

Effect of NF- κ B signaling pathway mediated by miR-711 on the apoptosis of H9c2 cardiomyocytes in myocardial ischemia reperfusion

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Abstract. – OBJECTIVE: The aim of this study was to explore the change of the expression of miR-711 in myocardial ischemia-reperfusion (I/R) injury and the possible mechanism.

MATERIALS AND METHODS: The cardiomyocyte model of I/R injury was constructed. Real-time quantitative fluorescence polymerase chain reaction (qRT-PCR) and Western blotting were used to detect the expression of miR-711 as well as the mRNA and protein levels of NF- κ B (p65). Flow cytometry, CCK-8 kit, and enzyme-linked immunosorbent assay (ELISA) were used to detect the apoptosis, cell viability, and the content of LDH and MDA, respectively.

RESULTS: Compared to control cells, the expression levels of miR-711, the mRNA, and protein levels of NF- κ B were higher in H9c2 cardiomyocytes of I/R, the apoptosis rate of H9c2 cardiomyocytes of I/R was higher, the levels of LDH and MDA were higher in the supernatant of cell culture, and the cell viability was lower. In comparison to the cells of I/R, the apoptosis rate of H9c2 cardiomyocytes of I/R plus miR-711 inhibitors was lower, the levels of LDH and MDA were lower in the supernatant of cell culture, and the cell viability was higher. In comparison to control cells, the expression level of Bcl-2 was lower, and the expression levels of Bax and Caspase-3 were higher in the cells of I/R. In comparison to the cells of I/R, the expression level of Bcl-2 was lower, and the expression levels of Bax and Caspase-3 were higher in the cells of I/R plus miR-711 inhibitors.

CONCLUSIONS: Overexpression of miR-711 could promote the expression of NF- κ B (p65) in the cardiomyocytes of I/R and accelerate the transportation of NF- κ B (p65) into the nucleus, thus promoting the apoptosis of cardiomyocytes.

Key Words:

miR-711, Ischemia-reperfusion, NF- κ B, Apoptosis.

ed coronary artery and recover normal blood flow, thus decreasing the mortality of acute myocardial infarction². However, ischemia-reperfusion (I/R) injury impacts the cardiac function and prevents the recovery of myocardial function. This is leading to worsened myocardial damage, arrhythmia, endothelial dysfunction and extended myocardial infarction³. Apoptosis is crucial in I/R injury and usually occurs in the early phase of the disease^{4,5}. Thus, it is very important to prevent the apoptosis of cardiomyocytes after I/R injury so as to improve its prognosis and treatment. miRNA (microRNA) is an endogenous non-coding single-stranded RNA with a length of approximately 22 nucleotides. It binds to the 3'-UTR of the mRNA of target gene specifically, and degrades the mRNA or inhibits the translation, thus regulating the expression of genes after transcription⁶. It was reported^{7,8} that miRNA regulates the proliferation, apoptosis, and angiogenesis during I/R injury. Therefore, it was closely associated with cardiovascular diseases. This work suggested that the abnormally expressed miR-711 plays an important role in I/R injury. By inhibition or over-expression of miR-711, we analyzed the effect of miR-711 on the expression of NF- κ B and explored the mechanism by which miR-711 affected myocardial I/R injury. Moreover, this work constructed the H9c2 cardiomyocyte model of I/R injury and explored the mechanism and role of miR-711 in the pathological apoptosis of cardiomyocytes in I/R injury. Further, we also clarified the pathological mechanism of I/R injury and provided theoretical evidence for clinical treatment of I/R injury.

