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MicroRNA-361 regulates apoptosis of cardiomyocytes after ischemic-reperfusion injury

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Abstract. – OBJECTIVE: To investigate the role and mechanism of microRNA-361 (miR-361) in apoptosis after myocardial ischemia-reperfusion (MI-R) injury.

MATERIALS AND METHODS: For the *in vivo* experiments, the mice model of MI-R injury was established, and miR-361 was up-regulated via lentivirus with miR-298 overexpression. The expression of miR-361 and Bcl-2 associated X protein (BAX) were detected via Real Time-quantitative Polymerase Chain Reaction (qPCR) and Western blot (WB), respectively. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect the apoptosis in myocardial tissues. MI-R injury was also simulated *in vitro* experiments, and the relationship between miR-361 and BAX was verified using Luciferase reporter vector. The effect of miR-361 on cardiomyocyte apoptosis was also detected at the cellular level.

RESULTS: *In vivo* experiments showed that the miR-361 expression was down-regulated at MI-R injury area. The up-regulation of miR-361 significantly decreased the expression of BAX, reduced the myocardial apoptosis and inhibited the mitochondrial apoptosis pathway protein expression, including the cytochrome-c (Cyt-C) and cleaved caspase-3. *In vitro* experiments revealed that BAX was a target gene of miR-361 and further proved that miR-361 could inhibit the cytochrome-c and cleaved caspase-3 expression, as well as reduce the myocardial apoptosis through BAX.

CONCLUSIONS: MiR-361 could improve the myocardial apoptosis through the target gene BAX in MI-R injury.

Key Words:
MicroRNA-361 (miR-361), Bcl-2 associated X protein (BAX), Myocardial ischemia-reperfusion (MI-R), Mitochondrial apoptosis pathway.

Introduction

At present, the incidence rate of coronary heart disease increases rapidly in China, making it a serious threat to the health of adults. The acute myocardial infarction is characterized by acute onset, high mortality and disability rates, which causes the most serious damage to myocardial tissues. Opening the infarction-related artery rapidly for reperfusion therapy has been the most effective method to save the dying myocardial cells. However, reperfusion therapy will aggravate the myocardial cell injury, namely the reperfusion injury[1]. Some studies[2] had even demonstrated that 50% of myocardial apoptosis might be related to the reperfusion injury. Thus, reducing the reperfusion injury-induced myocardial apoptosis could significantly attenuate the area of myocardial injury and improve the mid- and long-term cardiac function of patients with myocardial infarction[3,4].

Micro-ribonucleic acid (RNA) is a kind of short endogenous single-stranded non-coding RNA fragment with about 22 nucleotides in length, which can inhibit the post-transcriptional level of gene expression and is considered an important component of cellular regulatory network[5]. The basic mechanism of miRNAs in exerting a regulatory effect is as follows: the miRNA-induced silencing complex (miRISC) is formed and binds to the 3' untranslated region (3'UTR) of the target mRNA *via* complementary base pairing to inhibit or degrade the mRNA function, thus regulating the post-transcriptional level of the target gene. One miRNA could have hundreds of target mRNAs, and it was predicted that all miRNAs in one cell regulated at least 30% of the DNA expression[6]. Therefore, miRNAs might be involved in almost all cellular functional activities, such as growth, differentiation and apoptosis. MiRNAs have attracted much attention as a major participant in the regulation of cellular activities in recent years.

There was a growing interest in the important role of miRNAs in the cardiovascular field. At the

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same time, the role of miRNAs in the apoptosis of cardiomyocytes in MI-R injury has also been continuously monitored. MicroRNA 361 (miR-361) is an important component of the microRNA regulatory network. Recently, it has been reported to be associated with many diseases and involved in the operation of many mechanisms[7-10]. However, the effects of miR-361 on MI-R injury had been less mentioned. In this work, we explored the role of miR-361 in MI-R injury.

Materials and Methods

Animals and Establishment of the Mice MI-R Model

Male C57BL/6 mice (20-25 g) aged 4-6 weeks were fed freely at room temperature for 1 week to adapt. All mice were randomized for dividing into 4 groups: the Sham-operation group (Sham group, n=10), Myocardial ischemia reperfusion group (MI-R group, n=10), MI-R + Lv-miR-361 group (the lentivirus with miR-361 overexpression was injected through the tail vein 24 h before operation, n=15) and MI-R + Lv-NC group (the blank lentivirus was injected through the tail vein 24 h before operation, n=15). This study was approved by the Animal Ethics Committee of Maternity and Child Health Care of Zaozhuang Animal Center.

