# Inactivated pseudomonas aeruginosa protects against myocardial ischemia reperfusion injury via Nrf2 and HO-1

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**Abstract.** The current study investigated the protective effects of inactivated *pseudomonas aeruginosa* (IPA) on myocardial ischemia reperfusion injury (MIR/I) and the mechanisms governing this interaction. Left anterior descending coronary artery ligation was performed on rats for 30 min and reperfusion was performed for a subsequent 2 h. Rat hearts were obtained and the myocardial infarction area was determined using nitroblue tetrazolium. Myocardial cell apoptosis was determined using flow cytometry. Malondialdehyde (MDA) content, lactate dehydrogenase (LDH) activity, superoxide dismutase (SOD) activity and catalase (CAT) activities were assayed using the corresponding kits. Additionally, nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1) were assayed using western blot and immunofluorescence analysis. When compared with the model group, the results of IPA treatment revealed improved heart function, reduced myocardial infarction area and reduced endothelial cell apoptosis, which led to decreased LDH and MDA levels, and increased SOD and CAT levels in serum, and decreased LDH and MDA levels and increased SOD and CAT in myocardial tissues. Moreover, increased Nrf2 and HO-1 expression levels in the myocardial tissues were also observed at all concentrations of IPA. It was concluded that IPA pretreatment ameliorated MIR/I and reduced endothelial apoptosis and oxidative stress via the Nrf2/HO-1 pathway.

## Introduction

Coronary heart disease (CHD) is the most common cause of cardiovascular associated death worldwide. Acute myocardial infarction (AMI) is a critical and severe condition, which can occur following CHD, and exhibits the second highest mortality rate in the disease spectrum. The incidence of AMI has increased, with treatment options including thrombolysis and interventional therapy to restore blood perfusion, which can prevent mortality in patients with AMI. However, the presence of myocardial ischemia-reperfusion injury (MIR/I) affects the prognosis of these patients (1). The pathological mechanism of MIR/I is complex. However, oxidative stress, secondary myocardial cell apoptosis and abnormal cardiac function have been revealed to be associated with MIR/I pathophysiology (2).

Numerous studies have demonstrated that MIR/I is associated with the generation of reactive oxygen species (ROS), which impair the structure and function of the cell membrane (3,4). Phospholipids are rich in polyunsaturated fatty acids and constitute an essential part of cell membranes. Phospholipids are also susceptible to ROS and may result in a decrease in membrane phospholipids, increased membrane cholesterol and ratio of cholesterol/phosphate, thereby reducing membrane fluidity (5). Decreased membrane fluidity can affect membrane proteins via cross-linking or polymerization, promoting membrane damage (6,7). An imbalance between the production of free radicals during MIR/I and the inability to counteract or detoxify their harmful effects results in inflammation and oxidative stress (8).

The microbiota has been revealed to protect innate immunity and decrease inflammation and oxidative stress (9). Previous studies have demonstrated that bacterial preparations serve protective functions against inflammation (10,11). Chai *et al* (12) reported that inactivated pseudomonas aeruginosa (IPA) attenuated pulmonary hypertension, right ventricle hypertrophy and pulmonary vascular remodeling in rats by restraining the hypoxia-induced overactive transforming growth factor-β1/Smad signaling (12). The current study aimed to investigate the role of IPA in anti-oxidative stress, endothelial protection and the subsequent protective effects of IPA against MIR/I.

## Materials and methods

*Animal models.* A total of 40 specific pathogen-free male Sprague-Dawley rats (weight, 250-300 g) were equally divided into five groups. Rats were provided by The Experimental

