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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, an[illegible] 30a inhibitor group. The pathological cha[illegible] heart tissues in MIRI rats were detected [illegible]e-matoxylin-eosin (HE) staining. The levels o[illegible] rum aspartate aminotransferase (AST) and c[illegible] atine phosphokinase (CPK) in MIRI rats we[illegible] detected using the biochem[illegible]od. The content of serum malondia[illegible]yde[illegible]A) and superoxide dismutase (S[illegible] was d[illegible]ted *via* enzyme-linked immuno[illegible]nt as[illegible] Moreover, the apopto[illegible]is [illegible]rt [illegible]des RI rats was detecte[illegible] ter[illegible]eoxynucleotidyl transferase-[illegible]ated dUT[illegible]end labeling (TUNEL) sta[illegible] The protein [illegible]of ULK1 and Beclin-1 w[illegible]d *via* Weste[illegible]blotting.

RESULTS: [illegible]pare[illegible]n those in the Sham group, the [illegible]athological [illegible] of heart tissues was sev[illegible] the levels of s[illegible] AST and CPK were i[illegible]ased, the content [illegible] MDA was decrea[illegible] the co[illegible]t of SOD was increased, the ap[illegible]c rat[illegible] heart tissues was significantly in[illegible]d the [illegible]in levels of ULK1 and Beclin-[illegible]art tis[illegible] were also significantly [illegible]ased [illegible]de[illegible]up. Compared with those in [illegible]Mode[illegible] the pathological injury of the [illegible]rt tissue[illegible]as alleviated, the levels of serum [illegible] and CPK were declined, the content of M[illegible]ased, the content of SOD was de[illegible]ased, the apoptotic rate of heart tissues, and [illegible] protein levels of ULK1 and Beclin-1 in heart [illegible]s also significantly declined.

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

Key Words:
E[illegible], Autophagy, [illegible]rdial ischemia-reperfu[illegible]jury, Apoptosis, MiR-[illegible]a.

[illegible]roduction

Curre[illegible]ardiovascular disease has become [illegible]ne of the most important diseases threatening [illegible]ealth, whose morbidity rate, in partic[illegible]ar, [illegible] exceeded that of cancer in the last 2-3 decades[1]. 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, etc[5].

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-

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lived proteins and organelles through lysosomes, which is closely related to many physiological and pathological processes[8]. 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 function[9].

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 diseases[12].

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 expl[illegible] possible regulatory mechanism.

Materials and Methods

Reagents

Aspartate aminotransfer[illegible] (AST[illegible]nd creatine phosphokinase (C[illegible] kits, m[illegible]ldehyde (MDA) and supero[illegible]dis[illegible] enzyme-linked imm[illegible]sorb[illegible]ay (ELISA) kits and termin[illegible]eoxynucle[illegible] transferase-mediated dU[illegible]k end label[illegible]UNEL) kit were pur[illegible]ed Beyotime (Shanghai, China), 4',6-diamidino[illegible]nylindole (DAPI) dye, para[illegible]aldehyde, dia[illegible]enzidine (DAB) dye an[illegible]matoxylin-eosin (H[illegible]dye from Solarbio (B[illegible]ing, C[illegible]), streptozotocin (STZ) from Sig[illegible]ldric[illegible]st. Lo[illegible]s, MO, USA), ULK1, Beclin[illegible]β-actin[illegible]mary antibodies from [illegible]T (Da[illegible] MA[illegible]SA), and horse reddish [illegible]dase ([illegible]beled secondary antibodies fro[illegible]henyang [illegible]hlei Biotechnology Co., Ltd., (Sh[illegible] China).

Instruments

[illegible]he rapid mixer was purchased from Changz[illegible]atan 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 read[illegible] Biobase Group (Fremont, CA, USA[illegible] speed refrigerated centrifuge from [illegible]uge Strato (Hanau, Germany).

Rats

The male Sprague-D[illegible]ey rats [illegible] (220±20) g (license No[illegible]XK20[illegible]-0002) purchased from the [illegible]orator[illegible]imal Center of Shandong Univer[illegible]nd[illegible]y were[illegible] fed with clean regul[illegible]odde[illegible]ad fre[illegible]cess to food and wate[illegible]der the 12[illegible]lig[illegible]ark cycle and temper[illegible]of (24±2)°C.[illegible]study was approved [illegible]imal Ethics [illegible]ommittee of Weifang [illegible]ople's H[illegible]l Animal Center.

