Effect of miR-21/TLR4/NF-κB pathway on myocardial apoptosis in rats with myocardial ischemia-reperfusion


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Abstract. – OBJECTIVE: The aim of the study was to investigate the influences of micro ribonucleic acid (miR)-21 and downstream Toll-like receptor 4 (TLR4)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway on myocardial apoptosis induced by myocardial ischemia-reperfusion (I/R) injury in rats.

MATERIALS AND METHODS: Recombinant adeno-associated virus rAAV9-ZsGreen-pre-miR-21 and blank control virus were constructed. A total of 48 Sprague-Dawley (SD) rats were randomly divided into S1 group (open chest only), S2 group (transfection with blank virus + open chest), I/R1 group (transfection with blank virus + 6 d of myocardial I/R), and I/R2 group (transfection with miR-21 + 6 d of myocardial I/R). The cardiac function and myocardial infarct size of rats were evaluated in each group. Quantitative Polymerase Chain Reaction (qPCR) was applied to measure the expression level of miR-21 in the myocardium. The level of myocardial apoptosis in each group was detected through terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining. Western blotting was performed to determine the protein expression levels of B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax), Caspase-3, TLR4, and NF-κB in the myocardium. The content of interleukin-6 (IL-6) and IL-10 was measured using enzyme-linked immunosorbent assay (ELISA) kits.

RESULTS: The cardiac function of rats in I/R1 and I/R2 groups was significantly lower than that in S1 and S2 groups ($p<0.01$). Rats in I/R2 group had better cardiac function than those in I/R1 group ($p<0.01$). In I/R1 group, the level of myocardial apoptosis of rats was overtly increased compared with that in S1, S2, and I/R2 groups ($p<0.01$), while the expression level of miR-21 in myocardium was evidently lower than that in S1, S2, and I/R2 groups ($p<0.01$). Compared with S1, S2, and I/R2 groups, I/R1 group had markedly decreased Bcl-2/Bax expression level and IL-10 content and overtly elevated expression levels of Caspase-3, p-TLR4, p-NF-κB, and IL-6 content in the myocardium ($p<0.01$).

CONCLUSIONS: Myocardial I/R injury in rats leads to decreased expression of miR-21. The overexpression of miR-21 is able to effectively inhibit the TLR4/NF-κB pathway and reduce the level of myocardial apoptosis of rats and the release of inflammatory factors.

Key Words: MiR-21, Myocardial I/R, Apoptosis, TLR4/NF-κB pathway.

Introduction

Ischemia-reperfusion (I/R) injury refers to the severer injury to the body due to the reperfusion of blood after a period of ischemia. Some researchers$^{1,2}$ believe that the injury caused by reperfusion is much severer than that because of transient ischemia, so I/R injury has gradually become an urgent problem in clinical research. Moreover, myocardial I/R leads to further aggravated damage to ultrastructure, function, and metabolism of the myocardium, leading to irreversible pathological processes and thereby resulting in hypotension, arrhythmia, shock or even sudden death of patients$^{3,4}$.

Yao et al$^5$ showed that myocardial ischemia leads to inflammatory responses, in which the activation of complements, infiltration of neutrophils and production of reactive oxygen species destroy the integrity of endothelial cell structure and further produce toxic substances and aggravate the damage. Zhang et al$^6$ found that myocardial ischemia leads to decomposition of a large amount of adenosine triphosphate (ATP) in myocardial cells to produce substances (such as adenosine) that will be eluted during reperfusion, resulting in the
Effect of miR-21 on myocardial apoptosis

blocked synthesis of ATP and energy metabolic disturbance and further damaging myocardial cells. More and more attention is paid to the pathogenesis of I/R injury and related prevention and treatment measures in the medical filed, and methods preventing myocardial I/R injury are continuously studied. Ghosh et al manifested that the expression level of micro ribonucleic acid (miR)-21 is significantly reduced after I/R. Wang et al discovered that Toll-like receptor 4 (TLR4)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) plays an important role in the production of reactive oxygen species in I/R injury. However, the effect of miR-21/TLR4/NF-κB on myocardial apoptosis in rats with I/R has not been studied. In this work, a rat model of myocardial I/R injury was established, and the impact of miR-21/TLR4/NF-κB on myocardial apoptosis in rats with myocardial I/R was evaluated, providing a theoretical basis for further understanding of myocardial I/R injury.

