

Effect of exosome-carried miR-30a on myocardial apoptosis in myocardial ischemia-reperfusion injury rats through regulating autophagy

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Abstract. – OBJECTIVE: To explore the effect of exosome-carried micro-ribonucleic acid-30a (miR-30a) on myocardial apoptosis in rats with myocardial ischemia-reperfusion injury (MIRI) and its possible regulatory mechanism.

MATERIALS AND METHODS: The MIRI rat model was established *via* ligation of the left anterior descending coronary artery. A total of 30 Sprague-Dawley (SD) rats were randomly divided into Sham group, Model group, and miR-30a inhibitor group. The pathological changes in heart tissues in MIRI rats were detected *via* hematoxylin-eosin (HE) staining. The levels of serum aspartate aminotransferase (AST) and creatine phosphokinase (CPK) in MIRI rats were detected using the biochemical method. The content of serum malondialdehyde (MDA) and superoxide dismutase (SOD) was detected *via* enzyme-linked immunosorbent assay (ELISA). Moreover, the apoptosis of heart tissues in MIRI rats was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The protein levels of ULK1 and Beclin-1 were detected *via* Western blotting.

RESULTS: Compared with those in the Sham group, the pathological injury of heart tissues was severe, the levels of serum AST and CPK were increased, the content of MDA was decreased, the content of SOD was increased, the apoptotic rate of heart tissues was significantly increased, and the protein levels of ULK1 and Beclin-1 in heart tissues were also significantly increased in Model group. Compared with those in the Model group, the pathological injury of the heart tissues was alleviated, the levels of serum AST and CPK were declined, the content of MDA was increased, the content of SOD was decreased, the apoptotic rate of heart tissues, and the protein levels of ULK1 and Beclin-1 in heart tissues also significantly declined.

CONCLUSIONS: The exosome-carried miR-30a inhibitor can suppress the myocardial apoptosis in MIRI rats by reducing autophagy.

Key Words:

Exosomes, Autophagy, Myocardial ischemia-reperfusion injury, Apoptosis, MiR-30a.

Introduction

Currently, cardiovascular disease has become one of the most important diseases threatening human health, whose morbidity rate, in particular, has exceeded that of cancer in the last 2-3 decades¹. Ischemic heart disease or sudden heart attack are the most common cardiovascular disease in the world. With the development of medicine, the thrombolytic therapy and the direct percutaneous coronary intervention are dominated in the clinical treatment of myocardial infarction, which can alleviate the disease to some extent^{2,3}. However, restoring myocardial blood reperfusion may also aggravate the myocardial injury in the treatment of myocardial ischemia and infarction, which is called myocardial ischemia-reperfusion injury (MIRI)⁴. MIRI refers to the process where a large number of oxidative free radicals are produced after the recovery of blood supply in ischemic myocardium, leading to myocardial apoptosis, thus further aggravating the myocardial injury, and inducing large-area myocardial infarction and arrhythmia, etc⁵.

Therefore, the damage caused by myocardial ischemia is an important problem to be solved urgently during the treatment of MIRI, but the pathogenesis of MIRI is complex and diverse^{6,7}. Some studies have demonstrated that mitochondrial autophagy is closely related to the occurrence and the development of MIRI. Autophagy is a metabolic pathway that degrades long-

lived proteins and organelles through lysosomes, which is closely related to many physiological and pathological processes⁸. Mitochondria also release oxygen free radicals while producing energy, and the excessive accumulation of oxygen free radicals will attack mitochondrial DNA, leading to disorders of mitochondrial structure and function⁹.

The exosome is a research hotspot in the biomedical field in recent years, which is a nanometer-sized lipid microvesicle with endocytosis, about 30-200 nm in diameter and widely existing in such biological fluids as blood, urine, and saliva^{10,11}. Micro-ribonucleic acid (miRNA) carried by exosomes can regulate the gene expression, and participate in many vital activities, such as epigenetic modification, cell proliferation, differentiation and apoptosis, which plays an important role in the occurrence and development of cardiovascular diseases¹².

