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MiR-145-5p promotes myocardial cell apoptosis in rats with myocardial infarction through PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the influence of micro ribonucleic acid (miR)-145-5p on myocardial cell apoptosis in rats with myocardial infarction (MI) through the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) pathway.

MATERIALS AND METHODS: In this study, Sprague-Dawley rats were used as research objects to establish the acute MI model *in vivo*. Infarction tissues and non-infarction tissues were both collected from rats. The expression level of miR-145-5p was determined using quantitative Polymerase Chain Reaction (qPCR), and the pathological changes in myocardial tissues of rats were observed through hematoxylin-eosin (HE) staining. In addition, H9c2 rat myocardial cells were cultured under hypoxia or normal oxygen concentration to simulate hypoxia in MI tissues. The changes in the expression of miR-145-5p in H9c2 cells in normal oxygen and hypoxia were determined. Meanwhile, the ratio of apoptotic cells to viable cells, and the changes in the expressions of proteins B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), Caspase-3 and Caspase-9 were evaluated through flow cytometry assay and Western blotting, respectively. The expression levels of crucial proteins in the PI3K/Akt pathway were measured as well. Additionally, H9c2 cells were transfected with miR-145-5p control and miR-145-5p mimic to evaluate cell apoptosis.

RESULTS: QPCR results revealed that the expression level of miR-145-5p was substantially elevated in MI tissues ($p<0.05$). HE results indicated that the soma exhibited deformation after MI, suggesting that there were more necrotic and apoptotic cells. Compared with those cultured under normal oxygen concentration, H9c2 cells cultured in hypoxia environment exhibited significantly upregulated expression level of miR-145-5p, downregulated expression level of anti-apoptosis protein Bcl-2, upregulated level of pro-apoptosis protein Bax, activated Caspase-3 and Caspase-9, and downregulated expression level of functional proteins in the PI3K/Akt pathway ($p<0.05$). Furthermore, the expression levels of apoptosis-associated proteins significantly rose in H9c2 cells transfected with miR-145-5p mimic compared with those transfected with miR-145-5p control, showing statistically significant differences ($p<0.05$).

CONCLUSIONS: MiR-145-5p is notably raised in MI tissues of rats. After infarction, there are evidently more apoptotic myocardial cells. The expression of miR-145-5p is markedly elevated in H9c2 rat myocardial cells in hypoxia. Compared with those cultured in normal oxygen, H9c2 cells cultured in hypoxia showed increased apoptosis. The apoptosis of myocardial cells transfected with miR-145-5p mimic is notable higher than that of myocardial cells transfected with miR-145-5p control. Moreover, the expressions of active Akt and PI3K proteins decrease remarkably. The results of this study demonstrate that miR-145-5p inactivates the PI3K/Akt pathway to promote the apoptosis of MI cells.

Key Words:
MiR-145-5p, Myocardial infarction (MI), PI3K/Akt, Cell apoptosis.

Introduction

Acute myocardial infarction (AMI) has gradually become a major killer of cardiovascular disease, and is also a leading cause of death in patients with cardiovascular diseases[1]. After infarction, complex changes occur in myocardial cells, involving ischemia, hypoxia, necrosis, apoptosis and myocardial remodeling. Growing evidence has emphasized the significance of inflammatory responses and myocardial cell apoptosis in the pathogenesis of AMI. They are involved in regu-

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lating myocardial function impairment and heart failure[2,3]. The expression of pro-inflammatory mediators is upregulated in cardiac dysfunction. In particular, the level of TNF-α is significantly elevated in locally infarcted myocardium, leading to acute myocardial dysfunction and myocardial cell apoptosis[4]. Therefore, myocardial cell apoptosis treatment has been researched as the potential and efficacious way for the treatment of ischemic myocardial injury.

