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Mir-208 inhibits cardiomyocyte apoptosis in acute myocardial infarction in rats by targeting GATA4 to activate qk15 related signaling pathway

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Summary

Objective: The objective of this study was to investigate the potential role of mir-208 in blocking myocardial cell apoptosis during acute myocardial infarction (AMI) via activating the qk15-related signal pathway through targeting GATA4. Methods: Three groups of forty-five clean male Wistar rats were assigned at random: sham operated group (open chest only, without ligation of left anterior descending coronary artery), model group and low expression group (tail vein injection of mir-208 inhibitor after model establishment). Each group had 15 rats. The changes of myocardial function (MI) were measured by ECG. The changes of LDH in serum were measured by colorimetry. The infarct area was calculated. TUNEL method was used to detect cardiomyocyte apoptosis. Western blot was used to assess the expression levels of qk15, GATA binding protein 4, and caspase-3. Results: In contrast to the sham group, the expression level of mir-208, serum LDH level, MI area, apoptosis rate of myocardial cells and caspase-3 expression levels of rats in the model group were increased, and the expression levels of EF, SV, FS, GATA4 and QK15 were decreased. In contrast to the model group, the expression level of mir-208, serum LDH level, MI area, myocardial cell apoptosis rate and caspase-3 expression levels of rats in the mir-208 low-expression group were reduced, while the expression levels of EF, SV, FS, GATA4 and QK15 were significantly increased. Conclusion: Down regulation of mir-208 expression can inhibit apoptosis of myocardial cells in AMI, which may be achieved by targeting GATA4 to activate qki5 related signaling pathway.

Keywords: Mir-208, GATA4, qk15 related signaling pathways, AMI, cardiomyocyte apoptosis

Due to the improvement of living standards and the gradual aging of the population, the incidence rate of coronary heart disease worldwide is increasing every year. Acute myocardial infarction (AMI) is a kind of ischemic heart disease caused by acute coronary artery occlusion, characterized by high mortality and disability rates, which has seriously threatened people's lives and health (13). An increasing number of cardiovascular disorders may now be healed by surgery or other methods thanks to advancements in medical research and technology; yet, myocardial ischemia/reperfusion during surgery has become a major worry for surgeons. Ischemia-reperfusion injury may aggravate the damage of myocardial parenchyma and even cause myocardial failure (5). The key method of treating dying myocardium is to open the infarct-related arteries in a timely manner, thus restoring the supply of blood flow (8). Some studies have found that ischemic preconditioning or postconditioning can significantly reduce myocardial ischemia-reperfusion injury to a certain degree of life, but there are still numerous inconveniences in an actual clinical operation (2). Therefore, it is important to find drugs with protective tenancy to reduce myocardial ischemia-reperfusion injury. Ischemia-reperfusion damage has a complicated and unknown etiology. It has been found that the pathogenesis of myocardial ischemia/reperfusion injury may be closely related to impaired myocardial energy metabolism, vascular endothelial cell injury and apoptosis (12). It has been reported that apoptosis may be an important part of the pathogenesis of myocardial ischemia-reperfusion injury, which mainly occurs in the ischemic area, and the number of apoptotic cells is closely related to the degree of myocardial

injury and cardiac function after ischemia-reperfusion injury (4).

MiRNAs are non-coding RNAs that can control gene expression by binding to inhibit specific mRNA targets, and are an important post-transcriptional regulator that is crucial to the development and recurrence of heart illnesses (11). MiR-208 belongs to the family of miRNAs, and it is a cardiac tissue-specific expression miRNA, which may be involved in the signaling of cardiac arrhythmia, myocardial remodeling, signaling pathways of cardiac hypertrophy, and the conduction system of the heart (9). According to some research, miR-208 has a significant regulatory function in the onset and progression of cardiac disorders, and can inhibit cardiomyocyte apoptosis, but the mechanism of action related to the inhibition of apoptosis of cardiomyocytes in AMI by miR-208 is not clear (6). This study investigated the mechanism by which miR-208 prevents acute myocardial infarction cardiomyocytes from going through apoptosis.

Material and methods

Experimental animals. There were 45 clean-grade healthy male Wistar rats (purchased from Nanjing Junke Bioengineering Co., Ltd, production license SCXK (Ning) 2017-0001), weighing (221 ± 19) g. For a week, all rats were housed in an environment with a temperature of 23 ± 2°C to acclimate them, a humidity of 48 ± 15%, and alternating day and night for 12 h each. To obtain scientific research results and minimize the pain caused to animals, this experiment completely followed the 3R principles of *Reduce, Replacement and Refine*, and conformed to the relevant provisions of the national welfare ethics for experimental animals. This experiment was approved by Suzhou Ninth People's Hospital Animal Experimental Ethics Committee with the approval number of SNPH032.

