Influences of microRNA-451 on the expression of HMGB1 in myocardial cells and its mechanism in ischemia-reperfusion injury

Ruiye Qiu1*, Yajuan Zhang2, Jun Li1, Jun Wu1, Ruifeng He1

1 Department of Emergency, Zhuhai Integrated Traditional Chinese and Western Medicine Hospital, Zhuhai, 519020, Guangdong Province, China
2 Department of Intensive Care Unit, Zhuhai Integrated Traditional Chinese and Western Medicine Hospital, Zhuhai, 519020, Guangdong Province, China

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Introduction

Ischemia-reperfusion injury (IRI) refers to the progressive aggravation of original myocardial tissues caused by reperfusion after ischemia. IRI is related to the changes in molecules, cells, and tissues, such as cell apoptosis, inflammatory reaction, the activation of neutrophils, and oxidative stress (1,2). At present, the assessment and treatment of IRI is still one of the difficulties in clinical practice. Because the specific pathogenic mechanism of IRI is unclear, IRI is the hot topic of the current research into acute myocardial ischemia (3,4).

In recent years, high mobility group box 1 protein (HMGB1) plays a crucial role in the occurrence and progression of cardiovascular diseases as a new proinflammatory factor (5,6). The studies on IRI demonstrate that the expressions of proteins and messenger ribonucleic acid (mRNA) remarkably increased half an hour after myocardial cell hypoxia occurred (7,8). HMGB1 causes injuries to tissues by releasing proinflammatory factors (9,10). IRI can be obviously improved by different interventions of the activity of HMGB1 or its binding receptor (11,12). Hence, the verification of the influencing factors of HMGB1 up-regulation and the search for the key genes regulating HMGB1 expression can provide a new idea for the prevention of IRI (13).

microRNA is a small non-coding molecular RNA consisting of 18 to 25 nucleotides. It is involved in regulating gene expression and various biological processes of the body at the post-transcriptional level, such as cell differentiation, cell growth, and cell death (14,15). At present, multiple studies confirm that miRNAs get involved in the IRI process, such as miR-1, miR-29, miR-451, and miR-494. miRNA can regulate IRI by regulating different target genes (16,17). It is demonstrated that multiple may regulate HMGB1 expression. According to Fu et al. (18), miR-451 expression in the extracted myocardial tissues dramatically decreased in the rat IRI model. Therefore, HMGB1 may be the target gene of miR-451. The regulation of miR-451 can protect IRI through HMGB1.

Based on the above opinions, 30 Sprague-Dawley (SD) rats were selected for the construction of the I/R animal model. The research was aimed at investigating the regulatory mechanism of miR-451 in HMGB1 expression of I/R model rats and protection of I/R model rats by miR-451 up-regulation to provide an experimental basis for preventing and treating IRI clinically.

Materials and Methods

Experimental animal

30 selected specific pathogen-free (SPF) male rats were sourced from Hunan SJA Laboratory Animal Co., Ltd.. The weight of the selected rats ranged from 240g to 320g.
with an average of 265.7±24.3g. They were 8 weeks old. SD rats were fed adaptively for 3 days with a free diet and drinking. The temperature and humidity of the animal room were kept at about 23°C and about 48°C, respectively. Besides, day lighting and night lighting were alternated.

**Experimental grouping and processing**

30 SD rats were grouped into 5 (6 in each), including sham operation control (control), an IRI(I/R), an I/R+Ad-GFP, miR-451 up-regulation (I/R+Ad-miR-451), and a miR-451 down-regulation groups (I/R+Ad-asmiR-451).

Rats in the Control group were only subjected to surgical operation without being induced with IRI.

Initially, 6 injection sites were identified on the frontal wall of the left ventricle of rats in the I/R group. Then, 150 μL phosphatic buffer solution (PBS) was injected. After 3 days, the left anterior descending branch (LAD) was ligated. After 30 minutes, reperfusion was performed for 24 hours.

Secondly, 6 injection sites were identified on the frontal wall of the left ventricle of rats in the I/R+Ad-GFP group. Then, 150 μL LAd-GFP was injected. After 3 days, LAD was ligated. After 30 minutes, reperfusion was performed for 24 hours.

Next, 6 injection sites were identified on the frontal wall of the left ventricle of rats in the I/R+Ad-miR-451 group. Then, 150 μL Ad-miR-451 was injected. After 3 days, LAD was ligated. After 30 minutes, reperfusion was performed for 24 hours.

