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Effect of microRNA-208a on mitochondrial apoptosis of cardiomyocytes of neonatal rats

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ABSTRACT

Objective: To explore the effect and mechanism of microRNA-208a (miR-208a) in the mitochondrial apoptosis of cardiomyocytes of neonatal rats.

Methods: The primary cultured cardiomyocytes of neonatal rats were added into the hypoxia incubator for the hypoxia induction. The overexpression system for miR-208a of cardiomyocytes of neonatal rats was built. The flow cytometry assay was employed to detect the incidence of apoptosis in the over-expressed miR-208a. The mitochondrial staining technique was used to detect the change in the mitochondrial morphology of over-expressed miR-208a. The bioinformatic analysis was chosen to analyze and predict the target gene of *miR-208a*.

Results: Firstly, the primary culture system of cardiomyocytes of neonatal rats was successfully built. The miR-208a was over-expressed in cardiomyocytes of neonatal rats by miR-208a Mimics. Results of flow cytometry assay showed that the over-expressed miR-208a could significantly reduce the incidence of apoptosis; while results of mitochondrial staining indicated the change in the mitochondrial morphology of over-expressed miR-208a and the mitochondrial fission process was inhibited. In conclusion, it was supposed that miR-208a could inhibit the activation of mitochondrial fission process to keep the cardiomyocytes from apoptosis.

Conclusions: The over-expressed miR-208a can reduce the incidence of apoptosis in the cardiomyocytes of neonatal rats, significantly change the mitochondrial morphology and inhibit the mitochondrial fission process.

1. Introduction

According to epidemiological studies, the cardiovascular disease was one of highest incidence of the disease in the world [1,2]. Most of cardiovascular diseases, including myocardial infarction, angina and arrhythmia, were caused by the myocardial ischemia and anoxia. The ischemic/anoxic heart diseases had the high probability of disability and death, which severely harmed the human's health [3,4]. The cell apoptosis was the process of physiological death in cells, while the ischemic/anoxic heart diseases were accompanied with the abundant apoptosis death of cardiomyocytes [5–7]. However, the molecular biological mechanism for the incidence of ischemic/anoxic heart diseases has not been clear. The molecular biological mechanism of the hypoxia-induced cardiomyocytes apoptosis will be discussed in this paper.

The microRNAs (miRNA) is a kind of small non-coding single-strand RNA with the length of about 18–25 nucleotides [8,9]. The first miRNA (lin-4) was discovered by Lee *et al* in *Caenorhabditis elegans* using the method genetic analysis in 1993 [10,11]. Afterwards, the study on miRNA has become the hot spot in the fields of biological science and basic medicine in the past decade around the world. Several researches indicated that miRNA-208a was some kind of miRNA with

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the high expression in the cardiac muscular tissue and it possessed the tissue specificity [12,13]. It's still not clear whether miR-208a was involved in the regulation of ischemic/anoxic heart diseases.

2. Materials and methods

2.1. Laboratory animals

One or two days SPF-grade SD neonatal rats, which were purchased from Vital River Laboratories, were selected as the objects. The feeding, management and related experiment of animals were all strictly in accordance with Guide for the Care and Use of Laboratory Animals, which was published by the U.S. Department of Health and Human Services. The use of laboratory animals in this study was approved by Laboratory Animal Ethics Committee and the laboratory procedures were strictly complied with Guide for the Care and Use of Laboratory Animals that was published by U.S. Department of Health and Human Services. Its aim is to relieve the suffering of animals.

2.2. Isolation and culture of primary cardiomyocytes

One or two days SPF-grade SD neonatal rats were taken out and anaesthetized. 75% ethanol was used to disinfect the skin of rats. The skin of breast was cut open using the dissecting scissors. Then the heart was taken out using the dissecting tweezers and it was placed in the dish with the PBS buffer. The vessels on the surface of heart and the atrium were removed. Afterwards, the ventricle was cut into 1 mm^3 tissue block using the dissecting scissors. The tissue blocks were moved by the pipettes into the digestive fluid with 0.05% pancreatin and 0.025% collagenase II. It was then placed on the temperature-controlled magnetic stirrer and digested for 9 times at 37 °C, with 5 min at each time. The complete culture solution with the serum was used to stop the digestion. After the digestion, the digestive fluid was collected and centrifuged to get the cardiomyocytes. The complete culture solution with 15% fetal bovine serum was used to re-suspend the cells (the culture medium also contained 0.1 mmol/L BrdU). 200-mesh filter was used for the filtration. The technique of differential velocity adherent was performed for 90 min to remove the cardiac fibroblasts. Finally, cardiomyocytes were seeded on 6-well plate and it was placed in the cell culture incubator for 48 h of stationary culture. Afterwards, the fluid was replaced by the serum-free medium and it was moved to the hypoxic environment with 3% O_2–5% CO_2 (CO_2 incubator) for 12 h to take the hypoxic stimulation.