Modeling operation is simple: the experimental mice fasted for 12 h before the operation. Besides, the limb-lead electrocardiogram was recorded, and those with abnormal electrocardiogram were excluded. After mice were anesthetized with intramuscular injection of 2% pentobarbital sodium (90 mg/kg) and fixed, the oral tracheal tube was placed and connected to the rodent ventilator. The respiratory rate was adjusted to 80 beats/min and the electrocardiogram was recorded. An ~2 cm long longitudinal incision was made on the chest skin along the left mid-clavicular line, the parasternal muscle was clamped several times using hemostatic forceps and cut, and a small incision was bluntly separated in the 2nd-3rd intercostal space at the left of the sternum. Then, the thoracic cavity was opened, the pericardium was bluntly separated to expose the heart, and the left anterior descending artery (LAD) was ligated below the junction of the left auricle and pulmonary artery. The ST-segment elevation in the electrocardiogram indicated the successful modeling of myocardial infarction. In I/R group, the ligature was loosened 30 min after infarction, followed by reperfusion for 24 h. In the sham-operation group, LAD was threaded but not ligated, and the remaining operations were the same as those in the other groups.

Cell Culture and Treatment

The clean-grade Sprague-Dawley (SD) rats aged 1-2 days were provided by the Shandong University Animal Center and soaked in 75% medical alcohol. Then, the chest cavity was cut, and the heart was gently extruded using two fingers and cut off using sterile ophthalmic scissors. After trypsinization and differential adhesion, the cell suspension obtained was inoculated into a 12-well plate with Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and cultured in an incubator with 5% CO_2 at 37°C for 48 h.

The primary myocardial cells in good conditions were inoculated into a 60 mm culture dish (1×10^6 cells/bottle), cultured in the incubator with 5% CO_2 at 37°C for 24 h and synchronized with serum-free DMEM for 24 h before cell modeling. For cell model establishment, the cells were placed in an anoxia chamber pumped with a gas mixture of 95% N2-5% CO_2 for 6 h, then cultured into the pre-equilibrated DMEM containing 10% FBS for another 24 h. Three groups were set for *in vitro* research: miR-NC group (negative control, myocardial cells were transfected with miR-NC using Lipofectamine TM 2000 according to the instructions 48 h before molding), miR-361 mimics group (myocardial cells transfected by miR-361 mimics before molding) and mimics + BAX group (myocardial cells co-transfected by miR-361 mimics and LV-BAX).

Quantitative Polymerase Chain Reaction (qPCR) Analysis

The total RNA of tissues and cells was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and the expression level of miR-361 was detected *via* qPCR. With U6 as an internal reference, the expression level was calculated using the Ct method. The reaction conditions are as follows: 95°C for 5 min, 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, a total of 40 cycles. Primer sequences used in this study were as follows: BAX, F: 5'-CCAGGAACCCCTCCTTACTC-3', R: 5'-TGTCCGAAGGCTAGGGATGA-3'; microRNA-361, F: 5'-AACTAGGTAAACTCGACTG-3', R: 5'-GGCCTACGTGTCGTGGAGTCG-3'; U6:

F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Luciferase Reporter Assays

BAX was predicted to be the target gene of miR-361 by the TargetScan, miRDB and microRNA websites. The binding sequence of miR-361 at the 3'-end of BAX was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA), and the mutated BAX (Mut-type) and non-mutant BAX (WT-type) were connected with the pGL3-Basic Luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vectors with mutant or non-mutant BAX were then transfected into myocardial cells after lentivirus intervention on the 12-well plate according to steps in the Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China). Then, the luciferase activity was detected in a multi-function microplate reader.