Materials and Methods

Construction of Recombinant Adeno-Associated Virus

The recombinant adeno-associated virus was constructed using the technical service provided by Shanghai GenePharma Co., Ltd. (Shanghai, China) and with reference to the research method of Guo et al. Published miR-21 sequence was obtained, followed by design of miRNA and synthesis of pre-miR-21 fragment. Then, the synthesized pre-miR-21 fragment was subjected to annealing ligation. The plasmid pGene-si12-ccdB was digested with BsaI, and the vector plasmid was recovered and ligated with miR-21 annealing product. After transformation and identification, pAAV9-ZsGreen-pre-miR-21 was constructed, and the viral vector was introduced into 293AAV cells via calcium phosphate precipitation assay, followed by measurement of the biological titer. rAAV9-ZsGreen-pre-miR-21 was transfected into the ventricles of Sprague-Dawley (SD) rats by coronary injection. Rats in control group were injected with an equal amount of blank control virus.

Experimental Animals and Grouping

Male SD rats [purchased from Guangdong Medical Laboratory Animal Center, with the animal experiment license number: SCXK (Guangdong, China) 2015-0002] were fed in a clean animal feeding room for 7 days of environmental adaptation under the following environment: 12/12 h light/dark cycle, (22±2)°C, free to drink water and diet. Before the experiment, rats were fasted for 12 h. All experimental operations were carried out in accordance with the relevant provisions of the National Institutes of Health guide for the care and use of laboratory animals and were reviewed and approved by the Laboratory Animal Ethics Committee.

Experimental Grouping

Recombinant adeno-associated virus rAAV9-ZsGreen-pre-miR-21 and blank control virus were constructed. A total of 48 SD rats were randomly divided into SI group (n=12, open chest only) and S2 group (n=12, transfection with blank virus + open chest), I/R1 group (n=12, transfection with blank virus + open chest + anterior descending coronary artery ligation + 6 d of myocardial I/R), and I/R2 group (n=12, transfection with miR-21 + open chest + anterior descending coronary artery ligation + 6 d of myocardial I/R).

Establishment of Models of I/R Injury

After SD rats were adapted to the environment, they were anesthetized with 10% chloral hydrate and fixed in the supine position. Then, hairs were removed from the neck and chest with a depilatory cream. Next, neck skin was cut open, and neck muscles were bluntly separated to expose the trachea that was then cut open to connect a small animal ventilator (RWD Life Science, Shenzhen, China), followed by adjustment of the parameters of the ventilator. Thereafter, the left chest was cut open, and the muscles were bluntly separated. Then, tearing the pericardium to expose the heart, and the left anterior descending coronary artery was blocked using a 5-0 suture with a needle (white myocardium below inoculation position, weakened pulsation, and markedly elevated ST segment on the electrocardiogram indicated the successfully complete blocking of the anterior descending coronary artery). After 45 min of ischemia, the blood flow of the anterior descending branch was restored. After the heartbeat well, the chest was closed, the chest wall and skin were sutured, the ventilator was removed, and the neck skin was sutured and disinfected. After surgery, penicillin G (100,000 units/day) was consecutively given for 3 d to prevent infection. Rats were sacrificed at 6 d after reperfusion.
Cardiac Function Examination and Specimen Collection

Cardiac Function Examination

Rats in each group were anesthetized after 6 d of reperfusion. The ejection fraction (EF%), left ventricular systolic peak pressure (LVSP), and left ventricular end-diastolic pressure (LVEDP) of rats in each group were recorded and analyzed using a BL-420E+ biosignal collection system (Mindware, Gahanna, OH, USA) and an analysis device, so as to evaluate the cardiac function of rats in each group.