Micro ribonucleic acids (miRNAs) are a kind of short-stranded non-coding small RNAs in body fluid and cells. They have been found to play crucial roles in organisms. As siRNAs with extremely high abundance, miRNAs mainly bind to target genes, thereby inhibiting protein expression. Considering their merits, their roles in MI have attracted more and more attention[5]. MiRNAs serve as key regulators in various biological pathways, such as development, growth, homeostasis, immunoregulation and disease progression. MiRNAs participate in the pathogenesis of various cardiovascular diseases, including AMI[6-8]. Notably, miR-145 plays an important role in various malignancies, such breast cancer, esophageal squamous cell carcinoma and bladder cancer[9-11]. MiR-145 is downregulated in differentiated vascular smooth muscle cells (VSMCs), acting as a key regulator in the phenotype of VSMCs[12,13]. Additionally, a study has found that the proliferation and differentiation of immature VSMCs during maturation can be suppressed by the overexpression of miR-145[14]. Latest studies have demonstrated that miR-145 decreases rapidly in the heart after AMI[15]. MiR-145 is substantially downregulated in ischemic heart and cardiac fibroblasts to respond to hypoxia in organisms[16]. However, the relationship between myocardial cell apoptosis and the expression of miR-145 in patients with MI and the underlying regulatory mechanism have not been fully elucidated. Therefore, the aim of this research was to explore the influence of miR-145 on the apoptosis of MI rats and its mechanism.

Materials and Methods

Materials

Sprague-Dawley rats were purchased from and fed in the Animal Center of our hospital, H9c2 murine cardiomyocytes from American Type Culture Collection (ATCC; Manassas, VA, USA), quantitative Polymerase Chain Reaction (qPCR) kit from Roche (Basel, Switzerland), antibodies (1:1,000) from Shanghai Jinpan Biotech Co., Ltd. (Shanghai, China), and flow cytometry kit from Beijing Biosynthesis Biotechnology Co, Ltd. (Beijing, China). All reagents and consumables were ordered by the laboratory on a centralized basis.

Research Subjects and Grouping

Female laboratory rats were divided into two groups, including: healthy control (HC) group (n=10) and MI group (n=10). H9c2 cells were cultured in hypoxia, and then, assigned into Control group and Hypoxia group. Additionally, H9c2 cells were transfected with miR-145 control gene sequences as miR-145 control group or miR-145 mimic gene sequences as miR-145 mimic group. This study was approved by the Ethics Committee of Jinan Fourth People's Hospital.

Establishment of H9c2 Hypoxia Model

1 d before the experiment, H9c2 cells were inoculated into a culture bottle. When the cell density exceeded 60%, the bottle was replaced with a specially purchased anoxic medium, and the cells continued to be cultured in an incubator at 37°C. Meanwhile, the incubator was filled with 99.9% N_2, and the volume fraction of O_2 was monitored to ensure it was less than 1%.

Detection of Cell Apoptosis

The cells were normally sub-cultured, and the digestion of adherent cells was terminated using ET. Subsequently, the cells were transferred into a 10 mL centrifugal tube, followed by centrifugation at 1,300 rpm/min for 10 min. Cell deposits were retained and rinsed with the incubation buffer aspirated from the flow cytometry kit. Then, the mixture were transferred into 1.5 mL Eppendorf (EP, Hamburg, Germany) tubes and centrifuged at 4,000 rpm/min for 2 min. At the end of centrifugation, the cells were blown away using buffer, and separately added with 20 μL of AnnexinV-FITC (fluorescein isothiocyanate) and 4 μL of Propidium Iodide (PI) staining solution at the interval of 10 min. After the second addition of staining solution, the reaction was performed in dark for 10 min. Finally, the resulting reaction system (400 μL) was aspirated to a centrifuge tube and cell apoptosis was detected on a machine.

Hematoxylin-eosin (HE) Staining of Myocardial Tissues

Tissue specimens were obtained, sectioned and embedded in paraffin. At 1 d before the experiment, the sections were pre-heated at 65°C overnight. Briefly, the sections were de-paraffinized in three xylene cylinders containing different concentrations of xylene, dehydrated in ethanol with gradually increasing concentrations, slightly washed with deionized water for 4 times, and dried in air. Subsequently, the resulting tissues were spread on sterile slides and stayed above the flame of an alcohol burner for 15-30 s. Next, eosin Y-solution was added drop-wise and retained for 3 min to stain the cytoplasm. At the completion of cytoplasmic staining, the staining solution was diluted using distilled water to terminate the staining. After that, stained tissue sections were cleaned slowly using ethanol, and we added methylene blue dye solution in drops to stain the cell nuclei for 60 s. This staining was stopped using the same method as above. Finally, the sections were sealed for long-term preservation.