Introduction

Acute myocardial infarction is one of the most severe types of coronary heart disease with a high morbidity in the elderly population and the mortality of which is 10-15%¹. Thrombolysis and percutaneous coronary intervention could open the occluded

Materials and Methods

Cell Line and Reagents

H9c2 cardiomyocytes were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). DMEM (Dulbecco's Modified Eagle Medium) culture medium was purchased from

Hyclone (Logan, UT, USA). Trizol kit and Lipofectamine 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). One Step PrimeScript cDNA synthesis kit and SYBR® Premix Ex Taq™ were purchased from Dalian TaKaRa Biotechnology Co., Ltd., (Dalian, Liaoning, China). Polymerase chain reaction (PCR) enzyme and reagents were purchased from Beijing TransGen Biological Technology Co., Ltd (Beijing, China). CKK-8 kit and Annexin-V-FITC/PI apoptosis kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA kit was purchased from Wuhan Boshide Biological Engineering Co., Ltd (Wuhan, Hubei, China). The antibodies against Bax, Bcl-2, Caspase-3, NF-κB (p65), p-IκBα, IκBα, and β-actin, as well as secondary antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Instruments

PCR amplifier (ABI, Waltham, MA, USA); Horizontal and Vertical Gel Electrophoresis Apparatus (Beijing Six-One Instrument Factory, Beijing, China) and film-transferring system (Beijing Six-One Instrument Factory, Beijing, China). Flow cytometer (BD Biosciences, San Jose, CA, USA); multifunctional microplate reader (Thermo Fisher Scientific Co. Ltd., Waltham, MA, USA) and NanoDrop 2000 Ultramicro spectrophotometer (Thermo Fisher Scientific Co. Ltd., Waltham, MA, USA).

Construction of the Cardiomyocyte Model of I/R Injury

Sugar and serum-free culture medium were used as the hypoxic and ischemic solution. The sugar and serum-free culture medium were incubated with the mixed air of 1% O₂, 94% N₂, 5% CO₂ for at least 2 h to produce an air-saturated anoxic solution. H9c2 cardiomyocytes were cultured in a 6-well plate; the normal culture medium was replaced by hypoxic and ischemic solution. Then, the cells were incubated in a 37°C microaerobic incubator for 10 h. The hypoxic and ischemic solutions were discarded. The cells were washed with phosphate buffered saline (PBS) for 3 times and incubated with Dulbecco's Modified Eagle Medium (DMEM) high glucose. Then, the cells were supplemented with 10% fetal bovine serum (FBS) and cultured in an incubator (95% air and 5% CO₂) at 37°C for 2 h. The H9c2 cardiomyocyte model of I/R injury was constructed.

qRT-PCR

H9c2 cardiomyocytes were harvested. Total RNA was extracted with a Trizol kit (Thermo

Fisher Scientific, Waltham, MA, USA) and the concentration of RNA was measured by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized by a One Step PrimeScript cDNA Synthesis kit (TaKaRa Biotechnology Co., Ltd, Dalian, Liaoning, China). According to the instructions, the diluted cDNA was incubated with SYBR®Premix Ex Taq™ (TaKaRa Biotechnology Co., Ltd, Dalian, Liaoning, China), qRT-PCR was performed in an ABI 7500 Real-time PCR amplifier (ABI Company, Waltham, MA, USA). The gene of β-actin was used as internal reference/housekeeping gene.

Western Blotting

The protein sample was mixed with loading buffer and denatured in a 100°C water bath for 5 min and then loaded into the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Thermo Fisher Scientific, Waltham, MA, USA) for electrophoresis. Thereafter, the proteins were transferred to the polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was blocked with 5% skimmed milk at room temperature for 1 h and then incubated with primary antibody at 4°C overnight. After washing with Tris-buffered saline and Tween 20 (TBST) for 10 min × 3, the membrane was incubated with horseradish peroxidase (HRP) labeled secondary antibody at room temperature for 1 h. The membrane was washed TBST for 10 min × 3 again. Visualization was performed with an ECL chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, USA). The film was scanned and the band was quantified by ImageJ software (ImageJ, <https://imagej.nih.gov>).