Specimen Collection

After the experiment, the rats were anesthetized, and the coronary artery was ligated again. 4 mL 4% Evans blue was injected into the heart from the apex. The non-ischemic zone was stained blue, and the ischemic zone was not stained blue. Then, the heart was cut off and rinsed with ice physiological saline. Next, excess tissues were cut off to separate the left ventricle, followed by separation of the blue and non-blue zones and weighing of the non-blue zone. Tissues in the non-blue zone were placed in 1% 3-(4,5)-dimethylthiazol-(2,5)-diphenyltetrazolium-bromide (MTT) for 20 min of incubation at 37°C. The infarct zone was deep red, and the non-infarct zone was pale. The infarct zone was isolated and weighed. The size of myocardial infarction zone was calculated according to the following formula: size of myocardial infarction zone % = myocardial weight in the infarct zone / myocardial weight in the ischemic zone ×100%. After tissues were collected, some were prepared into paraffin sections for TUNEL staining, and some were frozen in liquid nitrogen and stored in a refrigerator at -80°C for long-term storage for subsequent molecular biology research.

Determination of the Number of Apoptotic Cells Via TUNEL Staining

The paraffin sections of myocardial tissues in each group were dewaxed, treated with freshly prepared 3% hydrogen peroxide solution for 10 min, washed with phosphate-buffered saline (PBS) for 3 times, and subjected to TUNEL staining in strict accordance with the instructions of the TUNEL kit (Beyotime, Shanghai, China). After staining, sections were added with anti-fluorescence quenching liquid for sealing, and the number of apoptotic myocardial cells in the ischemic zone was observed and calculated. The TUNEL-positive cells were yellow-green fluorescence (apoptotic cells).

Detection of MiR-21 Expression in Myocardium Through qPCR

The cardiac apex of rats in each group was taken out and weighed. Then, it was added with TRIzol (Invitrogen, Carlsbad, CA, USA) at a ratio of 100 mg: 1 mL, and homogenized using an ultrasound homogenizer until there was no visible tissue fragment. After repeated shaking for 10 times, it was left to stand for 10 min, added with 200 μL chloroform and let stand for 5 min, followed by centrifugation at 12000 rpm and 4°C for 10 min. Next, the supernatant was aspirated into a new centrifuge tube, added with an equal volume of isopropanol, mixed and centrifuged at 12000 rpm and 4°C for 10 min. Thereafter, the supernatant was discarded, 75% ethanol was newly prepared and added 1 mL to the centrifuge tube, followed by repeated shaking, washing of the precipitate and centrifugation at 10,000 rpm and 4°C for 5 min. Then, the supernatant was discarded, and the tube was uncovered to dry the precipitate naturally. After completely drying, the precipitate was dissolved with 30 μL diethylpyrocarbonate (DEPC) water to obtain RNA. The ratio of absorbance at 260 and 280 nm (A260/A280) and optical density (OD) value of obtained RNA were measured, and its integrity was detected via agarose electrophoresis.

A reaction system (25 μL) was prepared strictly according to the instructions of the reverse transcription kit (Wuhan Vazyme Biotech Co., Ltd., Wuhan, China): 2 μL total RNA, 1 μL Oligo (dT) 20, 1 μL 10 mM deoxyribonucleotide triphosphate (dNTP), 3 μL 0.1M DTT, and 18 μL DEPC water were added together and reacted at 37°C for 15 min, followed by incubation at 85°C for 5 min for reverse transcription.

The qPCR reaction system was prepared, followed by amplification using a qPCR instrument. The primers were synthesized by Invitrogen Corporation, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The sequences are shown in Table I. After the completion of PCR, the baseline and threshold values were set, and the relative expression level of miR-21 was calculated according to the formula of $2^{-\Delta\Delta Ct}$ and expressed as miR-21/GAPDH.