In this paper, therefore, the MIRI rat model was established by the ligation of the left anterior descending coronary artery to study the regulatory effect of exosome-carried miR-30a on myocardial apoptosis in MIRI rats and further explore its possible regulatory mechanism.

Materials and Methods

Reagents

Aspartate aminotransferase (AST) and creatine phosphokinase (CPK) kits, malondialdehyde (MDA) and superoxide dismutase (SOD) enzyme-linked immunosorbent assay (ELISA) kits and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit were purchased from Beyotime (Shanghai, China), 4',6-diamidino-2-phenylindole (DAPI) dye, paraformaldehyde, diaminobenzidine (DAB) dye and hematoxylin-eosin (HE) dye from Solarbio (Beijing, China), streptozotocin (STZ) from Sigma-Aldrich (St. Louis, MO, USA), ULK1, Beclin-1 and β -actin primary antibodies from CST (Danvers, MA, USA), and horse reddish peroxidase (HRP) labeled secondary antibodies from Shenyang Wanlei Biotechnology Co., Ltd., (Shenyang, China).

Instruments

The rapid mixer was purchased from Changzhou Jintan Youlian Instrument Institute (Changzhou, China), the protein electrophoresis apparatus and membrane transfer instrument from

Beijing Biopeony Co., Ltd. (Beijing, China), the fluorescence inverted microscope from Nikon (Tokyo, Japan), the microplate reader from Biobase Group (Fremont, CA, USA), and high-speed refrigerated centrifuge from Biofuge Strato (Hanau, Germany).

Rats

The male Sprague-Dawley rats weighing (220 $\pm$ 20) g (license No.: SCXK2009-0002) were purchased from the Laboratory Animal Center of Shandong University, and they were all fed with clean regular fodder and had free access to food and water under the 12/12 h light/dark cycle and temperature of (24 $\pm$ 2) $^{\circ}$ C. This study was approved by the Animal Ethics Committee of Weifang People's Hospital Animal Center.

Establishment of MIRI Rat Model by the Ligation of Left Anterior Descending Coronary Artery

The MIRI rat model was established by the ligation of the left anterior descending coronary artery. Firstly, the rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium and fixed on an operating table in a supine position. The electrode was inserted into limbs subcutaneously to monitor the condition of heart, and the ventilator was connected. An incision was made between the 3rd and 4th ribs on the left chest wall, the chest wall was bluntly separated layer by layer, and the left anterior descending coronary artery was ligated with suture, followed by reperfusion for 60 min. Reperfusion was performed when there was ST-segment elevation in electrocardiogram, and the ST-segment resolution to 1/2 and reddening of myocardium indicated the successful establishment of the MIRI rat model. Finally, the pathological changes in heart tissues in each group were detected *via* HE staining.

Determination of Serum AST and CPK Levels Using Biochemical Method

The whole blood was collected in each group and centrifuged, and the serum was retained for later experiments. AST was used to determine the degree of injury: the standards were diluted according to the instructions, and the standard curve was plotted to determine the concentration. 50 μ L of samples were added and incubated at 37 $^{\circ}$ C for 30 min. After the solution was spun dry, 50 μ L of enzyme indicator was added into each well, followed by incubation and washing.

Then, the developing solution was added, followed by incubation in a dark place for 15 min, and the stop buffer was added to terminate the reaction. Finally, the absorbance was measured at a wavelength of 450 nm using a microplate reader. The level of CPK was determined in the same way as above.

Determination of Serum MDA and SOD Content Via ELISA

The whole blood was collected in each group and centrifuged, and the serum was retained for later experiments. First, the MDA mother solution was dissolved by heating, the working solution was prepared, and the solution at a concentration of 1, 2, 5, 10, 20, and 50 μM was prepared using double distilled water to make the standard curve. The standards and samples were added according to the instructions and mixed evenly, followed by boiling water bath for 15 min. After the mixture was cooled to room temperature, it was centrifuged, and 200 μL of it was added into a 96-well plate. Finally, the absorbance was measured at a wavelength of 532 nm using the microplate reader. The content of SOD was determined in the same way as above.

