Aerobic exercise attenuates myocardial ischemia-reperfusion injury in rats by regulating the SIRT3/SOD2/NF-κB pathway

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Abstract

Objective: The purpose of this study was to investigate the effect of aerobic exercise on myocardial injury induced by I/ R in rats by regulating SIRT3/SOD2/NF-κB signaling pathway, and to provide theoretical guidance for clinical treatment of myocardial I/R injury.

Methods:SPF Male Sprague-Dawley(SD) rats were randomly assigned to 4 groups: Sham operation group(n=10), I/R group(n=10), Aerobic exercise group(n=10)and Aerobic exercise+κ-receptor antagonist group(Pro DTC group， n=10). The left anterior descending coronary artery(LAD) of rats was ligated and re-canalized to establish I/R rat model. Hematoxylin-eosin(HE) staining was performed to examine histological morphology in myocardial tissues of each group. The biological analysis was performed to measure cTnI、CK-MB、BNP levels in blood samples of each group. The expression levels of SOD2, TLR4, and p65 in myocardial tissues were measured by immunohistochemical assay. The influence of aerobic exercise on Beclin-1 、LC3II/I、SIRT3, TLR4, and phosphorylated p65 was measured by Western blotting.

Results: The result of histological morphology examination revealed that Aerobic exercise group exhibited integrated cardiac myofilament, less inflammatory cell infiltration, as much as significantly decreased cellular edema. Measurement of cTnI、CK-MB 、 BNP revealed that oxycodone post-treatment reduces the injury of myocardial tissues(P<0.05). Immunohistochemical staining results revealed that aerobic exercise clearly decreased the expression of TLR4 and p65, and increased the

expression of SOD2(P<0.05). Besides, Western blotting revealed that aerobic exercise down-regulated the expression of Beclin-1 、LC3II/I、TLR4 and phosphorylated p65, up-regulated the expression of SIRT3(P<0.05).

Conclusions: Aerobic exercise significantly improved myocardial I/R injury. The mechanisms may be associated with activating κ-receptor to regulate SIRT3/SOD2/NF-κB pathway.

Keywords: Aerobic exercise, Myocardial ischemia/reperfusion, autophagy， SIRT3, SOD2, NF-κB，Biochemical indicators.

Introduction

Acute Myocardial Infarction (AMI) is a common clinical disease of cardiovascular and cerebrovascular diseases, which refers to the acute coronary artery obstruction, so that part of the myocardium sustained ischemia and necrosis of an acute disease(1).The main cause of the disease is coronary atherosclerosis, with clinical symptoms of chest pain, chest tightness, hypotension, arrhythmias, and even shock.During the onset, patients are prone to anxiety, sadness, fear and other adverse emotions, which further affect the treatment effect(2).AMI is the leading cause of death and disability around the world, which is a significant public health problem(3). Nowadays, the most effective strategy to treat AMI is early coronary artery recanalization therapy to quickly restore occlusive coronary blood flow and reduce myocardial infarction area(4). However, half of the final infarct area induced by AMI results from reperfusion injury, namely myocardial ischemia/reperfusion(I/R) injury(5). There is no practical, clinically method to prevent irreversible damage caused by myocardial I/R injury(6). Therefore, alleviating myocardial I/R injury contributes to the treatment for ischemic cardiomyopathy. Myocardial I/R injury is associated with various factors, including autophagy、 oxidative stress, mitochondrial dysfunction, overload of calcium, and inflammation response. During myocardial I/R injury, the accumulation of massive intracellular reactive oxygen species (ROS) production in mitochondrion following released into cytoplasmic, which is called oxidative stress(7,8). Sequently, TLR4/NF-κB pathway was activated, which results in the release of inflammatory

cytokines, such as tumor necrosis factor α(TNF-α) and interleukin-1(IL-1), then damages myocardial cells and vascular endothelial cells indirectly. Sirtuins 3(SIRT3) is a nicotinamide adenine dinucleotide (NAD)+-dependent protein deacetylase highly expressed in mitochondrial content, involved in ROS clearance by deacetylating manganese superoxide dismutase(MnSOD, SOD2) and resulted in the protection of DNA copy numbers and genomic stabilities(9,10). Studies have shown that inhibiting the function of SIRT3 leads to mitochondrial dysfunction and loss of the inhibition of NF-κB pathway, which incurred various cardiovascular disorders, including myocardial I/R injury.