Finally, 6 injection sites were identified on the frontal wall of the left ventricle of rats in the I/R+Ad-asmiR-451 group. Then, 150 μL Ad-asmiR-451 was injected. After 3 days, LAD was ligated. After 30 minutes, reperfusion was performed for 24 hours.

**Construction of IRI model**

The rats underwent abdominal cavity anesthesia with 3% pentobarbital sodium (purchased from The BSZH Scientific Inc., Beijing). After tracheal intubation, mechanical ventilation was carried out. Chests were ruptured and opened with the third rib forcep. A hemostatic forcep was used to clamp the intercostal artery to stop bleeding. Next, the pericardium was torn to expose the heart. LAD was ligated and then fixed with 1% Evans blue dye was injected from the head side to the heart side via the jugular vein. After the staining, the heart was extracted and the cardiac atrium and right ventricle were removed. After that, the heart was cleansed with physiological saline and then frozen at -80°C for 30 minutes. The heart was taken out and the left ventricle was cut into 5 to 6 transverse sections with a thickness of 2mm along the major axis of the heart. Then, the transverse sections were put into 1% 2,3,5-triphenyltetrazolium chloride (TTC) and stored away from light at 37°C for incubation for 15 minutes. When the sections were stained red, cardiac tissues were rinsed with physiological saline and then fixed in 4% polyformaldehyde (POM) (SenBeiJia Biological Technology Co., Ltd.) overnight. Next, a computer-aided image analysis system (Image-Pro Plus3.0) was used for the measurement of the unischemic myocardial tissue area (blue area), infarction area (white area), and ischemic area (red and white area). Besides, the proportions of the areas of the infarction area and ischemic area in myocardial tissues were calculated to estimate the myocardial infarction area. Finally, hematoxylin-eosin staining (HE staining) was adopted for the morphological observation of myocardial tissues.

**Assessment of myocardial cell apoptosis by TdT-mediated dUTP nick end labeling (TUNEL) staining**

TUNEL cell apoptosis detection kit (purchased from Shuoheng Biotechnology Co., Ltd., Guangzhou) was used for the detection of the rupture of nucleus DNA during the early apoptosis of sectioned myocardial cells. Myocardial sections were placed into a constant-temperature oven at 65°C and then baked for 1 hour. Next, they were successively soaked in xylene I and II for 15 minutes, respectively and 100%, 95%, 85%, and 75% ethanol for 5 minutes. Myocardial sections were rinsed with running water for 10 minutes. Sections were incubated in a protease working solution for 20 minutes. Then, they were added with 50μL TUNEL reaction mixture and incubated away from light in a biochemical incubator at 37°C for 60 minutes. Next, myocardial sections were cleansed with PBS 3 times and then added with 50 μL transformed peroxidase (POD) solution. The myocardial sections were incubated at 37°C for 30 minutes. Then, myocardial sections were cleansed with PBS 3 times and added with 50 μL 3,3-diaminobenzidine (DAB) (purchased from Suzhou Yaoo Science Co., Ltd.) substrate. Myocardial sections were stained again with hematoxylin and then dehydrated, transparentized, and sealed for observation under a light microscope. Next, the cells with positive TUNEL results and all cells in 10 fields of view were counted. Besides, the myocardial cell apoptosis index was calculated.

**Measurement of the markers for myocardial tissue injury in serum (LDH and CK)**

Before and after the production of all rat I/R models, 2 mL of blood was extracted from the external jugular vein before ischemia and 24 hours after reperfusion. Then, the extracted blood was placed in a coagulation tube at room
temperature for 15 minutes. After that, it was centrifuged. The upper layer yellowish transparent serum was extracted and then frozen in a refrigerator at -80°C. According to serum LDH and CK detection kit (Zhejiang IKON Biotechnology Co., Ltd.) instructions, samples and reaction reagents were added. Besides, a microplate reader was utilized to measure absorbance value. The concentrations of LDH and CK samples were calculated.

