European Review for Medical and Pharmacological Sciences 2020; 24: 12896-12903

MiR-506 alleviates myocardial ischemia-reperfusion injury *via* targeting PI3K/AKT

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of micro ribonucleic acid (miR)-506 and phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT) pathway on myocardial ischemia-reperfusion injury (MIRI) in rats.

MATERIALS AND METHODS: A total of 90 healthy rats weighing 260-300 g were selected as research subjects, and divided into three groups, including: Control group (n=30), IR group (n=30), and miRNA treatment group (IR + miR-506 group, n=30). The model was successfully established via threading the coronary artery. The structural differences in myocardial tissues were observed via hematoxylin-eosin (HE) staining in each group. The mRNA expressions of miR-506 and PI3K in myocardial tissues were detected using fluorescence quantitative Polymerase Chain Reaction (qPCR). Meanwhile, AKT protein phosphorylation activity in myocardial tissues was detected as well. The apoptosis of myocardial tissues was detected via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. In addition, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in myocardial tissues were compared in each group.

RESULTS: In Control group, no structural abnormalities were found in myocardial tissues, and no inflammatory cells were observed. In IR group, myocardial tissues were arranged disorderly, and inflammatory cell infiltration was found. In IR + miR-506 group, myocardial tissue lesions were milder than those in the IR group. qPCR results indicated that the mRNA expressions of miR-506 and PI3K in myocardial tissues were statistically different among groups ($p<0.05$), with the lowest in the IR group. The expression of miR-506 was evidently higher in IR + miR-506 group than that in the Control group ($p<0.05$). However, the mRNA expression of PI3K was significantly higher in the Control group than IR + miR-506 group ($p<0.05$). There was a significant positive correlation between the expressions of miR-506 and PI3K in each group ($p<0.05$). The phosphorylation activity of AKT protein in IR + miR-506 group was markedly higher than the other two groups ($p<0.05$). In addition, TUNEL staining demonstrated that the apoptosis rate in Control group, IR group and IR + miR-506 group was only 1.3%, 20.3%, and 9.8%, respectively. SOD activity was remarkably stronger in the Control group (62.7 U/mg pro) than the other two groups ($p<0.05$). In addition, MDA content was remarkably higher in IR group (0.747 nmol/mg pro) than that in the other two groups ($p<0.05$).

CONCLUSIONS: MiR-506 is associated with myocardial injury in rats, which can alleviate myocardial injury through the PI3K/AKT signaling pathway.

Key Words:
MiR-506, PI3K/AKT pathway, Ischemia-reperfusion, Myocardial injury.

Introduction

With the constant improvement of people's living standards, the means to resist diseases have been greatly improved. However, the prevalence rate of high-risk diseases becomes increasingly higher[1]. Myocardial infarction is a kind of disease with high mortality rate, whose morbidity rate rises constantly around the world. Over the past decades, myocardial infarction seriously threatens people's normal lives. Therefore, its related treatment and theoretical studies have attracted widespread attention[2,3]. In clinic, the main treatment for myocardial infarction is drug therapy and surgery[4,5]. Although myocardial ischemia-reperfusion has a certain therapeutic effect in the clinical treatment of myocardial infarction, its success rate is still relatively poor. Meanwhile, the opposite effect can be observed in many cases. As a result, myocardial tissue and cell injury becomes worse, leading to the therapeutic effect far from satisfactory[6,7].

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With the development of modern molecular biology in recent years, the regulatory role of micro ribonucleic acids (miRNAs) in the pathological process of diseases has been gradually revealed[8]. In the treatment of myocardial infarction, the upregulation or downregulation of miRNAs is closely correlated with many functions and behaviors of myocardial cells, such as programmed death and metabolism[9]. In addition, isoproterenol regulates the biological process of cardiomyocyte hypertrophy mainly through the regulatory effect of miRNAs on target genes[10]. Therefore, it is noteworthy that the regulatory role of miRNAs in the treatment of myocardial infarction is mainly realized by affecting specific metabolic pathways. However, the influencing mechanism varies from miRNAs to miRNAs.

