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Research on cardioprotective effect of irbesartan in rats with myocardial ischemia-reperfusion injury through MAPK-ERK signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore whether the mechanism of Irbesartan (IRB) in the treatment of rats with myocardial ischemia-reperfusion injury (MIRI) was related to the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) signaling pathway.

MATERIALS AND METHODS: The rat model of MIRI was first successfully established. All rats were randomly divided into 5 groups, including the Sham group (sham operation control group, ligation only), Model group (MIRI rat model group), IRB12.5 group [low-dose IRB (12.5 mg/kg/d) group], IRB50.0 group [medium-dose IRB (50.0 mg/kg/d) group] and IRB200.0 group [high-dose IRB (200.0 mg/kg/d) group]. After treatment of IRB in MIRI rats, the activities of four myocardial enzyme indexes, including creatine kinase-MB (CK-MB), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and cardiac troponin T (cTnT), were detected *via* enzyme-linked immunosorbent assay (ELISA). The effect of IRB on myocardial apoptosis in MIRI rats was detected *via* Annexin V-fluorescein isothiocyanate/Propidium Iodide (FITC/PI) double staining. Meanwhile, the messenger ribonucleic acid (mRNA) levels of ERK, B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax) in myocardial cells after treatment of IRB were detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Furthermore, the protein levels of ERK and p-ERK were detected *via* Western blotting.

RESULTS: Different concentrations of IRB could protect myocardium from MIRI. IRB at doses of 50.0 mg/kg/d and 200.0 mg/kg/d could significantly downregulate myocardial enzyme indexes in MIRI ($p<0.01$). Meanwhile, both the doses could markedly inhibit myocardial apoptosis in MIRI rat model by regulating the expressions of apoptosis-related genes (Bcl-2 and Bax) ($p<0.01$), eventually improving myocardial pathological damage. At the same time, it could also significantly decrease the mRNA and protein levels of ERK in the MAPK-ERK signaling pathway ($p<0.05$).

CONCLUSIONS: The cardioprotective mechanism of IRB in MIRI rats may be related to the inhibition of the activation of the MAPK-ERK signaling pathway.

Key Words:
Irbesartan, MAPK-ERK signal transduction pathway, Myocardial ischemia-reperfusion injury.

Introduction

Acute myocardial infarction (AMI) is one of the most serious clinical manifestations of coronary artery diseases. Blood oxygen flow in the heart is blocked in AMI, leading to myocardial ischemia and hypoxic necrosis. Meanwhile, myocardial reperfusion will aggravate the original myocardial damage[1]. This pathophysiological process is defined as myocardial ischemia-reperfusion injury (MIRI) in clinic[2]. Current studies have demonstrated that the mechanism of MIRI is very complex. It is mainly related to enhanced oxidative stress caused by MIRI, myocardial apoptosis or autophagy, inflammatory response and corresponding energy metabolic disorder[3].

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Currently, it has been demonstrated that the molecular mechanism of MIRI regulation is very complex. Previous research mainly focused on signal transduction pathways related to cell growth and survival, such as the mitogen-activated protein kinase (MAPK) signal transduction pathway[4] and phosphatidylinositol 3-hydroxy kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signal transduction pathway[5]. The MAPK/ extracellular signal-regulated kinase (ERK) pathway is one of the three major MAPK signaling pathways with c-Jun N-terminal kinase (JNK) and p38 MAPK. Meanwhile, it is also the most studied pathway in searching for potential targeted drugs for the radical treatment of AMI. Therefore, it has already been a research hotspot in MIRI currently[6].

There are many types of targeted therapeutic drugs for AMI. The first choice is angiotensin-converting enzyme inhibitors (ACEI), followed by angiotensin receptor blockers (ARB) represented by Sartans or Ca^{2+} channel blockers (CCB) such as Verapamil. Single or combined medication is determined based on the condition and course of the disease. Irbesartan (IRB) is a long-acting ARB applied alone or in combination with other antihypertensive drugs in the treatment of hypertension[7], coronary atherosclerosis[8], heart failure[9] and diabetes[10]. However, its mechanism of action has not been systematically elucidated. In the present study, the MIRI model was established in rats to explore the cardioprotective role of IRB in MIRI *via* the MAPK-ERK signaling pathway.