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

The MIRI ra[illegible]odel was established by the li[illegible] of the [illegible] anterior descending coronary[illegible]y, the rats were anesthetized by intrap[illegible]neal injection of 3% pentobarbital [illegible]odium and fixed on an operating table in a su[illegible]ition. The electrode was inserted into [illegible]ubcutaneously 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 μL of samples were added and incubated at 37°C for 30 min. After the solution was spun dry, 50 μL 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 Tissue Via TUNEL Staining

The heart tissues were taken and 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 fluorescence labeling solution and 50 μL of TUNEL assay solution were added to each sample according to the instructions, followed by incubation for 1 h. Then, the sections were washed with PBS and sealed with 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) membrane (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 ECL developing solution was added and the bands were analyzed using ImageJ software.

Statistical Analysis

The data in each group were expressed as mean±standard deviation. Comparisons between groups was performed 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 ($^{\#}p<0.05$, $^{\#}p<0.05$).

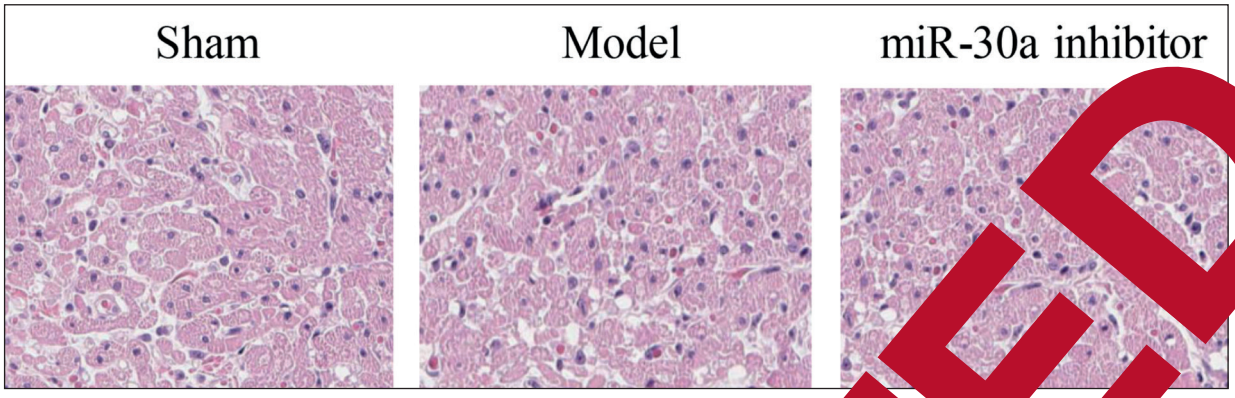

Figure 1. Changes in pathological morphology of myocardial cells in each group (2

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	4[illegible].31 ± 50.93
Model group	643.28 ± 59.3[illegible]	1205.35 ± 80.51*
miR-30a inhibitor group	458.54 ± 49.2	682.74 ± 79.86#

Note: *p<0.05: Model group *vs*. Sham group, #p<0.05: miR-30a inh[illegible]roup *vs*. Mo[illegible]roup.

Exosome-Carried MiR-30a Inhibito[illegible] Could Reduce Serum MDA Content [illegible] Increase SOD Activity in MIRI Rats

The serum MDA content and SOD activity each group were detected *via* EL[illegible] The resul[illegible] revealed that compared with [illegible]e Sham group, the serum MDA c[illegible]t wa[illegible]creased (*p<0.05), while the SOD [illegible]vity de[illegible] in the Model group (*p<0.05). C[illegible]ed [illegible] the Model group, th[illegible]rum [illegible] content declined (#p<0.05), w[illegible] the SOD [illegible]ty was increased in the m[illegible] inhibitor gr[illegible]<0.05) (Table II).

Exosom[illegible]arried MiR-3[illegible]hibitor Could[illegible]ibit Myocardial [illegible]optosis in MIR[illegible]ts

[illegible]opt[illegible] of m[illegible]cardial cells in heart tissue[illegible] group[illegible] detected *via* TUNEL staining. The red color represented the apoptotic [illegible] the blue color represented the nuclei [illegible]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

Tab[illegible] Serum M[illegible] content and SOD activity in each group (*p<0.05, #p<0.05).