Western Blotting

Cell deposits were collected, and the total proteins were extracted on ice. Absorbance at the wavelength of 560 nm was determined to calculate protein concentration. A total of 10 μL of total proteins were separated by electrophoresis and transferred onto membranes at 300 mA for 1 h. After sealing with skimmed milk powder at 25°C for 2 h, the membranes were washed with phosphate buffered saline-tween (PBST) and incubated with primary antibodies at 4°C for 16 h. Next, the membranes were washed again with PBST for 10 min × 3 times and incubated with the corresponding secondary antibodies for 2 h in dark. Immuno-reactive bands were finally scanned and quantified using an Odyssey membrane scanner (Seattle, WA, USA).

Fluorescence qPCR

1 mL of samples (serum/plasma) or cell deposits were taken to extract total RNAs using the conventional method. The concentration of extracted RNAs was then determined. Subsequently, the RNAs were reversely transcribed according to the instructions of a kit (Roche, Basel, Switzerland). Briefly, the total system was added with RNAs, and No. 5, No. 6 and No. 7 reagents. After heating at 65°C for 10 min, No. 2, No. 3, No. 4 and No. 1 reagents were added, followed by heating at 50°C for 60 min and 85°C for 5 min to obtain complementary deoxyribonucleic acids (cDNAs). Next, the concentration of cDNAs was determined. After that, cDNAs were diluted to 500 ng/μL and we added buffer, primers, cDNA and water to obtain a 20 μL reaction system. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used in this study were as follows: MiR-145-F ATTTCGCTGCTCCATTTA; MiR-145-R ATTTCGCTGCTCCATTTA. U6-F CTCGCTTCGGCAGCACA, U6-R AACGCTTCACGAATTTGCGT.

Transfection of H9c2 Cells With MiR-145-5p Mimic and Control Gene Sequences

At 1 d before the experiment, H9c2 cells were seeded into 6-well plates at a density of 3.0×10^5 cells/well. MiR-145-5p mimic and control transfection reagents were prepared in advance. After growing to an appropriate density 24 h later, cell transfection was performed. After an appropriate volume of viruses was selected in accordance with MOI, miR-145-5p mimic and control reagents were added separately. Transfected cells were cultured in an incubator for use.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. *t*-test and mono-factor analysis were performed when appropriate. $p<0.05$ was considered statistically significant.

Results

Expression Level of MiR-145-5p in Tissues and Pathological Morphology of Infarction Tissues in Rats After MI

Compared with healthy myocardial tissues, the expression level of miR-145-5p was substantially upregulated in infarcted myocardial tissues ($p<0.05$). Moreover, the soma of infarcted myocardial cells showed deformation, and large numbers of necrotic tissues and significaltly increased apoptotic cells were observed under a microscope ($p<0.05$) (Figure 1).

Changes in MiR-145-5p Expression Level and Apoptosis in H9c2 Cells After Hypoxia Treatment

Compared with those in Control group, H9c2 cells in Hypoxia group exhibited evidently up-