Western Blot (WB) Analysis

The protein was extracted from myocardial tissues and cells after treatment and the concentration was quantitatively detected using bicinchoninic acid method (BCA; Pierce, Waltham, MA, USA). A total of 30 μg protein samples were loaded for electrophoresis, transferred onto the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) and sealed with 5% skim milk powder for 90 min. After that, the samples were incubated with the primary antibody [anti-BCL2, anti-BAX, anti-cytochrome c, anti-cleaved caspase-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Abcam, Cambridge, MA, USA) (1:1000)] at 4°C overnight and incubated again with the secondary antibody (1:2000) at room temperature for 90 min. Finally, the color was developed *via* chemiluminescence instrument and the results were analyzed *via* Image-J software. GAPDH was used as the internal reference.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick end Labeling (TUNEL) Detection

After the mice were euthanatized *via* excessive injection of anesthetics, the heart was immediately removed, washed with normal ice saline, fixed with 4% paraformaldehyde for 4 h and dehydrated with 30% sucrose at 4°C overnight. The heart specimen was taken from 1 mm below the ligature to the apex, embedded in optimal cutting temperature compound (OCT), frozen at -21°C, sliced into 5 μm-thick sections using the LEICA-CM1900 freezing microtome and stored at -21°C. For each piece of frozen section, the DNA3-OH in the nucleus was labeled *via* TUNEL, and the apoptotic cells were displayed *via* in situ fluorescent labeling. Apoptotic index (AI) (%) = number of apoptotic myocardial nucleus/total number of myocardial nucleus × 100%.

Flow Cytometry Detection

After cell transfection, the single cell suspension was prepared *via* hypoxia/reoxygenation treatment. After being washed twice with pre-cooled Phosphate-Buffered Saline (PBS) at 4°C, the collected cells were resuspended with 500 μL binding buffer, and labeled with 5 μL Annexin V-fluorescein isothiocyanate (FITC) and 5 μL propidium iodide (PI), followed by reaction in a dark place and detection of apoptosis *via* flow cytometry immediately after 15 min.

Statistical Analysis

Statistical analysis was performed with the Student's *t*-test or *F*-test. All *p*-values were two-sided and $p<0.05$ was considered significant and analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

The Expression of MiR-361 in MI-R Area in Different Groups of Mice

At the beginning of the experiment, qPCR was used to detect the expression of miR-361 in the MI-R area of mice. Compared with that in the sham-operation group, the expression of miR-361 in the MI/R treatment groups was decreased significantly. On the other hand, the miR-361 expression in the miR-361 lentivirus treated group was higher than that in the miR-NC lentivirus group, suggesting that it was feasible to increase the expression of miR-361 by lentivirus (Figure 1).

Overexpression of MiR-361 Could Inhibit Myocardial Apoptosis

TUNEL staining was performed in our study to assess apoptosis in myocardial tissue. As shown in Figure 2A, we could find that there was almost no apoptotic cell in the myocardial tissue of the Sham group, while the cardiomyocytes of the MI/R treated group had a considerable amount of

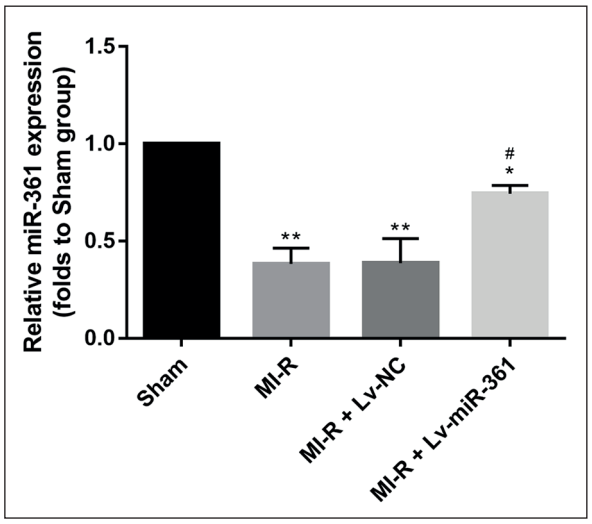

Figure 1. The expressions of miR-361 in myocardial ischemic-reperfusion (MI-R) injury area in different groups (***p<0.001 compared with the Sham group; $^{\#}p$<0.05 *vs.* MI group).

apoptosis. However, mitigation of apoptosis was observed in the myocardium of the mice overexpressing miR-361.