In this study, under different treatment conditions, the structure of myocardial tissues was detected and compared. The mRNA expressions of miR-506 and phosphatidylinositol 3-hydroxy kinase (PI3K) in myocardial tissues were analyzed as well. The protein kinase B (AKT) protein phosphorylation activity was detected. Moreover, the apoptosis of myocardial tissues was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Our research aimed to systematically clarify the effects of miR-506 and PI3K/AKT pathway on myocardial ischemia-reperfusion injury (MIRI) in rats.

Materials and Methods

Animals and Reagents

Healthy rats fed in The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University in 2018 were selected as research subjects and protected from other diseases. At 3 d before the experiment, 90 healthy rats of weighing 260-300 g were divided into three groups, including: Control group (n=30), IR group (n=30), and miRNA treatment group (IR + miR-506 group, n=30). The superoxide dismutase (SOD) kit (Solarbio, Beijing, China, BC0107) and malondialdehyde (MDA) kit (YX-C-A401) were purchased from Guizhou WLJL Biological Co., Ltd. (Guiyang, China). Image J software was used for analysis of protein gray level. This study was approved by the Animal Ethics Committee of The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University Animal Center.

Modeling and Sample Preparation

Rats in each group were first fixed, and the treatment site was sterilized with 75% alcohol. After the chest was cut open with a scalpel, the coronary artery was only carefully threaded to prevent tissue damage. In IR group, after ischemia for 20 min, the coronary artery was perfused with blood at the same concentration for 80 min. In IR + miR-506 group, miR-506 (80 mg/g) was injected *via* the caudal vein, followed by ischemia-reperfusion. During the operation, the environment must be strictly disinfected to avoid contamination. After modeling, the abdomen of rats in each group was sterilized with 75% alcohol to prevent bacterial contamination. After eligible disinfection, the heart was carefully taken with tweezers to avoid physical damage. Next, they were immediately rinsed with normal saline to remove the blood on the surface, followed by storage at -80ºC for use.

Detection of Structural Changes in Myocardial Tissues in Each Group Via Hematoxylin-Eosin (HE) Staining

Collected tissue samples were taken from liquid nitrogen and fixed with formalin to prevent cell autolysis after death. 24 h later, formalin was washed away with running water for a certain period of time, followed by tissue dehydration with alcohol at different concentrations for 2 h per gradient. Paraffin blocks were boiled in an electric furnace, and myocardial tissues were then embedded in the paraffin prepared. After paraffin solidification, the tissues were sliced into 6 μm-thick sections, fixed on a glass slide, and dried in a drying machine (OTS, MedChem Express, Monmouth Junction, NJ, USA). The structural changes in myocardial tissues of rats were finally observed and photographed under an optical microscope (EVO MA 15/LS 15, Zeiss, Oberkochen, Germany).

Determination of mRNA Expressions of MiR-506 and PI3K in Myocardial Tissues in Each Group

Total RNA was extracted from myocardial tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity of extracted RNA was detected using an ultraviolet spectrophotometer (AA63/UV1800, Shanghai, China). Next, RNA with eligible purity was subjected to reverse transcription (RT) as follows: 10 μL of buffer, 1 μL of primers, 2 μL of RNA template and 1 μL of reverse transcriptase was added in Eppendorf

(EP; Hamburg, Germany) tubes, with diethyl pyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China) added to make the total volume of 20 μL. RT conditions were as follows: reaction at 42°C for 60 min and heating at 90°C for 10 min. After that, qPCR was performed. The reaction system included 10 μL of fluorescent dye, 0.1 μL of forward primers (10 μM), 0.1 μL of reverse primers (10 μM) and 1 μL of cDNA, and dH_2O was added until the total volume reached 20 μL. q-PCR conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The expressions of miR-506 and PI3K were finally determined using $2^{-\Delta\Delta Ct}$ method. U6 was used as the internal reference in the quantitative analysis of miR-506 expression, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference in the quantitative analysis of the PI3K expression. Primer sequences used in qPCR were designed by Guizhou WLJL Biological Co., Ltd. (Guiyang, China), as shown in Table I.