	MDA (nmoL/mg protein)	SOD (U/mg protein)
[illegible]am group	3.14 ± 0.86	31.46 ± 2.17
[illegible]l group	9.26 ± 1.02*	6.79 ± 0.92*
[illegible]a 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.

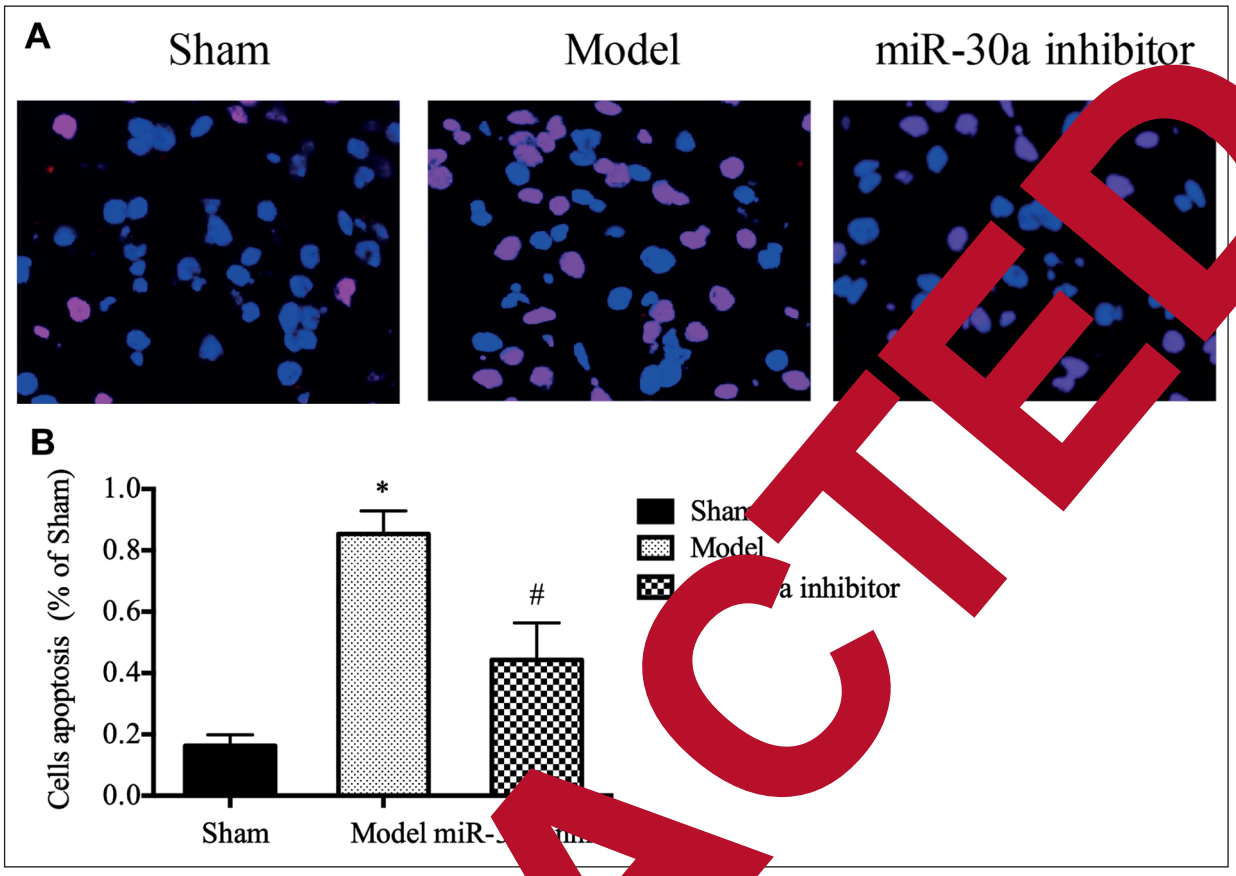

Figure 2. Myocardial apoptosis in heart tissues in each group. ***A,*** TUNEL staining, ***B,*** statistical diagram of myocardial apoptosis ($^{*}p<0.05$, $^{\#}p<0.05$).

bands are shown in Figure 3A. According to the statistical analysis using Image-Pro Plus 6.0 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.

