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MiR-328 inhibits cell apoptosis and improves cardiac function in rats with myocardial ischemia-reperfusion injury through MEK-ERK signaling pathway

H.-K. YE[1], H.-H. ZHANG[1], Z.-M. TAN[2]

[1]Department of Critical Care Medicine, Jining No. 1 People's Hospital; Affiliated Jining No. 1 People's Hospital of Jining Medical University, Jining Medical University, Jining, China
[2]Cardiology Intensive Care Unit, Jining No. 1 People's Hospital; Affiliated Jining No. 1 People's Hospital of Jining Medical University, Jining Medical University, Jining, China

Huaikuo Ye and *Haohua Zhang* contributed equally to this work

Abstract. – OBJECTIVE: To explore the influences of micro ribonucleic acid (miR)-328 on rats with myocardial ischemia-reperfusion (IR) injury through the methyl ethyl ketone (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway.

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly assigned into the sham group (n=12), model group (n=12), and miR-328 group (n=12). The model of myocardial IR injury was established by ligating the left anterior descending coronary artery, without any intervention in the model group, while 200 μL of miR-328 antagomir was intravenously injected before modeling in the miR-328 group. The activity of the serum myocardial enzymes lactate dehydrogenase (LDH) and creatine kinase-muscle/brain (CK-MB) was determined *via* ELISA to assess the cardiac function in the three groups of rats, and the mRNA expression level of miR-328 in myocardial tissues was measured through real-time fluorescence qRT-PCR in the sham group, model group, and miR-328 group. TUNEL staining was performed to detect apoptotic cells, and the levels of myocardial apoptosis-associated protein Caspase-3 and phosphorylated MEK1/2 (p-MEK1/2) and p-ERK1/2 proteins were determined using Western blotting.

RESULTS: Compared with the sham group, the model group exhibited increased activity of LDH and CK-MB, miR-328 expression level, apoptotic cells, the relative expression level of Caspase-3, and protein levels of p-MEK and p-ERK, with statistically significant differences ($p<0.05$). Besides, in comparison with the model group, miR-328 group showed a decreased activity of LDH and CK-MB, miR-328 expression level, the relative expression level of Caspase-3, and protein levels of p-MEK and p-ERK, displaying statistically significant differences ($p<0.05$).

CONCLUSIONS: MiR-328 modulates the MEK-ERK signaling pathway to inhibit cell apoptosis and improve the cardiac function in rats with myocardial IR injury.

Key Words:
MiR-328, MEK-ERK signaling pathway, Cardiac function, Myocardial ischemia-reperfusion.

Introduction

Myocardial ischemia and reperfusion (IR), a severe cardiovascular disease, is one of the leading causes of deaths worldwide[1], and ischemic heart disease is normally attributed to the insufficiency of blood flowing into the myocardium[2]. According to Zhang et al[3], myocardial IR-induced cell apoptosis aggravates the myocardial injury, and myocardial IR injury is associated with the death of myocardial cells. Myocardial apoptosis during IR injuries can result in irreversible damage to cardiac function. Extracellular signal-regulated kinases (ERKs), a member of the family of ubiquitous and conservative serine/threonine protein kinases, are involved in cell proliferation, differentiation, and apoptosis. The ERK pathway can induce the conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II and promote the generation of beclin-1 protein and autophagosomes. Some studies[4,5] have demonstrated that the myocardial IR-induced autophagy and apoptosis can be reduced through the methyl

Corresponding Author: Ziming Tan, MM; e-mail: sandy1412@sina.com

ethyl ketone (MEK)/ERK/early growth response 1 (EGR1) pathway. Micro ribonucleic acids (miRNAs), as endogenous non-coding small RNAs, can bind to the 3' untranslated region of messenger RNAs (mRNAs) to repress the transcription and translation and regulate gene expression[6]. MiRNAs are able to regulate the pathological processes of multiple heart diseases. Spannbauer et al[7] have proved that the level of miR-328 is increased in rats with myocardial IR, and that miR-328 over-expression suppresses cell proliferation and migration and regulates the apoptosis-associated signaling pathways[8].