Materials and Methods

Materials

Laboratory Animals

A total of 50 healthy adult male Sprague-Dawley (SD) rats weighing 180-220 g and aged 10-12 weeks were purchased from the Laboratory Animal Center of Beijing University of Traditional Chinese Medicine. All rats were fed in accordance with the health and use standards of laboratory animals. This study was approved by the Animal Ethics Committee of Beijing University of Traditional Chinese Medicine Animal Center.

Drug

IRB was purchased from Shanghai QT Biotechnology Co., Ltd., China (CAS No.: 138402-11-6, purity ≥98%, Shanghai, China).

Reagents and Instruments

Rat creatine kinase-MB (CK-MB) kit (GOY-S1163; Shanghai Goybio Technology Co., Ltd. , Shanghai, China), rat aspartate aminotransferase (AST) kit (9000-97-9), rat lactate dehydrogenase (LDH) kit (YS-4097) and rat cardiac troponin T (cTnT) kit (YS-3489; Shanghai YS Biotechnology Co., Ltd., Shanghai, China), and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis double staining kit (KGA105; Shanghai KeyGEN Biotechnology Co., Ltd., Shanghai, China). Main instruments in Reverse Transcription-Polymerase Chain Reaction (RT-PCR): spin-column total ribonucleic acid (RNA) extraction kit (DP419; Tiangen, Beijing, China), PrimeScript™ RT reagent kit (RR036A; TaKaRa, Otsu, Shiga, Japan), and TB Green™ Premix Ex Taq™ II quantitative PCR (qPCR) kit (RR820A; Takara, Otsu, Shiga, Japan). Related antibodies in Western blotting: rabbit monoclonal antibodies of phosphorylated ERK (p-ERK) (Thr202/Tyr204) (#4370), ERK (#4695) and β-actin (#4970; Cell Signaling Technology, Danvers, MA, USA), horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG (BA1054; Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), multi-lead electrophysiological recorder (iWorx, Dover, NH, USA), SpectraMax Paradigm multi-functional microplate reader (Molecular Devices, Silicon Valley CA, USA), FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), StepOne Plus Real Time fluorescence qPCR instrument (ABI Applied Biosystems, Foster City, CA, USA), and electrophoresis apparatus and gel imaging system (Bio-Rad, Hercules, CA, USA).

Methods

Grouping and Drug Administration

All healthy adult SD rats (n=50) fed routinely were randomly divided into 5 groups, including the Sham group (sham operation control group, ligation only), Model group (MIRI rat model group), IRB12.5 group [low-dose IRB (12.5 mg/kg/d) group], IRB50.0 group [medium-dose IRB (50.0 mg/kg/d) group] and IRB200.0 group [high-dose IRB (200.0 mg/kg/d) group]. An equal amount of 0.9% NaCl was administered intragastrically in the Sham group and Model group. Meanwhile, an equal amount of IRB (12.5 mg/kg/d, 50.0 mg/kg/d and 200.0 mg/kg/d) was administered intragastrically in rats of IRB groups once a day for 7 consecutive days.