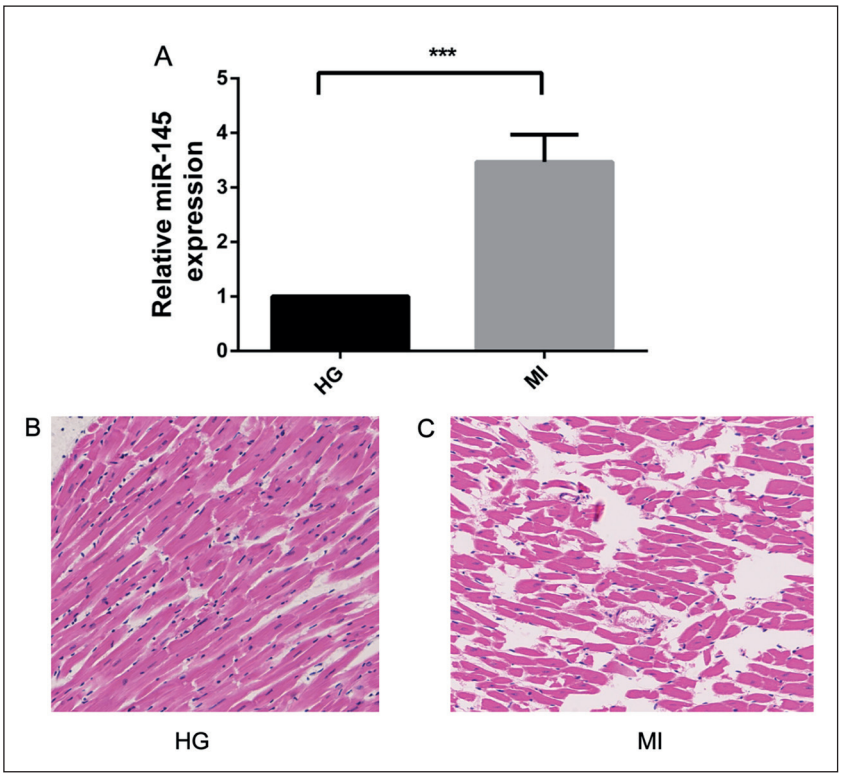

Figure 1. Expression level of miR-145-5p, and pathological morphology of infarction tissues in HC and MI groups. **A,** Expression level of miR-145-5p in tissues in HC group and MI group: Compared with that in HC group, the expression level of miR-145-5p is elevated notably in tissues in MI group ($p<0.01$). **B,** and **C,** HE staining results in HC and MI groups: In comparison with HC group, MI group has more necrotic and apoptotic cells (magnification: 40×).

regulated expression level of miR-145-5p, downregulated expression level of anti-apoptosis protein B-cell lymphoma 2 (Bcl-2), and upregulated expression level of pro-apoptosis protein Bcl-2 associated X protein (Bax) ($p<0.05$). After hypoxia treatment, the expression level of miR-145-5p remarkably increased and the apoptosis was enhanced in H9c2 cells ($p<0.05$). These results suggested that in MI tissues, hypoxia could induce the increase in miR-145-5p expression, and that miR-145-5p was correlated with apoptosis (Figure 2).

Apoptosis Was Enhanced in H9c2 Cells After Overexpression of MiR-145-5p

H9c2 cells were transfected with miR-145-5p mimics to overexpress miR-145-5p in myocardial cells. Apoptosis-associated marker proteins were detected in this study. It was found that compared with miR-145-5p control group, Caspase-3 and Caspase-9 were activated in miR-145-5p overexpression group. Meanwhile, the expression of Bcl-2 remarkably declined, whereas Bax significantly increased in miR-145-5p mimic group ($p<0.05$). According to the results of flow cytometry, the proportion of apoptotic cells in the right upper quadrant in miR-145-5p mimic group was significantly higher than that in miR-145-5p control group (the right upper quadrant and the left lower quadrant represented the number of apoptotic cells and normal cells, respectively) ($p<0.05$). All these findings implied that upregulated expression level of miR-145-5p could promote apoptosis (Figure 3).

The Influence of MiR-145-5p on Apoptosis Was Related to the Inactivation of the PI3K/Akt Signaling Pathway

The content of phosphorylated (p)-Akt and p-PI3K decreased significantly in miR-145-5p mimic group compared with miR-145-5p control group ($p<0.05$). However, there were no statistically significant differences in Akt and PI3K between the two groups ($p>0.05$). The above results suggested that the overexpression of miR-145-5p in H9c2 cells could weaken the activity of the PI3K/Akt pathway, thereby facilitating cell apoptosis (Figure 4).