Detection of Cell Apoptosis by Flow Cytometry

The H9c2 cardiomyocytes in logarithmic phase were collected and digested with trypsin. The cells were re-suspended and diluted to a density of 1×10⁵ cells/mL. An aliquot of 2 mL cells was inoculated in a 6-well plate. After induction of I/R injury, the percentage of cell apoptosis was measured by a Annexin-V-FITC/PI kit according to the instruction: the cells (2×10⁵) were re-suspended with 100 μL 1 × binding buffer, incubated with 2.5 μL Annexin-V-FITC and shook with 5 μL propidium iodide (PI) in the dark for 15 min. Then 400 μL 1 × binding buffer were added. The cells were gently shaken and the percentage of cell apoptosis was measured by a flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA).

Detection of Cell Proliferation Activity

The cells in logarithmic phase were collected and cultured in a 96-well plate (5×10^4 cells/mL, 200 μ L/well). The cells were incubated for 12 h. Induction of I/R injury: briefly, 10 μ L CCK-8 solution was added to each well and incubated for 3 h. The optical absorption was measured at 450 nm in a microplate reader and recorded as value A. The proliferation activity equals A of the treatment group/A of the control group.

LDH and MDA Levels in the Supernatant of Cell Culture Medium

The levels of LDH and MDA in the supernatant of cell culture were measured by ELISA (Abcam, Cambridge, MA, USA). ELISA was performed according to the instructions: the supernatant of cell culture and different levels of standard were added into the 96-well plate (100 μ L/well), which were then sealed with parafilm and incubated at 37°C for 90 min. The antibody (100 μ L/well) was added and incubated for 60 min. The enzyme-binding solution (100 μ L/well) was added

and further incubated for 30 min. The plate was washed 4 times and the optical absorption was measured at 450 nm in a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data were represented by mean $\pm$ SD, the inter-group difference was analyzed by *t*-test, * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ were considered as significant difference.

Results

Expression of miR-711 and NF- κ B in H9c2 Cardiomyocytes of I/R injury

The expression level of miR-711 was higher in H9c2 cardiomyocytes than in control cells (Figure 1A). Both the expression levels of mRNA and proteins of NF- κ B were increased (Figure 1B and Figure 1C), indicating that both miR-711 and NF-