Determination of AKT Protein Phosphorylation Activity in Myocardial Tissues in Each Group

An appropriate number of myocardial tissues were taken using an electronic balance, and were manually ground with liquid nitrogen until they were completely smashed. Radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and protease inhibitor were added to extract total target protein (AKT protein). The concentration of extracted protein was measured using the Bradford method. Subsequently, the protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes using a membrane transfer machine (SDE102). After sealing at room temperature for 30 min, the membranes were incubated with primary antibodies of p-AKT, AKT, and GAPDH at 4°C overnight. On the next day, the membranes were incubated again with horseradish peroxidase-labeled rabbit anti-mouse secondary antibodies, followed by washing for 3 times. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) method. Finally, AKT protein phosphorylation activity was calculated using the band gray value.

Detection of Apoptosis of Myocardial Tissues Using TUNEL Staining Under Different Treatment Conditions

Collected tissue samples were taken from liquid nitrogen and fixed with formalin. Then, the tissues were sliced into 6 μm-thick sections and air dried. The apoptosis was detected strictly according to the instructions of TUNEL staining kit (Sigma-Aldrich, St. Louis, MO, USA). Under a high-power microscope, the total number of cells and the number of apoptotic cells were counted in 6 non-repeated fields of view randomly selected in each section. Apoptosis rate was finally calculated as follows: apoptosis rate (%) = number of apoptotic cells/total number of cells × 100%.

Determination of SOD Activity and MDA Content in Myocardial Tissues Under Different Treatment Conditions

The samples in each group were taken, homogenized, and centrifuged. Subsequently, the supernatant was collected. SOD activity and MDA content were determined according to the instructions of relevant kits. Absorbance was finally measured using a spectrophotometer.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis. The mRNA

Table I. Primer sequences in fluorescence qPCR.

Primer	Sequence
PI3K-F	5'CAAGGAAAAATGTTCTATTGAAGAA3'
PI3K-R	5'TGTGCCTGTCACCTATAACCAAGA3'
GAPDH (internal reference)-F	5'GGAGATTACTGCCCTGGCTCCTA3'
GAPDH (internal reference)-R	5'GACTCATCGTACTCCTGCTTGCTG3'
miR-506-F	5'TCTCCCAACCCTTGTACCAGTG3'
miR-506-R	5'TGGTGTCGTGGAGTCG3'
U6 (internal reference)-F	5'CTCCGTCATGTGCTGTGACTG3'
U6 (internal reference)-R	5'CCAGGGCAGCTACGGTTTC3'