Discussion

With the improvement of modern medical techniques, the mortality rate of AMI has declined considerably in the last decades. However, after

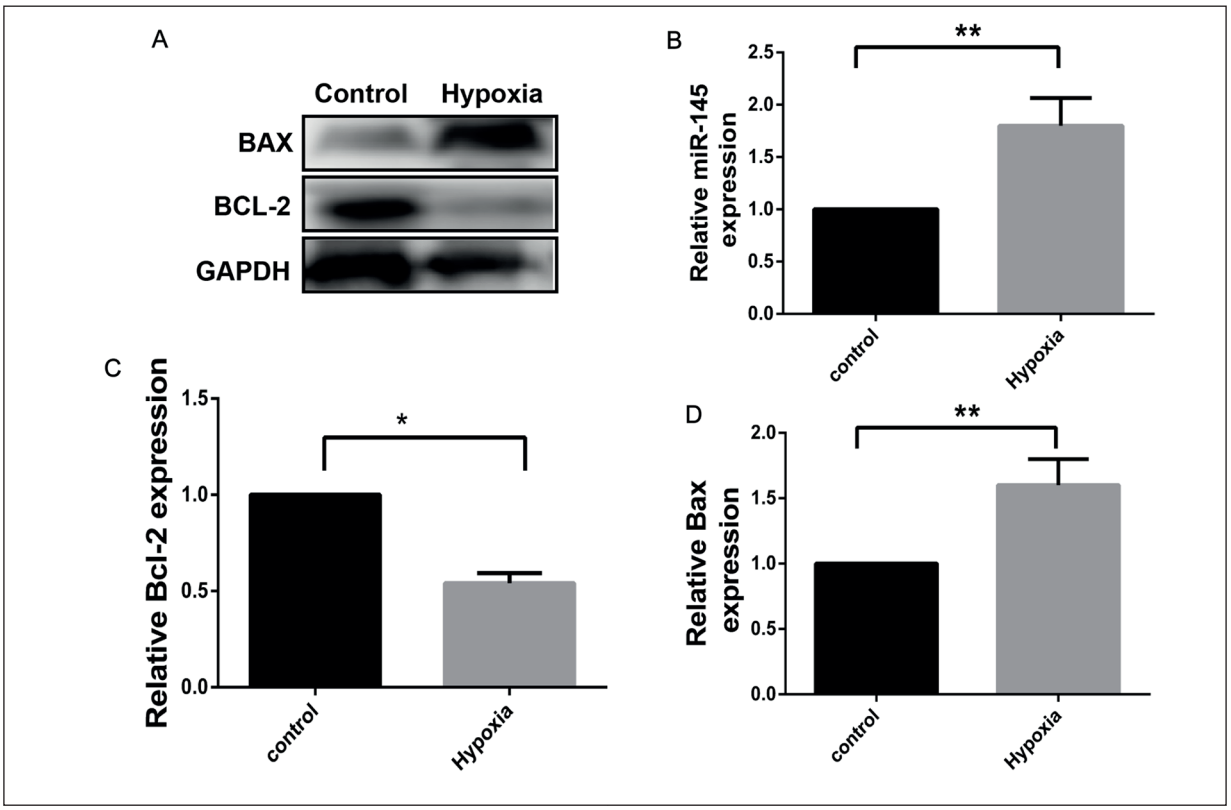

Figure 2. Changes in miR-145-5p and apoptosis-associated proteins in H9c2 cells in Control and Hypoxia groups. **A,** Western blotting results of apoptosis-associated proteins Bcl-2 and Bax. **B,** Expression level of miR-145-5p: Compared with that in Control group, the expression level of miR-145-5p rises remarkably in Hypoxia group ($p<0.01$). **C,** and **D,** Statistical results of apoptosis-associated proteins Bcl-2 and Bax: Hypoxia group has a notably higher expression level of pro-apoptosis protein Bax ($p<0.01$), and a markedly lower expression level of anti-apoptosis protein Bcl-2 than Control group ($p<0.05$).