Establishment of MIRI Rat Model

The MIRI model was established according to the methods of Zhu et al[11] and Ottani et al[12]. Meanwhile, the methods were modified to meet laboratory conditions. The specific operation was as follows: after preoperative fasting for solids and liquids for 12 h, rats were anesthetized *via* intraperitoneal injection of 3% pentobarbital sodium (25 mg/kg), and fixed on the operating table in the supine position. The leads of the multi-lead electrophysiological recorder were connected to the limbs of rats to record an electrocardiogram (ECG). Then, the rats were disinfected with alcohol routinely, and the hair on the neck and chest was shaved off. After that, the chest skin and muscle were cut along the 4th intercostal space of the left chest, and the ribs were cut off to expose the heart. Next, the pericardium was carefully cut and ligated at the proximal end of the left anterior descending coronary artery (LAD), inducing signs of myocardial ischemia. ECG was observed in all rats within 30 min. Significant ST-segment elevation >0.1 mV or T wave elevation in ECG indicated successful LAD ligation. Except for the Sham group, in which the only operation was performed without ligation to induce simple myocardial ischemia, the slipknot was loosened after ligation for 30 min and reperfusion was conducted for 2 h in the remaining 4 groups. Finally, significant ST-segment depression was observed in ECG, indicating that successful establishment of MIRI model in rats.

Detection of Effect of IRB on Serum Myocardial Enzyme Activity in MIRI Rats via ELISA

After reperfusion for 2 h, rats in each group were executed, and whole blood samples were collected from rats. According to the instructions of CK-MB, AST, LDH and cTnT kits, whole blood samples were placed at room temperature for 20 min, followed by centrifugation at 3000 rpm for 20 min at room temperature. Subsequently, clear serum isolated was collected. The absorbance of CK-MB, AST, LDH and cTnT at 450 nm was measured using a multi-functional microplate reader. The standard curves were plotted, and the concentrations of CK-MB, AST, LDH and cTnT in each sample were calculated.

Detection of Effect of IRB on Apoptosis in MIRI Rats via Annexin V-FITC/PI Double Staining

About 5 g of fresh myocardial tissue was taken from each group, and residual impurities were washed away with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA). The tissues were ground into the single cell suspension, followed by centrifugation twice at 1000 rpm for 10 min at room temperature. Subsequently, connective tissues were filtered out through a 300-mesh screen. Simple myocardial cells were collected, and cell concentration was adjusted to 5×10^5 cells/mL. After that, myocardial cells were gently re-suspended with 1 × Binding Buffer (500 μL), and transferred into a 4 mL flow tube. Then, the cells were mixed evenly with 5 μL of FITC-labeled Annexin V, followed by incubation in a dark place at room temperature for 15 min. 5 μL of PI was added for incubation on ice for 5 min in the dark. Flow tubes were gently tapped before detection to prevent counter wells from being blocked by cell precipitates. Finally, myocardial apoptosis in each group was analyzed using FACS Calibur flow cytometer within 30 min.

Detection of Effect of IRB on Messenger RNA (mRNA) Expression Levels of ERK, B-Cell Lymphoma-2 (Bcl-2) and Bcl-2-Associated X Protein (Bax) in MIRI Rats via RT-PCR