The ability of the body to complete daily activities or exercise under the condition of aerobic metabolism is called aerobic exercise capacity.In 2016, the American Heart Association ranked aerobic exercise capacity as the fifth vital sign along with respiration, blood pressure, pulse, and body temperature (11),it shows that aerobic exercise capacity is a basic indicator of physical health.AMI, cardiac ejection function is reduced, the body can cause slight activity heartbeat speed, shortness of breath, fatigue and weakness, showing movement intolerance.To improve cardiac function and aerobic capacity after AMI, early cardiac rehabilitation for AMI was initiated in the United States in the early 1960s.The five prescriptions for cardiac rehabilitation are:Drugs 、 exercise 、 smoking cessation 、 nutrition and psychology,exercise therapy is the key factor to relieve fatigue and improve aerobic exercise ability and quality of life in patients with myocardial infarction.However, the mechanism of its role is not yet fully clear.It has been reported in the literature that aerobic exercise training can enhance the autophagy activity of myocardial cells in normal rats and improve the aerobic exercise ability (12,13).However, there are few reports on the effect of aerobic exercise on autophagy activity in AMI.Therefore, in this study, the rats with AMI were taken as the research object to observe the molecular mechanism of low-intensity exercise improving myocardial injury in rats with AMI by regulating SIRT3/SOD2/NF-κB pathway and cardiomyocyte autophagy.

Materials and Methods

Animals

A total of 40 SPF Sprague-Dawley(SD) rats(SPF Biotechnology, Shanxi, China) aged 8-10 weeks, weighed 230-250g, raised in Experimental Animal Center of Yanan University. All rats were routinely raised in a clean and quiet environment, free to eat and drink water, with a feeding temperature of 25℃, relative humidity of 50%. Before the experiment, rats were fasted for 12h and drank water freely. All rats were randomly assigned to 4 groups(n=10 in each, including Sham operation group(n=10), I/R group(n=10), Aerobic exercise group(n=10)and Oxycodone+κ-receptor antagonist group(Pro DTC，n=10). There were no statistically significant differences in body, age, and weight among rats of five groups. This study was authorized by the Ethics Committee of the Experimental Animal Center at Yanan University(YADX20210128).

Specific operation procedures were as follows(14):The rats were anesthetized with 50mg/kg pentobarbitone sodium(Meck, Japan) intraperitoneally and fixed in the dorsal position on the special operating table. Limbs of rats were subcutaneously connected to SP2006 ECG analysis system(Softron, Shanghai, China) to monitor lead II. Subcutaneous local anesthesia with 2% lidocaine hydrochloride(Shen zhenzhiyao, Guangdong, China) was injected to operate a mid-neck incision for dissociating and cutting open trachea. DW-2B small animal artificial respirator(Xian, Shanxi, China) was connected with a tracheal tube for positive pressure ventilation. The thorax was opened from the third and fourth intercostal space, and the left anterior descending coronary artery (LAD) was ligated with 6-0 suture 2mm below the left auricle to induce local MI. The suture was loosened 30min after ischemia, and reperfusion was conducted for 180min. Rats in Sham operation group were performed above operation without silk suture ligation. Rat in Morphine group, Oxycodone group, and Oxycodone group+κ-receptor antagonist group were respectively injected with morphine(Shengluzhiyao, China) 1.5mg/kg, oxycodone(Hamol Umited, Britain) 0.5 mg/kg, oxycodone 0.5 mg/kg + κ-receptor antagonist Pro DTC (MedChemExpres, America) 0.5 mg/kg 5min before reperfusion. After reperfusion, rats were sacrificed. Left ventricular myocardial tissues were removed and placed in a liquid nitrogen tank. After slowly thawing and washing away the blood with PBS, tissue can be used.

Sports training program

3days after the operation, the rats performed adaptive exercises on the treadmill,treadmill speed 5～10m/min,exercise for 10 minutes/d.Endurance exercise was started one week after surgery, and the exercise program was as follows (15)： In the first week, the running speed of the platform was 10～15m/min, and the running time gradually increased from 10min/d to 30～40min/d.In the second week, the speed of the platform was 10～15m/min and increased to 50～60min/d during running.In the third to fourth week, the platform speed was 15～20m/min, and increased to 50～60 min during running,5d/week. In the myocardial infarction exercise group, 2rats were withdrawn due to disease, and the remaining 8 rats were tested for follow-up items.

Histological Examination

Myocardial tissue was removed from rats at the end of the experiment and fixed in 4% paraformaldehyde solution(Saiguobio, Guangzhou, China) for 24h. Tissues were dehydrated and embedded with paraffin blocks than cut into 5μm thick sections. After dewaxed, sections were stained with hematoxylin and eosin and blocked with neutral gum. After that, the sections were observed and photographed with a light microscope to determine the histological morphology of myocardial cells, cardiac interstitial tissues and myofilaments.

Measurements of cTnI、CK-MB and BNP

At the end of the experiment, the blood of rats was collected from the abdominal aorta in order to detect the level of cTnI、CK-MB and BNP using cTnI、CK-MB and BNP analysis kit(Beckmancoulter, America). According to the manufacture instructions, UniCel DxI 800 Acess Immunoassay System(Beckmancoulter, America) was used for this analysis.