Therefore, the rat model of myocardial IR injury was established in this investigation to explore the influences of miR-328 on the cell apoptosis and cardiac function in rats with myocardial IR injury *via* the MEK/ERK signaling pathway and elucidate the mechanism of action of miR-328 on myocardial IR injury.

Materials and Methods

Animal Experiments and Grouping

A total of 36 specific pathogen-free Sprague-Dawley rats weighing (280±20) g conformed to the feeding standards for laboratory animals, and they were adaptively fed at 5-6 rats/cage for 1 week, followed by modeling and intervention. All animal programs were based on the Guide for the Care and Use of Laboratory Animals stipulated by the National Academy Press. This study was approved by the Animal Ethics Committee of Jining Medical University Animal Center.

Main Reagents and Instruments

The main reagents were: miR-328 antagomir (Shanghai Genechem Co., Ltd., Shanghai, China), phosphorylated MEK (p-MEK) and p-ERK primary antibodies (CST), DM4000B LED microscope (Leica, Wetzlar, Germany), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit and BCA protein assay kit (Shanghai Beyotime Biotechnology, Shanghai, China), AceQ quantitative Polymerase Chain Reaction (qPCR) SYBR Green Master Mix kit and HiScript II Q RT SμperMix for qPCR (+gDNA wiper) kit (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China), fluorescence qPCR instrument (ABI 750, Applied Biosystems, Foster City, CA, USA) and Image Lab image analysis system.

Modeling and Grouping

The 36 rats were divided into the sham group, modeling group, and miR-328 group using a random number table. The model of myocardial IR injury was prepared through ligating the coronary artery in the model group and miR-328 group[9]. After fasting for 12 h prior to operation, the rats were intraperitoneally injected with 3.0% pentobarbital for anesthesia and fixed, and then, the trachea was separated and cut open. An animal respirator was connected intraoperatively to maintain mechanical ventilation with the tidal volume of 20 mL/kg and respiratory rate of 60 breaths/min. The chest capacity was opened between the 3rd and 4th lib of the left sternum to expose the heart and ligate the lower part of its left anterior descending coronary artery. Blood flow was restored after ischemia for 30 min, and blood was taken from the heart 2 h later to establish the model of myocardial IR injury. Subsequently, the rats were sacrificed, and the heart was removed and stored for later use. Before the modeling of myocardial IR injury, 200 μL of miR-328 antagomir was injected into the tail veins in the miR-328 group, and only the heart was exposed without vascular ligation in the sham group.

Determination of the Activity of Serum Myocardial Enzymes Via Enzyme-Linked Immunosorbent Assay (ELISA)

Following the completion of modeling, blood was drawn from the abdominal aorta of rats in each group. Then, the collected blood samples were centrifuged, and the serum was extracted. The activity of lactate dehydrogenase (LDH) and creatine kinase-muscle/brain (CK-MB) was determined using the kit, and the absorbance was measured through colorimetry using a spectrophotometer to calculate the content of CK-MB and LDH.

Detection of Messenger RNA (mRNA) Expression of MiR-328 Via quantitative Reverse Transcription (qRT)-PCR

The total RNAs were extracted from the myocardial tissues of rats in each group, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs), with the primer sequences as follows: miR-328: Forward: 5'-CUGGCCCUCUC-UGCCCUUCCGU-3', Reverse: 5'-GGGGGGCA-GGAGGGGCUCA-3', and U6: Forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTCACGAATTTGCGT-3'. PCR amplification was performed using a reaction system (20 μL) in an amplifier under the following condi-

tions: pre-denaturation at 94°C for 10 min, denaturation at 94°C for 15 s, and annealing at 60°C for 15 s, for 35 cycles. The primers were designed and synthesized by Fuzhou Jingrui Biotechnology (Fuzhou, China). Finally, the relative mRNA expression level of miR-328 in the myocardial tissues of rats was calculated in each group.