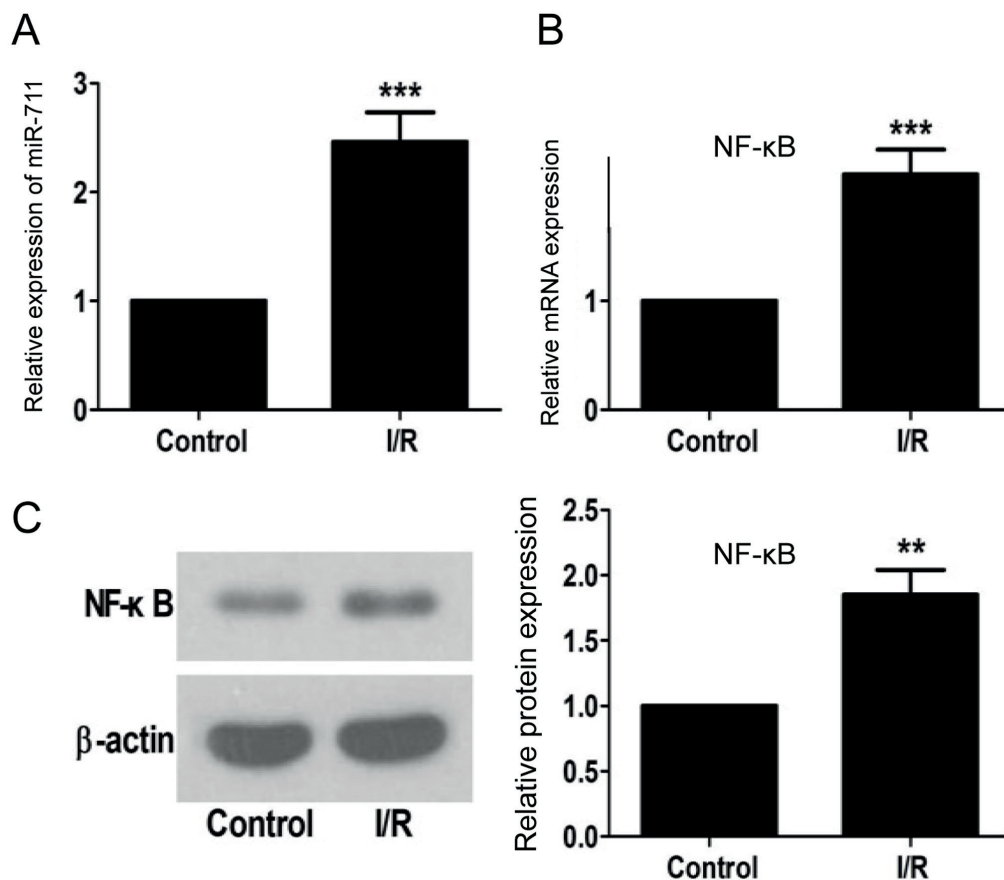


Figure 1. The expression of miR-711 and NF- κ B in H9c2 cardiomyocytes after I/R injury. **A**, Expression level of miR-711; **B**, mRNA expression of NF- κ B; **C**, Protein expression of NF- κ B.