Immunohistochemical Assay

Myocardial tissues were slowly thawed out, fixed in 4% paraformaldehyde solution, paraffin-embedded, sectioned, and routinely dewaxed to water. The sections were heated at high pressure in PBS and cooled down. 3% methanol hydrogen peroxide was added to each section to inhibit the activity of endogenous peroxidases. After blocking with bovine serum albumin(Bovogen, Australia) for 60min, sections

were incubated respectively with primary antibodies: SOD2(1:200 dilution, bio-tool technology, China), TLR4(1:50 dilution, Abcam, America), and NF-κB p65(1:200 dilution, GeneTex, America). GT anti-RB/HRP (1:200 dilution, RedpartyTech, China) was added, followed by color development with diaminobenzidine. Five samples were randomly selected from each group, and six fields were randomly selected for each sample. Photography was finally performed with magnification ×400 light microscopes. Mean optical density (MOD = IOD/Area) was used in this study for statistical analysis.

Western Blotting Assay

Myocardial tissues were resolved with RIPA Lysis Buffer(Jianfeibio, China). Then the lysis solution was centrifuged, and the supernatant was aspirated and separately sub-packaged into EP tubes. According to the instruction, mitochondrial isolation kit(Inventbiotechnologies, America) was used to isolate mitochondria. Bicinchoninic acid(BCA) kit was to detect the concentration of extracted proteins. After the loading concentration was normalized at 40 μg per well, the extracted proteins were separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Epizyme, Shanghai, China). Blocked the membranes with bovine serum albumin for 60min, then probed with corresponding primary antibodies including TLR4(1:500 dilution, Abcam, America), NF-κB p65(1:1000 dilution, GeneTex, America), Phospho-NFκB p65 (Ser536)(1:800 dilution, Affinity, America), β-Actin(1:10000 dilution, Abclonal, China) for cytoplasm and membrane proteins, and SIRT3(1:1000 dilution, Affinity, America), Beclin-1(1:5000 dilution, bio-tool technology, China) ， LC3II/I(1:5000 dilution, bio-tool technology, China) for mitochondrial proteins at 4℃ overnight. After incubation with GT anti-RB/HRP(1:1000 dilution, RedPartyTech, China) for 60min, the protein bands were visualized with enhanced ECL enlighted reagent(RedPartytech, China). β-Actin was used as a loading control for cytoplasm and membrane proteins. The ratio of target proteins to loading control was used in this study for statistical analysis.

Statistic Analysis

SPSS 23.0(IBM, American) was used for statistical analysis. The numerical data

were represented as the Mean±SD. Comparisons of more than two groups were assessed by one-way analysis of variance (ANOVA) and a post hoc LSD test. Statistical significance was assigned at *P* values less than 0.05.

Results

Effect of low intensity aerobic exercise on myocardial histology in AMI rats

In the experiment, the changes of myocardial fibers in each group were observed by HE staining.Sham operation group: the shape of myocardial fibers is clear and neatly arranged,there is neither obvious cell edema nor inflammatory cell infiltration was observed;I/R group:Myocardial fibers are clearly broken, arranged chaotically, and cell nuclei escape，obvious cell edema and dark contraction bands were observed；All these effects were reduced in Aerobic exercise group and Pro DTC group. Compared with the I/R group,Aerobic exercise group:Myocardial fiber rupture was significantly reduced and nucleus ejection decreased.The changes of myocardial fiber in Pro DTC group and I/R group were similar without significant difference （***Figure.1***）.The results suggest that Aerobic exercise can improve myocardial fiber arrangement disorder caused by I/R and reduce fiber breakage.

The effect of aerobic exercise on cTnI, CK-MB and BNP of rats in each group

The biological analysis was measured to determine the injury of myocardial tissues(***Figure.2***).Aerobic exercise treatment alleviates myocardial I/R injury measured by decrease the expression level of cTnI、CK-MB and BNP compared with other groups(*P<0.05*). Also, there is significant difference between I/R group and Aerobic exercise group (*P<0.05*),but there is no significant difference between I/R group and Pro DTC group(*P>0.05,****Figure.2A、B、C****).The results suggest that aerobic exercise can improve myocardial infarction by decreasing the levels of cTnI、CK-MB and BNP in I/R rats.