treatment, there is still a high risk of post-AMI complications, such as heart failure and cardiac death. Meanwhile, the complications of cardiovascular disease remain hard to be predicted[17,18]. In AMI, persistent inflammatory responses and necrosis are the two most evident features in ischemic tissues. They may be mutually enhanced in MI-induced heart damage, ultimately resulting in heart failure. Therefore, elucidating the possible underlying molecular mechanism is of great significance for formulating the treatment strategy of ischemic heart diseases. It has been largely demonstrated the importance of miRNAs in ischemic heart disease. A large number of studies have focused on the role of miR-145 in malignant tumors. However, the exact effect of miR-145 on human heart has not been fully clarified. Xu et al[19] showed that overexpression of miR-145-3p protects against rat myocardial infarction by targeting PDCD4. However, Wu et al[20] found that miR-145-5p induces apoptosis after ischemia-reperfusion by targeting dual specificity phosphatase 6. In addition, the physiological significance of changes in miR-145 level in ischemic injury remains to be elucidated. As the apoptosis of myocardial cells is closely correlated with the prognosis, it is of great significance to further evaluate the role of miR-145 in the apoptosis of AMI for its treatment in the future.

In the present study, the rat MI model was first established, and myocardial tissues were collected from MI and HC groups. HE staining and real-time fluorescence qPCR assays were performed to observe the changes in myocardial cell morphology and miR-145-5p expression in the two groups. It was verified that the expression of miR-145-5p was considerably upregulated in myocardial tissues in MI group compared with that in HC group ($p<0.01$). HE staining results showed that in infarcted tissues, the boundary between myocardial cells was blur, and the cells exhibited swelling and deformation. Meanwhile, many of the cells suffered from necrosis and apoptosis, with inflam-