Main instruments and reagents. Biological tissue roaster (Shanghai Precision Instrumentation Co., Ltd, model: YD-AB); pathology sectioning machine (Shanghai Jumo Medical Instrument Co., Ltd, model: QP420); electronic balance (Shanghai Precision Instrumentation Co., Ltd, model: FA1604B); thermostatic water bath box (Changzhou Jintan Youlian Instrument Research Institute, Model: HH-600); electron microscope (Pumice Optical Science and Technology Co., Ltd., Model: VP-LZ-650105); –80°C ultra-low-temperature refrigerator (Beijing Aeris Bio-technology Co., Ltd., Model: DW-86L626); TUNEL Apoptosis Detection Kit [Beckman Coulter Trading (China) Co., Ltd]; rabbit anti-human Caspase-3 polyclonal antibody (Shanghai Maicang Biotechnology Co., Ltd.); hematoxylin-eosin staining (Shanghai Gudo Biotechnology Co., Ltd.); miR-208 inhibitor (BIOMARCO Biotechnology Co., Ltd.).

Grouping. Establishment of AMI rat model: the rats were anesthetized, fixed on the working table, type tracheal intubation connected to small animal ventilator. Chest skin preparation, longitudinal incision of the skin in the left edge of the sternum at the 3rd-4th intercostal space, in turn, longitudinal march, and finally the pericardium will be cut open. At the base of the roughly 2 cm connected to a mixture, the heart is exposed to the left anterior descending branch of the coronary artery, which is passed through a plastic tube which will be mixed tightly, and then the chest cavity quickly closed; cardiac ligature about 45 min to pull out the plastic tube to restore the blood flow.

The rats were split into three groups at random: the model group, the miR-208 low-expression group (miR-208 inhibitor was injected into the tail vein after the rat model was established), and the sham operation group (only opening the chest without ligating the left anterior descending branch of the coronary artery). Each group consisted of fifteen rats.

Observation indexes. After successful modeling, rats in each group had their cardiac function assessed by echocardiography.

In each group, 3 mL of heart blood was extracted, centrifuged, and the supernatant was taken to determine the changes in serum lactate dehydrogenase (LDH) levels in each group by colorimetric method.

After the rat heart ischemia-reperfusion model was made, myocardial tissues were taken and put into a refrigerator at –20°C for approximately half an hour, cut into thin slices about 2 mm thick, and then put into a tetramethylethylenediamine phosphate buffer at 37°C for 15 min. Ischemic tissues appeared white, and non-ischemic tissues were red, and the extent of myocardial infarction was calculated.

The rats were put to death, the rat hearts were removed, washed with saline, and the myocardial tissues were taken from about 2 mm distal to the ligature and stored in an ultra-low-temperature refrigerator at –80°C for spare parts.

A tissue block of about 5 mm was cut off, fixed using formaldehyde solution, paraffin cut blocks were routinely made, deparaffinized using xylene, dehydrated with ethanol, washed with distilled water. Add 50 µL TUNEL assay solution to the tissue specimen, incubate in 37°C incubator with light protection for 1 h, and rinse with phosphate buffer solution for 3 times, each time for 3 min. Cardiomyocyte apoptosis was observed using a microscope.

Protein blotting was used to assess Caspase-3 expression in the rat cardiac tissues of each group.

Protein blotting was used to assess the expression levels of GATA-binding protein 4 (GATA4) and QK15 in the cardiac tissues of the rats in each group.

Statistical methods. In this study, the measurement data was compared between groups using the independent sample t-test; the paired sample t-test was used to compare the data before and after the treatment; the differences between the groups were compared at each time point using the independent sample t-test; and the differences between the groups in terms of time were compared using the LSD-t-test. The statistical data in this study were analyzed using SPSS17.0 software, and the results were statistically significant at $P < 0.05$.

