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Propofol relieves inflammation in MIRI rats by inhibiting Rho/Rock signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of propofol on myocardial ischemia-reperfusion injury (MIRI) and its mechanism by establishing *in vivo* rat models.

MATERIALS AND METHODS: Sprague-Dawley rats were selected for the construction of MIRI models in vivo. All rats were divided into three groups, including sham operation group (Sham operation), MIRI group and MIRI + propofol group. At 2 h after reperfusion, myocardial tissues and blood samples were collected from rats. The expression levels of serum lactic dehydrogenase (LDH) and creatine kinase-MB (CK-MB), as well as serum interleukin-6 (IL-6), IL-10 and tumor necrosis factor-α (TNF-α), were measured in each group of rats, respectively. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay was employed to detect the apoptosis of myocardial cells. Additionally, the messenger ribonucleic acid (mRNA) and protein expressions of Ras homolog gene family, member A (RhoA) and Rho-associated coiled-coil-containing protein kinase 2 (Rock2) were determined via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting, respectively.

RESULTS: (1) The expression levels of serum LDH and CK-MB were significantly lower in MIRI + propofol group than those in MIRI group ($p<0.05$). (2) In comparison with MIRI group, MIRI + propofol group exhibited significantly reduced serum IL-6 and TNF-α levels ($p<0.01$) and elevated serum IL-10 level ($p<0.01$). (3) Compared with MIRI group, the apoptosis of myocardial cells was remarkably reduced in MIRI + propofol group after IRI ($p<0.05$). (4) The mRNA and protein expressions of RhoA and Rock2 were significantly lower in MIRI + propofol group than those in MIRI group ($p<0.05$).

CONCLUSIONS: Propofol relieves MIRI and inflammation, reduces the level of oxidative stress and represses I/R-induced myocardial cell apoptosis in MIRI rats by inhibiting the activity of the Rho/Rock signaling pathway.

Key Words:

Myocardial ischemia-reperfusion (MIRI), Propofol, Rho/Rock signaling pathway, Apoptosis

Introduction

Ischemic heart disease is a leading cause of morbidity and mortality in the world[1]. In the case of myocardial ischemic injury, especially acute myocardial infarction, restoring blood supply, i.e., reperfusion therapy, is considered as the best approach to save the endangered myocardium. However, reperfusion itself sometimes can aggravate myocardial injury clinically, also known as myocardial ischemia-reperfusion injury (MIRI)[2]. Fatal reperfusion injury, which is a potential harm of MIRI, is defined as myocardial injury caused by the recovery of coronary blood flow after ischemic attacks. It may eventually result in the death of myocardial cells[3]. MIRI is a major obstacle for reperfusion therapy. Preventing myocardial cell death after ischemia is the best method to control myocardial ischemic injury. Previous studies have indicated that oxidative stress is a leading factor of the onset and progression of many pathological states, especially MIRI[4]. In addition, oxidative stress induced by reactive oxygen species (ROS) is considered as a vital initiator of MIRI[5].

During MIRI, the loss of most cells occurs through necrosis. However, the apoptosis of myocardial cells is a process of cell death controlled by a series of procedures, which is distinct from cell necrosis. Myocardial cell apoptosis starts presently after ischemia. It is amplified by reperfusion and can partially lead to somatic death of myocardial cells[6]. The latest evidence has denoted that the apoptosis of myocardial cells and inflammation are markers of MIRI[7]. In addition, mitigating inflammation during reperfusion after ischemic injury has been proven to be helpful[8]. Recent researches have demonstrated that Ras homolog gene family (Rho)-associated coiled-coil-containing protein kinase (Rock) is one of the effectors of small G protein Rho. Meanwhile, Rho/Rock is a molecular switch able

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to function in smooth muscle contraction, cell adhesion, motility and cytokinesis[9]. Increasing studies have manifested that Rocks play a crucial role in mediating myocardial cell apoptosis and IRI-induced inflammation[10,11]. They have also been considered as potential therapeutic targets for cardiovascular diseases[12,13]. Suppressing the activity of Rocks is able to protect the heart from IRI[8]. For instance, inhibiting Rock by fasudil, an inhibitor of Rock, exerts a beneficial effect against IRI[14].