Effects of MiR-361 on BCL2/BAX Ratio and Apoptotic Pathway

The mitochondrial apoptotic pathway is probably the pathway that miR-361 affects. As many studies had confirmed the important role of mitochondrial apoptosis pathway in MI-R injury[11,12], the expression of Bcl-2 and BAX, which were restricted to each other in the Bcl-2 family, was first studied. The Bcl-2/BAX ratio was significantly lower in the MI/R treatment group than the Sham group, while it was moderated in the Bcl-2/BAX ratio in the MI/R+Lv-miR-361 group compared with other MI/R surgical groups. Cytochrome-c (Cyt-C) and cleaved caspase-3 are characteristic signs of apoptosis[13]. We found that, consistent with the ratio of Bcl-2/BAX, miR-361 could significantly reduce the expression levels of Cyt-C and cleaved caspase-3 stimulated by MI/R (Figure 2B).

BAX Was a Target Gene of MiR-361

The TargetScan, miRDB and microRNA online prediction websites were used to predict the potential targets for miR-361. We were pleasantly surprised to find that BAX had a binding target for miR-361 at the 3'UTR, which indicated that BAX was a potential target for miR-361 (Figure 3A). To verify whether miR-361 could target BAX, we immediately conducted a Luciferase reporter gene experiment. The results showed that the transfection of miR-361 overexpressed lentivirus significantly reduced the fluorescence expression of the pGL3-Basic vector with WT-BAX, but did not reduce the fluorescence expression of the pGL3-Basic vector with Mut-BAX (Figure 3B). This was a serious explanation of the regulation of miR-361 on BAX, and it also explained why miR-361 could have a great impact on the proportion of Bcl-2/BAX.

The MiR-361/BAX Axis Was Measured In Vitro

To further investigate the role of the miR-361/BAX axis in MI-R injury, we overexpressed miR-361 or/and BAX in cardiomyocytes *in vitro*, and simultaneously performed hypoxia-reoxygenation experiments to simulate MI-R damage. As expected, miR-361 overexpression significantly inhibited BAX expression, while miR-361 appeared to had no impact on Bcl-2 expression (Figure 4B). Subsequently, the downstream apoptotic pathway protein (Cyt-C, cleaved caspase-3) was also detected, and the results showed the blocking effect of miR-361 on apoptosis (Figure 4B). In the flow cytometry results, the decline in apoptosis was also observed in the miR-361 overexpression group (Figure 4A). However, in the group co-transfected with miR-361 and BAX, apoptosis was markedly revived. This result reflected the anti-apoptotic effect of miR-361/BAX in MI-R injury.

Discussion

MiRNAs are closely involved in the regulation of pathophysiological processes, such as angiogenesis, cardiac development, myocardial hypertrophy, heart failure and reperfusion injury[14]. There are about 150-200 kinds of miRNAs expressed in normal myocardium, and many of these dysregulated miRNAs will be dynamically changed in the case of cardiac stress, especially MI-R[15,16]. At present, the latest domestic and foreign studies have proved the correlation between some miRNAs and MI-R injury. Xu et al[17] reported that miR-1 (pro-apoptotic effect) and miR-133 (anti-apoptotic effect) played opposite roles in the oxidative stress-induced apoptosis of H9C2 cells. Ren et al[18] found that the expression of miR-320 significantly declined in the mouse model of reperfusion *in vitro* and *in vivo* through the high-throughput miRNA chip analysis. The area of

