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MiR-34a promotes myocardial infarction in rats by inhibiting the activity of SIRT1

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Abstract. – OBJECTIVE: To investigate the effect of micro ribonucleic acid (miR)-34a regulating silent information regulator 1 (SIRT1) on myocardial infarction (MI) rats.

MATERIALS AND METHODS: A total of 30 male, 8-week-old rats were divided into three groups, including: sham group (M group), MI group and MI + miR-34a treatment group (miR group). Tissue morphology in the MI region was observed via hematoxylin-eosin (HE) staining. Myocardial apoptosis in the three groups was detected via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Furthermore, the protein levels of SIRT1, B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax) in myocardial cells were detected via Western blotting.

RESULTS: Compared with M group, left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) increased significantly in MI group and miR group ($p<0.05$), while left ventricular ejection fraction (LVEF) and fractional shortening (FS) decreased obviously ($p<0.05$). The results of HE staining showed that the inflammatory infiltration of myocardial cells and intercellular collagen fibers significantly increased, and the neuronal damage was remarkably aggravated in MI group and miR group when compared with M group ($p<0.05$). Compared with MI group, myocardial necrosis, inflammatory cell infiltration and intercellular collagen fibers all increased significantly in miR group ($p<0.05$). Moreover, the results of TUNEL assay revealed that myocardial apoptosis rate in MI group [(21.35±3.12)%] was remarkably higher than that of M group [(9.53±1.17)%]. Meanwhile, it was significantly higher in miR group [(42.38±3.44)%)] than that of MI group, displaying statistically significant differences ($p<0.05$). The number of apoptotic cells increased obviously in MI group when compared with M group, while it decreased significantly in MI group when compared with miR group ($p<0.05$). Besides, the protein levels of SIRT1 and Bcl-2 in myocardial tissues in miR group were remarkably lower than those of M group and MI group ($p<0.05$). Furthermore, the protein level of Bax in miR group was higher than that of M group and MI group, and there were statistically significant differences ($p<0.05$).

CONCLUSIONS: Overexpression of miR-34a inhibits the activity of SIRT1, thereby promoting the apoptosis of MI.

Key Words:
MiR-34a, SIRT1, Myocardial infarction (MI), Apoptosis.

Introduction

Myocardial infarction (MI) has become the major cause of death in China[1]. The therapy, percutaneous coronary intervention and coronary artery bypass grafting, greatly save the lives of MI patients. Meanwhile, they can also reduce the mortality rate of 1.3 billion of population in China[2]. There are still some serious influencing factors, such as myocardial injury caused by myocardial ischemia reperfusion, as well as changes in the original substances in the heart and heart morphology. Secondary myocardial apoptosis is closely related to myocardial injury caused by myocardial ischemia reperfusion. Moreover, MI process is closely associated with the increase and decrease of myocardial apoptosis or chronic heart failure after MI. Micro ribonucleic acids (miRNAs) are a kind of non-coding single-stranded small RNAs with 20-25 nucleotides in length. Previous studies have demonstrated that[3] cell growth and apoptosis are very likely to be regulated by miR-34a. A variety of tumor-associated miRNAs have been found so far, indicating that miRNAs play crucial roles in the occurrence of tumors. Among them, miR-34a is considered as a tumor-suppressor miRNA. It has been demonstrated that miR-34a can inhibit the expression of target genes, playing an important role in the development of cancers[4]. However, whether miR-34a regulates the growth and

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apoptosis of MI remains to be further elucidated. Silent information regulator 1 (SIRT1) is a nicotinamide adenine dinucleotide-dependent histone deacetylase that plays a significant role in promoting cell proliferation and apoptosis[5,6]. Wollert et al[7] have shown that SIRT1 significantly reduces hepatic parenchymal cell damage caused by ischemia reperfusion after liver transplantation. Meanwhile, Nakazawa et al[8] have pointed out that activating the SIRT1 signaling pathway protects myocardial cells in rats. In this study, the effects of miR-34a regulating SIRT1 on cardiac function and myocardial cells in rats were studied. Our findings might help to provide a new direction for the clinical diagnosis and treatment of MI.