Propofol, an antioxidant anesthesia-inducing agent, is capable of preventing IRI[15]. It can alleviate both local IRI of rat heart *in vivo*[16], and MIRI in patients in a dose-dependent manner[17]. Moreover, propofol exerts antioxidant effects in rat myocardial cells and macrophages. This is related to the inhibition of oxidative stress-related enzymes, including inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), and neutrophil cytoplasmic factor[18,19]. Previous studies have demonstrated that propofol plays multiple roles in different cell types through various signaling pathways. However, the effects of propofol on human myocardial cells through the Rho/Rock signaling pathway have not been fully elucidated. In this study, the effects of propofol on MIRI through the Rho/Rock signaling pathway were explored using rat models of MIRI *in vivo*. It was uncovered that propofol inhibited myocardial cell apoptosis, oxidative stress and inflammation by impeding the Rho/Rock signaling pathway. All our findings might help to provide a more sufficient theoretical basis for propofol as a potential drug in the treatment of MIRI.

Materials and Methods

Construction of MIRI Models in Rats

A total of 30 Sprague-Dawley rats were randomly divided into 3 groups and anesthetized with pentobarbital, followed by exposure of the heart. Next, the heart was exposed without ligation of left anterior descending coronary artery (LAD) in Sham group. In MIRI group, the LAD was sutured with a silk thread for 30 min, followed by reperfusion for 2 h. In MIRI + propofol group, 5 mg/kg propofol was injected *via* femoral vein before the ligation of LAD until the end of reperfusion. Afterwards, heart tissues were collected for further analysis. This investigation was approved by the Animal Ethics Committee of Chengde Medical College Animal Center.

Measurement of Serum Levels of Lactic Dehydrogenase (LDH) and Creatine Kinase-MB (CK-MB)

Serum levels of LDH and CK-MB were measured to evaluate the degree of myocardial cell injury. At 3 h after reperfusion, serum LDH activity was detected *via* spectrophotometry. Similarly, serum CK-MB was quantified according to the manufacturer's instructions of an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA).

Detection of Serum Inflammatory Factors

ELISA was conducted as per the instructions of an ELISA kit (eBioscience, San Diego, CA, USA). Briefly, the ELISA kit was taken out and equilibrated at room temperature for 20 min. Anticoagulant blood of each rat was centrifuged at 4000 rpm for 15 min, followed by collection of the serum. Serum levels of inflammatory factors interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) were finally detected by the double antibody sandwich method.

Determination of Adenosine Triphosphate (ATP) Content

ATP content (nmol or μmol/mg of protein) in heart tissues was determined in strict accordance with an enhanced ATP detection kit (Beyotime, Shanghai, China).

Assessment of Oxidative Stress

The content of malondialdehyde (MDA) produced by the degradation of polyunsaturated lipids was measured to reflect the level of oxidative stress. MDA reacted with thiobarbituric acid (TBA), a TBA reactive substance (TBARS), to form a 1:2 MDA-TBA adduct absorbed at 532 nm. Therefore, the number of TBARS was proportional to that of MDA. Finally, the concentration of TBARS was calculated based on standard curves.

Detection of Apoptosis Via Terminal Deoxynucleotidyl Transferase-Mediated c Triphosphate-Biotin Nick End Labeling (TUNEL) Assay

The left ventricular heart was first taken and embedded in paraffin. Subsequently, the paraffin block was sliced into sections (5 μm in thickness), hydrated, and added with Triton X-100 for cell membrane permeabilization. Next, apoptotic myocardial cells were detected by TUNEL stain-

ing according to the manufacturer's instructions of an *in situ* cell death detection kit (Roche). TUNEL signal was observed, and TUNEL-positive cells were finally counted under a fluorescent microscope (NIKON ECLIPSE TI-SR, Tokyo, Japan).

Determination of Changes in Messenger Ribonucleic Acid (mRNA) Levels Through Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was first extracted from heart tissues. The quality and concentration of extracted RNA were determined using NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit. Next, the mRNA expression levels of RhoA and Rock2 were quantified using a SYBR Green PCR kit (Promega, Madison, WI, USA) and determined by the 7500-Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method. Primers used in qRT-PCR were shown in Table I.