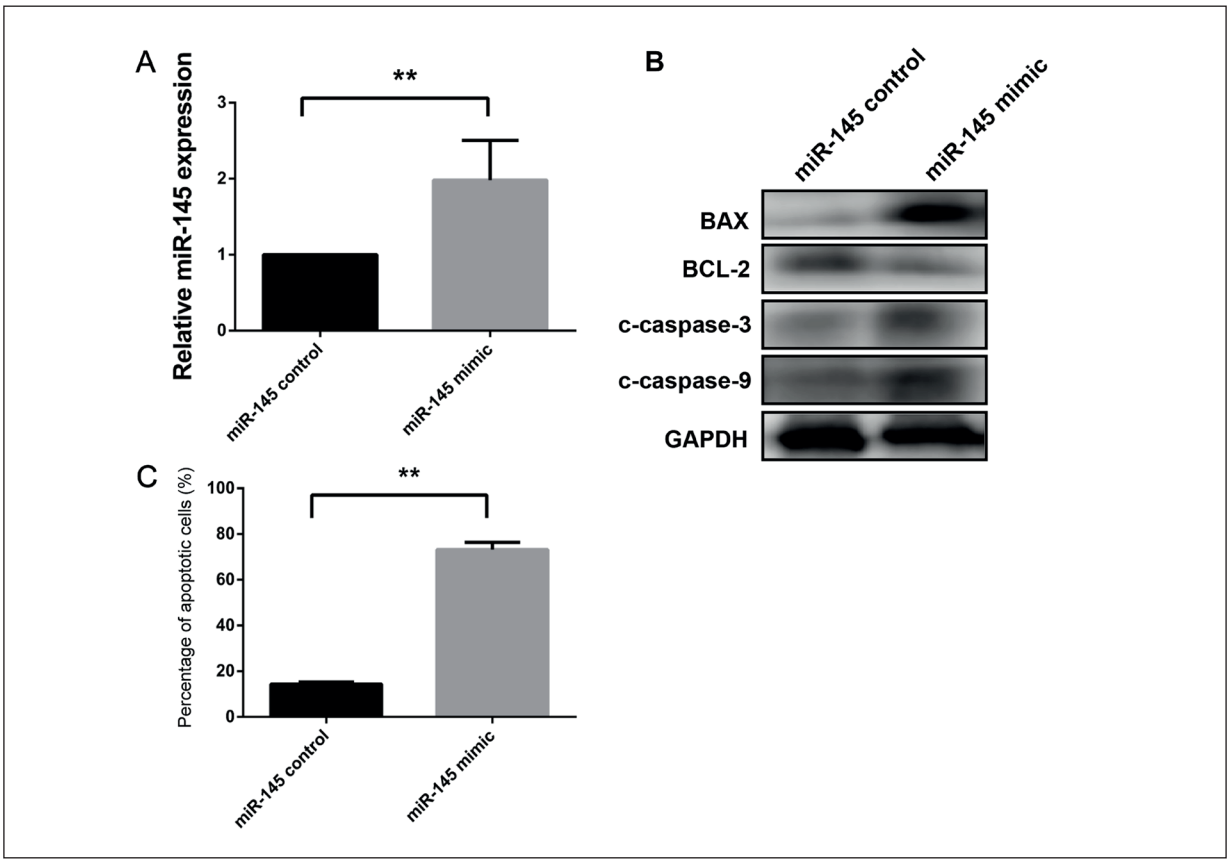

Figure 3. Influence of miR-145-5p overexpression on H9c2 cell apoptosis. **A,** Expression level of miR-145-5p: After transfection with miR-145-5p mimic and control, the expression level of miR-145-5p in H9c2 cells in miR-145-5p mimic group is notably higher that in miR-145-5p control group ($p<0.05$). **B,** Results of Western blotting: compared with those in miR-145-5p control group, Caspase-3 and Caspase-9 are activated, the expression of Bcl-2 is reduced, and the expression Bax is elevated in miR-145-5p mimic group. **C,** and **D,** Flow cytometry results: the apoptosis is remarkably enhanced in miR-145-5p mimic group compared with that in miR-145-5p control group ($p<0.01$).

matory cell infiltration observed in foci. This suggested a certain correlation between miR-145 and cell apoptosis. Subsequently, H9c2 cells were subjected to hypoxia treatment to simulate MI environment. After hypoxia treatment for 12 h, it was discovered that the expression level of miR-145 in Hypoxia group was substantially higher than that in Control group ($p<0.05$). The apoptosis in hypoxia environment was detected as well. The results showed that the apoptosis remarkably increased, consistently with the pathological phenomenon in infarcted tissues. In other words, due to frequent ischemia and hypoxia, massive myocardial cell death can be observed in foci in MI patients. Such death is pathological, and the apoptosis of myocardial cells is not reversed. After upregulating the expression level by transfection of miR-145-5p mimic into H9c2 cells, it was found that after hypoxia treatment, the expression of pro-apoptosis protein Bax was notably elevated, and the content of anti-apoptosis protein Bcl-2 was remarkably reduced in miR-145-5p mimic group. Flow cytometry manifested that the proportion of apoptotic cells was evidently elevated as well. This suggested that the apoptosis of infarcted myocardial cells was associated with the expression level of miR-145-5p in cells. Moreover, miR-145-5p could induce the apoptosis of myocardial cells, which might be reversed by reducing the content of miR-145-5p. The PI3K/Akt pathway has been found extensively involved in cellular physiological processes, especially cell proliferation and apoptosis[21]. Bcl-2 protein is an important downstream molecular signal of the PI3K/Akt pathway, which can resist cell apoptosis. Inactivation of the PI3K/Akt pathway can trigger the recruitment of Bcl-2 protein and