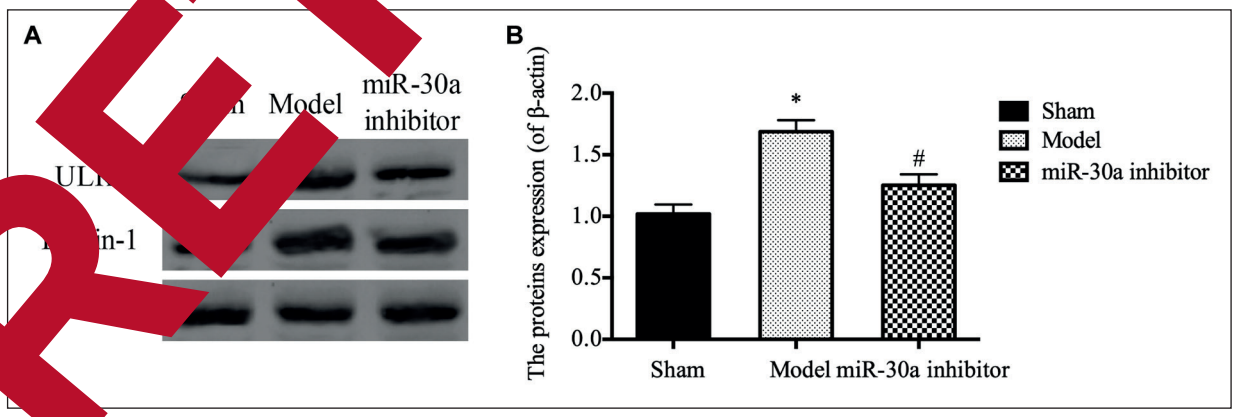

Figure 3. Expressions of autophagy-related proteins in heart tissues in each group. ***A,*** Western blotting bands, ***B,*** statistical diagram of bands ($^{*}p<0.05$, $^{\#}p<0.05$).

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 MIRI[13]. 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 workers[14]. 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 damage[15].

The autophagy is an ancient vital phenomenon widely existing in eukaryotic organisms. The mitochondrial autophagy is a selective auto[phagy] that exerts a protective effect on damage[d mito]chondria and maintains cell homeostasis [in t]he case of damage to intracellular mitochond[ria]. Mitochondria are the main "factory" of cell[ular] aerobic respiration, which sup[plies] energy [for] the body through oxidative [phosphory]lation. At the same time, the mitoch[ondr]ria a[re] also the command center for ap[opt]osis. M[ore]over, the autophagy exerts its effect [throu]gh [Beclin]-1 autophagy regulator [Beclin]-1.

The exosomes [are] derived *in* [vesicles] and form in the vesicles. [Exo]somes with [a] diameter of 30-110 nm [are] i[mpor]tant functional and play key roles in [cel]lular su[rveil]lance and signal transduction[18]. [The] exosomes c[ontain] a large number of acti[ve] substances, such as mRNA, miRNA, prote[ins] and li[pids]. The secretion of exosomes is [closel]y rel[ated] to cellular physiological and pathol[ogical] condition[s]. Almost all cells can release exo[somes] an[d] stress changes will occur [in the] comp[osition] of exosomes under different physiological a[nd] pathological conditions[19]. According to recent studies, exosomes are considered [as a] mechanism for miRNA transmission, which transmits different types of signaling molecules to the recipient cells and participate in [cellu]lar exchange and the transmission among cells. Besides, the latest studies have found that exosomes play important roles in the physiological and pathological processes of cardiovascular diseases. For example, Luo et al[20] found that exosome-carried miR-126 can protect the [endo]thelial cell, resist myocardial apoptosis a[nd] the inflammatory response, thereby pr[otec]ting myocardial reperfusion injury.

In this paper, the MIRI rat mo[del] was established by the ligation of the [left] anterior descending coronary artery, and t[he] pathological changes in heart tissues in rat[s] were de[tec]ted *via* HE staining. It was foun[d] that the [miR-]30a inhibitor could improve the pa[thol]ogical changes and morphology. Then t[he] serum [LDH] and CPK levels in each group w[ere] detected [by] the biochemical method, and [the] results showed [that] the miR-30a inhibitor [can] significantly reduce the serum AST and CPK co[ntent]. Moreover, the serum MDA content and SOD [activ]ity 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 miR-30a inhibitor could markedly lower the apoptosis rate. Finally, the expression levels of autophagy-related proteins were detected by 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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