Measurement of Related Protein Levels Through Western Blotting

Myocardial tissues in each group of rats were homogenized and added with an appropriate amount of lysis buffer for ultrasonic lysis. Total protein was extracted, and protein concentration was measured by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Next, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After that, the membranes were incubated with RhoA antibody (Abcam, Cambridge, MA, USA, diluted at 1:2000) and Rock2 antibody (Abcam, Cambridge, MA, USA, diluted at 1:2000) at 4°C overnight. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted at 1:1000) at room temperature for 1 h. Enhanced chemiluminescence (ECL) plus Western blotting reagent was used for color development.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was utilized for statistical analysis. Experimental data were expressed as mean ± standard deviation. Measured data were checked and analyzed by *t*-test (two groups). $p<0.05$ was considered statistically significant.

Results

Propofol Reduced the Release of Serum Biochemical Marker Enzymes in MIRI Rats

As shown in Figure 1, IRI resulted in the injury of myocardial cells, manifested as significantly increased levels of such serum biomarkers as LDH and CK-MB in MIRI group ($p<0.01$). Serum levels of LDH and CK-MB were evidently lower in MIRI + propofol group than those in MIRI group ($p<0.05$).

Effects of Propofol on Inflammation in MIRI Rats

As shown in Figure 2, MIRI group exhibited significantly elevated serum levels of IL-6 and TNF-α, and reduced serum level of IL-10 ($p<0.05$), suggesting IRI-induced inflammation in heart tissues. Compared with MIRI group, MIRI + propofol group exhibited remarkably reduced serum levels of pro-inflammatory factors IL-6 and TNF-α and elevated level of the anti-inflammatory factor IL-10 ($p<0.05$).

Table I. Primer sequences of qPCR.

Indicator	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
RhoA	AGCTTGTGGTAAGACATGCTTG	GTGTCCCATAAAGCCAACTCTAC
Rock2	TTGGTTCGTCATAAGGCATCAC	TGTTGGCAAAGGCCATAATATCT

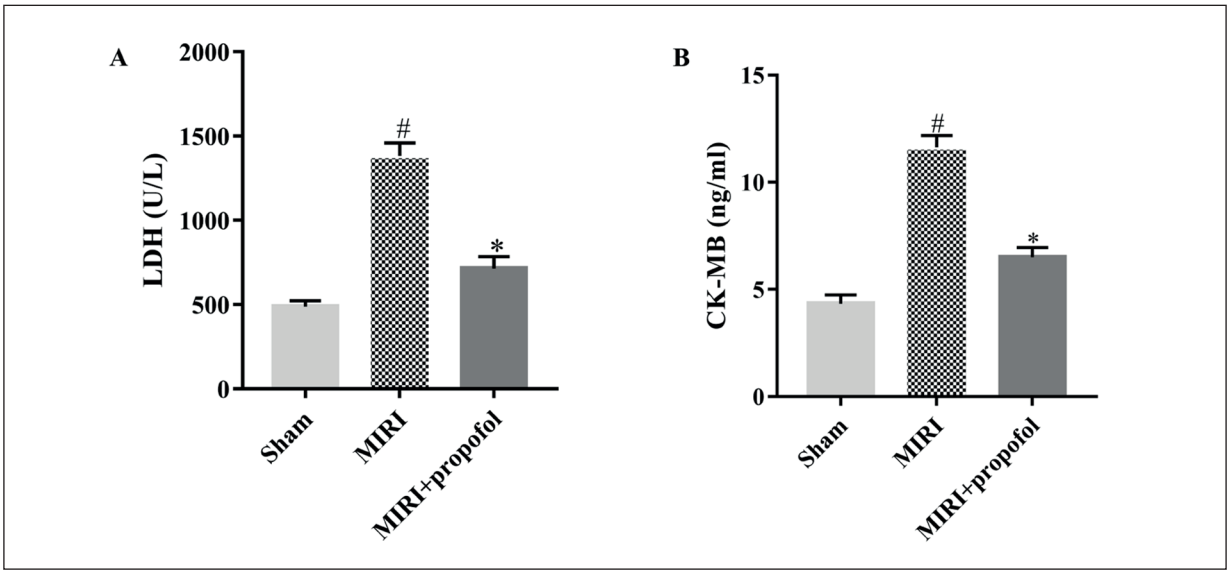

Figure 1. Propofol decreased serum biochemical marker enzymes in MIRI rats. #$p<0.01$ *vs.* Sham group, *$p<0.05$ *vs.* MIRI group.