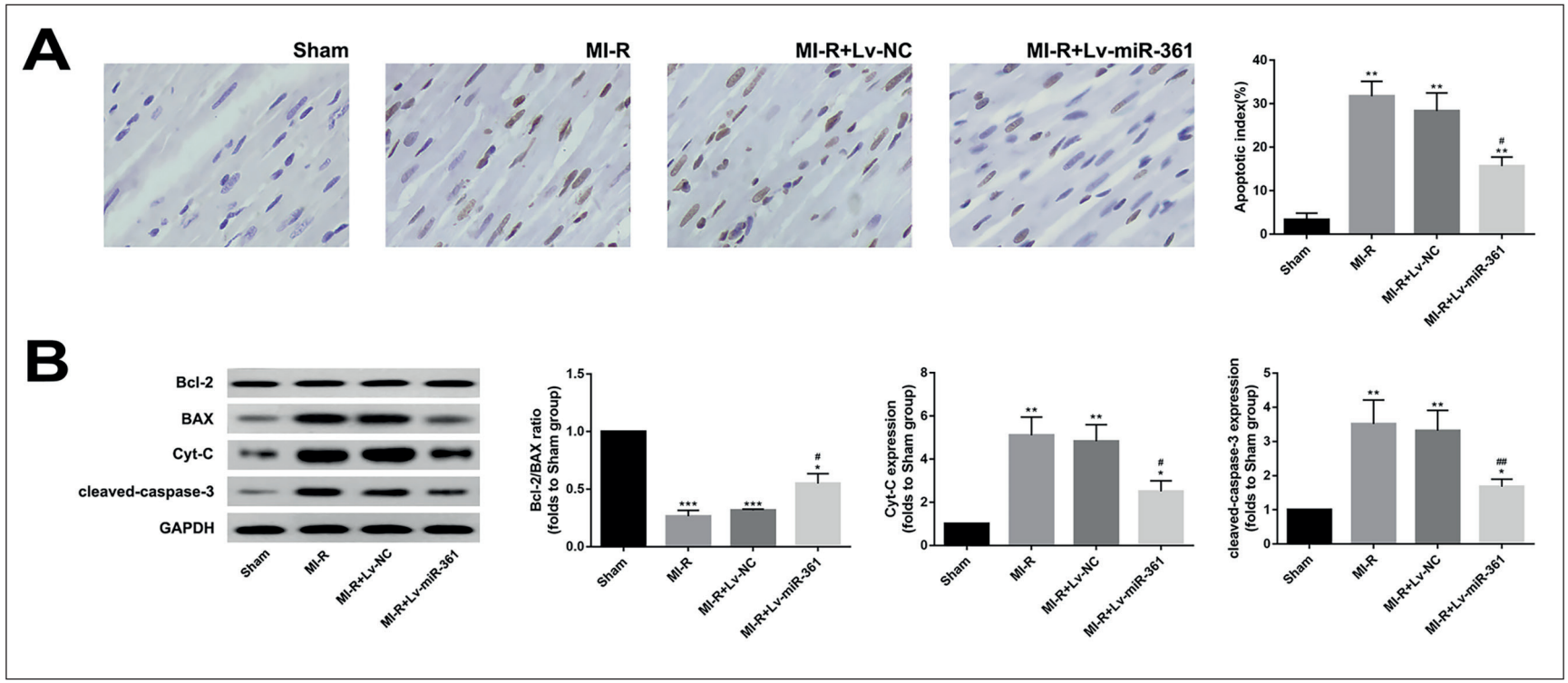

Figure 2. Up-regulation of miR-361 decreased the myocardial apoptosis *in vivo*. ***A***, Images of the myocardial cell stained by TUNEL assay *in vivo*. Photomicrographs were taken at 400× magnification. Apoptotic cardiomyocyte nuclei appear brown stained, histogram shows the percentage of TUNEL-positive cells (brown staining). ***B***, Protein expression of mitochondrial apoptosis pathway *in vivo* determined by Western blot. Data were presented as means ± standard deviations. (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the Sham group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs*. MI+Lv-NC group).