Measurement of malondialdehyde (MDA) content and superoxide dismutase (SOD) activity

100 mg myocardial tissues were extracted from all groups and then added with physiological saline. Then, they were placed into a glass homogenizer and stirred into 10% myocardial tissue homogenate. Next, the myocardial tissue homogenate was centrifuged on ice at 4,000 rpm/min for 15 minutes. According to MDA and SOD kit (Nanjing Jiancheng Bioengineering Institute) instructions, xanthine oxidation and thiobarbituric acid methods were adopted to measure SOD activity and the content of MDA, which were set as the indexes for the expressions of oxygen free radical and lipid hydroperoxide in myocardial tissues (equations 1 and 2).

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\text{Activity of SOD in tissue (U/mg)} = \frac{[(\text{Control tube absorbance}) - (\text{Measure tube absorbance})] \times 50\%}{(\text{Total volume of reaction solution} \times \text{Sample loading} (\text{mL}))} / \text{Tissue protein content (mg/mL)}
\]

\[
\text{MDA content in tissue (nmol/mg)} = \frac{[(\text{Measure tube absorbance}) - (\text{Absorbance of blank tube})]}{[\text{Standard tube absorbance}] \times (\text{Absorbance of standard tube})} / \text{Tissue protein content (mg/mL)}
\]

Detection of the expressions of miR-451 and HMGB1mRNA using real-time polymerase chain reaction (PCR) method

After liquid nitrogen was extracted and quickly frozen, 100mg myocardial tissues in the ischemic area were frozen at -80°C in a refrigerator and then cut into pieces on ice. After that, the myocardial tissues were added with 1mL Trizol solution and homogenized with a glass homogenizer. Next, total RNA was extracted for quantification and purity identification. A complementary RNA (cRNA) chain was synthesized and the Real-time PCR method was used for the detection of the expressions of miR-451 and HMGB1mRNA in myocardial tissues. The results were expressed as 2^{-ΔΔCt}. The quantification expression of mRNA was calculated.

Detection of the expressions of HMGB1 and Caspase-3 proteins in myocardial tissues by Western Blotting method

After liquid nitrogen was extracted and quickly frozen, 100mg myocardial tissues in the ischemic area were frozen at -80°C in a refrigerator and then cut into pieces on ice. After that, they were added with radioimmunoprecipitation assay (RIPA) cell lysis solution containing 1mL phenylmethylsulfonyl fluoride (PMSF) and homogenized on a glass grinder. Next, HMGB1 and Caspase-3 antibodies were used to detect the contents of HMGB1 and Caspase-3. Western Blotting was employed for the analysis. GAPDH expression was set as an internal standard. Besides, relative expressions of HMGB1 and Caspase-3 were calculated.

Statistical methods

Excel was used to set up a database for all research data. SPSS21.0 was employed for data analysis. Measurement data were expressed as mean±standard deviation (X±s). Enumeration data were analyzed with χ² test and denoted by percentage (%). P<0.05 revealed that differences indicated statistical significance.

Results

Measurement of myocardial infarction area

Figure 1 compares the measurements of myocardial infarction areas in various groups. As it revealed, after 24-hour reperfusion, the myocardial infarction area induced by rat myocardial injury in the I/R + Ad-miR-451 group reduced more remarkably than that in the I/R and I/R + Ad-GFP groups (P<0.05). No apparent change was detected in the myocardial infarction area inl/I/R + Ad-asmiR-451 group (P>0.05).

Inflammatory infiltration of myocardial tissues

The inflammatory infiltration of sample tissues was analyzed, as demonstrated in Figure 2 below. It was demonstrated that myocardial cell staining was normal among the rats in the Control group. Besides, the cells were arranged regularly and tightly. The nucleus had clear boundaries without obvious infiltration of inflammatory cells and injured areas. In contrast, myocardial cells were disordered with...
different levels of swelling and blurred boundaries of the nucleus in I/R and I/R+Ad-GFP groups. Myocardial cells showed mild swelling with the infiltration of a few neutrophils in the I/R+Ad-miR-451 group. Besides, myocardial cells were disordered and the infiltration of neutrophils increased in the I/R+Ad-asmiR-451 group.

Detection of rat myocardial cell apoptosis by TUNEL method

After 24-hour reperfusion, sporadic apoptotic cells were detected in the Control group. Apoptosis indexes in the other four groups were all superior to those in the Control group (P<0.05). Versus that in I/R and I/R+Ad-GFP groups, apoptosis indexes of myocardial cells in the I/R+Ad-miR-451 group decreased (P<0.05). Myocardial cell apoptosis indexes in the I/R+Ad-asmiR-451 group indicated no statistical significance (P>0.05). These results are displayed in Figure 3 below.