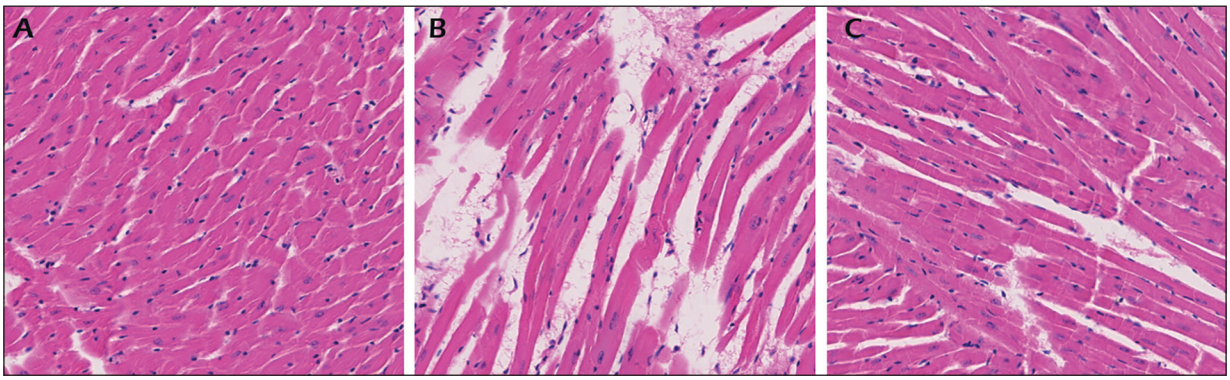

Figure 1. Structural changes in myocardial tissues *via* HE staining in each group. (magnification: 200×). **A,** Control group. **B,** IR group. **C,** IR + miR-506 group.

expression, AKT protein phosphorylation activity and proportion of apoptotic cells were expressed as mean ± standard deviation (`χ±s). The differences in indexes among the three groups were compared using univariate analysis. The correlation between the expressions of miR-506 and PI3K in myocardial tissues in each group was analyzed using Pearson correlation analysis (α=0.05). $p<0.05$ was considered statistically significant.

Results

Structural Changes in Myocardial Tissues in Each Group

The structural changes in myocardial tissues were observed in each group. In the Control group, no structural abnormalities were found in myocardial tissues, and no inflammatory cells were observed (Figure 1A). In the IR group, the myocardial tissues were disorderly arranged, with inflammatory cell infiltration (Figure 1B). In IR + miR-506 group, the myocardial tissue lesions were milder than those in IR group. Meanwhile, no evident structural disorder or necrosis of myocardial tissues was observed (Figure 1C).

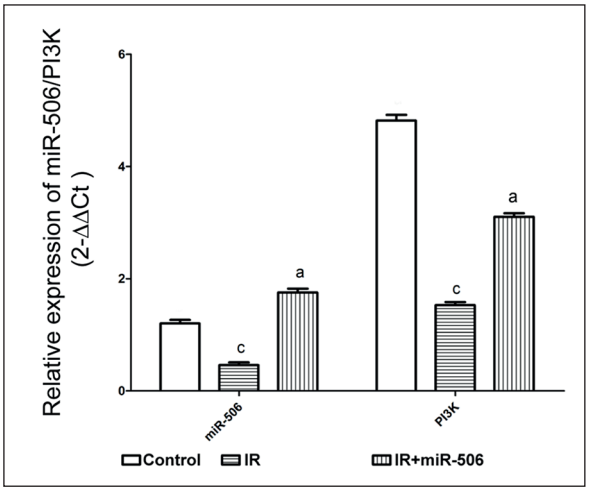

Figure 2. Expressions of miR-506 and PI3K mRNA in myocardial tissues in each group. c: There is a significant difference compared with Control group ($p<0.05$). a: There is a significant difference compared with IR group ($p<0.05$).

Expressions of MiR-506 and PI3K mRNA in Myocardial Tissues in Each Group

qPCR (Figure 2) results demonstrated that the mRNA expressions of miR-506 and PI3K in myocardial tissues exhibited evident differences among groups ($p<0.05$). In IR group, the expressions of miR-506 (0.47) and PI3K mRNA (1.54) were significantly lower than those in the Control group and IR + miR-506 group ($p<0.05$). The expression of miR-506 in myocardial tissues was remarkably higher in IR + miR-506 group (1.74) than the Control group (1.21) ($p<0.05$). However, the expression of PI3K mRNA was remarkably higher in the Control group (4.81) than that in IR + miR-506 group (3.11) ($p<0.05$). There was a significant positive correlation between the expressions of miR-506 and PI3K mRNA in each group ($p<0.05$). In addition, the correlation coefficient in the Control group, IR group and IR + miR-506 group was 0.743, 0.612, and 0.677, respectively (Table II).