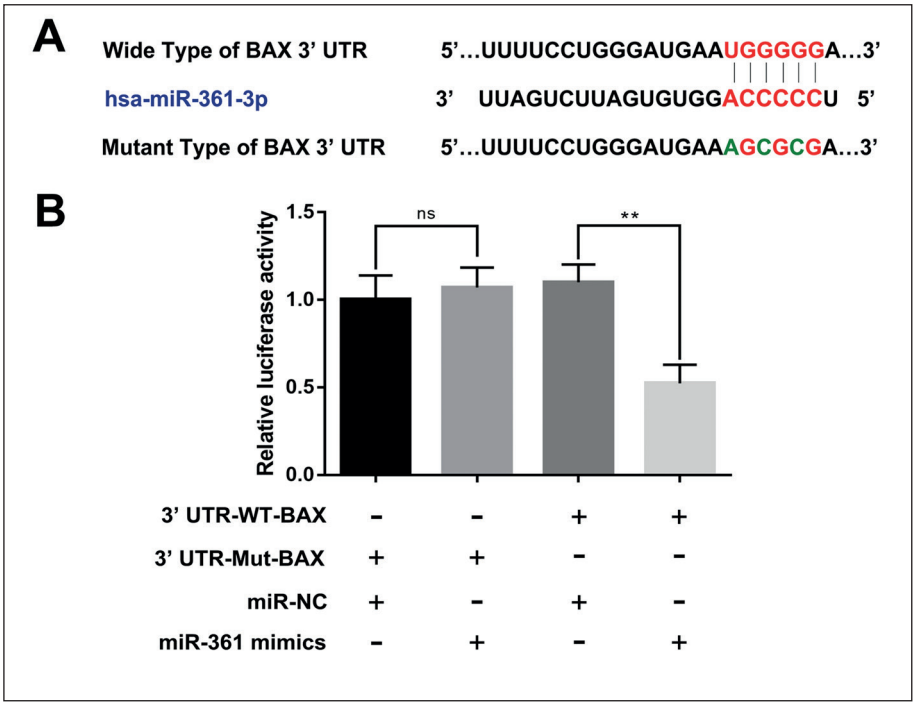

Figure 3. BAX is a direct and functional target of miR-361. ***A***, Diagram of putative miR-361 binding sites of BAX. ***B***, Relative activities of Luciferase reporters (**$p<0.01$).

MI-R injury could be reduced *in vivo* after mice were treated with miR-320 antisense strand, and it was proved that heat shock protein 20 (Hsp20) was its target gene. These effects make miRNAs a research hotspot in modern science.

There are mainly three apoptosis signal transduction pathways, which are the death receptor pathway, the mitochondrial pathway and the endoplasmic reticulum stress pathway. The MI-R injury-induced apoptosis is mainly regulated by the mitochondrial pathway. Gustafsson et al[19] argued that within the first few minutes after reperfusion, the mitochondrial permeability transition pore (mPTP) or major outer membrane protein opened, the cytochrome C[20] and apoptosis-inducing factor[21] were released, which resulted in the cascade activation of the downstream death-associated kinase, Caspase[22], and ultimately led to the myocardial apoptosis.

Apoptosis is a highly-programmed cell death mode, in which the B-cell lymphoma-2 (Bcl-2) family proteins are key regulators[23]. The changes in mitochondrial outer membrane permeability can lead to apoptosis, which was directly controlled by anti-apoptotic and pro-apoptotic Bcl-2 family proteins. Bcl-2 and BAX was a pair of important balance factors with opposite effects. Bcl-2 can inhibit the opening of mPTP, thereby preventing the pre-apoptotic factors in the mitochondrial intermembrane space from entering the cytoplasm. However, Bax promotes the opening of mPTP. The slight dynamic change of the Bcl-2/Bax ratio affects mitochondrial permeability, thus regulating apoptosis[24]. In some known heart protection mechanisms, such as ischemic preconditioning and ischemic postconditioning, increasing the Bcl-2/Bax ratio and preventing the opening of mPTP could reduce apoptosis[25,26].

The number of myocardial cells lost after acute and chronic myocardial infarction was the most important factor affecting the prognosis. Reperfusion therapy could lead to apoptosis and more extensive loss of cells. The myocardial reperfusion injury was studied in this research, to find an optimized reperfusion therapeutic regimen, reduce the secondary damage to reperfusion injury and protect the myocardial tissue cells to the maximum degree, thus laying a foundation for further exploration of the internal mechanism of reperfusion injury. Therefore, clarifying the rule of regulation of miRNAs on apoptosis in myocardial reperfusion injury was conducive to the in-depth understanding of the mechanism underlying reperfusion injury and searching new intervention strategies.