Comparison of the expressions of immune cells of rats in all groups

It was found that the Control group exhibited lower expressions of CD3+, CD4+, and CD4+/CD8+ to other groups (P<0.05). Relative to those in I/R and I/R+Ad-GFP groups, CD3+, CD4+, and CD4+/CD8+ in I/R+Ad-miR-451 group decreased (P<0.05). CD8+ in I/R, I/R+Ad-GFP, I/R+Ad-miR-451, and I/R+Ad-asmiR-451 groups were all inferior to that in Control group (P<0.05). Besides, CD8+ in I/R+Ad-asmiR-451 group didn’t change significantly relative to that in I/R and I/R+Ad-GFP groups (P>0.05) (Figure 4).

Influences of miR-451 on the concentrations of serum LDH and CK

In this work, the impacts of miR-451 on the concentrations of serum LDH and CK were analyzed and summarized in Figure 5 below. After 24-hour reperfusion, the concentrations of LDH and CK in I/R and I/R+Ad-GFP groups enhanced based on those in the Control group (P<0.05). Based on those in I/R and I/R+Ad-GFP groups, LDH and CK concentrations in I/R+Ad-miR-451 group reduced (P<0.05). LDH and CK concentrations in I/R+Ad-asmiR-451 increased without statistical significance (P>0.05).

Influences of miR-451 on MDA content and SOD activity in myocardial tissues

Following a 24-hour reperfusion, MDA contents in I/R and I/R+Ad-GFP groups obviously enhanced relative to those in Control group, while SOD activity apparently decreased (P<0.05). Relative to those in I/R and I/R+Ad-GFP groups, MDA content and SOD activity improved in I/R+Ad-miR-451 group (P<0.05). MDA content enhanced while SOD activity declined in I/R+Ad-asmiR-451 group (P>0.05). The above descriptions are depicted in Figure 6.

Influences of miR-451 on the expressions of HMGB1 and Cleaved-caspase3 in myocardial tissues

After 24-hour reperfusion, the expressions of HMGB1 and Cleaved-caspase3 in I/R and I/R+Ad-GFP groups...
obviously increased based on those in Control group ($P<0.05$). Based on those in I/R and I/R+Ad-GFP groups, HMGB1 and Cleaved-caspase3 in I/R+Ad-miR-451 group remarkably reduced ($P<0.05$). HMGB1 and Cleaved-caspase3 in I/R+Ad-asmiR-451 group enhanced ($P>0.05$). The results described herein are explained in Figure 7.

Expressions of miR-451 in myocardial tissues in all groups and influences of the expressions of miR-451 in myocardial tissues on the expression of HMGB1mRNA

Relative to that in Control group, miR-451 in I/R, I/R+Ad-GFP, I/R+Ad-miR-451, and I/R+Ad-asmiR-451 groups apparently reduced ($P<0.05$). Versus that in I/R and I/R+Ad-GFP groups, miR-451 in I/R+Ad-miR-451 group notably enhanced ($P<0.05$). Versus that in I/R and I/R+Ad-GFP groups, miR-451 in I/R+Ad-asmiR-451 group notably reduced ($P<0.05$).

Versus that in Control group, HMGB1mRNA in other groups apparently improved ($P<0.05$). Relative to that in I/R and I/R+Ad-GFP groups, HMGB1mRNA in I/R+Ad-miR-451 group obviously decreased ($P<0.05$). Versus that in I/R and I/R+Ad-GFP groups, HMGB1mRNA in I/R+Ad-asmiR-451 group remarkably enhanced ($P<0.05$) (Figure 8).

Discussion

With the development and progress of vascular reca

Figure 6. Influences of miR-451 on MDA content (A) and SOD activity (B). Note: The comparison in Control group revealed *$P<0.05$. The comparison with those in I/R and I/R+Ad-GFP groups indicated $P<0.05$.

Figure 7. Expressions of HMGB1 and Cleaved-caspase3 proteins of rats in all groups. (A) Relative expressions of HMGB1 and Cleaved-caspase3 in all groups. (B) Comparison of the expressions of HMGB1 among all groups. (C) Comparison of the expressions of Cleaved-caspase3 among all groups. Note: The comparison with the expressions of HMGB1 and Cleaved-caspase3 in Control group demonstrated *$P<0.05$. The comparison with HMGB1 and Cleaved-caspase3 in I/R and I/R+Ad-GFP groups indicated $P<0.05$.