Materials and Methods

Laboratory Animals

In this experiment, 30 healthy male disease-free rats (aged about 8 weeks old and weighing about 260 g) were purchased from the Laboratory Animal Center of Changhai Hospital. All rats were fed adaptively at 24°C, with free access to food and water for 1 week. This study was approved by the Animal Ethics Committee of Changhai Hospital Animal Center.

Reagents and Instruments

Instruments and reagents used in this experiment included: polyvinylidene difluoride (PVDF) membrane (Whatman) (Selleck, Houston, TX, USA), hematoxylin-eosin (HE) staining kit (Maxim, Fuzhou, China), polymerase chain reaction (PCR) kit, 96-well plate and sealing membrane (ABI, Applied Biosystems, Foster City, CA, USA), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit (Beyotime, Shanghai, China), Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS) (Sangon, Shanghai, China), rabbit anti-rat B-cell lymphoma-2 (Bcl-2) polyclonal antibody (Shanghai Hufeng Chemical Co., Ltd., Shanghai, China), ultrasonic imager (Philips, Eindhoven, The Netherlands), and gel electrophoresis imaging analysis system (Bio-Rad, Hercules, CA, USA).

Animal Grouping and Establishment of MI Model

30 male rats were divided into three groups, including: sham group (M group), MI group and MI + miR-34a treatment group (miR group). The baseline data were the same in rats of the three groups. In MI group and miR group, the left anterior descending coronary artery was ligated. The observation of local cardiac ischemia and ST-segment elevation in electrocardiogram indicated successful establishment of MI model in rats. No treatment was performed in rats of M group (control group). MiR-34a was intravenously injected in rats of miR group. Meanwhile, an equal dose of normal saline was injected in those of M group and MI group. After injection for 1 week, subsequent experiments were conducted.

Cell Culture and Grouping

Rat H9C2 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37°C in an incubator with 5% CO_2 for 3 consecutive days. Subcultured cells were then inoculated in 6-well plates. After that, the cells were transfected with 5 μL of FAM-miRNA wrapped with 5 μL of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) in strict accordance with the instructions of transfection reagent.

Detection of Cardiac Function

The rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium the day after the last administration. Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF) and fractional shortening (FS) in echocardiogram were measured for 3 times using the ultrasonic imager. The average value was calculated for analysis.

HE Staining

After the detection of cardiac function (LVEDD, LVESD, LVEF and FS), the rats in the three groups were executed and treated as follows. MI tissues were taken under aseptic conditions and fixed with 4% formaldehyde for 1 day. Subsequently, the tissues were sliced into 4 μm-thick coronal sections, followed by HE staining. Finally, tissue morphology in the infarction region was observed.

Detection of Myocardial Apoptosis in Three Groups via TUNEL

Prepared paraffin sections were first stained with TUNEL, and myocardial apoptosis in the three groups was detected according to the instructions of TUNEL kit. Five uncrossed

and non-repeated fields of view were randomly selected for each section. Next, the sections were observed under a high-power optical microscope. Dark brown granules indicated apoptosis. Myocardial apoptosis rate = number of apoptotic cells/total number of cells × 100%.

Detection of Protein Levels of SIRT1, Bax and Bcl-2 in Rats

Total protein was first extracted from 100 mg of myocardial tissues using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) in each group. Subsequently, the concentration of extracted protein was detected according to the instructions of bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were then separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (50 μg/well) and transferred onto PVDF membranes. After sealing with 5% skim milk powder for 2 h, the membranes were washed with Tris buffered saline and Tween-20 (TBST) and incubated with primary antibodies at 4°C for 24 h. Next, the membranes were incubated with corresponding secondary antibody at 22°C for 1 h. Image development and exposure was performed *via* ECL. The relative expressions of proteins were calculated after analysis.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Cardiac function, myocardial apoptosis, SIRT1, Bax and Bcl-2 proteins were compared among M group, MI group and miR group using *t*-test. Univariate analysis was performed for data analysis among different groups. The results were expressed as mean ± standard deviation ($\overline{x}$±s). $p<0.05$ was considered statistically significant.

Table I. Primer sequences.