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Animal Center of General Hospital of Central Theater Command (Wuhan, China). The current study was performed in strict accordance with the recommendations in The Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of China. The protocol was also approved by the Institutional Animal Care and Use Committees of Wuhan Army General Hospital (permit no. SCXK-20080047). Rats were provided with food and water *ad libitum*, 12-h light/dark cycle and constant temperature (25˚C) and humidity (50%). Rats were acclimatized to these conditions for ~1 week. All rats were then divided into five groups of 8 rats under the same housing conditions: A control group, a model group, a MIR/I+IPA (low; $10^6$ cfu/kg) group, a MIR/I+IPA (medium; $10^7$ cfu/kg) group and a MIR/I+IPA (high; $10^8$ cfu/kg) group. Rats were then anaesthetized with 2% sodium pentobarbital (40 mg/kg; intraperitoneal injection) and fixed on an operating table in supine position with rubber bands to fix limbs. The trachea was separated and tracheal intubation was performed, whilst connected to a DHX-150 animal ventilator (Chengdu Instrument Factory). The respiratory rate was adjusted to 60-80/min and ventilation volume to 20 ml/kg. The chest was opened 3 mm away from left margin of the chest bone and the heart was exposed. Ligation of the left anterior descending coronary artery was performed for 30 min. This was then released for 2 h. This surgical protocol was performed on all of model and the three IPA treatment groups. Sham surgery with no coronary artery ligation was performed in the control group. IPA was intravenously administered once per day for 1 week prior to the surgery in the groups with various IPA treatments. An equal volume of 0.5% saline was administered to the control and model groups once per day for a week. Model reliability was assessed by continuously monitoring rat electrocardiograms. ST segment elevation was considered to indicate the presence of myocardial ischemia, with 1/2 drop of ST indicating reperfusion success. The heart ejection fraction (EF), fractional shortening (FS) and left ventricle inner diameter at systole (LVIDs) were obtained using Philips iU22 Color Doppler Ultrasound Diagnostic Instrument (Philips Healthcare) prior to and after coronary artery ligation. A total of 25% of the rats (n=2) were euthanized with sodium pentobarbital following ligation procedure owing to acute heart failure or malignant arrhythmia (13).

*Determination of serum and heart catalase (CAT), superoxide dismutase (SOD), lactate dehydrogenase (LDH) and malondialdehyde (MDA) protein expression.* After reperfusion, blood was extracted from the femoral artery of all groups and left for 30 min. To obtain serum, blood was subsequently centrifuged at 300 x g for 15 min. Total protein was extracted from myocardial tissues of the infarct area using RIPA buffer according to the manufacturer's protocol (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) and protein concentrations were determined using the bicinchoninic acid method. CAT (cat. no. A007), LDH (cat. no. M002), SOD (cat. no. A001-2) activities and MDA (cat. no. A003-2) contents were determined using kits in accordance with the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute).

*Myocardial infarction area.* After sacrifice, rat hearts were obtained and rinsed in PBS buffer. Ventricular tissues were

subsequently obtained and half of the ventricular tissues were cut into five sections of equal thickness (2-3 mm). The pieces were added into 0.1% NBT solution (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) at 37˚C for 15 min. Normal myocardium was indicated by a blue color, while the infarcted myocardium was colorless. Digital camera imaging and ImagePro plus 7.0 analysis software (Media Cybernetics, Inc.) were used to calculate the ventricular infarction area and ventricular total area. The myocardial infarction rate was the ratio of infarcted myocardium area compared with the total myocardium area of five sections (%).

*Apoptosis assay.* Myocardial tissues from the infarct area were collected by digestion with a pancreatic enzyme. Cells were then resuspended in PBS at a concentration of $1x10^6$ cells/ml. Apoptosis was assessed via Annexin V-FITC and propidium iodide (PI) staining using the Annexin V-FITC/PI Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol followed by flow cytometry analysis, as previously described (14). A total of $1x10^5$ cells were added to a 5 ml culture tube and 5 $\mu$l Annexin V-FITC and 10 $\mu$l of PI were added. The tube was vortexed and incubated for 15 min at room temperature in the dark. A total of 400 $\mu$l 1X binding buffer was added to each tube. The stained cells were analyzed using flow cytometry (BD Accuri C6 cytometer and BD Accuri C6 Software; BD Biosciences).