Detection of Apoptosis of Heart Tissues Via TUNEL Staining

The heart tissues were taken, sliced into sections, equilibrated at room temperature, washed with PBS, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.3% Triton X-100 for 15 min. 5 μL of terminal deoxynucleotidyl transferase (TdT) solution, 45 μL of fluorescence labeling solution and 50 μL of TUNEL assay solution were added into each sample according to the instructions, followed by incubation for 1 h. Then, the sections were washed with PBS and sealed with an anti-fluorescence quencher. Finally, the staining was observed under an inverted fluorescence microscope.

Detection of ULK1 and Beclin-1 Protein Levels Via Western Blotting

The heart tissues were taken in each group and centrifuged, and the supernatant was retained. The protein was extracted using the protein lysis buffer, and the protein concentration was determined. Then the protein was loaded and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) until the sample ran to the bottom. The protein was transferred onto a polyvinylidene difluoride (PVDF) mem-

brane (Roche, Basel, Switzerland), sealed with 5% bovine serum albumin (BSA) for 1 h, washed with Tris-Buffered Saline and Tween 20 (TBST) and incubated with ULK1 and Beclin-1 primary antibodies at 4°C overnight. In the next day, the protein was incubated again with HRP-labeled secondary antibodies at room temperature for 1 h and washed with TBST. Finally, the DAB developing solution was added, and the band was analyzed using ImageJ software.

Statistical Analysis

The data in each group were expressed as mean $\pm$ standard deviation. Comparison between groups was done using one-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). One-way analysis of variance was performed using Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ suggested a significant difference.

Results

Exosome-Carried MiR-30a Inhibitor Could Improve Morphology of Myocardial Cells in MIRI Rats

The heart tissues in each group were dissected and stained with HE and the morphology of myocardial cells was observed. As shown in Figure 1, the myocardial cells had a clear boundary and outline, and the matter in the nucleus was arranged evenly, without infarction lesions in the Sham group. In the Model group, myocardial necrosis occurred, myocardial cells were swollen and enlarged with a diffused outline and unclear boundary, and there were hemorrhage and inflammatory cell infiltration. In the miR-30a inhibitor group, the pathological morphology of cells was significantly improved compared with that in the Model group.

Exosome-Carried MiR-30a Inhibitor Could Reduce Serum AST and CPK Levels in MIRI Rats

The serum AST and CPK levels in each group were detected using the biochemical method. As shown in Table I, the serum AST and CPK levels were significantly increased in the Model group compared with those in the Sham group ($*p < 0.05$, $*p < 0.05$), while they significantly declined in the miR-30a inhibitor group compared with those in Model group ($\#p < 0.05$, $\#p < 0.05$).

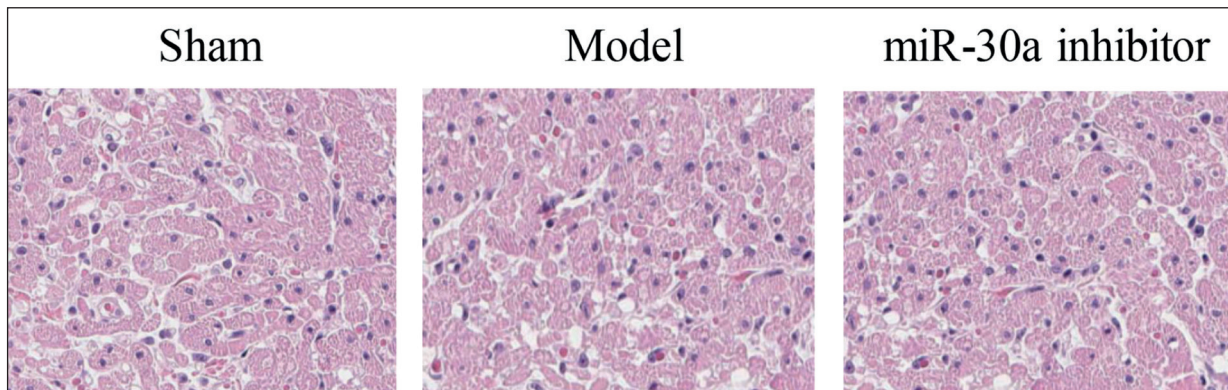


Figure 1. Changes in pathological morphology of myocardial cells in each group (20×).