Figure 8. Expressions of miR-451 in myocardial tissues in all groups and the influences of the expressions of miR-451 in myocardial tissues on the expression of HMGB1mRNA. (A) miR-451 solubility curves. (B) miR-451 amplification curves. (C) HMGB1mRNA solubility curves. (D) miR-451 amplification curves. (E) Expressions of miR-451 in myocardial tissues in all groups. (F) Influences of miR-451 expression on HMGB1mRNA expression. Note: The comparison with the expressions of miR-451 and HMGB1mRNA in Control group showed *$P<0.05$. The comparison with miR-451 and HMGB1mRNA in I/R and I/R+Ad-GFP groups revealed $P<0.05$.
al. (24) verified that miR-451 regulated the expression of the target gene CUGBP in the form of a gene cluster in the myocardial cells of myocardial ischemia-reperfusion rat models. Consequently, the downstream anti-apoptotic gene COX was regulated and the myocardial cell apoptosis rate was reduced. In addition, pre-processing of myocardial cells with mimetic agent miR-451 could remarkably inhibit the expression of target gene Rac-1 to down-regulate the activity of oxidase NAPDH and avoid the injury of oxygen-free radicals (25-27). The research results demonstrated that the expressions of HMGB1 mRNA and its proteins both decreased after the up-regulation of miR-451 in myocardial ischemia-reperfusion rat models (P<0.05). HE staining was used for the observation of infiltration of inflammatory cells in myocardial tissues among I/R model rats. Different levels of the infiltration of inflammatory cells occurred in myocardial tissues among the rats in all groups other than Control group after the successful construction of myocardial ischemia-reperfusion models. Relative to that in I/R and I/R+Ad-GFP groups, myocardial infarction area of I/R+Ad-miR-451 group reduced (P<0.05). Based on those in Control group, LDH and CK and MDA contents in other groups decreased, while SOD activity improved, which was consistent with the study outcomes obtained by Jia et al. (28). The above finding indicated that up-regulating miR-451 expression could protect myocardial cells by down-regulating HMGB1. The protection mechanism might be the alleviation of inflammatory reactions, the reduction in oxidative stress injury, or the inhibition of cell apoptosis.

Active and effective treatment methods can prevent and treat myocardial ischemia-reperfusion, improve surgical success rate, and accelerate the rehabilitation of patients. Inflammatory reaction and immunology mechanisms are the key mechanisms of IRI. Neutrophil is the main inflammatory cell in IRI. However, the latest study shows that lymphocyte plays a significant role in IRI. Hence, early effective inhibition of lymphocyte aggregation can prevent the occurrence of IRI. According to the research results, the expressions of CD3+, CD4+, and CD4+/CD8+ in I/R, I/R+Ad-GFP, I/R+Ad-miR-451, and I/R+Ad-asmiR-451 groups were all superior to those in Control group (P<0.05). Versus those in I/R and I/R+Ad-GFP groups, CD3+, CD4+, and CD4+/CD8+ in I/R+Ad-miR-451 group reduced (P<0.05). It was indicated that I/R models were successfully constructed and the accumulation of CD3+ and CD4+ were accelerated. Up-regulating miR-451 expression could inhibit the aggregation of lymphocytes CD3+ and CD4+, reduce IRI, and protect myocardial cells.

The limitation of this research lies in the markedly upshifted HMGB1 proteins in I/R+Ad-asmiR-451 group relative to that in I/R and I/R +Ad-GFP groups without statistical significance, which may be caused by the small sample size of animals included in the experiment.

The research was aimed at investigating the regulatory mechanism of miR-451 in HMGB1 among rats with IRI and the protection of I/R rat model by the up-regulation of miR-451 expression. It was verified that miR-451 could regulate HMGB1 mRNA and the expressions of its proteins at the transcriptional level. The up-regulation of miR-451 could inhibit HMGB1 expression, reduce IRI, and protect myocardial cells, which might be achieved by improving oxidative stress injuries of cells and inhibiting cell apoptosis. The limitation of this research lies in the small sample size of experimental animals, which may result in the lack of statistical significance. In follow-up research, the sample size needs to be expanded for further investigation and verification of the accuracy of the conclusion.

References