*Western blot analysis.* The total protein from infarct areas of myocardial tissues was extracted using RIPA buffer. A total of 75 $\mu$g protein was mixed with 5X loading buffer and separated using SDS-PAGE. The protein was then transferred to PVDF membranes and incubated with 5% skim milk powder at 37˚C for 1 h and rabbit anti-rat GAPDH (cat. no. BM1623; 1:1,000), nuclear factor erythroid 2-related factor 2 (Nrf2; cat. no. AB76026; 1:500) and heme oxygenase 1 (HO-1; cat. no. M00253-2; 1:500) antibodies at 4˚C overnight (all from Boster Biological Technology). After washing with TBST three times, the membrane was incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibodies (cat. no. BA1056; 1:1,000; Boster Biological Technology) at room temperature for 1 h. Signals were subsequently detected using ECL-0012 chemiluminescence detection kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) and analyzed using a ChemiDocXRS chemiluminescence imaging system (Bio-Rad Laboratories, Inc). Densitometry analysis was performed using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.).

*Immunofluorescence.* Myocardial tissues from the infarct area were collected by digestion with pancreatic enzyme. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized by 3% $H_2O_2$ for 10 min, blocked with normal goat serum (Wuhan Boster Biological Technology, Ltd.) for 20 min at 37˚C, and then incubated with rabbit anti-rat Nrf2 antibodies (cat. no. AB76026; Boster Biological Technology) overnight at 4˚C and washed with 0.2% Tween 20/PBS prior to and following incubation with goat anti-rabbit secondary antibodies, Cy3 conjugate (cat. no. BA1032; Boster Biological Technology) for 1 h at room temperature. The sections were counterstained

with DAPI (100 ng/ml) at room temperature for 15 min. The slides were sealed with the anti-fluorescence quenching agent (cat. no. AR1109; Wuhan Boster Biological Technology, Ltd.). and subsequently analyzed using fluorescence microscopy (magnification, x200). The integral optical density values of Nrf2 were measured using the CMIAS-8 color pathological image analysis system (purchased from Institute of Biological Engineering, PLA Air Force General Hospital).

*Statistical analysis.* SPSS 15.0 (SPSS, Inc.) software was used for statistical analysis. The experimental data are presented as the mean ± standard deviation (x±s). Statistical comparisons were performed using a Student's t-test for comparison between two groups and a one-way ANOVA for comparison among multiple groups, followed by a Duncan's multiple range test. P<0.05 was considered to indicate a statistically significant result.

## Results

*IPA alleviates myocardial infarction and inhibits myocardial apoptosis.* EF, FS and LVIDs were examined prior to and after coronary artery ligation. As presented in Table I, EFs and FS in the IPA group were significantly higher compared with the model group. However, LVID values in all IPA treatment groups revealed a significant reduction compared with the model group. No statistical differences were exhibited among groups treated with different concentrations of IPA.

The results demonstrated that the myocardial infarction area in the model group was 33.67±4.53% and that this was reduced to 30.07±2.99% in MIR/I+IPA (low), 14.88±3.12% in MIR/I+IPA (medium) and 18.69±3.41% in MIR/I+IPA (high) groups (Fig. 1A). The results presented in Fig. 1B demonstrated that apoptosis was significantly increased in the model group compared with the control group while apoptosis in all IPA groups were decreased significantly. Moreover, IPA treatments of $10^7$ and $10^8$ cfu/kg exhibited better protective effects against myocardial infarction and apoptosis than IPA treatment of $10^6$ cfu/kg.

*IPA reduces oxidative stress.* After 30 min of myocardial ischemia and 2 h of reperfusion, the level of MDA and LDH in the serum and myocardial tissues of the model group increased significantly and the activities of CAT and SOD decreased significantly compared with the control group. Compared with the model group, all IPA groups revealed a significant decrease in serum MDA and LDH at various concentrations (Fig. 2A). However, in myocardial tissues of infarct area, only IPA at $10^6$ and $10^7$ cfu/kg exhibited a significant reduction (Fig. 2B; P<0.05). Furthermore, serum CAT significantly increased in all IPA treatment groups and SOD activities significantly increased at $10^6$ cfu/kg in serum (Fig. 2A). However, CAT and SOD only increased at $10^6$ and $10^7$ cfu/kg IPA in myocardial tissues of infarct area compared with the model group (Fig. 2B).