Table I. Serum AST and CPK content in each group (* $p < 0.05$, # $p < 0.05$).

List	AST (U/L)	CPK (U/L)
Sham group	289.32 ± 39.73	482.31 ± 50.93
Model group	643.28 ± 59.39*	1205.35 ± 80.51*
miR-30a inhibitor group	458.54 ± 49.27#	682.74 ± 79.86#

Note: * $p < 0.05$: Model group vs. Sham group, # $p < 0.05$: miR-30a inhibitor group vs. Model group.

Exosome-Carried MiR-30a Inhibitor Could Reduce Serum MDA Content and Increase SOD Activity in MIRI Rats

The serum MDA content and SOD activity in each group were detected *via* ELISA. The results revealed that compared with those in the Sham group, the serum MDA content was increased (* $p < 0.05$), while the SOD activity declined in the Model group (* $p < 0.05$). Compared with those in the Model group, the serum MDA content declined (# $p < 0.05$), while the SOD activity was increased in the miR-30a inhibitor group (# $p < 0.05$) (Table II).

Exosome-Carried MiR-30a Inhibitor Could Inhibit Myocardial Apoptosis in MIRI Rats

The apoptosis of myocardial cells in heart tissues in each group was detected *via* TUNEL

staining. The red color represented the apoptotic cells and the blue color represented the nuclei (Figure 2A). The statistical results showed that the apoptotic rate of myocardial cells in heart tissues was obviously increased in the Model group compared with that in the Sham group (* $p < 0.05$), while it was significantly decreased in the miR-30a inhibitor group compared with that in the Model group (# $p < 0.05$) (Figure 2B), indicating that the miR-30a inhibitor can significantly inhibit myocardial apoptosis in MIRI rats.

Exosome-Carried MiR-30a Inhibitor Could Reduce Protein Levels of ULK1 and Beclin-1 in Myocardial Tissues in MIRI Rats

The levels of autophagy-related proteins ULK1 and Beclin-1 in heart tissues in each group were detected *via* Western blotting, and the protein

Table II. Serum MDA content and SOD activity in each group (* $p < 0.05$, # $p < 0.05$).

List	MDA (nmol/mg protein)	SOD (U/mg protein)
Sham group	3.14 ± 0.86	31.46 ± 2.17
Model group	9.26 ± 1.02*	6.79 ± 0.92*
miR-30a inhibitor group	7.35 ± 0.53#	18.41 ± 2.05#

Note: * $p < 0.05$: Model group vs. Sham group, # $p < 0.05$: miR-30a inhibitor group vs. Model group.