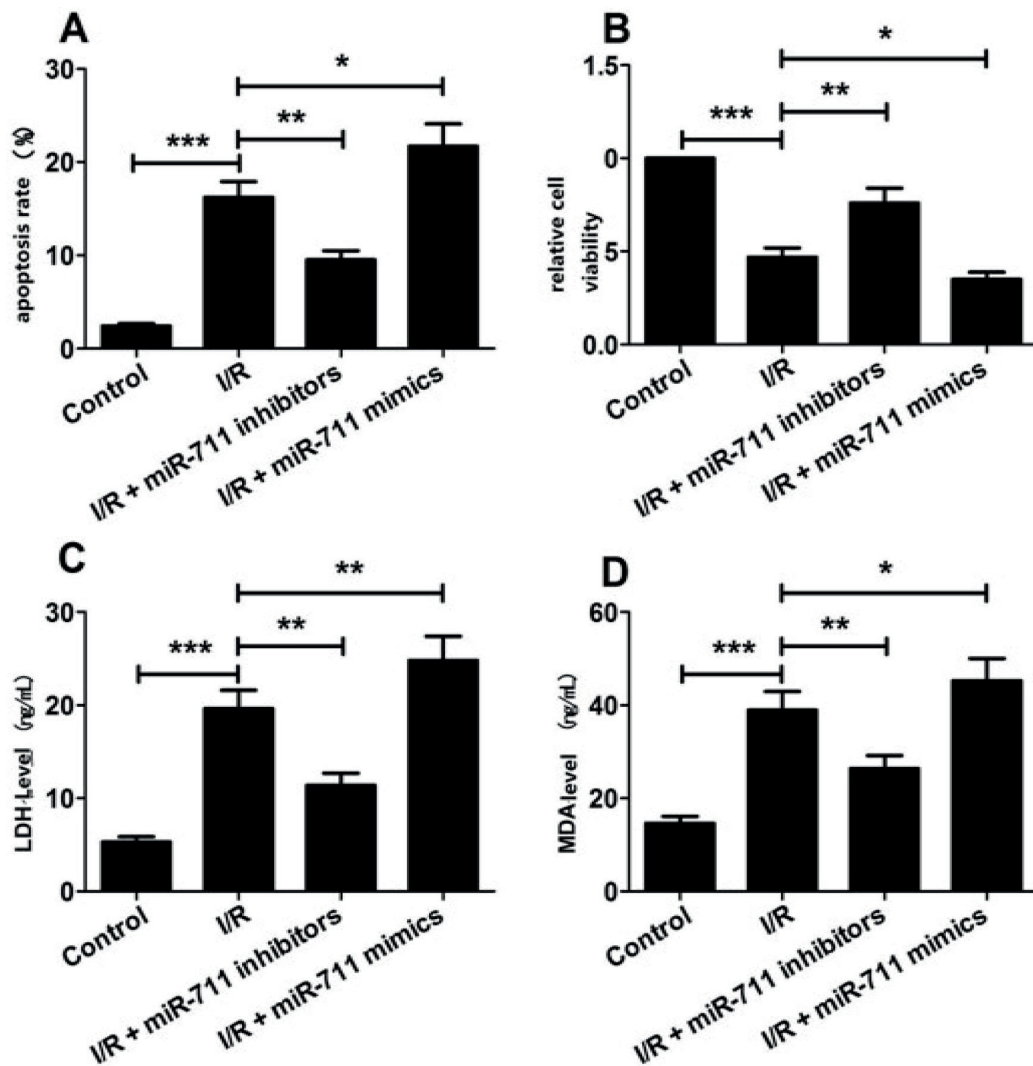


Figure 2. The effects of miR-711 on the apoptosis of H9c2 cardiomyocytes induced by I/R injury. *A*, Apoptosis rate of H9c2 cardiomyocytes; *B*, Cell viability; *C* and *D*, Levels of LDH and MDA.

κ B may be involved in the I/R injury of cardiomyocytes.

miR-711 Promoted the Apoptosis of H9c2 Cardiomyocytes Induced by I/R Injury

In comparison to control cells, the apoptosis rate of H9c2 cardiomyocytes after I/R injury was higher (Figure 2A), both the levels of LDH and MDA were higher, and the cell viability was lower (Figure 2B). In comparison to the cells of I/R injury, the apoptosis rate of H9c2 cardiomyocytes of I/R injury plus miR-711 inhibitors was lower, both the levels of LDH and MDA were lower, and the cell viability was higher. In comparison to the cells of I/R injury, the apoptosis rate of H9c2 cardiomyo-

cytes of I/R injury plus miR-711 mimics was higher, both the levels of LDH and MDA were higher (Figure 2C-D), and the cell viability was lower.

Effects of miR-711 on the Expression of Apoptosis-Related Proteins Bax, Bcl-2, and Caspase-3

In comparison to control cells, the expression levels of Bcl-2, Bax, and Caspase-3 in cells of I/R injury were lower, higher and higher, respectively. In comparison to the cells of I/R injury, both the expression levels of Bax and Caspase-3 in the cells of I/R injury plus miR-711 mimics were higher, the expression level of Bcl-2 was lower. In comparison to the cells of I/R injury, both the expression levels of Bax and Caspase-3 in the cells of

I/R injury plus miR-711 inhibitors were lower, the expression level of Bcl-2 was higher.

miR-711 Promoted the Apoptosis of H9c2 Cardiomyocytes Induced by I/R Injury Through Activating NF- κ B

As shown in Figure 4A, the expression level of NF- κ B (p65) in the cells of I/R injury was higher than in control cells. In comparison to the cells of I/R injury, the expression level of NF- κ B (p65) in the nucleus of the cells of I/R injury plus miR-711 inhibitors was lower, while the expression levels of NF- κ B (p65) in the cytoplasm was higher. In comparison to the cells of I/R injury, the expression levels of NF- κ B (p65) in the nucleus of the cells of I/R

injury plus miR-711 mimics were higher, while the expression levels of NF- κ B (p65) in the cytoplasm were lower. The expression level of p-I κ B α was higher and the expression level of I κ B α was lower after I/R injury. Inhibition of the expression of miR-711 could reverse the change of the expression of both p-I κ B α and I κ B α induced by I/R injury (Figure 4B). These results indicated that miR-711 was involved in the apoptosis of H9c2 cardiomyocytes induced by I/R injury through activating NF- κ B.