*IPA increases the expression of Nrf2 and HO-1.* When compared with the control group, the model group Nrf2 protein expression increased significantly (P<0.05; Fig. 3A), and nuclear translocation of Nrf2 also increased (Fig. 3C). Compared with the model group, Nrf2 protein expression in all IPA groups

Table I. Echocardiographic analysis.

| Groups | EF (%) | FS (%) | LVIDs (mm) |
|---|---|---|---|
| Control | 72.44±3.65 | 41.56±2.87 | 3.92±0.68 |
| MIR/I | 41.23±3.21[a] | 15.32±3.16[a] | 6.23±0.57[a] |
| MIR/I +$10^6$ IPA | 56.37±4.67[b] | 25.57±2.65[b] | 4.93±0.89[b] |
| MIR/I +$10^7$ IPA | 55.78±3.09[b] | 28.62±1.98[b] | 4.42±0.53[b] |
| MIR/I +$10^8$ IPA | 58.79±4.98[b] | 28.49±2.62[b] | 4.11±0.25[b] |

[a]P<0.01 vs. Control; [b]P<0.05 vs. MIR/I. MIR/I, myocardial ischemia reperfusion injury; IPA, *pseudomonas aeruginosa*; EF, ejection fraction; FS, fraction shortening; LVIDs, left ventricle inner diameter at systole.

significantly increased (Fig. 3A), and Nrf2 nuclear translocation also increased (Fig. 3C). No significant differences in Nrf2 protein expression levels and nuclear translocation were determined among groups treated with different concentrations of IPA. HO-1 protein expression in the model group significantly increased compared with the control group (Fig. 3B). Compared with the model group, HO-1 protein expression in all treatment groups significantly increased (Fig. 3B) in all IPA treatment groups and IPA at $10^7$ exhibited stronger promoting effects.

## Discussion

CHD is currently more prevalent in the elderly, causing increased rates of morbidity and mortality rates among these patients and implicating the use of medical resources in CHD treatment (15). Repeated MIRI can cause further damage to the ischemic myocardium (16). A number of studies have suggested that the excessive generation of ROS serves a central role in myocardial injury (17,18). A free radical scavenging system exists, which includes antioxidant enzymes such as SOD, peroxidase, CAT and antioxidants including vitamins C and E (19). The excessive production of free radicals, particularly ROS, often impairs the structure and function of cells (20). Although the use of antioxidants in the treatment of MIR/I has been the focus of a variety of studies (21), an effective, clinically useful treatment is yet to be determined.

Myocardial apoptosis intervention could be used in the recovery of MIR/I (22,23). The present study demonstrated that during MIR/I, EFs and FS decreased and LVID and apoptosis increased. IPA treatments of $10^7$ and $10^8$ cfu/kg exhibited increased protective effects and improved heart function, endothelial cell injury and apoptosis. These results are consistent with a previous report, which demonstrated that IPA promoted cell proliferation and inhibited apoptosis in bronchial epithelial cells (7). Inactivated IPA at treatments of $10^7$ and $10^8$ cfu/kg exhibited increased protective effects on heart function and anti apoptotic effects compared with IPA treatment of $10^6$ cfu/kg.