2.3. Transfection of cardiomyocytes of neonatal rats

As the chemosynthetic small fragments of nucleotides, the miR-208a Mimics has the same nucleotide sequence with miR-208a, which can over-express miR-208a *in vivo*; while Scramble is the small fragments of nucleotides with the random order of bases that acts as the control. After 48 h, the primary cardiomyocytes of neonatal rats were transfected according to the following procedures: in a well of 24-well plate, 20 pmol fragments of nucleotides were dissolved in 50 μL serum-free medium; then 1 μL transfection reagent Lipofectamine RNAiMAX was dissolved in 50 μL serum-free medium and placed at the room temperature for 5 min; the liquid in two Eppendorf tubes was mixed and let it stand for 20 min. During that period, the culture medium in 24-well plate was replaced by the serum-free medium and 400 μL medium was added in each well. Finally, the mixed solution was added in the corresponding well of 24-well plate, with 100 μL in each well. It was placed in the incubator for 6 h of culture. Then the culture medium in 24-well plate was replaced by the normal medium. After 24 h of culture, it continued the further operations. For instance, it was replaced by the serum-free medium and moved to the hypoxic environment with 3% O_2–5% CO_2 (CO_2 incubator) for 12 h to take the hypoxic stimulation.

2.4. Real-time quantitative PCR

Extraction of RNA: the miRNA mini kit, which was purchased from Beijing BLKW Biotechnology, was used to extract the miRNA from cardiomyocytes and it should be operated strictly in accordance with the instruction manual. Synthesis of cDNA: the reverse transcription kit that purchased from Promega was used for the reverse transcription of miRNA and it should be operated strictly in accordance with the instruction manual. It should be specially noted that the primer of reverse transcription had the specificity of miRNA. The reaction system was incubated at 42 °C for 15 min. Then samples were heated at 95 °C for 5 min and placed at 0–5 °C for 5 min. PCR Amplification: 1 μL cDNA template was used for the fluorescent and quantitative RT-PCR amplification of *miR-208a* gene and *U6* gene. SYBR 15 μL system included the target miRNA and reference (U6) of each sample for Real-time PCR reaction. $2^{-\triangle\triangle Ct}$ was used for the data analysis.

2.5. Western blot assay

The protein lysis buffer was used for the lysis of cardiomyocytes of neonatal rats. The total protein was extracted and its concentration was measured using BCA method. The proper amount of protein was taken for 5% polyacrylamide gel electrophoresis. When the bromophenol blue was moved to the bottom of gel, the electrotransfection was performed and the protein was transferred to PVDF film. It was put in the 5% skim milk room for 1 h. Then the primary antibodies of BAX (purchased from Santa Cruz, No. sc-493), BCL-2 (purchased from Santa Cruz, No. sc-783) and GAPDH (purchased from Santa Cruz, No. sc-25778) were added at 4 °C over night. Then it was incubated by the secondary antibody for 2 h and ECL (purchased from Millipore) was used for the luminescence.

2.6. Detection of apoptosis rate of cardiomyocytes by flow cytometry

After the certain period of culture, cells were digested by the pancreatin and centrifuged at 800 rmp/min for 5 min. It was then washed by pre-cooled PBS and centrifuged. The operation was repeated twice. Afterwards, the binding buffer was used to re-suspend the cells and separate them into single ones. Then annexin and PI were added in the cell suspension. It was incubated in a dark place and at room temperature for 15 min. Cells were slightly shook and then the flow cytometry was used to detect the apoptosis rate.

2.7. Mitochondrial staining

Cells were seeded on confocal dish. Mitotracker staining agent that was purchased from Invitrogen was diluted by the serum-free medium with the proportion of 1: 5 000. DAPI that was purchased from Roche was diluted by the serum-free medium with the proportion of 1: 5 000. Then it was placed in the incubator at 37 °C for 10 min. At the time of staining, the culture medium was removed from the confocal dish. The dish was washed by the serum-free medium three times. Then the prepared staining solution was added in the confocal dish in a dark place and it was incubated at the room temperature for 10 min. After removing the staining solution, it was washed by PBS three times and then photographed under the laser confocal microscopy that was purchased from Olympus.

2.8. Statistical treatment

All experiments were repeated three times at least. The experimental data was expressed by mean ± SD. SPSS was used for the statistical treatment. The one-way analysis of variance was employed for the comparison among groups and $P < 0.05$ referred to the statistical difference.