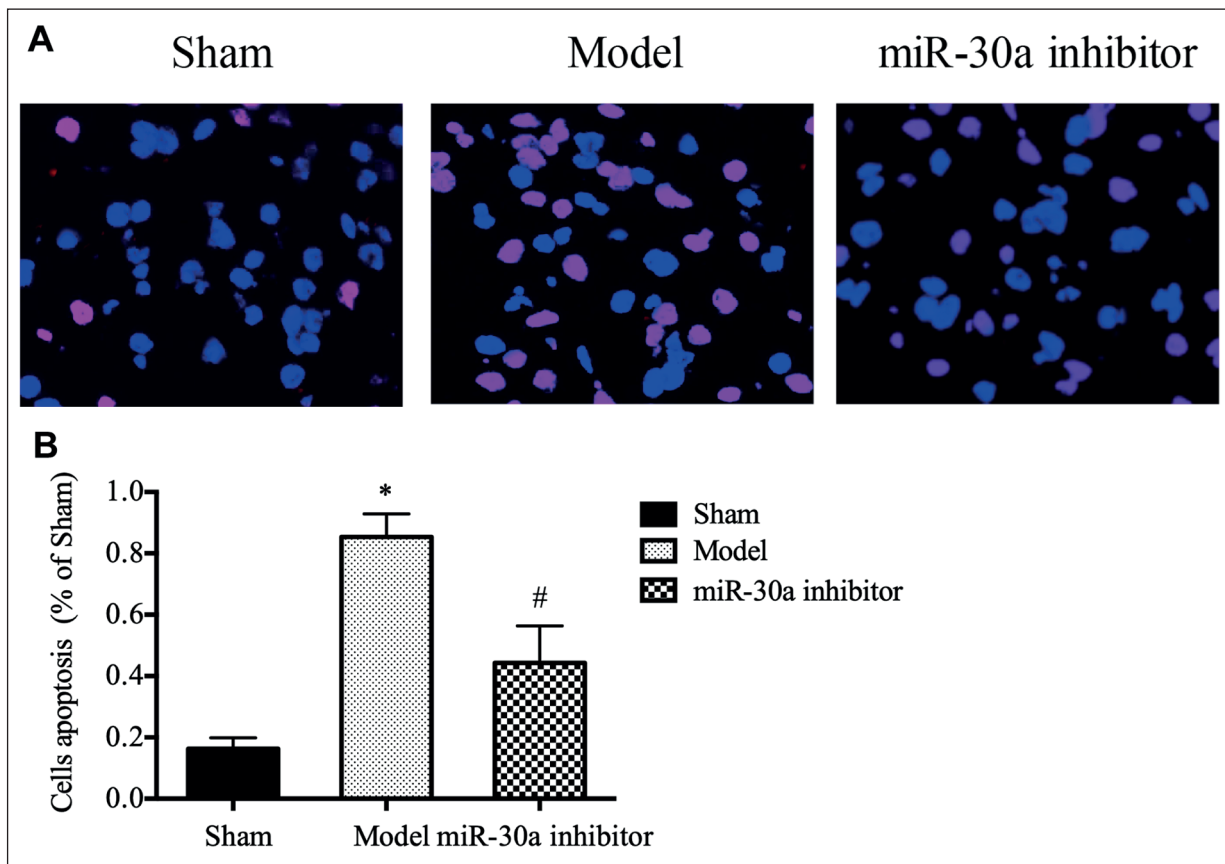


Figure 2. Myocardial apoptosis in heart tissues in each group (40 $\times$). **A**, TUNEL staining, **B**, statistical diagram of myocardial apoptosis (* p <0.05, # p <0.05).

bands are shown in Figure 3A. According to the statistical analysis using ImageJ software, the protein expressions of ULK1 and Beclin-1 in heart tissues were increased in the Model group compared with those in the Sham group

(* p <0.05), while they declined in the miR-30a inhibitor group compared with those in the Model group (# p <0.05) (Figure 3B), suggesting that the miR-30a inhibitor reduces myocardial apoptosis through inhibiting autophagy.

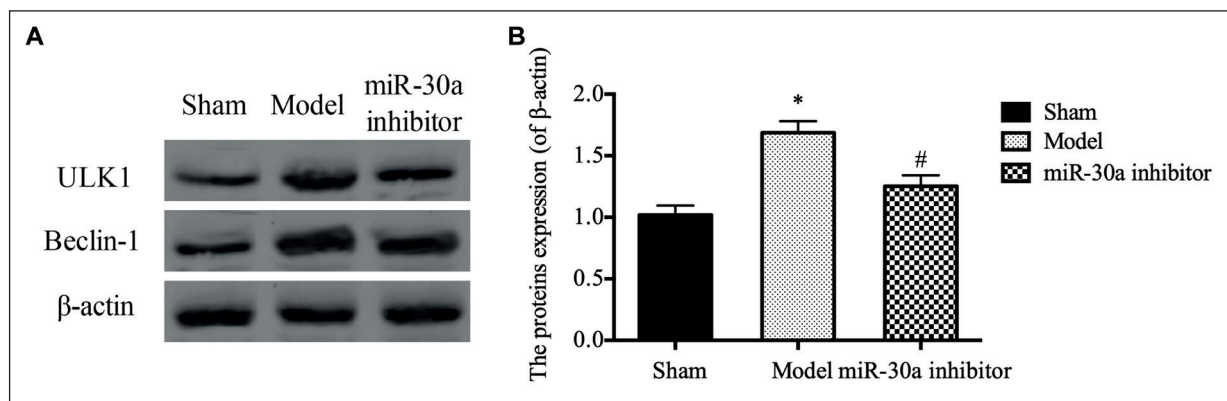


Figure 3. Expressions of autophagy-related proteins in heart tissues in each group. **A**, Western blotting bands, **B**, statistical diagram of bands (* p <0.05, # p <0.05).