Effects of Propofol on Oxidative Stress in MIRI Rats

Oxidative stress level rose significantly in MIRI group, manifested as an increase in MDA level in the serum and heart tissues ($p<0.05$). However, MDA level was significantly lower in MIRI + propofol group than that in MIRI group ($p<0.05$) (Figure 3).

Effects of Propofol on ATP Level in Heart Tissues in MIRI Rats

ATP level in heart tissues was significantly lower in MIRI group than that in Sham group ($p<0.05$). However, it was remarkably higher in MIRI + propofol group than that in MIRI group ($p<0.05$) (Figure 4).

Effects of Propofol on Apoptosis of Myocardial Cells in Heart Tissues in MIRI Rats

The number of apoptotic myocardial cells in heart tissues increased significantly in MIRI group ($p<0.05$). However, it was smaller in MIRI + propofol group than that in MIRI group ($p<0.05$) (Figure 5).

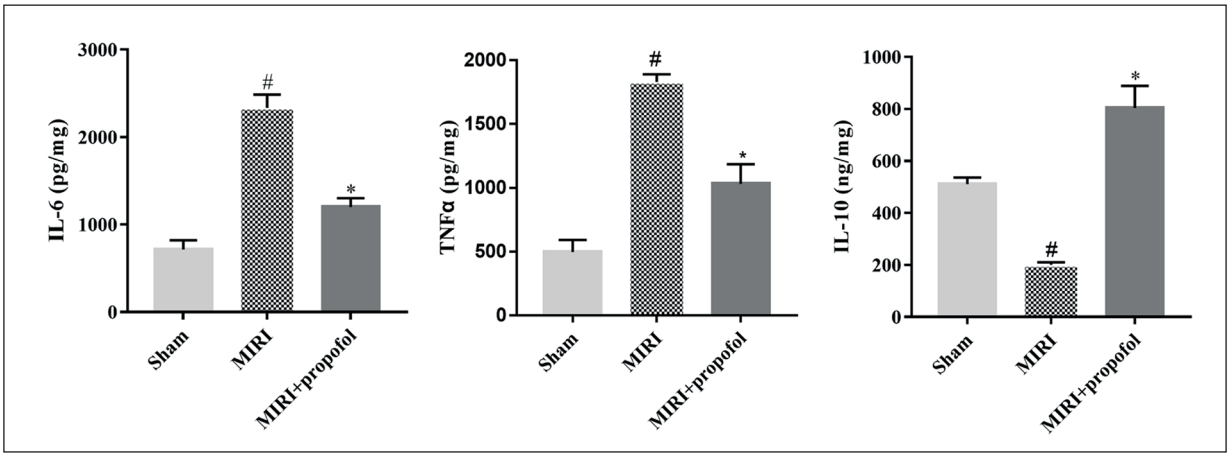

Figure 2. Propofol relieved inflammation in MIRI rats. #$p<0.05$ *vs.* Sham group, *$p<0.05$ *vs.* MIRI group.