Total RNA in myocardial tissues of rats in each group was extracted using spin-column assay. Double-stranded RNA was then reversely transcribed into stable single-stranded complementary deoxyribose nucleic acid (cDNA) according to the instructions of pre-mixed RT reaction buffer. Like the template, cDNA was added into RT-PCR reagent to prepare the reaction system (20 μL), followed by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) using the StepOne PCR instrument. Specific PCR amplification conditions were as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s (40×). Primer sequences used in this study were as follows: ERK-F: 5'-GAACTCCAAGGGCTATACCAAGT-3', ERK-R: 5'-GGAGGGCAGAGACTGTAGGTAGT-3', Bcl-2-F: 5'-CCGGGAGATCGTGATGAAGT-3', Bcl-2-R: 5'-ATCCCAGCCTCCGTTATCCT-3', Bax-F: 5'-GTGGTTGCCCTCTTCTACTTTG-3', Bax-R: 5'-CACAAAGATGGTCACTGTCTGC-3', β-actin-F: 5'-TGACAGGATGCAGAAGGAGA-3', β-actin-R: 5'-TAGAGCCACCAATCCACACA-3'. β-actin was used as an internal reference. The relative mRNA expression levels of ERK, Bcl-2 and Bax in each group were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Left ventricular myocardial tissues were first placed on ice and fully lysed with 300 μL of radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) for 30 min. Then, the mixture was repeatedly pipetted, followed by centrifugation at 12000 rpm and 4°C for 15 min. Total protein was collected and stored at -20°C for subsequent use. The concentration of extracted protein in each group was quantified using the bicinchoninic acid (BCA; Pierce, Waltham, MA, USA) method. An equal amount of protein was separated *via* 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, target bands were cut according to the marker, and the proteins were transferred onto a membrane under constant current (200 mA) for 90 min. After sealing with Tris-Buffered Saline and Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA) containing 5% skim milk or 5% BSA (used for phosphorylated antibody) for 2 h, the membranes were incubated with target primary antibodies of ERK, p-ERK and internal reference β-actin (1:1000) at 4°C overnight/at normal temperature for 2 h. After washing with TBST on a shaking table 3 times (10 min/time), the membranes were incubated again with Horseradish Peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody (1:5000) at room temperature for 2 h. Next, the membranes were washed again with TBST on a shaking table 3 times (10 min/time). Enhanced electrochemiluminescence (ECL) solution (Beijing 4A Biotech Co., Ltd., Beijing, China) was added to detect target protein bands using the gel imager. Optical density was analyzed using Image-Pro Plus 6.0 image analyzer to quantify the proteins (Media Cybernetics, Silver Springs, MD, USA).

Statistical Analysis

GraphPad Prism 5.0 software (La Jolla, CA, USA) was used for all statistical analysis and plotting. Experimental results were expressed as mean ± standard deviation ($\bar{\chi}$±s). One-way analysis of variance was performed to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Effect of IRB on Serum Myocardial Enzyme Activity in MIRI Rats

As shown in Table I and Figure 1, the activity of serum myocardial enzymes in the Model group increased to different degrees when compared with the Sham group. The results showed that CK-MB, LDH and cTnT increased significantly ($p<0.001$), while AST showed an increasing trend ($p<0.05$). These findings indicated that the MIRI model was successfully established in rats. After IRB intervention, the activity of serum myocardial enzymes declined markedly when compared with the Model group ($p<0.05$, $p<0.01$ or $p<0.001$), indicating that IRB could alleviate myocardial injury caused by MIR.

Effect of IRB on Apoptosis in MIRI Rats

As shown in Table II and Figure 2, the apoptotic rate in the Model group [(53.31±6.72)%] was significantly higher than that of the Sham group [(7.29±2.52)%] ($p<0.001$). Besides, the apoptotic rates of IRB groups [(38.63±4.67)%, (16.55±2.79)% and (13.06±3.64)%] were all markedly declined when compared with the Model group, and the differences were statistically significant ($p<0.001$). The above findings suggested that IRB could protect myocardial cells from apoptosis in MIRI rats to a certain extent.

Effects of IRB on mRNAExpression Levels of ERK, Bcl-2 and Bax in Myocardial Cells in MIRI Rats

After reperfusion for 2 h, the changes in relative mRNA expression levels of ERK, Bcl-2

Table I. Effect of IRB on serum myocardial enzymes in MIRI rats ($\bar{\chi}$±s).

Group	CK-MB (U/L)	AST (U/L)	LDH (U/L)	cTnT (μg/L)
Sham	896.33±227.80	145.87±27.83	494.84±230.35	0.06±0.01
Model	1992.83±367.30***	178.68±32.72*	1448.57±221.70***	0.31±0.04***
IRB12.5	1711.83±128.28$^{\#}$	167.27±16.51	1173.57±217.84$^{\#}$	0.15±0.01$^{\#\#\#}$
IRB50.0	1092.67±245.21$^{\#\#\#}$	129.30±19.52$^{\#\#}$	665.03±162.67$^{\#\#\#}$	0.14±0.01$^{\#\#\#}$
IRB200.0	941.50±223.24$^{\#\#\#}$	100.25±16.57$^{\#\#\#}$	530.88±120.88$^{\#\#\#}$	0.13±0.01$^{\#\#\#}$

*$p<0.05$, ***$p<0.001$ vs. Sham; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* Model (n=6).