Determination of Apoptosis Rate of Myocardial Cells Via TUNEL Assay

All groups of rats were anesthetized through intraperitoneal injection of 5% pentobarbital sodium and perfused with 4% paraformaldehyde for fixation, followed by removal of the heart. Then, the heart was soaked, fixed overnight, routinely embedded in paraffin, and prepared into 5 μm-thick coronary sections. Following deparaffinization, dehydration in gradient alcohol, antigen retrieval in citric acid, and permeabilization working solution were added dropwise to cover the tissues, and they were incubated at room temperature for 20 min and added with protease K in drops. Then, the obtained tissues were washed with phosphate-buffered saline (PBS) for 3 times, added dropwise with TUNEL assay solution, incubated in an incubator at 37°C in the dark for 60 min, and washed using PBS for 3 times again. Finally, the sections were sealed using antifade mounting medium, and observed under a fluorescence microscope.

Detection of the Protein Expression in Myocardial Tissues Via Western Blotting

The model rats were killed to taken out the heart, and then, the proteins were extracted from the myocardial tissues. After concentration determination, the proteins were incubated with primary antibodies at 4°C overnight, and with the horseradish peroxidase (HRP)-labeled secondary antibodies at 37°C for 1 h. Next, the membranes were washed, and reacted with chemiluminescence reagent for complete development. With β-actin as an internal reference, the relative expression level of each protein was the ratio of the grayscale of the target protein bands to that of β-actin protein bands, and the grayscale was analyzed using Image-J software in 3 valid bands from each group.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 24.0 software (IBM Corp., Armonk, NY, USA) was employed for statistical analysis. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA, followed by post-hoc test (Least Significant Difference). All the data were expressed as mean ± SD (standard deviation), and $p<0.05$ suggested that the difference was statistically significant.

Results

Comparison of Myocardial Enzyme Activity Among the Three Groups

As shown in Figure 1, compared with that in the sham group, the activity of serum LDH and CK-MB in rats was enhanced in the model group, showing a statistically significant difference ($p<0.05$), and in comparison with that in the model group, the activity was weakened in miR-328 group ($p<0.05$), suggesting that miR-328 can improve the activity of myocardial enzymes in rats with myocardial IR.

MRNA Expression of MiR-328 in Rats in Each Group

Compared with the sham group, the model group exhibited an evident increase in the expression level of miR-328 ($p<0.05$), and the expression level of miR-328 in miR-328 group was substantially lower than that in the model group ($p<0.05$) (Figure 2), implying that miR-328 serves as an important molecular therapeutic target for rats with myocardial IR.

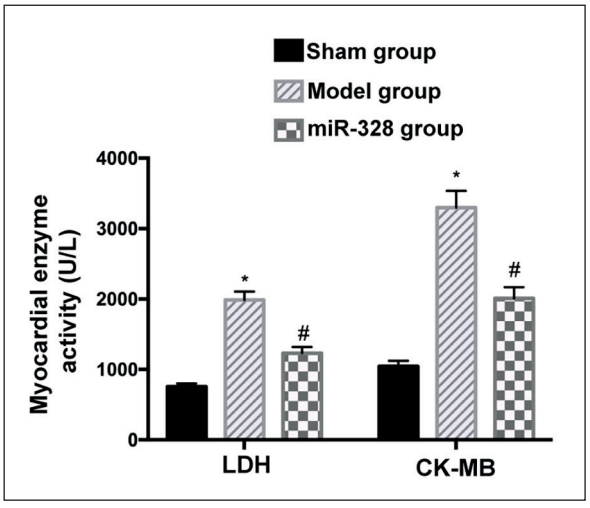

Figure 1. Comparison of myocardial enzyme activity among the three groups. Note: $p^{*}<0.05$ *vs.* sham group, and $p^{\#}<0.05$ *vs.* model group.