Discussion

Cardiovascular disease is a problem faced by the world, in which acute myocardial infarction is one of the most important diseases which is seriously endangering human life health. The medical technology and quality have been improved in the recent years, but the recovery of reperfusion after a series of treatments will aggravate the myocardial injury, namely MIRI¹³. Therefore, the research and development of drugs that can safely and effectively alleviate and treat MIRI have become a primary task to be solved urgently by researchers and medical workers¹⁴. The heart is composed of different cells, such as fibroblasts, myocardial cells and macrophages, and a mutual regulatory network system is formed among these cells, making the pathogenesis of MIRI complex. Nowadays, it is believed that MIRI is closely related to the release of oxygen free radicals, a too high concentration of intracellular calcium ion and an endoplasmic reticulum stress damage¹⁵.

The autophagy is an ancient vital phenomenon widely existing in eukaryotic organisms. The mitochondrial autophagy is a selective autophagy that exerts a protective effect on damaged mitochondria and maintains cell homeostasis in the case of damage to intracellular mitochondria¹⁶. Mitochondria are the main “factory” of cellular aerobic respiration, which supplies energy to the body through oxidative phosphorylation. At the same time, the mitochondria are also the command center for apoptosis. Moreover, the autophagy exerts its effect through regulating the autophagy regulator Beclin-1¹⁷.

The exosomes are derived *in vivo* and form in the vesicles, and exosomes with a diameter of 30-110 nm have important functions and play key roles in cellular substance and signal transduction¹⁸. The exosomes contain a large number of active substances, such as mRNA, miRNA, proteins, and lipids. The secretion of exosomes is closely related to cellular physiological and pathological conditions, almost all cells can release exosomes, and stress changes will occur in the components of exosomes under different physiological and pathological conditions¹⁹. According to recent studies, exosomes are considered as a new mechanism for miRNA transmission, which transmits different types of signaling molecules to the recipient cells and participate in the signal exchange and the transmission among cells. Besides, the latest studies have found that exosomes play important roles in the physiolog-

ical and pathological processes of cardiovascular diseases. For example, Luo et al²⁰ found that exosome-carried miR-126 can protect the myocardial cell, resist myocardial apoptosis and alleviate inflammatory response, thereby preventing myocardial reperfusion injury.

In this paper, the MIRI rat model was established by the ligation of the left anterior descending coronary artery, and the pathological changes in heart tissues in rats were detected *via* HE staining. It was found that the miR-30a inhibitor could improve the pathological changes in morphology. Then the serum AST and CPK levels in each group were detected using the biochemical method, and the results showed that the miR-30a inhibitor could significantly reduce the serum AST and CPK content. Moreover, the serum MDA content and SOD activity were determined *via* ELISA, and it was found that the miR-30a inhibitor could reduce serum MDA content and increase SOD activity. To further study the myocardial apoptosis in MIRI rats, the apoptosis was detected *via* TUNEL assay, and the results manifested that the miR-30a inhibitor could markedly lower the apoptosis rate. Finally, the expression levels of autophagy-related proteins were detected *via* Western blotting, and the results showed that the miR-30a inhibitor could significantly reduce the protein levels of ULK1 and Beclin-1. The above findings indicate that exosome-carried miR-30a inhibitor can suppress the myocardial apoptosis through down-regulating the protein levels of ULK1 and Beclin-1.

Conclusions

We found that the exosome-carried miR-30a inhibitor can suppress the myocardial apoptosis in MIRI rats through reducing autophagy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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