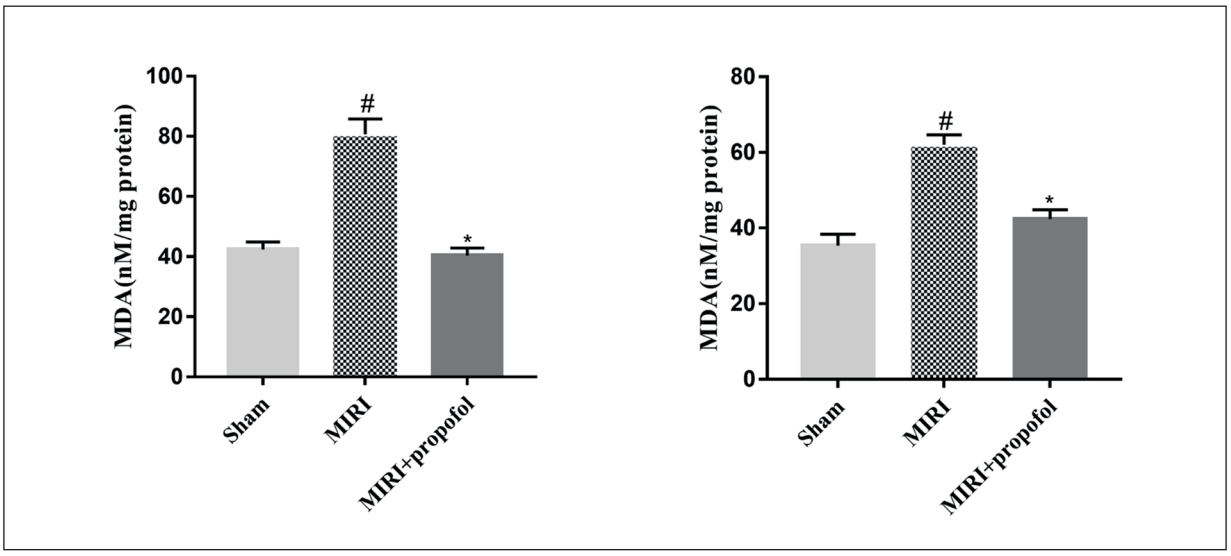

Figure 3. Propofol decreased oxidative stress level in MIRI rats. #$p<0.05$ *vs.* Sham group, *$p<0.05$ *vs.* MIRI group.

Propofol Repressed the Activation of Rho/Rock Signaling Pathway in MIRI Rats

The mRNA expressions of RhoA and Rock2 in heart tissues were remarkably elevated in MIRI group ($p<0.05$), whereas they were lower in MIRI + propofol group than those in MIRI group ($p<0.05$) (Figure 6). Besides, the protein expressions of RhoA and Rock2 in heart tissues rose significantly in MIRI group ($p<0.05$). However, they were markedly lower in MIRI + propofol group than those in MIRI group ($p<0.05$) (Figure 7).

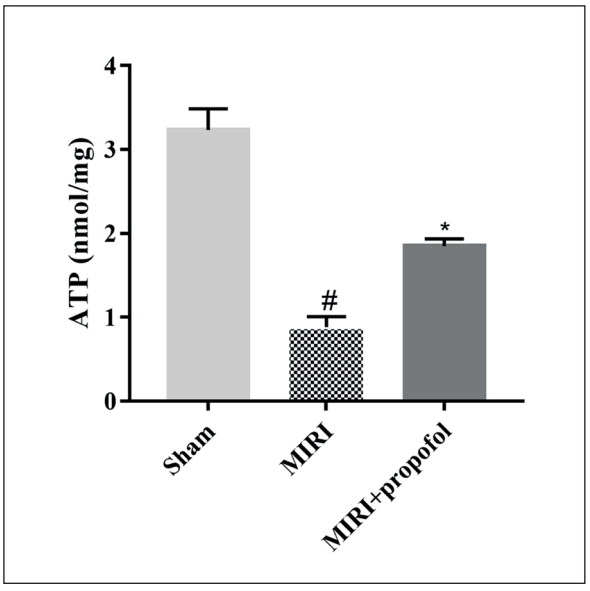

Figure 4. Effects of propofol on ATP level in heart tissues. #$p<0.05$ *vs.* Sham group, *$p<0.05$ *vs.* MIRI group.

Discussion

In this study, it was discovered that (1) propofol relieved myocardial injury in MIRI rats by reducing the release of serum LDH and CK-MB. (2) Propofol alleviated myocardial injury in MIRI rats by promoting the production of ATP in heart tissues. (3) Propofol facilitated the secretion of serum cytokine inhibitor IL-10 and inhibited the release of inflammatory factors IL-6 and TNF-α to suppress the inflammation in MIRI rats, thus mitigating MIRI. (4) Propofol participated in the protective effect against IRI and inhibited the apoptosis of myocardial cells in MIRI rats by inhibiting the Rho/Rock signaling pathway. The activation of the pathway was essential for the activation of the oxidative stress process in MIRI.

