enriched in MIRI, which is also the reason why the amount of ROS in the rats in the model group in this study was still higher than that in the sham group and normal group. In contrast, after morphine preconditioning, ROS dropped significantly and the Nrf2 expression was increased significantly in the MFC group, which were opposite to the results in the MOA group after adding the Nrf2/ARE inhibitor. The above results indicate that morphine preconditioning can protect against MIRI via the Nrf2/ARE signalling pathway and remarkably activate the Nrf2/ARE pathway to exert an antioxidant effect.

CONCLUSION

To sum up, the Nrf2/ARE pathway is involved in morphine preconditioning to alleviate MIRI in rats with HF induced by doxorubicin hydrochloride. In addition, morphine can remarkably activate the Nrf2/ARE pathway and exert an antioxidant effect. However, further research is needed to investigate whether there are other pathways involved in this process.

SIGNIFICANCE STATEMENT

This study discovers the role of the Nrf2/ARE pathway that can be beneficial for rats with HF induced by doxorubicin hydrochloride. This study will help the researcher to uncover the critical area of MIRI treatment that many researchers were not able to explore. Thus, a new theory on the role of the Nrf2/ARE pathway in HF may be arrived at.

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