Gene		Primer sequence
SIRT1	F	5'-GGAGGAGCTGGATTTGGGACTGAT-3'
	R	5'-GGTGGAACAATTCCTGTACCTGCACA-3'
Bax	F	5'-TCCACCAAGAAGCTGAGCGAG-3'
	R	5'-GTCCAGCCCATGATGGTTCT-3'
Bcl-2	F	5'-TTCTTTGAGTTCGGTGGGGTC-3'
	R	5'-TGCATATTTGTTTGGGGCAGG-3'
β-actin	F	5'-CAGAGCCTCGCCTTTGCCGATC-3'
	R	5'-GGCCTCGTCGCCCACATAGG-3'

Results

Comparison of Cardiac Function Among Groups

According to the comparison of cardiac function, compared with M group, LVEDD and LVESD increased significantly, while LVEF and FS decreased significantly in MI group and miR group ($p<0.05$). Compared with MI group, LVEDD and LVESD significantly increased, while LVEF and FS were remarkably reduced in miR group ($p<0.05$) (Table II).

HE Staining in Three Groups

HE staining showed that the inflammatory infiltration of myocardial cells and intercellular collagen fibers increased significantly, whereas myocardial damage was aggravated in MI group and miR group when compared with M group ($p<0.05$). Compared with MI group, myocardial necrosis, inflammatory cell infiltration and intercellular collagen fibers all significantly increased in miR group ($p<0.05$) (Figure 1).

Comparison of Rat Myocardial Apoptosis via TUNEL Assay

The results of TUNEL assay revealed that myocardial apoptosis rate in MI group [(21.35±3.12) %] was significantly higher than that of M group

Table II. Comparison of cardiac function among groups ($\overline{x}$±s).

Group	n	LVESD (mm)	LVEDD (mm)	LVEF (%)	FS (%)
M group	10	3.36±0.42	5.58±0.64	87.61±1.59	36.26±5.08
MI group	10	4.27±0.50[a]	7.44±0.49[a]	66.65±5.11[a]	32.43±2.62[a]
miR group	10	6.52±0.38[ab]	10.08±0.65[ab]	48.87±4.47[ab]	23.88±3.21[ab]
F		5.435	6.84	4.48	4.536
p		<0.001	<0.001	<0.001	<0.001

Note: [a]$p < 0.05$ *vs.* M group, [b]$p<0.05$ *vs.* MI group.

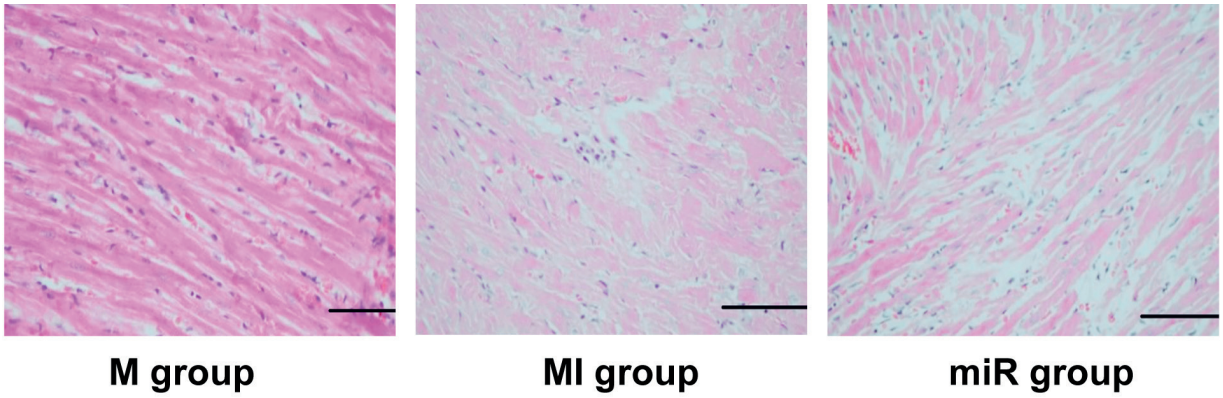

Figure 1. HE staining of myocardium in three groups of rats (×200).

[(9.53±1.17) %] (p<0.05). However, it was significantly higher in miR group [(42.38±3.44) %)] compared to that of MI group as well (p<0.05). The number of apoptotic cells significantly increased in MI group when compared with M group (p<0.05). However, it decreased remarkably in MI group when compared with miR group, showing statistically significant differences (p<0.05) (Figure 2 and 3).