Measurement of Related Protein Expression Via Western Blotting

Myocardial tissues were taken out, added with radioimmunoprecipitation assay (RIPA)
Effect of miR-21 on myocardial apoptosis

7931

Lyse solution (Beijing TDY Biotech Co., Ltd., Beijing, China) at a volume ratio of 1:1, 1% protease inhibitor and 1% phosphatase inhibitor, and subjected to ultrasound homogenate until no fragment of tissue was observed with naked eyes. Then, tissues were centrifuged at 12000 rpm and 4°C for 10 min, and the supernatant, namely total protein, was aspirated and quantified by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Next, protein loading buffer with the same concentration was prepared, followed by loading, electrophoresis and membrane transfer. Thereafter, 5% skim milk powder was freshly prepared for blocking for 2 h. Then, target band was cut and incubated with primary antibodies [Bcl-2 (diluted at 1:1000, CST, Danvers, MA, USA), Bax (diluted at 1:1000, CST, Danvers, MA, USA), Caspase-3 (diluted at 1:1000, CST, Danvers, MA, USA), TLR4 (diluted at 1:1000, CST, Danvers, MA, USA) and NF-κB (diluted at 1:1000, CST, Danvers, MA, USA)] and GAPDH antibodies (internal reference antibody, diluted at 1:1000, CST, Danvers, MA, USA) at 4°C overnight. After rewarming, target band was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (diluted at 1:5000, Shanghai Yinhai St. Biotechnology Co., Ltd., Shanghai, China) for 1 h and added with enhanced chemiluminescence (ECL) solution. Lastly (Thermo Fisher Scientific, Waltham, MA, USA), images were acquired by fluorescence development technique.

Determination of the Content of Inflammatory Factors

The content of interleukin-6 (IL-6) and IL-10 in myocardial tissues in each group was determined in strict accordance to the instructions of the IL-6 and IL-10 enzyme-linked immunosorbent assay (ELISA) kits (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China).

Statistical Analysis

In this research, data were expressed as mean ± standard deviation and analyzed using statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance was applied for comparison among groups. If the variance was equal, Bonferroni method was employed for pairwise comparison. If the variance was not equal, the Welch method was used for analysis. \( p < 0.05 \) suggested that the difference was statistically significant.

Results

Cardiac Function Evaluated in Each Group

The EF%, LVSP, and LVEDP of rats in each group were recorded, and the size of myocardial infarction zone was calculated. Compared with those in S1 and S2 groups, the EF% and LVSP in I/R1 and I/R2 groups were significantly decreased \((p < 0.01, p < 0.05)\) (Figure 1A, 1B), and the decreases in I/R1 group were overtly larger than those in I/R2 group \((p < 0.01)\). In I/R1 and I/R2 groups, the LVEDP and myocardial infarction size% of rats were clearly elevated compared with those in S1 and S2 groups \((p < 0.01, p < 0.05)\) (Figure 1C, 1D). Rats in I/R2 group had significantly lowered EF% and LVSP compared with those in I/R1 group \((p < 0.01)\).

Level of Rat Myocardial Apoptosis Determined in Each Group

TUNEL staining was used to detect the apoptosis level of rat myocardial cells in each group, and the results showed that the number of TUNEL-positive cells in myocardial cells in I/R1 and I/R2 groups was clearly higher than that in S1 and S2 groups \((p < 0.01, p < 0.05)\), and it was significantly reduced in I/R2 group compared with that in I/R1 group \((p < 0.01)\) (Figure 2A, 2B).

Expression Level of MiR-21 in Myocardial Cells in Each Group

The expression level of miR-21 in myocardial tissues of rats in each group was detected by qPCR. The results revealed that the expression level of miR-21 in myocardial tissues of rats in I/R1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>MiR-21</td>
<td>5’AGGCCCTCTGAACCCTAAG3’</td>
<td>5’CCAGAGGCATACAGGGACAAC3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’CTGACGGGAAGCTCCTGG3’</td>
<td>5’TCCGATGCCTTGCCTACTAC3’</td>
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group was significantly lower than that in S1, S2, and I/R2 groups \((p<0.01)\) (Figure 3).

**Expression Levels of Apoptotic Proteins in Myocardial Tissues of Rats in Each Group**

Western blotting was used to detect the expression levels of apoptosis-related proteins in myocardial cells of rats in each group (Figure 4A), and the results manifested that compared with those in S1 and S2 groups, Bcl-2/Bax in myocardial cells of rats in I/R1 and I/R2 groups was overtly decreased \((p<0.01, p<0.05)\) (Figure 4B), but Caspase-3 was significantly increased \((p<0.01, p<0.05)\) (Figure 4C). I/R2 group had clearly elevated Bcl-2/Bax \((p<0.01)\) and evidently reduced Caspase-3 \((p<0.01)\) in myocardial cells of rats in comparison with I/R1 group.