Discussion

In the clinical treatment of cardiac ischemia, the I/R injury after the recovery of coronary blood flow

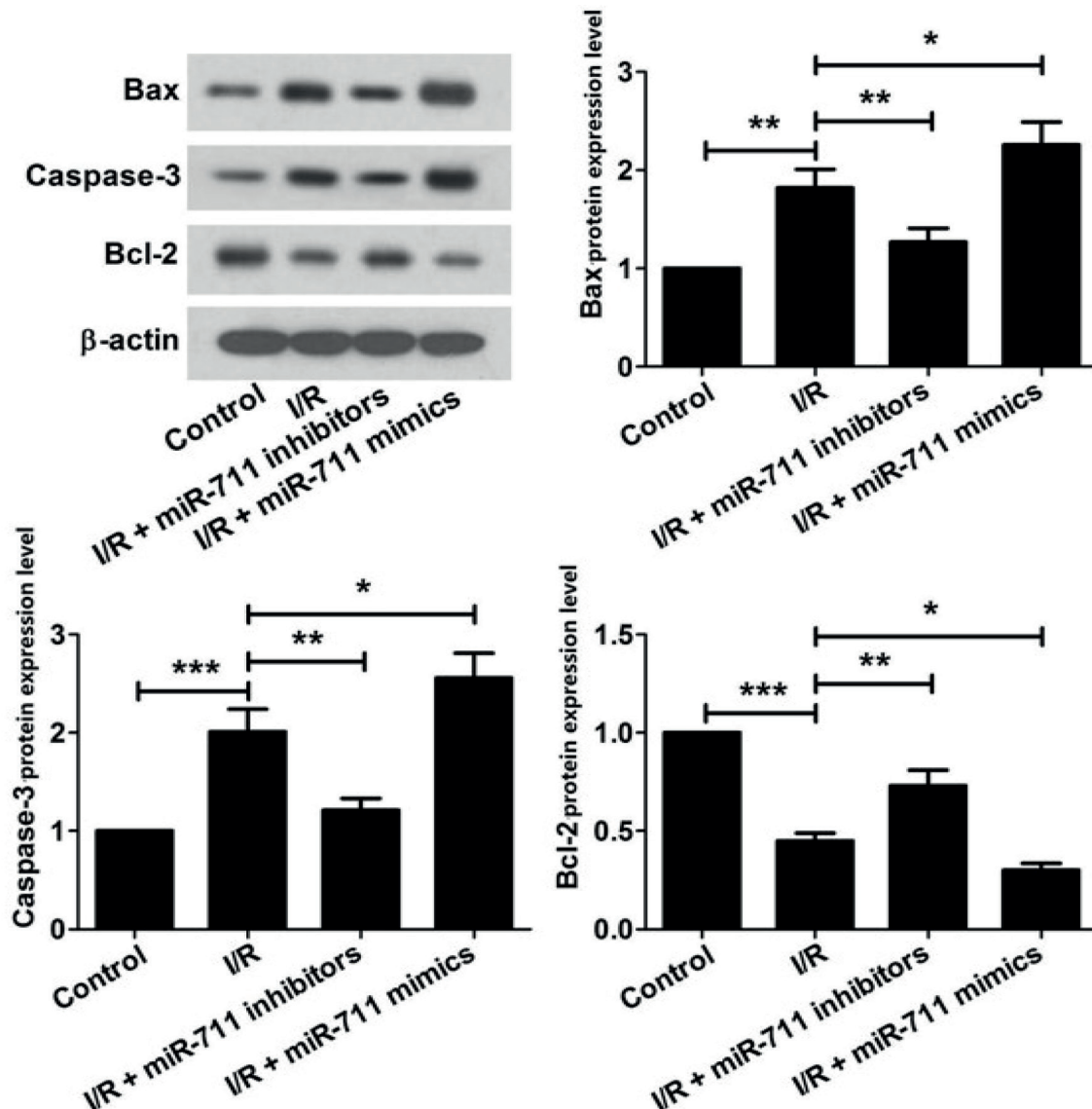


Figure 3. The effects of miR-711 on the expression of Bax, Bcl-2, and Caspase-3.