AKT Protein Phosphorylation Activity in Myocardial Tissues

As shown in Figure 3, AKT protein phosphorylation activity was markedly higher in IR + miR-506 group (0.81) than the other two groups ($p<0.05$), followed by Control group (0.53) and IR group (0.19) ($p<0.05$).

Table II. Correlation between expressions of miR-506 and PI3K mRNA in myocardial tissues in each group.

	Control group	IR group	IR + miR-506 group
Pearson correlation coefficient	0.743	0.612	0.677
Significance level	$p < 0.05$	$p < 0.05$	$p < 0.05$

Apoptosis Detected Under Different Treatment Conditions

TUNEL staining indicated that the apoptosis of myocardial tissues had evident differences among the three groups ($p<0.05$, Figure 4A). The apoptosis of myocardial tissues was the most severe in IR group, followed by IR + miR-506 group and Control group. According to the statistical results (Figure 4B), the proportion of apoptotic cells in IR group (34.2%) was markedly higher than that in IR + miR-506 group (19.6%), with the lowest in Control group (5.3%) ($p<0.05$).

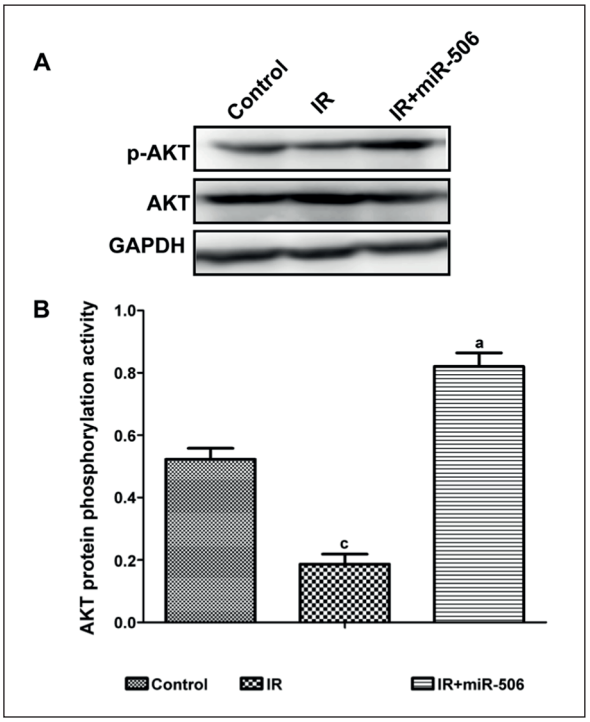

Figure 3. AKT protein phosphorylation activity in myocardial tissues in each group. **A,** Bands of AKT protein phosphorylation activity in myocardial tissues in each group. **B,** Comparison of AKT protein phosphorylation activity in myocardial tissues in each group. c: There is a significant difference compared with Control group ($p<0.05$). a: There is a significant difference compared with IR group ($p<0.05$).

SOD Activity and MDA Content in Myocardial Tissues Under Different Treatment Conditions

SOD activity in myocardial tissues was remarkably stronger in the Control group (62.7 U/mg pro) than that in IR + miR-506 group (43.1 U/mg pro), with the weakest in IR group (29.5 U/mg pro) ($p<0.05$, Figure 5A). Besides, statistically significant differences were observed in MDA content among the three groups (Figure 5B). The results indicated that MDA content was the highest in IR group (0.747 nmol/mg pro), followed by IR + miR-506 group (0.382 nmol/mg pro) and Control group (0.186 nmol/mg pro) ($p<0.05$).