MiR-361 was the core of our study, which was significantly decreased in the heart of the mice after MI-R treatment. The low expression of miR-361 was accompanied by a significant increase in myocardial apoptosis. As another focus of this

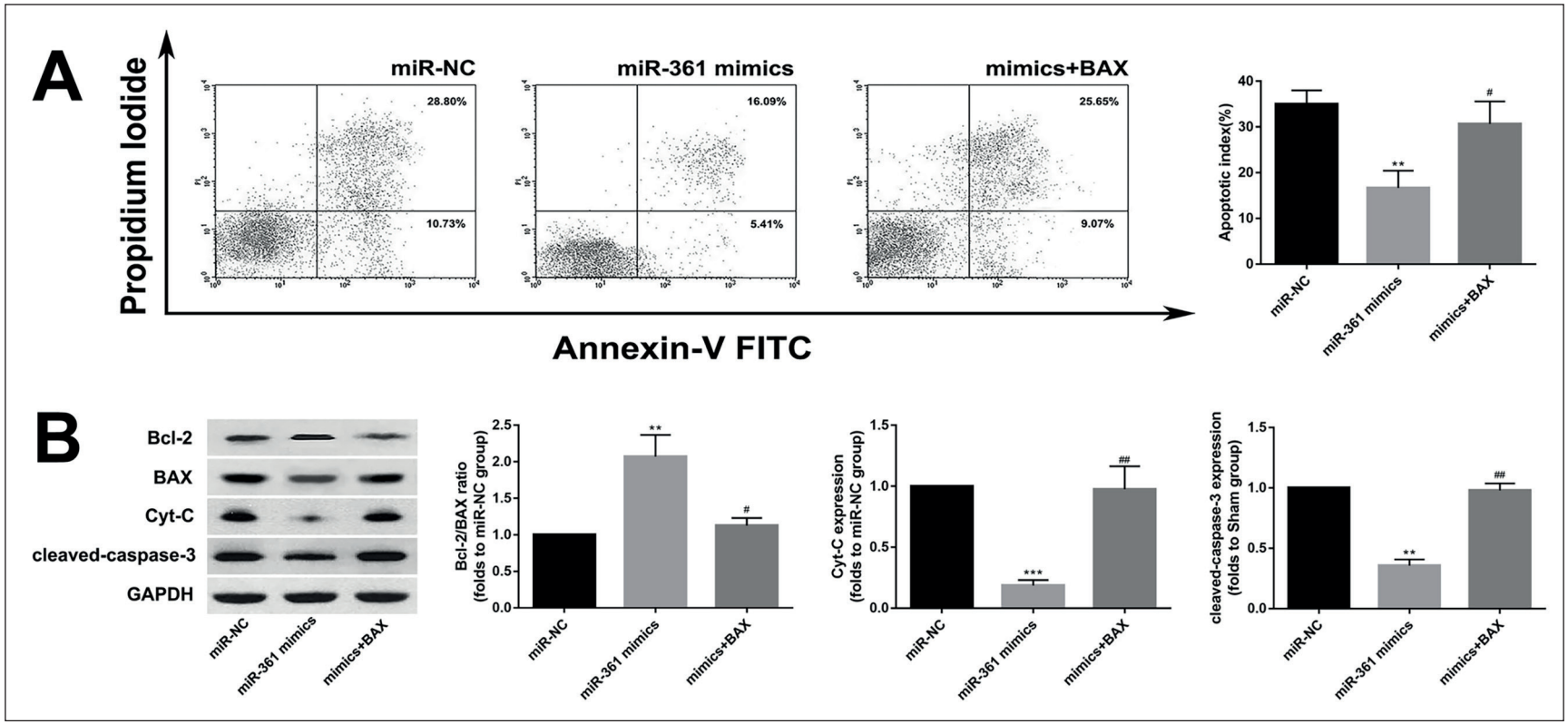

Figure 4. Up-regulation of miR-361 inhibited the expressions of BAX, reduced the myocardial apoptosis and attenuated the protein expression of cytochrome-c and cleaved caspase-3. ***A,*** Apoptosis level of myocardial cells detected by flow cytometer. ***B,*** Protein expression of mitochondrial apoptosis pathway *in vitro* determined by Western blot. ***C,*** Data were presented as means ± standard deviations. (**$p<0.01$, ***$p<0.001$ *vs.* NC group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* Mimics group).

experiment, the mitochondrial apoptotic pathway was discovered as a downstream pathway of miR-361 and involved in the anti-apoptotic effect of miR-361. BAX of the BCL-2 family, as a downstream target of miR-361, had been shown to be a functional target of miR-361 affecting MI-R injury *in vitro*.

Conclusions

We found that miR-361 could improve the myocardial apoptosis through the target gene BAX in MI-R injury. This study might be the theoretical basis for the targeted molecular therapy which was used to prevent and treat the MI-R injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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