MDA and LDH, which are biological products of ROS and lipid peroxidation, indicate the degree of lipid peroxidation in the body and subsequently indirectly reflect the degree of myocardial injury (24). SOD and CAT are the main antioxidant enzymes responsible for the mobilization of free radicals (25). The present
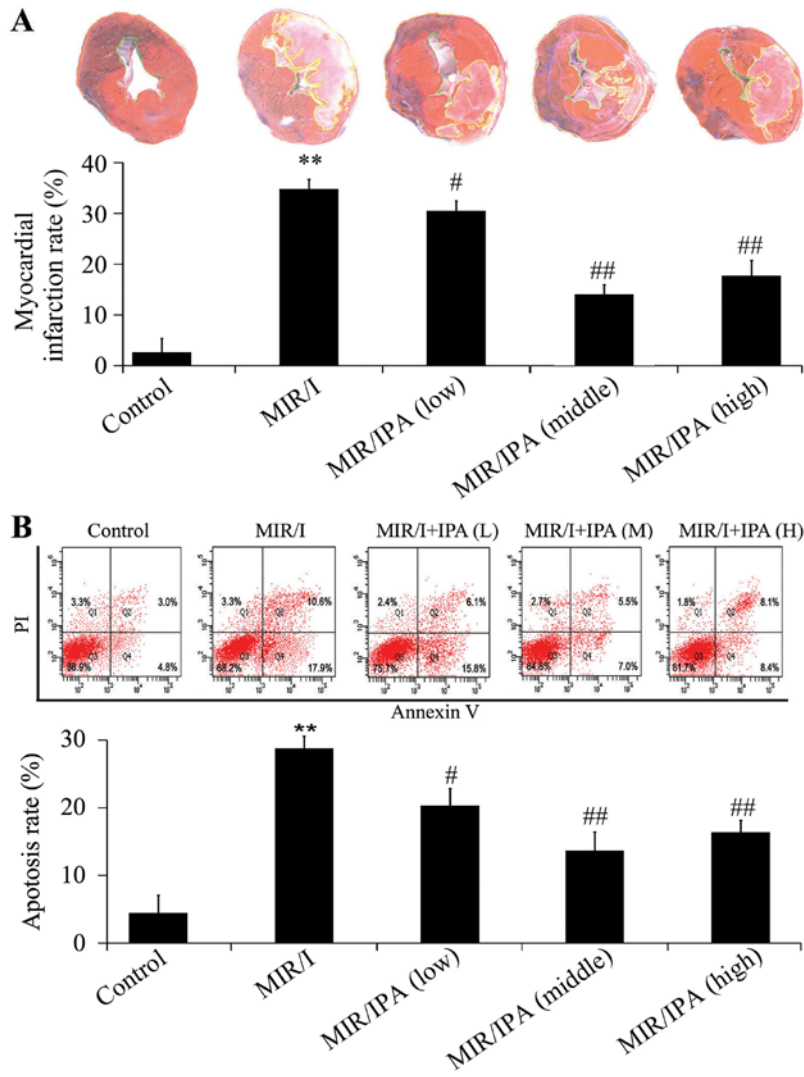
Figure 1. Myocardial infarction rate and the apoptosis of myocardial cells. (A) Nitro Blue Tetrazolium staining. (B) flow cytometry. n=6 for model group/n=8 for IPA groups. **P<0.01 vs. the control group; #P<0.05 and ##P<0.01 vs. MIR/I. MIR/I, myocardial ischemia reperfusion injury; IPA, *pseudomonas aeruginosa*; (L), low; (M), medium; (h), high.
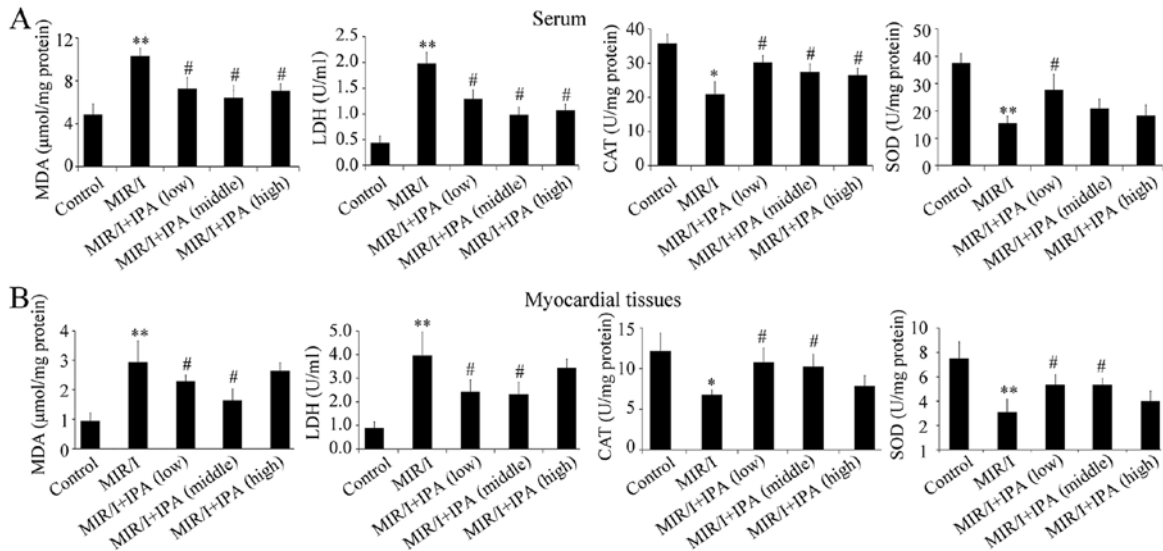