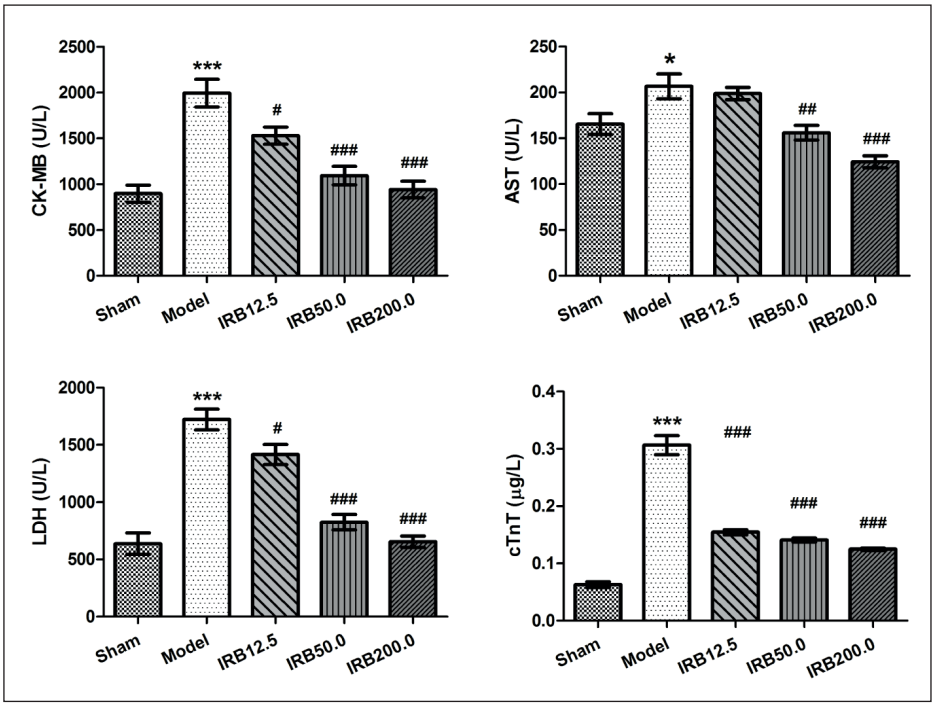

Figure 1. Effect of IRB on serum myocardial enzymes in MIRI rats ($\bar{\chi}$±s). *p<0.05, ***p<0.001 *vs.* Sham; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs.* Model (n=6).

and Bax were detected *via* RT-PCR. The results revealed that compared with the Sham group, the mRNA expression levels of ERK and Bax were significantly higher (p<0.001). However, the mRNA expression level of Bcl-2 was remarkably lower in the Model group (p<0.05), indicating that apoptosis occurred after MIR. After IRB treatment, the relative mRNA expression levels of ERK and Bax declined markedly. However, the mRNA expression level of Bcl-2 displayed an increasing trend in a dose-dependent manner, and there was a significant difference between IRB 200.0 group and Model group (p<0.001; Table III and Figure 3). The above results further demonstrated that IRB could inhibit myocardial apoptosis caused by MIRI.

Table II. Myocardial apoptosis in MIRI rat model detected via Annexin V-FITC/PI double staining ($\bar{\chi}$±s).