**Expression Level of TLR4/NF-κB in Myocardial Cells of Rats**

The expression levels of p-TLR4 and p-NF-κB in myocardial cells of rats were measured in each group using Western blotting, and it was found that the expression levels of p-TLR4 and p-NF-κB in myocardial cells of rats were significantly enhanced in I/R1 and I/R2 groups compared with those in S1 and S2 groups \((p<0.01)\), and the two indicators in I/R2 group were clearly lower than those in I/R1 group \((p<0.01)\) (Figure 5).

**Expression Levels of Inflammatory Factors in Myocardial Cells of Rats**

The ELISA kit was used to detect the content of inflammatory factors in myocardial cells of rats in each group. The results showed that in com-
Effect of miR-21 on myocardial apoptosis

Comparison with S1 and S2 groups, I/R1 and I/R2 groups had significantly increased level of IL-6 (\(p<0.01\)) (Figure 6A) and evidently reduced IL-10 content (\(p<0.01\)) (Figure 6B) in myocardial cells of rats. I/R2 group had significantly decreased IL-6 content and overtly increased IL-10 content in myocardial cells of rats compared with those in I/R1 group (\(p<0.01\)).

Discussion

Myocardial I/R injury refers to that the reversible damage due to insufficient supply of oxygen and energy caused by myocardial ischemia is further aggravated due to the perfusion of blood flow when ischemia is restored, which even leads to necrosis of tissue and cell structures and dysfunction of cells\(^{[0,1]}\). Jin et al\(^{[2]}\) showed that the apoptosis in I/R injury is mainly mediated by the mitochondrial pathway, i.e., I/R leads to changes in mitochondrial transmembrane potential and increased mitochondrial permeability, further mediating the apoptosis. It has been confirmed that inhibiting the apoptotic protein Bcl-2 and the pro-apoptotic protein Bax can affect mitochon-

Figure 2. Level of myocardial apoptosis in rats in each group determined via TUNEL staining. A, Micrograph, and B, Car-togram, scale bar=50 μm. The number of TUNEL-positive cells in myocardial cells in I/R1 and I/R2 groups is clearly higher than that in S1 and S2 groups, and it is evidently decreased in I/R2 group compared with that in I/R1 group, **\(p<0.01\), *\(p<0.05\) vs. S1 group, ##\(p<0.01\) vs. I/R1 group.

Figure 3. Expression level of miR-21 in myocardial cells in each group measured by qPCR. Rats in I/R1 group have distinctly lowered expression level of miR-21 in myocardial tissues in comparison with S1, S2 and I/R2 groups, **\(p<0.01\) vs. S1 group, ##\(p<0.01\) vs. I/R1 group.
drial transmembrane potential, most mitochondrial pathway-mediated apoptosis, and abnormal expressions of Bcl-2 and Bax in cells\textsuperscript{13}. This study found that the expression level of Bcl-2/Bax was significantly decreased after I/R in rats, but the expression level of apoptosis-related protein Caspase-3 was overtly increased, indicating that myocardial apoptosis is evidently increased after myocardial I/R. At the same time, this conclusion was further confirmed by TUNEL staining. The above results suggest that the apoptosis induced by myocardial I/R injury may be associated with mitochondria-mediated apoptosis pathway.