Discussion

Myocardial infarction is a common cardiovascular disease, whose morbidity rate is increasing worldwide. Statistics have shown that its incidence also shows a gradually younger trend[11,12]. Myocardial infarction is mainly treated with drug therapy, followed by surgery. Despite good efficacy, the success rate of surgery is relatively low[13]. Currently, the therapeutic methods of myocardial infarction have attracted widespread attention from many international researchers. However, those methods with excellent efficacy and high success rate are still at the exploratory stage[14,15]. Myocardial infarction often leads to the dysfunction of myocardial tissues. In the present study, no structural abnormalities were found in myocardial tissues, and no inflammatory cells were observed in the Control group. In IR group, myocardial tissues were arranged disorderly, and there was inflammatory cell infiltration. In IR + miR-506 group, myocardial tissue lesions were milder than those in the IR group. The above findings were similar to the research results of Glatz et al[16]. In addition, the apoptosis rate of myocardial tissues was compared *via* TUNEL staining. The results found that the apoptosis rate was the highest in IR group, followed by IR + miR-506 group and Control group. Our findings indicated once again that miRNA played a positive role in inhibiting myocardial injury.

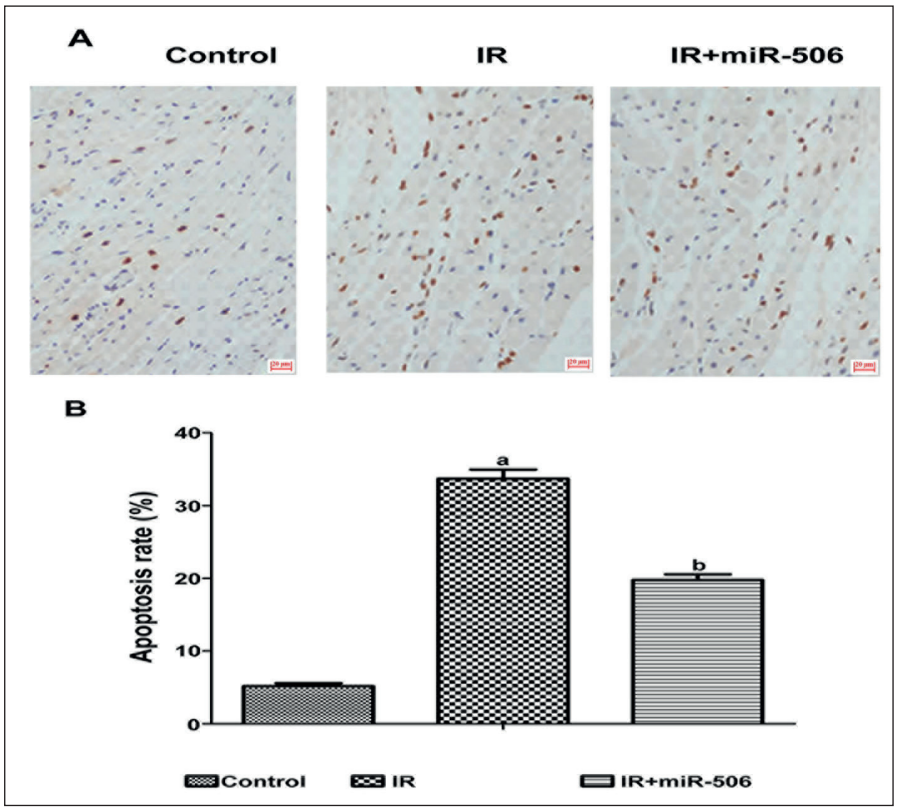

Figure 4. Apoptosis of myocardial tissues under different treatment conditions. **A,** Apoptosis of myocardial tissues (magnification: 200×). **B,** Comparison of apoptosis rate of myocardial tissues. a: There is a significant difference compared with Control group (p<0.05). b: There is a significant difference compared with IR group (p<0.05).