Group	Apoptotic rate (%)
Sham	7.29±2.52
Model	53.31±6.72***
IRB12.5	38.63±4.67$^{\#\#\#}$
IRB50.0	16.55±2.79$^{\#\#\#}$
IRB200.0	13.06±3.64$^{\#\#\#}$

***p<0.001 *vs.* Sham; $^{\#\#\#}p$<0.001 *vs.* Model (n=6).

Effects of IRB on Expression Levels of MAPK-ERK Pathway-Related Proteins in Myocardial Cells in MIRI Rats

After reperfusion for 2 h, the MAPK-ERK signal transduction pathway was studied *via* Western blotting (Figure 4A). The content of p-ERK, the active form of MAPK-ERK signaling molecule, in the Model group increased significantly when compared with the Sham

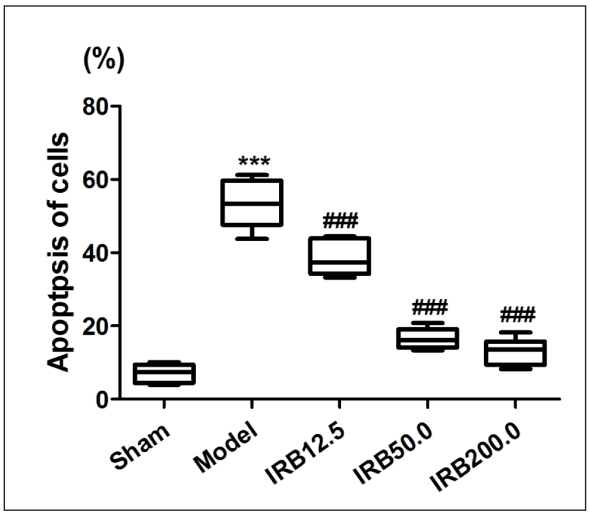

Figure 2. Myocardial apoptosis in MIRI rats detected via Annexin V-FITC/PI double staining ($\bar{\chi}$±s). ***p<0.001 *vs.* Sham; $^{\#\#\#}p$<0.001 *vs.* Model (n=6).

Table III. Effects of IRB on mRNA expression levels of ERK, Bcl-2 and Bax in myocardial cells in MIRI rats ($\bar{\chi}$±s).

Group	ERK	Bcl-2	Bax
Sham	0.48±0.26	1.90±0.65	0.25±0.25
Model	1.00±0.00***	1.00±0.00*	1.00±0.00***
IRB12.5	0.74±0.21	1.45±0.51	0.84±0.14
IRB50.0	0.62±0.16##	1.87±0.72	0.63±0.15##
IRB200.0	0.51±0.19###	2.67±0.70###	0.35±0.26###

*p<0.05, ***p<0.001 *vs.* Sham; #p<0.05, ##p<0.01, ###p<0.001 *vs.* Model (n=6).

group (Figure 4A and 4C). This suggested that MIR could induce the activation of the MAPK-ERK signal transduction pathway. After IRB treatment, the protein expression level of p-ERK declined with increased dose (p<0.05). However, the protein expression level of ERK was not affected when compared with the Model group (Figure 4A, 4B & 4C). Besides, p-ERK/ERK ratio in the Model group was significantly higher than that of the Sham group (p<0.01), whereas was remarkably declined in IRB groups compared with the Model group. Meanwhile, its phosphorylation level declined with the increase of IRB doses (p<0.05, p<0.01 or p<0.001; Figure 4A and 4D). The above findings further indicated that IRB could inhibit the activation of the MAPK-ERK signaling pathway in MIRI rats.