Figure 2. Activities of MDA, LDH, CAT and SOD in serum and in myocardial tissues assayed using biochemical kits. (A) The activities of MDA, LDH, CAT and SOD in serum. (B) The activities of MDA, LDH, CAT and SOD in myocardial tissues. n=6 for model group/n=8 for IPA groups. **P<0.01 and *P<0.05 vs. the control; #P<0.05 vs. MIR/I. MDA, malondialdehyde; LDH, lactate dehydrogenase; CAT, catalase; SOD, superoxide dismutase; MIR/I. MIR/I, myocardial ischemia reperfusion injury; IPA, *pseudomonas aeruginosa*.
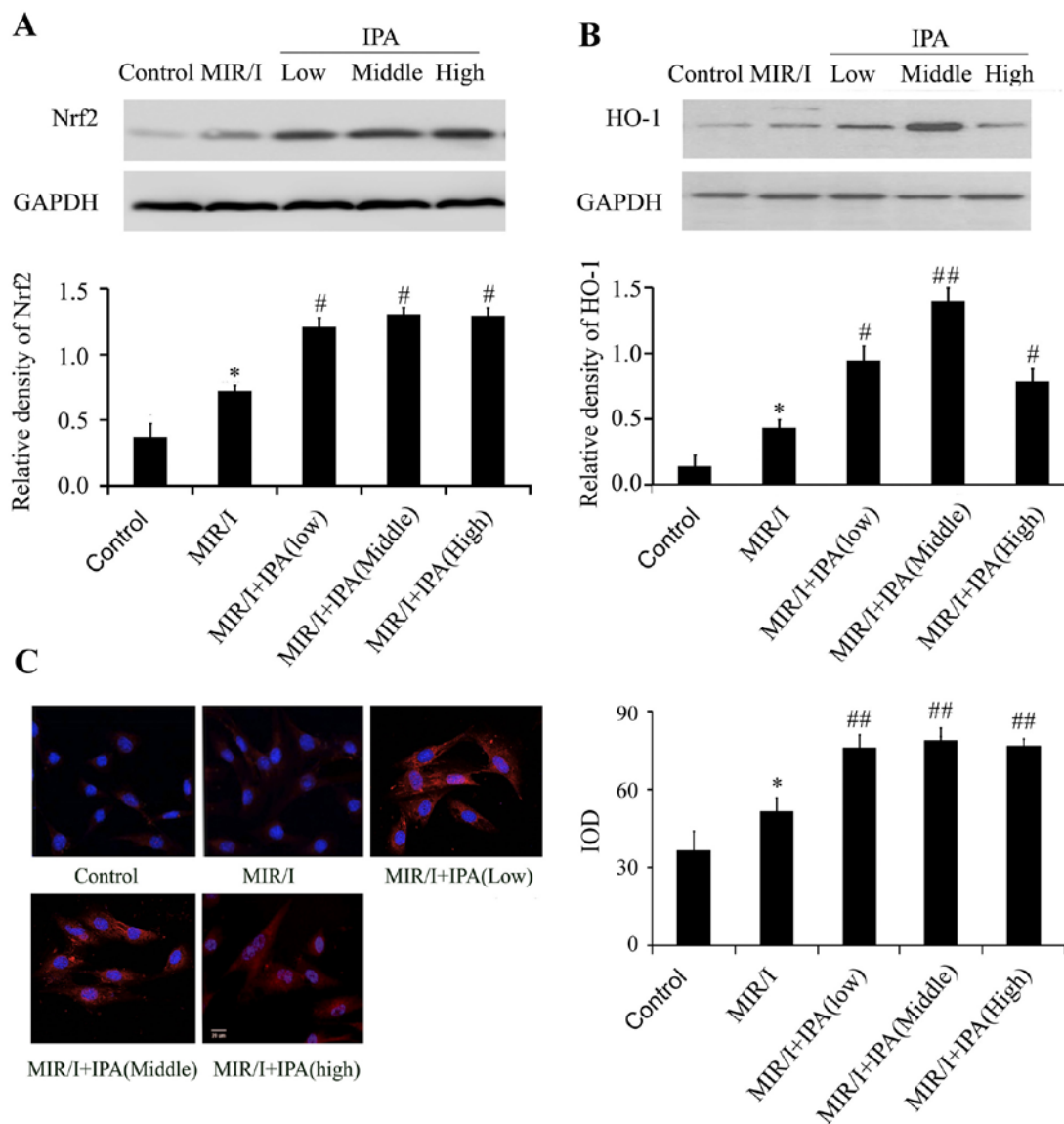
Figure 3. Nrf2 and HO-1 expression. (A) Nrf2 expression was assessed using western blot analysis. (B) Western blotting was performed to detect HO-1expression. n=6 for model group/n=8 for IPA groups. (C) The nuclear translocation of Nrf2 was assessed using immunofluorescence.*P<0.05 vs. Control; #P<0.05 and ##P<0.01 vs. MIR/I. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; MIR/I, myocardial ischemia reperfusion injury; IPA, *pseudomonas aeruginosa*; IOD, integral optical density.