Effects of miR-34a on Protein Levels of SIRT1, Bax and Bcl-2 in Myocardial Cells Detected via Western Blotting

The results of Western blotting revealed that the relative protein expressions of SIRT1 and Bcl-2 in rat myocardial tissues were remarkably lower in miR group than those of M group and MI group (p<0.05). However, the protein level of Bax in miR group was significantly higher than that of M group and MI group, and there were statistically significant differences (p<0.05) (Table III and Figure 4).

Discussion

MI is a common critical disease in clinic. In recent years, its morbidity rate has increased significantly with no age limit[9], seriously affecting the body and family of MI patients. At present, PCI, drug and bypass surgery are the main treatments for ischemic heart diseases such as MI. This can restore the blood flow of ischemic myocardium and reduce the range of MI as soon as possible. However, studies[10,11] have found that reactive oxygen species are produced and neutrophils are activated after myocardial ischemia-reperfusion. Ultimately, this leads to inflammation

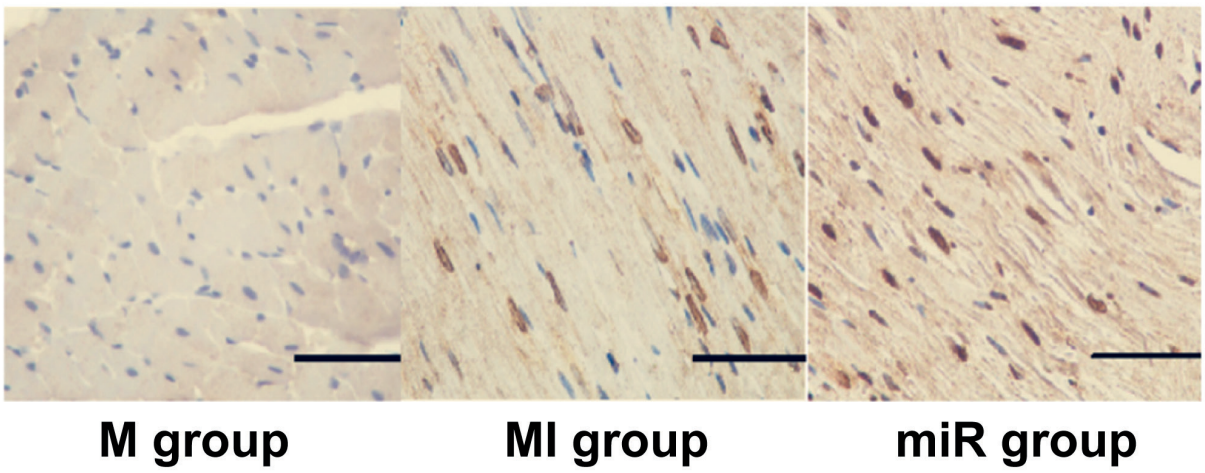

Figure 2. TUNEL results of rat myocardial apoptosis (×200).

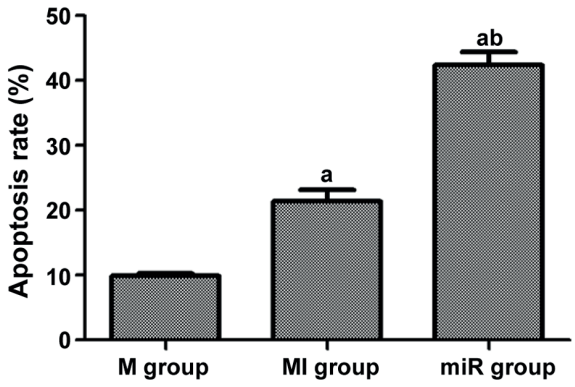

Figure 3. Comparison of rat myocardial apoptosis. Note: [a] $p<0.05$ *vs.* M group, [b] $p<0.05$ *vs.* MI group.