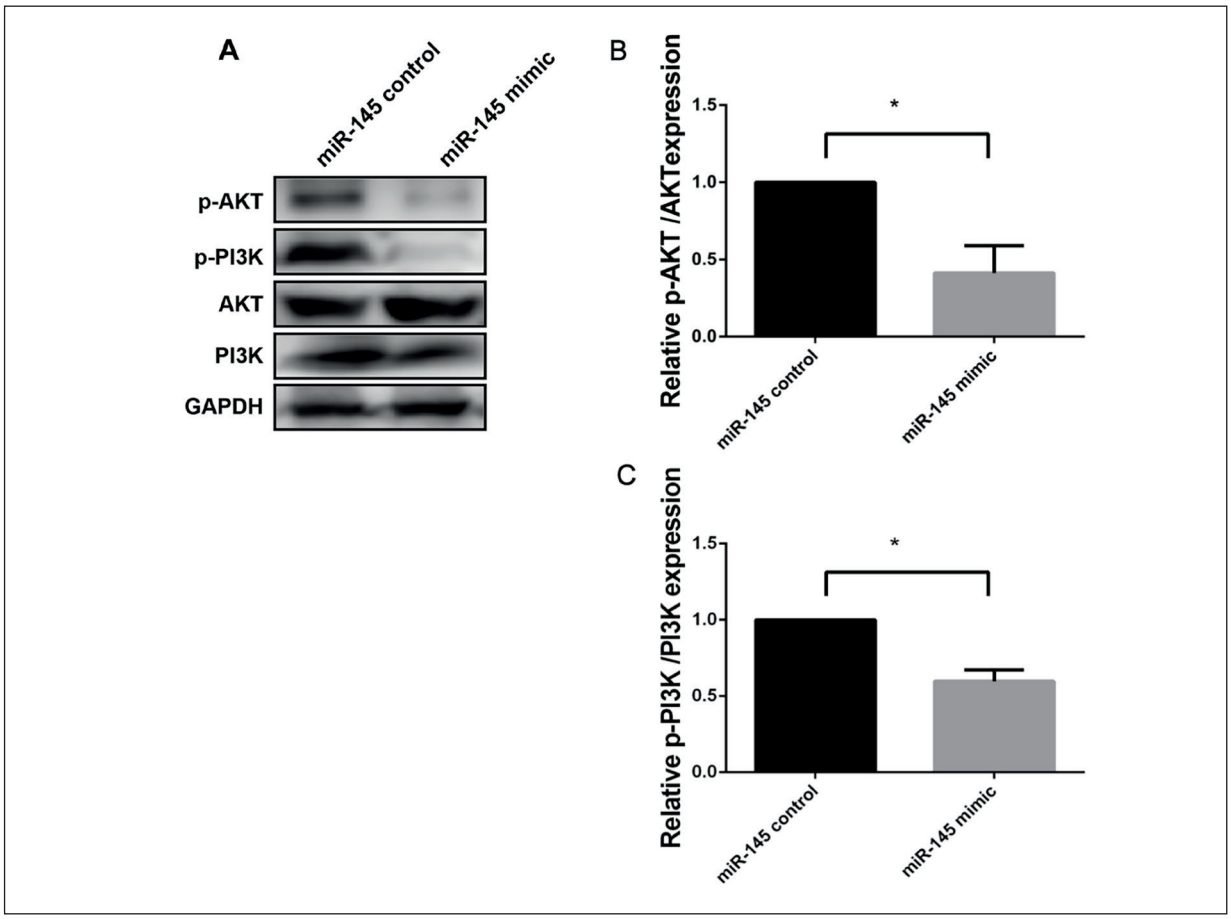

Figure 4. The pro-apoptosis effect of miR-145-5p is associated with the inactivation of the PI3K/Akt pathway. **A,** Compared with those in miR-145-5p control group, the expression levels of p-Akt and p-PI3K are lowered in miR-145-5p mimic group. **B,** and **C,** P-Akt/Akt and p-PI3K/PI3K decline in miR-145-5p mimic group, implying that once miR-145-5p is raised, the activity of the PI3K/Akt pathway is suppressed ($p<0.05$).

de-phosphorylation of Bax, thereby promoting cell apoptosis[22]. Additionally, the PI3K/Akt/Bcl-2 signaling pathway is implicated in tumor formation, osteoarthritis, cardiovascular disease, and other diseases[23,24]. In the present study, declined expression levels of p-Akt and p-PI3K were observed in miR-145-5p mimic group. In other words, the increase in miR-145-5p expression in myocardial cells can enhance the inhibition on p-PI3K and p-Akt. Ultimately, this can inactivate the proteins with important functions in the PI3K/Akt pathway and this pathway itself, and accelerate cell apoptosis. In summary, the results of the present study corroborate that miR-145-5p can affect myocardial cell apoptosis by inactivating the PI3K/Akt signaling pathway. However, we failed to verify whether miR-145-5p binds to mRNAs to affect the PI3K/Akt pathway. In subsequent research, the downstream target genes will continue to be screened out using biological prediction software. Meanwhile, the regulatory mode between miR-145-5p and target genes will be determined through researches so as to understand how miR-145-5p exerts a pro-apoptosis effect in MI more clearly.

Conclusions

Briefly, the novelty of this study was that miR-145-5p inactivates the PI3K/Akt pathway to promote the apoptosis of MI cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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