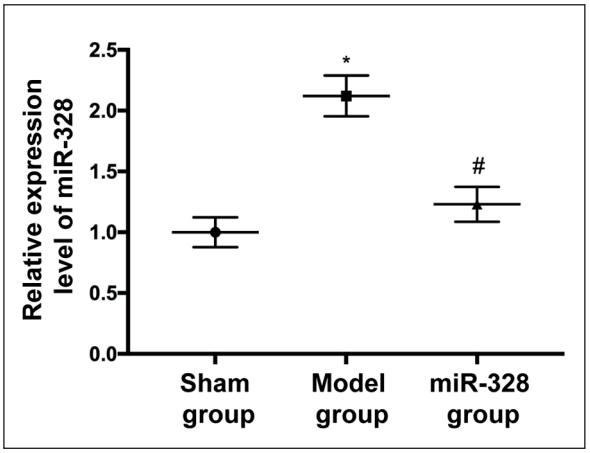

Figure 2. Relative mRNA expression level of miR-328 in each group of rats. Note: $p^{*}<0.05$ *vs.* sham group, and $p^{\#}<0.05$ *vs.* model group.

Comparison of Myocardial Apoptosis Rate Among all Groups of Rats

The cell apoptosis rate in both model group and miR-328 group was notably higher than that in the sham group ($p<0.01$), and it was markedly lower in the miR-328 group than that in the model group ($p<0.01$) (Figures 3-4), illustrating that miR-328 can decrease the rate of myocardial apoptosis in rats with myocardial IR.

Protein Expression Level in Myocardial Tissues in all Groups of Rats

As shown in Figures 5, 6, and 7, compared with the sham group, the model group showed remarkable increases in the protein expression levels of p-MEK and p-ERK ($p<0.05$) and Caspase-3, and in comparison with the model group, miR-328 group had lowered protein expression levels of p-MEK and p-ERK ($p<0.05$) and a decreased expression level of Caspase-3, suggesting that miR-328 can regulate the MEK-ERK pathway in rats with myocardial IR to inhibit cell apoptosis.

Discussion

As one of the leading causes of sudden cardiac death, myocardial IR can cause myocardial ischemia and necrosis, induce myocardial infarction, arrhythmia and contractile dysfunction, and its morbidity and mortality rates are on the rise, with an extremely poor prognosis. Therefore, the understanding of the mechanism of myocardial IR injury has vital clinical implications for exploring more effective treatment methods. Myocardial IR is induced by the temporary decline in oxygen delivered to tissues secondary to acute arterial occlusion, followed by rapid restoration of blood flow. Such a process brings about a series of injuries that result in myocardial cell dysfunction and death, namely the tissue hypoxia-induced primary ischemic injury and the second injury mode after reperfusion, and the mitochondria-based energy supply disorder, calcium homeostasis, cell apoptosis, and autophagy play major roles in the pathogenesis. The relief of IR-induced myocardial injury has received wide attention, and the protection against myocardial IR injury is the potential treatment target[10].

MiRNAs are a class of non-coding RNAs, whose products are 20-22 nt sequences that can simultaneously regulate the expressions of several genes. MiRNAs act as small RNAs negatively regulating post-transcriptional gene expression by inhibiting mRNA translation or promoting mRNA degradation, and in the heart, they modulate reactions in IR to facilitate cell survival[11]. Some miRNAs have been detected to be involved