Results and discussion

Comparison of miR-208 expression level in rats of each group. It has been found that myocardial sustained ischemia for a period and then re-establishment of blood perfusion will bring new damage to cardiac tissues such as decline in cardiac functioning and malignant arrhythmia episodes, i.e. myocardial ischemia/reperfusion injury, which has caused serious impact on both patients and their families (3). Consequently, it

is crucial to investigate the pathogenesis of myocardial ischemia-reperfusion injury in order to reduce its occurrence while restoring blood perfusion to ischemic tissues as early as possible. Changes in miR-208 expression have been reported to occur not only during cardiac development, but also in certain medical conditions (15). When the miR-208 gene is absent, the heart experiences abnormal conduction, which induces arrhythmia. Rats in the model group had greater levels of miR-208 expression than those in the sham-operated group, whereas rats in the miR-208 low expression group had lower levels of miR-208 expression than those in the model group. miR-208 expression level can be used as a potential marker of myocardial injury. See Table 1.

Tab. 1. Comparison of miR-208 expression levels ($\overline{x} \pm s$)

Group	Number	miR-208
Sham-operated group	15	0.099 ± 0.014
Model group	15	0.738 ± 0.141
Low expression group	15	0.425 ± 0.182
F		86.36
P		< 0.001

Comparison of changes in myocardial function of rats in each group. The levels of EF, SV, and FS in the model group were lower than those in the sham-operated group; conversely, the levels of these factors were greater in the miR-208 low-expression group than in the model group. See Table 2.

Tab. 2. Comparison of changes in myocardial function ($\overline{x} \pm s$)

Group	Number	EF (%)	SV (%)	FS (%)
Sham-operated group	15	70.45 ± 1.71	0.47 ± 0.13	35.23 ± 0.78
Model group	15	44.29 ± 2.08	0.26 ± 0.12	15.49 ± 0.77
Low expression group	15	53.95 ± 2.45	0.36 ± 0.10	23.03 ± 1.75
F		594.15	12.02	1047.23
P		< 0.001	< 0.001	< 0.001

Explanations: EF – Ejection fraction; FS – Fraction of short axis shrinkage; SV – Stroke volume

Comparison of serum LDH levels in rats of each group. Rats in the model group had greater serum LDH levels than those in the sham-operated group, whereas rats in the miR-208 low expression group had lower serum LDH levels than those in the model group. See Table 3.

Tab. 3. Comparison of serum LHD levels ($\overline{x} \pm s$)

Group	Number	LDH (U/L)
Sham-operated group	15	1078.66 ± 110.58
Model group	15	2107.21 ± 152.22
Low expression group	15	1678.63 ± 83.07
F		283.97
P		< 0.001

Comparison of MI area of rats in each group. Rats in the model group had a greater area of MI than those in the sham-operated group, whereas rats in the miR-208 low expression group had a smaller area of MI than those in the model group. See Figure 1, Table 4.

Tab. 4. Comparison of MI area in rats in each group ($\overline{x} \pm s$)

Group	Number	MI area (%)
Sham-operated group	15	0
Model group	15	36.64 ± 2.10
Low expression group	15	25.06 ± 2.02
F		15.392
P		< 0.01

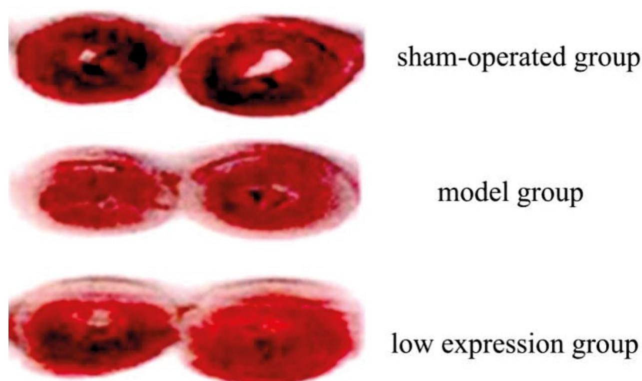
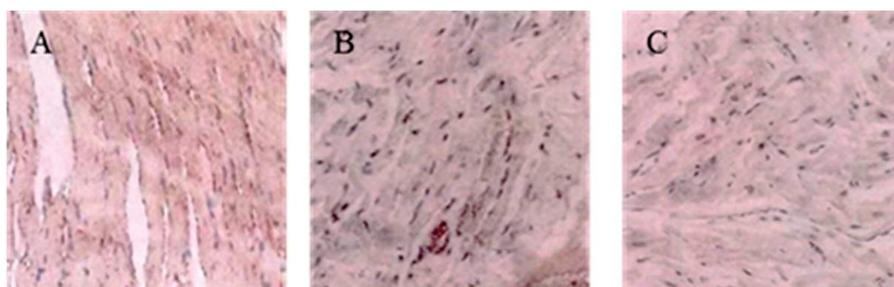