3. Results

3.1. Primary culture of cardiomyocytes of neonatal rats

For the separated cardiomyocytes of neonatal rats, the Western blot technique was used to detect the expression of important molecules α-MHC and β-MHC in cardiomyocytes, as shown in Figure 1 Results showed that α-MHC and β-MHC in cardiomyocytes of neonatal rats had the tendency of high expression. Meanwhile, the expression of α-MHC and β-MHC was detected in the cardiac fibroblasts of control group. It indicated that the primary culture system of cardiomyocytes of neonatal rats was successfully built, which laid the foundation for the further works.

3.2. Overexpression system of miR-208a

miR-208a Mimics was transfected into cardiomyocytes of neonatal rats. The real-time quantitative PCR was used to detect the expression of miR-208a. As shown in Figure 2, compared with the cardiomyocytes with the transfected Scramble fragments (the nucleotide fragment with the random order of bases and as the control for miR-208a Mimics), the expression of miR-208a in the transfection group of miR-208a Mimics was increased by nearly ten times.

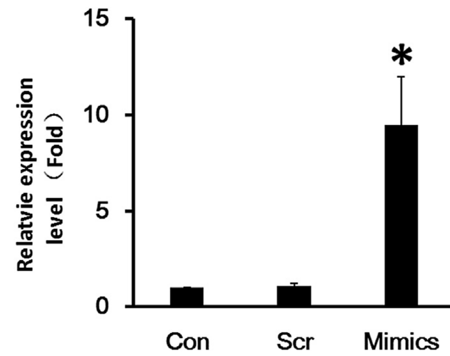

Figure 2. Overexpression of miR-208a in cardiomyocytes of neonatal rats. Con: no transfected nucleotide fragment; Scr: fragment of transfection control group; Mimics: overexpression fragment of transfected miR-208a ($^{*}P < 0.05$).

3.3. Effect of overexpression of miR-208a on expression of BCL-2 and BAX

BCL-2 and BAX are two important proteins in the apoptosis, where BCL-2 is to inhibit the apoptosis and BAX to promote the apoptosis. As shown in Figure 3, results of Western blot assay indicated that, under the hypoxic stimulation, the expression of BCL-2 was significantly increased after the overexpression of miR-208a, while the expression of BAX was significantly decreased, which indicated that miR-208a could inhibit the apoptosis.

3.4. Effect of overexpression of miR-208a on apoptosis

The flow cytometry was used to detect the apoptosis rate. As shown in Figure 4, under the hypoxic stimulation, the number of annexin positive cells was significantly reduced, while the number of normal cells was increased, which indicated that the overexpression of miR-208a could significantly inhibit the occurrence of hypoxia-induced apoptosis.

3.5. Effect of miR-208a on mitochondrial morphology of cardiomyocytes

The mitochondrial fission was closely related to the apoptosis. As shown in Figure 5, the mitochondrial morphology in the normal cardiomyocytes of neonatal rats was regular,

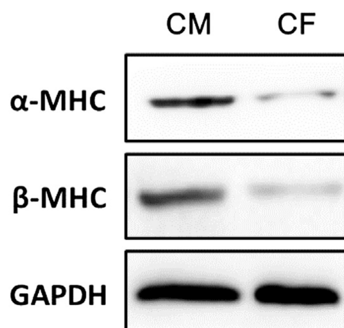

Figure 1. Expression of α-MHC and β-MHC in cardiomyocytes. CM: cardiomyocytes; CF: cardiac fibroblasts.

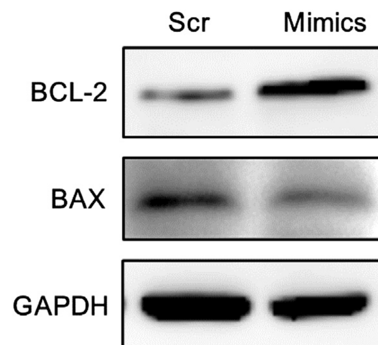

Figure 3. Change in expression of BCL-2 and BAX in miR-208a overexpression group and control group.

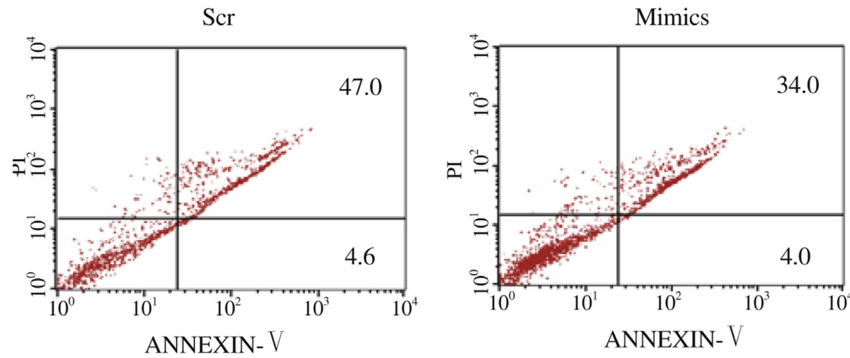

Figure 4. Apoptosis rate in miR-208a overexpression group and control group. Right upper quadrant and right lower quadrant were annexin V positive cells.