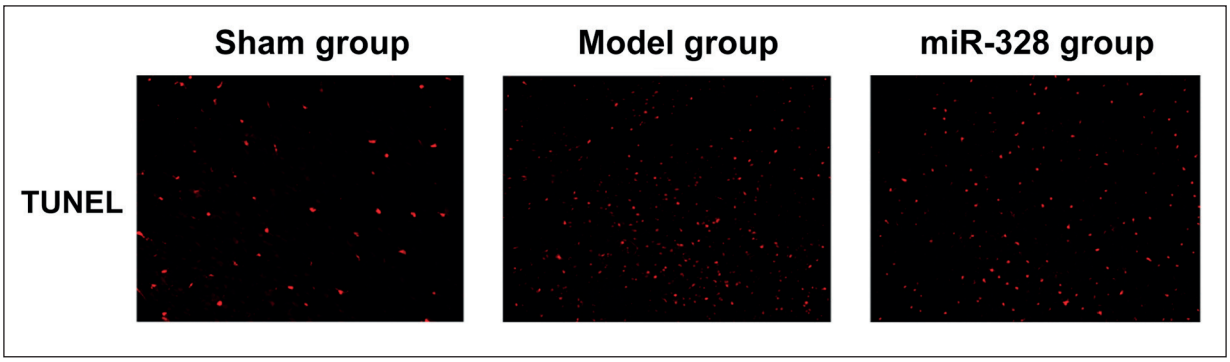

Figure 3. Comparison of myocardial apoptosis in rats among all groups (immunofluorescence ×200).

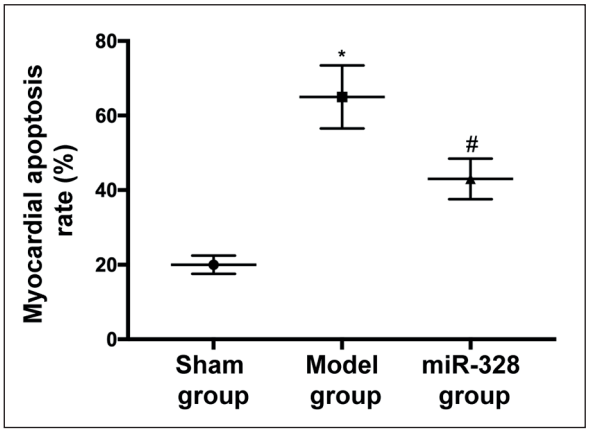

Figure 4. Comparison of myocardial apoptosis rate among all groups of rats. Note: $p^{*}<0.05$ *vs.* sham group, and $p^{\#}<0.05$ *vs.* model group.

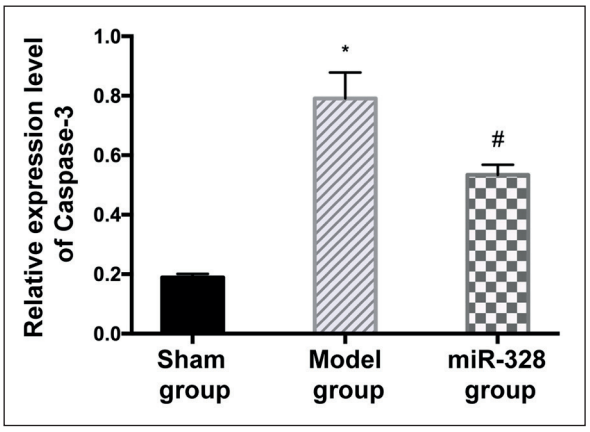

Figure 6. Comparison of relative expression level of Caspase-3 among all groups of rats.

in the regulation of myocardial ischemia[12,13]. The expression of miRNAs is complex and variable before, during and after myocardial IR. Before ischemia, there are protective miRNAs applied for local and remote pre-treatment of the myocardium and can protect the myocardium during ischemic events mainly through the mitochondrial protection mechanism and inhibition of cell apoptosis. After infarction, miRNAs can reduce cell apoptosis and inflammation, ultimately repressing fibrosis. MiRNAs play a vital role in regulating the myocardial IR injury cascades. Besides, the pretreatment with miR-155 and miR-130a before permanent coronary artery ligation can reduce infarction area and enhance left ventricle function. Thus, regulating miRNAs is a promising treatment strategy[14-16]. Li et al[17] have shown that the expression of miR-328, a miRNA, is involved in atrial fibrillation and atrial electrical remodeling and it plays a crucial role in