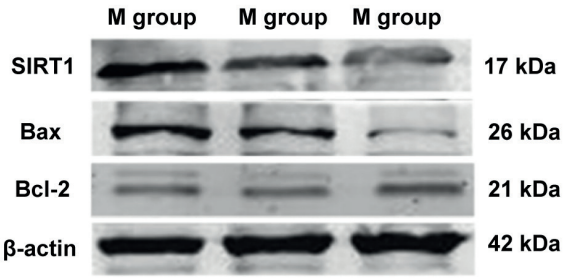

Figure 4. Protein levels of SIRT1, Bax and Bcl-2 in myocardial cells.

and myocardial apoptosis. It has been found that some treatment means can effectively improve the prognosis of MI patients so far. However, the regeneration of myocardial cells is very difficult, and myocardial apoptosis is even accelerated. The pathogenesis of myocardial apoptosis in MI is very complicated. This process requires to be regulated and controlled jointly by a variety of apoptosis genes. Among them, Bcl-2 and Bax are considered as important genes that can regulate and control myocardial apoptosis[12]. The balance between the content and function of Bcl-2 and Bax is an important mechanism for cell death or survival after apoptosis stimulus. Therefore, how to reduce the apoptotic myocardial cells, protect the structure and function of myocardial cells and lower the area of danger region around infarction has been a research hotspot in cardiovascular field in recent years. In the present study, a MI model was successfully established in rats. The results showed that compared with M group, LVEDD and LVESD significantly increased, while LVEF and FS decreased in MI group and miR group ($p<0.05$). Compared with MI group, LVEDD and LVESD increased obviously, while LVEF and FS significantly decreased in miR group ($p<0.05$). The results of HE staining showed that the inflammatory infiltration of myocardial cells and intercellular collagen fibers significantly increased, and the myocardial damage was aggravated in MI group and miR group when compared with M group ($p<0.05$). Compared with MI group, the myocardial necrosis, inflammatory cell infiltration and intercellular collagen fibers all significantly increased in miR group ($p<0.05$). Moreover, the results of TUNEL assay revealed that myocardial apoptosis rate in MI group [(21.35±3.12)%] was significantly higher than that in M group [(9.53±1.17)%], while it was higher in miR group [(42.38±3.44)%)] than that of MI group ($p<0.05$). The number of apoptotic cells increased remarkably in MI group when compared with M group. However, it evidently decreased in MI group when compared with miR group, showing statistically significant differences ($p<0.05$). Some studies have demonstrated that miR-34a promotes myocardial apoptosis[13,14]. Al-Herz *et al*[15] have found that down-regulating the expression of miR-34a inhibits the apoptosis and restores the functional activity of ischemic myocardial cells. Therefore, it is speculated that miR-34a is involved in apoptosis caused by myocardial ischemia and reflects the level of apoptosis. The protein levels of SIRT1, Bax and Bcl-2 in

Table III. Protein levels of SIRT1, Bax and Bcl-2 in rat myocardial cells in each group.

Group	n	SIRT1	Bax	Bcl-2
M group	10	0.58±0.09	0.38±0.4	0.68±0.07
MI group	10	0.42±0.12[a]	0.75±0.08[a]	0.44±0.09[a]
miR group	10	0.28±0.07[ab]	0.89±0.04[ab]	0.35±0.05[ab]
F		5.365	8.163	3.799
p		<0.001	<0.001	<0.001

Note: [a] $p < 0.05$ *vs.* M group, [b] $p<0.05$ *vs.* MI group.

rat myocardial tissues were also detected in this study. The results showed that the protein levels of SIRT1 and Bcl-2 in rat myocardial tissues were remarkably lower in miR group than those of M group and MI group. However, the protein level of Bax was higher in miR group than that of M group and MI group, showing statistically significant differences. The above findings indicated that myocardial apoptosis was promoted by SIRT1 protein through inhibiting the expression of Bcl-2 and promoting the expression of Bax. Autophagy refers to the process of phagocytosis of self-cells and self-renewing. However, its excessive activation will accelerate apoptosis. Previous evidence[16,17] has suggested that SIRT1 is a target gene of miR-34a. Meanwhile, it is closely related to cell growth and apoptosis and plays an important role in diseases[18]. In addition, SIRT1 has been reported to play a key role in cell growth and apoptosis[19]. Both Bcl-2 (an important anti-apoptotic gene) and Bax (a pro-apoptotic gene) play important roles in regulating apoptosis[20].

Conclusions

We found that the overexpression of miR-34a inhibits the activity of SIRT1, thereby promoting the apoptosis in MI.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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