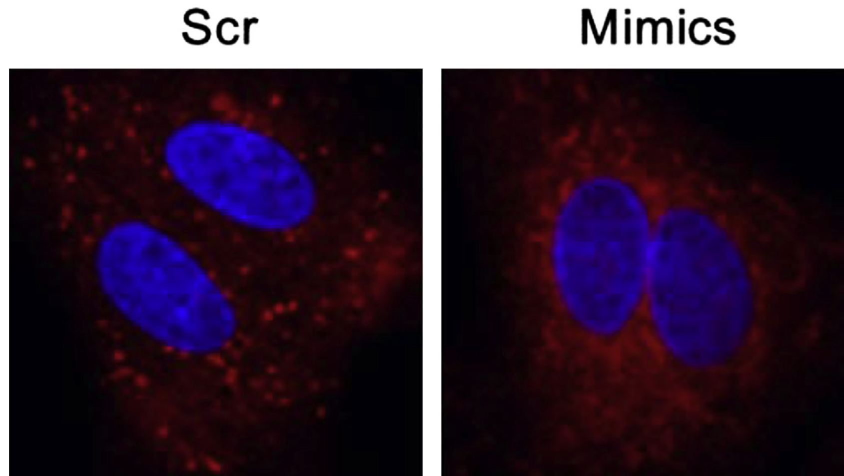

Figure 5. Change in mitochondrial morphology in miR-208a overexpression group and control group.

without the point accumulation, namely mitochondrial fission. But under the hypoxic stimulation, the mitochondrial fission was significantly increased, which indicated the occurrence of apoptosis. However, in the miR-208a overexpression group, the number of cells with the mitochondrial fission was significantly decreased, which indicated the decreased apoptosis rate in another way.

4. Discussion

In recent years, the study on miRNA in the heart diseases has become the hot topic for the study on the mechanism of heart diseases. According to previous researches, miRNA was involved in the physiological process of apoptosis, electrical conduction change, cardiomyocytes regeneration and cardiac remodeling during the myocardial ischemia and anoxia [14–17]. Besides, several researches have explained the mechanism of many miRNAs in the acute myocardial ischemia, which provide the reference for the treatment of heart diseases through the molecular drugs.

In addition, miRNA may also become the important biological marker for the myocardial injury, which would greatly contribute to the early diagnosis and prevention of acute myocardial infarction [18,19]. Lawrie *et al* found the miRNA in the human serum in 2008 [20]. Since then, a great number of researches have explained that the miRNA in the circulating blood could stably exist in the extracellular fluids such as serum and plasma [21,22]. miR-208a is the cardiac-specific miRNA, which can regulate the physiological and pathological process of generation, development and damage repair; and its content is stable in the blood [23–26]. But in patients with the heart diseases, the expression abundance of miR-208a in the serum was significantly increased, as an effective biological marker for the early diagnosis of heart diseases [12,27,28].

In this study, the primary cultured cardiomyocytes of neonatal rats *in vitro* was cultured in the hypoxia incubator and the mode for the cardiomyocytes of neonatal rats anoxia was built. Afterwards, the transfected small fragments of nucleotides of miR-208a Mimics was to overexpress miR-208a and then the related indicators of apoptosis were detected. Main assays included the western blot detection of BCL-2 and BAX, flow cytometry after double straining of annexin V/PI and Mito-tracker to observe the mitochondrial morphology. These three assays all confirmed that the over-expressed miR-208a could effectively inhibit the occurrence of apoptosis. Though there have been abundant data about the anti-apoptosis function of miR-208a, but its specific molecular biological mechanism has not been clear.

According to the bioinformatic analysis, it's found that miR-208a and miR-499 possessed the same seed sequence. Wang *et al* reported that miR-499 could inhibit the dephosphorylation of calcineurin-induced Drp1 protein, reduce the accumulation of Drp1 in the mitochondria and inhibit the activation of Drp1-induced mitochondrial fission, in order to keep the cardiomyocytes from apoptosis [29]. According to Targetscan bioinformatic websites [30,31], it's predicted that miR-208a could be complementarily paired with 3′UTR region of *calcineurin* mRNA, which indicated that miR-208a might also inhibit

the calcineurin, inhibit the activation of mitochondrial apoptosis pathway, and thus inhibit the apoptosis. The focus of our further study would be seeking the target gene of miR-208a and the determination whether miR-208a actually inhibits the apoptosis by acting on such target gene.

In conclusion, the high expression of cardiac-specific miRNA in the heart and its abnormal expression in the apoptosis of cardiomyocytes indicate its unique biological function. It is thus possible to improve the symptoms by changing the expression of these miRNAs, which provide the new opportunity for the treatment of heart diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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