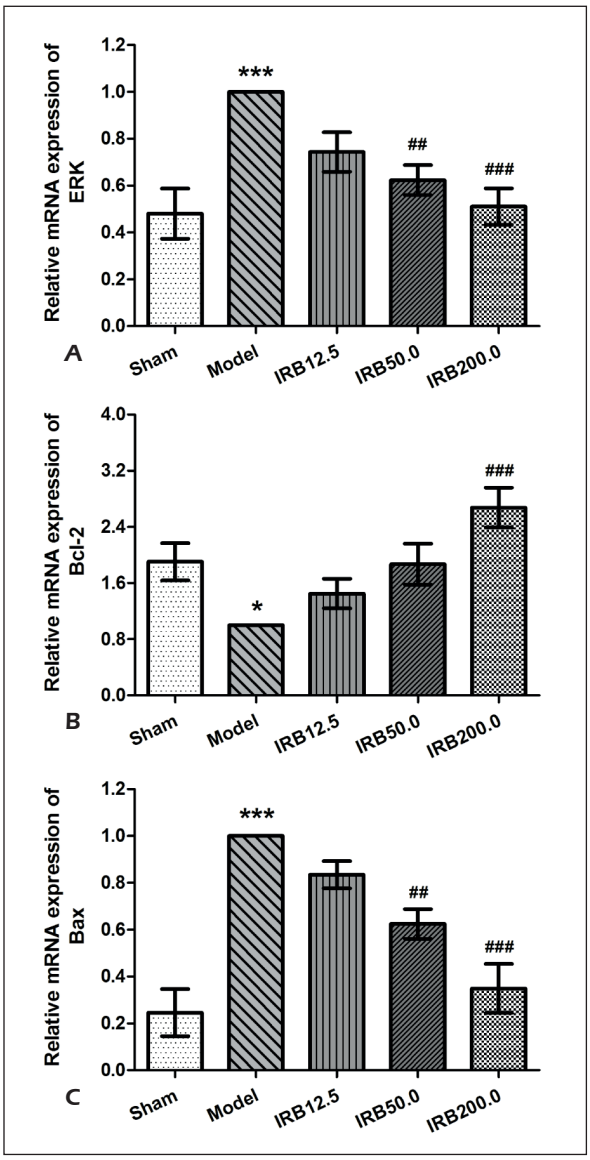

Figure 3. Effects of IRB on mRNA expression levels of ERK, Bcl-2 and Bax in myocardial cells in MIRI rats ($\bar{\chi}$±s). *p<0.05, ***p<0.001 *vs.* Sham; ##p<0.01, ###p<0.001 *vs.* Model (n=6).

Discussion

Decreased myocardial cell viability and ventricular remodeling are probably the major causes of death in AMI patients due to heart failure[13]. With the extensive application of emergency thrombolysis, emergency interventional therapy and coronary artery bypass surgery, the mortality rate of AMI patients has been greatly reduced. Meanwhile, the survival rate of patients significantly increases[1]. In the present work, it was found that in MIRI, myocardial necrosis occurred and a variety of myocardial enzymes were released into the blood. This could eventually increase the activity of serum myocardial enzymes, which was consistent with the results of Chen et al[14]. After pretreatment with IRB, the activity of serum myocardial enzymes (including CK-MB, AST, LDH and cTnT) in MIRI model was significantly declined, thereby reducing myocardial cell injury. Myocardial apoptosis was detected *via* flow cytometry in the study as well. The results showed that the apoptotic rate increased significantly in MIRI model after LAD ligation. However, it was remarkably declined after IRB intervention, further indicating that IRB