study revealed that IPA decreased LDH and MDA levels and increased SOD and CAT activities in myocardial tissues and serum samples, confirming the myocardial protection of IPA through the inhibition of lipid peroxidation and increased ROS removal. However, these results demonstrated that, in myocardial tissues of infarct area, IPA at $10^6$ and $10^7$ cfu/kg exhibited increase effects against oxidative stress. Oxidative stress refers to the process of oxidative damage caused by excessive ROS production, reduced scavenging capacity and an imbalance between the oxidation and antioxidant system (26). Nrf2 is an important transcription factor that is associated with regulating oxidative stress response. In oxidative stress, Nrf2 is translocated into the nucleus and binds with the antioxidant response element, initiating the transcription of detoxifying enzymes and antioxidant enzyme gene expression, including HO-1, which protects the body from ROS (27,28). The continuous expression of HO-1 in endothelial cells can reduce cell inflammatory damage and apoptosis that is caused by ischemia (29,30). Therefore, Nrf2

and HO-1 expression indicate the protective effects against cell injury. Inactivated IPA can significantly promote Nrf2 and HO-1 expression in rats exhibiting MIR/I, indicating that IPA induces protection against apoptosis and oxidative stress.

IPA treatments of $10^7$ and $10^8$ cfu/kg in the current study exhibited an increased protective effect against myocardial damage and apoptosis. IPA at $10^6$ and $10^7$ cfu/kg exhibited an increased effect against oxidative stress. Although IPA at $10^7$ and $10^8$ cfu/kg exhibited a stronger effect on the promotion of Nfr2 and HO-1, the activation of Nrf2 and HO-1 expression began to decrease at $10^8$ cfu/kg. Combined with the results of anti-apoptosis and antioxidant effects, the highest protective effect of IPA was exhibited by the $10^7$ cfu/kg treatment group. IPA exhibited a protective effect in the concentration range that was selected in the current study. However, further studies are still required to further investigate the effective treatment doses and detailed underlying mechanisms of IPA protection against MIR/I.

In conclusion, IPA pretreatment improves heart function, reduces endothelial apoptosis and oxidative stress, which is induced by MIR/I, via the Nrf2/HO-1 pathway.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

ZZ, ZT, WZ and JL performed the experiments. BL designed the current study and performed statistical analysis. ZZ drafted the manuscript for critical and intellectual content. SD made substantial contributions to study conception and revised the manuscript.All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All animal experiments and procedures were approved by The Institutional Animal Care and Use Committees of Wuhan Army General Hospital (permit no. SCXK-20080047).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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