Recently, miRNAs have been widely explored in the treatment of myocardial infarction. In 2016, Zhou et al[17] found that miRNAs could protect against tissue fibrosis after myocardial infarction by affecting the GATA pathway. Their findings undoubtedly laid a solid foundation for the treatment of muscle fibrosis. In addition, Meder et al[18] have found that the expressions of miR-30 and miR-145 are significantly correlated with the infarction area of myocardial tissues. Therefore, they speculate

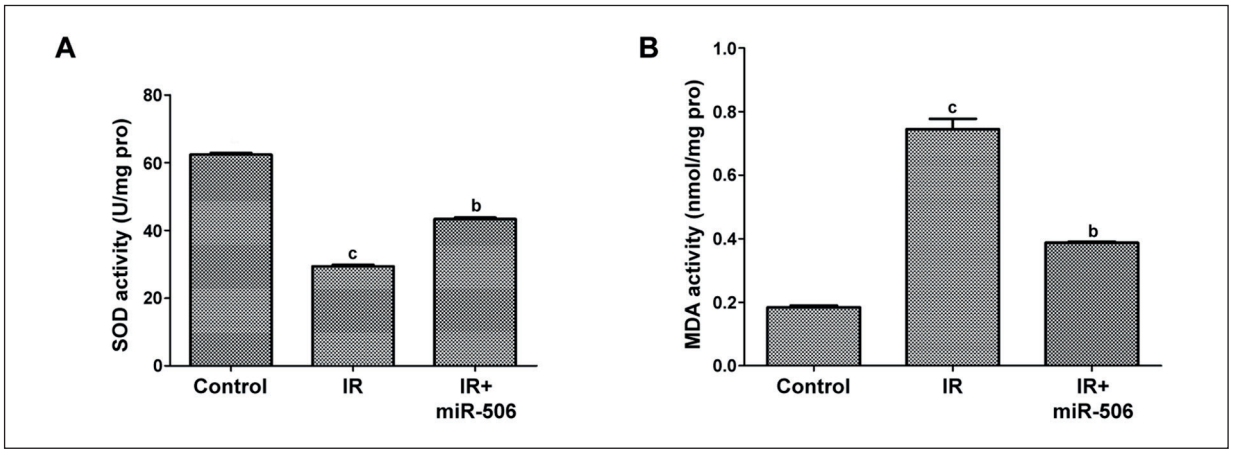

Figure 5. SOD activity and MDA content in myocardial tissues under different treatment conditions. **A,** Comparison of SOD activity in myocardial tissues. **B,** Comparison of MDA content in myocardial tissues. c: There is a significant difference compared with Control group (p<0.05). b: There is a significant difference compared with IR group (p<0.05).

that miRNAs, especially those from peripheral blood, are likely to act as new markers in the treatment of cardiovascular diseases. In this study, the expressions of miR-506 and PI3K mRNA in myocardial tissues were compared using fluorescence qPCR. The results manifested that the expressions of miR-506 and PI3K mRNA in myocardial tissues had evident differences among groups ($p<0.05$), with the lowest expressions observed in the IR group. The expression of miR-506 was evidently higher in IR + miR-506 group than that in the Control group. However, the expression of PI3K mRNA was evidently higher in the Control group than that in IR + miR-506 group. The correlation analysis indicated that there was a significant positive correlation between the expressions of miR-506 and PI3K mRNA in each group. Besides, AKT protein phosphorylation activity in myocardial tissues was detected in each group. It was observed that AKT protein phosphorylation activity was the highest in IR + miR-506 group ($p<0.05$), followed by Control group and IR + miR-506 group. The above findings were consistent with the research results of Lv et al[19] who showed that the effect of miRNAs on myocardial tissues could be realized through many metabolic pathways. The activity of AKT, as the main regulator of PI3K, has been found directly related to myocardial injury. In this study, we found that SOD activity was remarkably stronger in the Control group than the other two groups. Meanwhile, MDA content was remarkably higher in the IR group than the other two groups ($p<0.05$). The above results also reflected the effect of miR-506 on myocardial tissues of rats from another aspect. In conclusion, the research on the effect of miRNAs on myocardial tissues has just begun. The exploration of more pathways will offer effective means for the treatment of myocardial infarction[20].

Conclusions

In summary, miR-506 is associated with myocardial injury in rats, which can also alleviate myocardial injury through the PI3K/AKT signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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