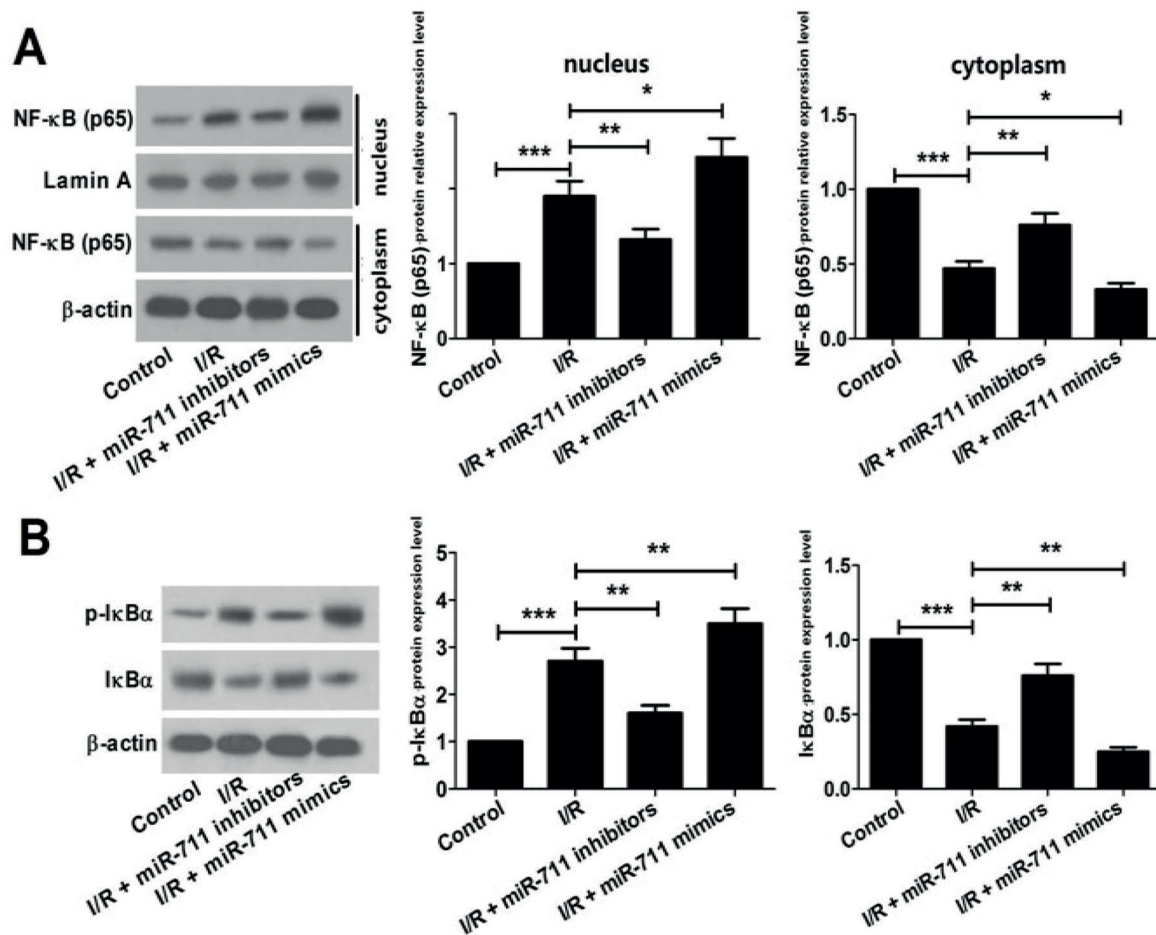


Figure 4. miR-711 promoted the apoptosis of H9c2 cardiomyocytes induced by I/R injury through NF-κB activation. **A**, The expression level of NF-κB (p65) in the nucleus of the cells of I/R injury plus miR-711 inhibitors was lower, while the expression levels of NF-κB (p65) in the cytoplasm was higher. **B**, Inhibition of the expression of miR-711 could reverse the change of the expression of both p-IκBα and IκBα induced by I/R injury.