Myocardial I/R injury leads to the infiltration of a large number of inflammatory factors that can tightly adhere to vascular endothelial cells and damage the vascular endothelium\textsuperscript{14}. TLR4 is highly expressed in myocardial cells and microvascular endothelial cells and it is able to promote the expression of interferon-\(\beta\) and activate NF-\(\kappa\)B through the MyD88-dependent pathway. Under conditions such as ischemia and hypoxia, phosphorylated TLR4 is increased, and TLR4 is activated, further elevating the phosphorylation of NF-\(\kappa\)B and activating the TLR4/NF-\(\kappa\)B pathway, thereby increasing the content of inflammatory factors\textsuperscript{15,16}. Chung et al\textsuperscript{17} found that TLR4 plays a crucial role in the production of reactive oxygen species and the infiltration of neutrophils in rats with myocardial ischemia. In this investigation, the expression levels of p-TLR4 and p-NF-\(\kappa\)B in myocardium were significantly increased after 6 d of myocardial I/R in rats, which led to a clear increase in the content of inflammatory factor (IL-6) and an evident decrease in the content of inflammatory inhibitor (IL-10). The above results imply that myocardial I/R injury can increase the expression levels of p-TLR4 and p-NF-\(\kappa\)B, activate the TLR4/NF-\(\kappa\)B pathway, and aggravate the damage of inflammatory factors, thus damaging myocardial cells and further deepening the level of myocardial apoptosis.

MiR-21 is expressed in various tissues, and its expression is the highest in the heart. It is a highly specific miRNA in the heart, which can

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**Figure 4.** Expression levels of apoptotic proteins in myocardial tissues of rats in each group. **A,** Compared with those in S1 and S2 groups, Bcl-2/Bax in myocardial cells of rats in I/R1 and I/R2 groups is overtly decreased, but Caspase-3 is significantly increased. **B-C,** I/R2 group has clearly elevated Bcl-2/Bax and evidently reduced Caspase-3 in myocardial cells of rats in comparison with I/R1 group, **\(\ast\)\(p<0.01\), \(\ast\ast\)\(p<0.05\) vs. S1 group, **\(\#\#\)\(p<0.01\) vs. I/R1 group.
Effect of miR-21 on myocardial apoptosis

**Figure 5.** Expression level of TLR4/NF-κB in myocardial cells of rats in each group. *A,* I/R1 and I/R2 groups have evidently higher expression levels of p-TLR4 and p-NF-κB in myocardial cells of rats than S1 and S2 groups. *B-C,* The expression levels of p-TLR4 and p-NF-κB in myocardial cells of rats in I/R2 group are overtly lowered compared with those in I/R1 group, **p<0.01 vs. S1 group, ##p<0.01 vs. I/R1 group.

**Figure 6.** Expression levels of inflammatory factors in myocardial cells of rats in each group. *A,* In comparison with S1 and S2 groups, I/R1 and I/R2 groups have significantly increased level of IL-6 and evidently reduced IL-10 content in myocardial cells of rats. *B-C,* The content of IL-6 in myocardial cells of rats in I/R2 group is significantly lower than that in I/R1 group, but the content of IL-10 is overtly higher than that in I/R1 group, **p<0.01 vs. S1 group, ##p<0.01 vs. I/R1 group.
participate in the degradation of target gene mRNA and effectively inhibit or promote the translation of target proteins\textsuperscript{11,12}. TLR4 is a target gene of miR-21, and miR-21 acts on TLR4 to mediate apoptosis in a variety of physiological and pathological processes. Cheng et al\textsuperscript{20} found that cerebral I/R significantly reduces the expression level of miR-21, and the overexpression of miR-21 effectively decreases the release of inflammatory factors in nerve cells, benefitting the recovery of neurological function. In this study, miR-21 was transfected into the ventricle of rats via plasmid transfection, and a rat model of myocardial I/R injury was constructed. The results showed that rats with overexpressed miR-21 had effectively improved cardiac function, significantly decreased the level of myocardial apoptosis and expression levels of apoptotic proteins, and effectively suppressed the release of inflammatory factors. Besides, the overexpression of miR-21 effectively reduces the expression levels of p-TLR4 and p-NF-κB, inhibits the TLR4/NF-κB pathway, and relieves myocardial cell injury induced by myocardial I/R injury.

Conclusions

We found that myocardial I/R injury in rats leads to decreased expression of miR-21 and induces myocardial apoptosis. The overexpression of miR-21 inhibits the TLR4/NF-κB pathway and reduces the level of myocardial apoptosis in rats, the expression levels of apoptotic proteins and the release of inflammatory factors, thereby protecting myocardial cells.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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Effect of miR-21 on myocardial apoptosis


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