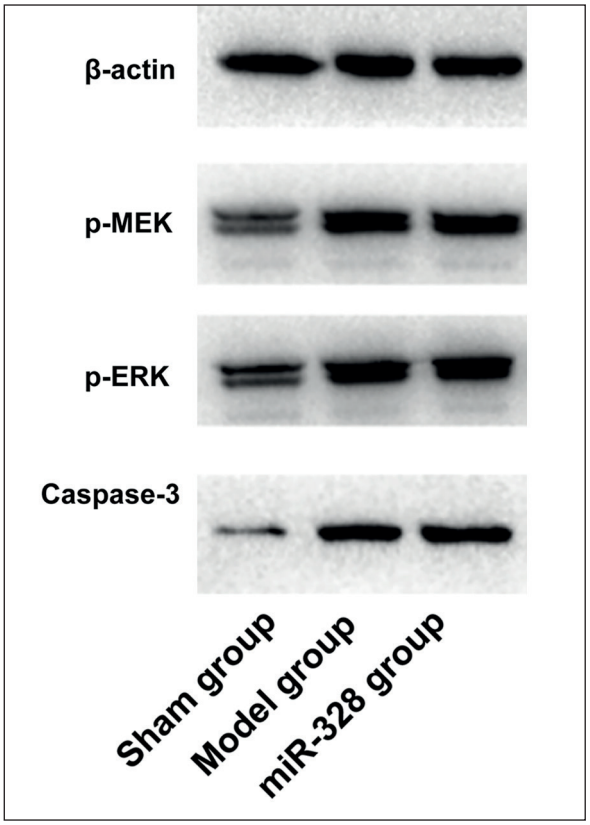

Figure 5. Comparisons of myocardial apoptosis-associated proteins among all groups of rats.

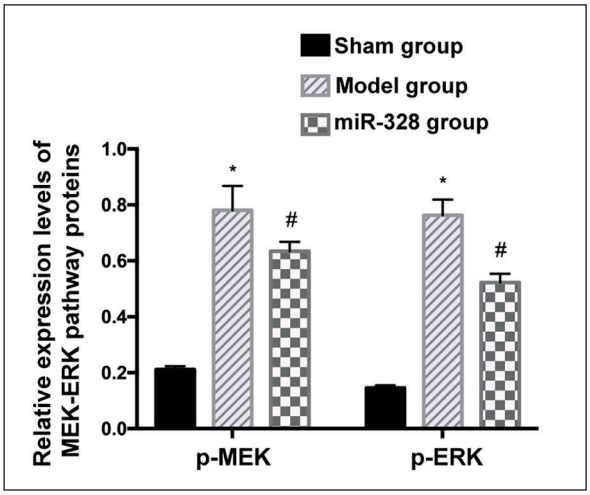

Figure 7. Comparisons of relative expression levels of MEK-ERK pathway-related proteins among all groups of rats. Note: $p^{*}<0.05$ *vs.* sham group, and $p^{\#}<0.05$ *vs.* model group.

the atria. The regulation of fibroblasts has been researched to verify whether miR-328 derived from myocardial cells promotes cardiac fibrosis. Cardiac fibrosis is characterized by the process that cardiac fibroblasts aberrantly differentiate, thereby causing the deposition of extracellular matrix in the myocardial interstitium, which is the typical pathological feature of myocardial IR. Meanwhile, cardiac fibrosis will be accompanied by a variety of cardiovascular diseases, such as myocardial infarction, cardiac hypertrophy, and atrial fibrillation. The important synergy of cardiac fibroblasts and myocardial cells helps maintaining the homeostasis in the body, and the specific miR-328 overexpression in myocardial cells triggers intensive fibrosis, thereby promoting the production of collagen and inducing fibrosis[18].

Wang et al[19] showed that the ERK-MEK signaling pathway is activated after myocardial IR, and that the expression of EGR-1 is substantially downregulated after the activation of the pathway is blocked by inhibitors, thus weakening Caspase-3 activity, reducing TUNEL-positive myocardial cells and alleviating myocardial injury. However, the novelty of our paper was that we first discovered the role of mir-328 in animal models of myocardial ischemia reperfusion injury and discussed its molecular mechanism. In the present study, miR-328 was overexpressed in rats with myocardial IR, and it was found that miR-328 activated the MEK-ERK signaling pathway to reduce myocardial apoptosis rate.

Conclusions

In summary, miR-328 inhibits cell apoptosis and improves cardiac function in rats with myocardial IR injury by regulating the MEK-ERK signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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