is important⁹. Thus, it was of clinical significance to clarify the molecular mechanism of I/R injury. This study detected the change of the expression of miR-711 and NF-κB in cardiomyocytes of I/R injury, and analyzed the effects of miR-711 on the apoptosis of H9c2 cardiomyocytes and the mechanism. Moreover, we found that the over-expression of miR-711 could promote the apoptosis of H9c2 cardiomyocytes through activating NF-κB. Recent studies showed that the expression of miRNAs was tissue-specific. The expression levels of miR-21, miR-1, miR-133a and miR-296 were specifically high in cardiomyocytes. These miRNAs were involved in heart development, apoptosis of cardiomyocytes, myocardial remodeling and myocardial hypertrophy^{10,11}. The regulation of miRNA in I/R injury have gained interest. It has been confirmed that miRNAs were involved in the patholo-

gy of I/R injury of the heart^{12,13}. It was reported that miR-1 could promote the release of LDH, up-regulated the expression of Caspase-3, promoted the apoptosis of cardiomyocytes and worsened the I/R injury in mouse model¹⁴. miR-613 can target the expression of programmed cell death 10 (PDCD10) to inhibit the apoptosis of cardiomyocytes induced by I/R injury¹⁵. Sabirzhanov et al¹⁶ found that the up-regulation of miR-711 promoted the apoptosis of nerve cells after traumatic brain injury. This study showed that the expression level of miR-711 was up-regulated in H9c2 cardiomyocytes of I/R injury. To further investigate the effects of miR-711 on the apoptosis of cardiomyocytes induced by I/R injury, we up-regulated and down-regulated the expression of miR-711 through the transfection of miR-711 mimics and inhibitors, respectively. We also showed that miR-711 could promote cell apoptosis

and that over-expression of miR-711 could promote the expression of Bax and Caspase-3 and decrease the expression of Bcl-2. When the expression of miR-711 was inhibited, the apoptosis rate was decreased. Both the expression levels of Bax and Caspase-3 were decreased, and the expression of Bcl-2 was increased. NF- κ Bs are a group of transcription factors in the Rel protein family, and homodimer or heterodimer composed of two polypeptide chains¹⁷, i.e. p50 and p65. In normal cells, I κ B α is anchored with NF- κ B through the anchored protein, thus NF- κ B is stored in the cytoplasm rather than in the nucleus; as a result, the activity of NF- κ B is inhibited. In case of external stimulation, I κ B α was rapidly phosphorylated and degraded, then activated NF- κ B entered the nucleus and regulated the inflammation and apoptosis¹⁸. A previous report¹⁹ showed that the NF- κ B was activated in the rat model of I/R injury, and that inhibited the activity of NF- κ B and reduced the apoptosis of cardiomyocytes after I/R. Also, the inhibition of activation of NF- κ B can inhibit the up-regulation of pro-inflammatory genes and the expression of matrix metalloproteinases in cardiomyocytes; thus, relieve the contractile impairment induced by I/R²⁰. Carvedilol could inhibit the TLR4/NF- κ B signal pathway and reduce the apoptosis of H9c2 cardiomyocytes induced by I/R injury; thus, it was used to protect the heart²¹. Consistent with previous studies, this work found that the apoptosis rate of H9c2 cardiomyocytes was higher and the expression level of NF- κ B (p65) was higher after I/R injury. This research showed that inhibited expression of miR-711 could decrease the activity of NF- κ B; also, over-expression of miR-711 could increase the activity of NF- κ B. All together, these results demonstrated that miR-711 was involved in the apoptosis of H9c2 cardiomyocytes induced by I/R through activating NF- κ B.

Conclusions

We found that miR-711 was involved in I/R injury in the cell model of I/R injury *in vitro*. miR-711 could worsen the apoptosis of cardiomyocytes through the up-regulation of the expression of NF- κ B, and plays important roles in I/R injury. This study indicated that miR-711 could be a potential target of clinical treatment of I/R injury in future.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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