Generally, it is recognized that ROS produced during reperfusion plays a key role in IRI by directly attacking large numbers of cellular molecules[20]. Propofol, a commonly used intravenous anesthetic with the chemical composition of 2,6-diisopropylcresol, has a strong antioxidant effect. In recent years, propofol

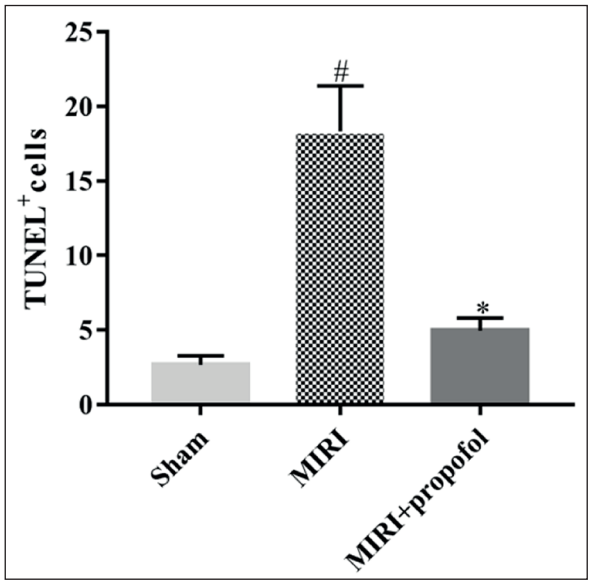

Figure 5.Effects of propofol on apoptosis of myocardial cells in heart tissues. $^{\#}p<0.05$ *vs.* Sham group, $^{*}p<0.05$ *vs.* MIRI group.

has been applied in the clinical treatment of IRI[21]. Evidence suggests that increased free radicals are one of the leading causes of ischemic tissue damage[22]. MDA, an important free radical, is widely used as an indicator of free radical-mediated lipid peroxidation damage[23]. It has been discovered that MDA tends to increase in various diseases[24]. In this study, MDA level increased significantly after MIRI. However, such an increase was reversed by propofol to a considerable extent, confirming the antioxidant effect of propofol on MIRI. ATP, a coenzyme (nucleoside triphosphate) in cells, protects the myocardium and vascular endothelial cells during I/R of heart tissues[25]. Of note, it has been reported that propofol functions in renal I/R models by activating ATP-sensitive potassium channels in I/R-induced apoptosis[26]. Therefore, increased ATP level in MIRI + propofol group may indicate that propofol exerts a protective effect against MIRI. Several studies[27,28] have reported that the elevated level of oxidative stress in ischemic myocardium can damage the integrity of myocardial cell membranes and release injury markers LDH and CK-MB into serum. CK-MB is a sensitive marker in patients with myocardial infarction after acute ischemia and acute myocardial ischemia[29]. In patients with acute myocardial infarction receiving thrombolytic therapy, the serum concentration of CK-MB reaches peak at 10 h after ischemia. Meanwhile, it has a certain relationship to the largest myocardial infarction indexes at 5-7 d after reperfusion[29]. A similar phenomenon has been observed in rat models of MIRI, and CK-MB level after ischemia reaches the maximum score after reperfusion[30]. In this study, it was observed that the levels of LDH and CK-MB were significantly elevated in rat models of MIRI. However, propofol treatment significantly limited the increase in the markers of MIRI in rat serum. These results suggest that propofol