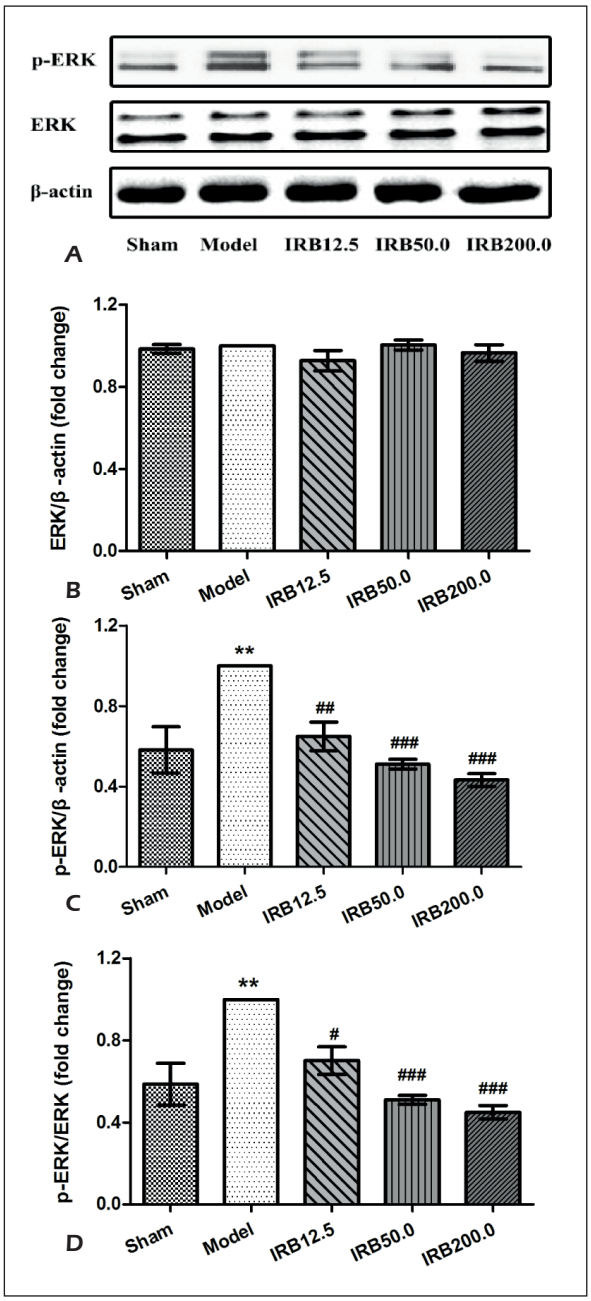

Figure 4. Effects of IRB on protein expression levels of ERK in rats ($\bar{\chi}$±s). **p<0.01 *vs.* Sham; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs.* Model (n=3).

could improve myocardial cell injury and have a certain preventive effect on myocardial injury in ischemia-reperfusion rats.

A large amount of evidence has demonstrated that the MAPK signal transduction pathway is associated with myocardial apoptosis[15-18]. MAPK cascade is needed in the process of extracellular stimuli binding to cell membrane surface receptors and transmitting signals into the cell membrane. Currently, the ERK signaling pathway is the most studied MAPK related pathway. It is involved in regulating multiple biological processes, such as survival, growth and death of various cells and inflammation-related immune response[19]. So far, studies have demonstrated that inhibiting the ERK pathway enhances reoxygenation and inhibits apoptosis and inflammatory response in myocardial cells. Moreover, this protects myocardium from secondary injury caused by ischemia-reperfusion to some extent[6]. Liu et al[20] have indicated that in the AMI rat model, the ratio of p-ERK/ERK ratio increases. Meanwhile, Baicalin can effectively inhibit the excessive phosphorylation of ERK. In the present study, p-ERK/ERK ratio in the Model group was significantly higher than that in the Sham group. The results indicated that MIRI could activate the MAPK-ERK signaling pathway, which was consistent with the results of Duan et al[21]. IRB could protect myocardial cells from IRI to some extent. In addition, the downstream molecule ERK of the MAPK-ERK signal transduction pathway, can transmit the upstream cascade signals into the nucleus upon the stimuli. Eventually, this regulates the expressions of various apoptosis-related genes in the nucleus, such as Bcl-2, Bcl-xL, Bax, Bad and Bim. Bcl-2 and Bax are key genes determining whether the apoptotic signaling pathway is activated after receiving extracellular stimulus signals in cells. Moreover, it was found in this study that in IRB groups, the relative mRNA expression of ERK was down-regulated. Meanwhile, the activity of p-ERK was markedly inhibited, thereby suppressing Bax expression and increasing Bcl-2 expression. The above results demonstrated that IRB could intervene in the expressions of Bcl-2 and Bax by regulating the activation of the ERK pathway. Furthermore, IRB could reduce myocardial apoptosis caused by ischemia-reperfusion, thereby maintaining and recovering the function of myocardial cells in rats as much as possible.

Conclusions

The cardioprotective mechanism of IRB in MIRI rats may be related to the inhibition on the activation of the MAPK-ERK signaling pathway. However, its effect target remains to be further studied.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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