Fig. 1. Extent of MI in each group of rats

Comparison of cardiomyocyte apoptosis in rats of each group. Some studies have confirmed that apoptosis occurs in ischemic myocardial tissue during early myocardial ischemia-reperfusion. The level of apoptosis can reflect the severity of ischemia-reperfusion injury to a certain extent (7). Caspase-3, an asparaginase-cleaved cysteine protease, is a recognized key protease activated at the early stage of apoptosis, which plays an important role in apoptosis as well as the final executing factor of apoptosis (10). Rat cardiomyocyte apoptosis rate and Caspase-3 expression level were both higher in the model group when compared to the sham-operated group; conversely, in the miR-208 low-expression group, rat cardiomyocyte apoptosis rate and Caspase-3 expression level were lower when compared to the model group. The results of our study showed that down-regulation of miR-208 level could significantly improve cardiomyocyte apoptosis. See Figure 2, Figure 3, and Table 5.

Comparison of GATA4 and QK15 expression levels in myocardial tissues. GATA4, a member of the GATA family of zinc-finger transcription factors, was first identified in the heart, is closely related to the normal development of the embryonic heart, and can regulate the growth, differentiation, and apoptosis of cardiomyocytes; in addition, GATA4 can also regulate cell survival (14). It was found in congenital heart disease that miR-208 was able to regulate GATA4 expression, promote cardiomyocyte apoptosis and inhibit



A: sham operation group
B: model group
C: miR-208 low expression group

Fig. 2. Comparison of apoptosis of rat cardiomyocytes

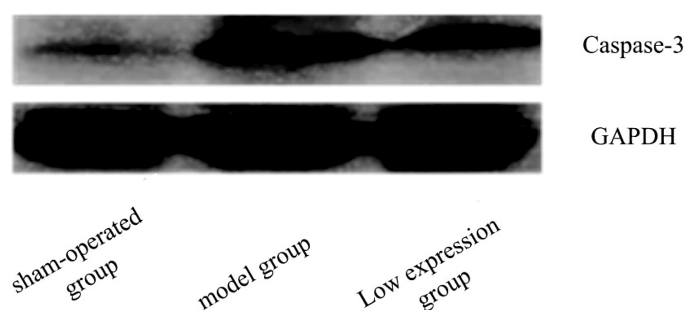


Fig. 3. Caspase-3 expression in myocardial tissues of rats in each group

Tab. 5. Comparison of apoptosis rate of rat cardiomyocytes in each group ($\bar{x} \pm s$)

Group	Number	Apoptosis rate (%)
Sham-operated group	15	4.98 ± 0.26
Model group	15	29.59 ± 2.61
Low expression group	15	15.11 ± 2.62
F		500.91
P		< 0.001

proliferation, thus promoting congenital heart disease. QK15 proteins are a class of proteins with signaling functions that can bind to mRNAs and are expressed in high abundance in the brain and cardiac muscle. It was found (1) that QKI5 was able to inhibit cardiomyocyte hypertrophy. Compared with the sham-operated group, the GATA4 and QK15 in rats of the model group were lower; compared with the model group, the GATA4 and QK15 in rats of the miR-208 low-expression group were higher. See Figure 4.

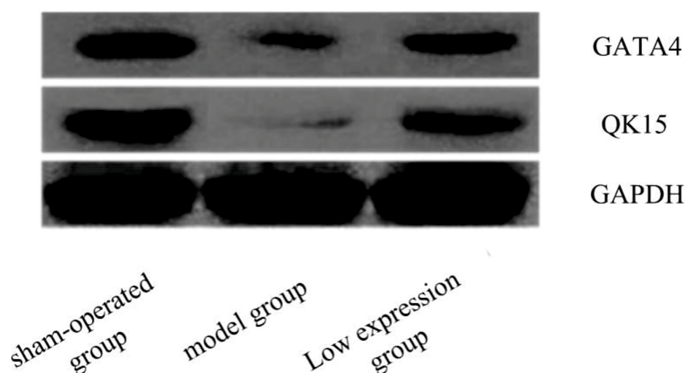


Fig. 4. GATA4 and QK15 in rat myocardial tissues

In conclusion, down-regulation of miR-208 expression can inhibit apoptosis in AMI cardiomyocytes, which may be achieved by targeting GATA4 to activate QK15-related signaling pathway.

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