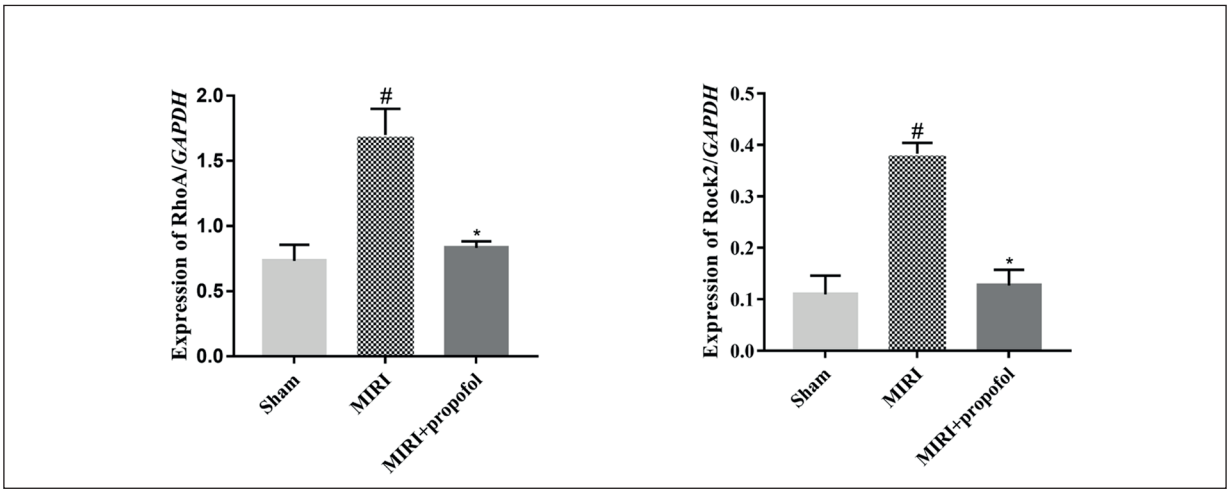

Figure 6. Propofol decreased mRNA expressions of RhoA and Rock2 in MIRI rats. $^{\#}p<0.05$ *vs.* Sham group, $^{*}p<0.05$ *vs.* MIRI group.

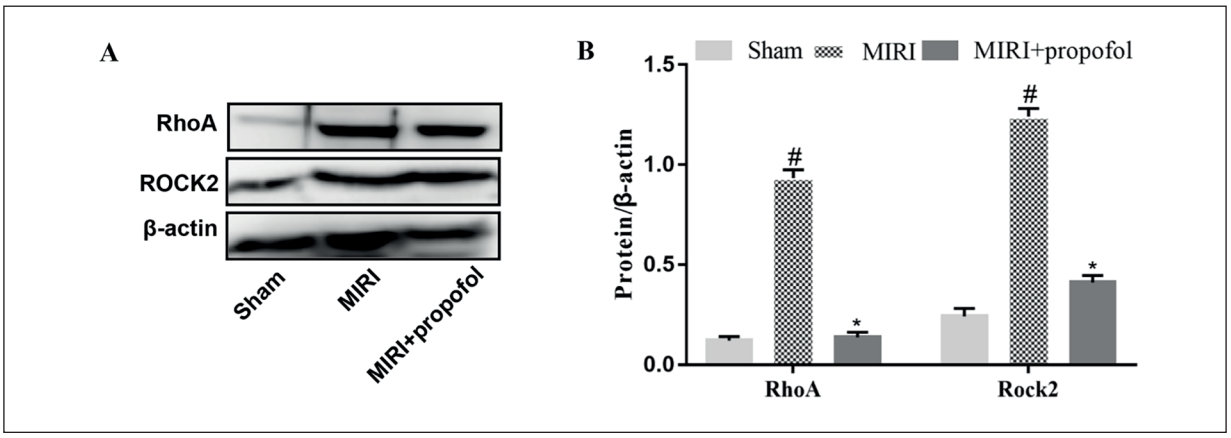

Figure 7. Propofol lowered protein expressions of RhoA and Rock2 in MIRI rats. $^{\#}p<0.05$ *vs.* Sham group, $^{*}p<0.05$ *vs.* MIRI group.

relieves ischemic heart injury in MIRI rats. Besides, propofol treatment repressed myocardial cell apoptosis caused by I/R and promoted the production of cytokine inhibitor IL-10. It was speculated that propofol treatment could suppress the release of inflammatory factors IL-6 and THF-α by promoting the production of IL-10, thus alleviating inflammation in MIRI rats.

In the present study, the results revealed that propofol protected against MIRI. However, its underlying mechanism remains unclear. Increasing studies have manifested that inhibiting the Rho/Rock pathway exerts a cardioprotective effect against myocardial infarction[31]. Therefore, it is speculated that the protective effect of propofol may be associated with the Rho/Rock pathway, and the specific mechanism needs to be further studied. The novelty of this study was that all our findings may lay a theoretical foundation and a scientific basis for the application of propofol in the treatment of MIRI. Furthermore, this study also proposes the possibility of Rho/Rock as a potential therapeutic target of MIRI.

Conclusions

Propofol relieves MIRI and inflammation, reduces the level of oxidative stress and represses I/R-induced myocardial cell apoptosis in MIRI rats by inhibiting the activity of the Rho/Rock signaling pathway. The protective effect of propofol may be associated with the Rho/Rock pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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