The expression of SOD2、TLR4 and p65 in myocardial tissues of each group

In order to further detect the expressions of SOD2 、 TLR4 and p65 in the myocardial tissues of each group,immunohistochemical staining and the immunohistochemical assay were used in this experiment. The results of Immunohistochemical staining showed:Compared with Sham Operation group, I/R

group rat myocardial cells all showed positive tests for SOD2 、 TLR4 and p65(***Figure.3-1B***、***3-2B***、***3-3B***、***3-4B***)，the positive expression rate was 100%.The results of Pro DTC group and I/R group were similar(***Figure.3-1D***、***3-2D***、***3-3D***、***3-4D***)， all showed signs of early myocardial ischemia with enhanced eosinophilic staining, cytoplasmic staining and uneven staining, and positive staining of focal myocardial tissue was observed.But aerobic exercise group myocardial nuclei are scattered positive(***Figure.3-1C***、***3-2C***、***3-3C***、***3-4C***)，other groups were negative（***Figure.3-1A***、***Figure.3-2A***、***Figure.3-3A***、***Figure.3-4A***）.

The results of immunohistochemical assay showed:Compared with Sham Operation group, I/R group： the expression of TLR4 and p65 was significantly increased, while SOD2 was significantly decreased，there is a significant difference between the two groups (P<0.05);Aerobic exercise group: the expression of TLR4 and p65 was significantly suppressed, while SOD2 was significantly fortified compared with I/R group (P<0.05). However, there is no significant difference between I/R group and Pro DTC group(P>0.05,***Figure.3-4A***、***3-4B***、***3-4C***、***3-4D***). The results revealed that Aerobic exercise treatment could inhibit the expression of TLR4 and p65 while promoting the expression of SOD2 in rats myocardial tissue,to attenuates the damage to the myocardial tissue.

Effects of aerobic exercise on the expression of Beclin-1 、LC3II/I、SIRT3、TLR4 and phosphorylated p65 in myocardial tissue of rats in each group

To further quantify the protein changes in SIRT3/SOD2/NF-κB pathway, Western blotting was applied in this study to detect the expression levels of Beclin-1 、LC3II/I、SIRT3, TLR4 and phosphorylated p65 in myocardial tissues of each group. The expression level of Beclin-1、LC3II/I、TLR4 and phosphorylated p65 were clearly up-regulated while the expression level of SIRT3 was down-regulated in I/R group(P<0.05,***(Figure.4A)***). However, compared with I/R group and Pro DTC group,the expression of TLR4 and phosphorylated p65 of Aerobic exercise group were inhibited while the expression of SIRT3 was enhanced (P<0.05). Moreover, Aerobic exercise can regulate these three molecules effectively and Beclin-1 expression and LC3II/Iratio of autophagy related gene (P<0.05). There is no

significant difference between I/R group and Pro DTC group(P>0.05,***Figure.4B***、***4C***、***4D***、***4E***、***4F***). Therefore, we hypothesized that Aerobic exercise alleviates myocardial I/R injury in rats by regulating SIRT3/SOD2/ NF-κB pathway and autophagy.

Discussion

Autophagy plays a key role in myocardial ischemia and myocardial injury during acute myocardial infarction.At the same time, autophagy is also considered to be“ a double-edged sword”. Early moderate autophagy is beneficial to the body and can delay the process of myocardial infarction. Late excessive autophagy is unfavorable to the body and can accelerate the process of myocardial infarction.In this experiment, aerobic exercise can down-regulate the expression of autophagy-related gene Beclin-1 and the ratio of LC3II/I, indicating that the effect of aerobic exercise in reducing ischemic cardiomyocytes is closely related to inhibiting autophagy(16).

AMI is a decrease in myocardial contractility and increased cardiac load caused by acute exacerbations or acute onset of left heart function abnormalities, leading to a sudden drop in acute cardiac output and an increase in peripheral circulatory resistance, which in turn causes pulmonary circulatory congestion and acute pulmonary congestion and pulmonary edema ,Accompanied by organ and tissue hypoperfusion and cardiogenic shock(17,18).The onset of AMI is sudden and the disease progresses very rapidly. If the treatment is not timely, it can seriously affect the prognosis of the patient. The mortality rate of the patient is extremely high. Therefore, it is of great significance to predict and evaluate the prognosis of AMI patients. (19,20).cTnI、CK-MB and BNP are all closely related to AMI. The detection of these indicators may help predict the condition and prognosis of AMI patients (21).In this experiment,the expression levels of cTnI、CK-MB and BNP in myocardial tissue of rats in aerobic exercise group were significantly decreased, suggesting that aerobic exercise can be used as an effective auxiliary method for clinical treatment of AMI.

In this experiment,the expression level of Beclin-1 、 LC3II/I 、 TLR4 and phosphorylated p65 were clearly up-regulated while the expression level of SIRT3

was down-regulated in I/R group.The above experimental results allow us to boldly make the following assumptions with scientific basis. The hypothesis diagram is as follows(Figure.5):This study provides the first evidence that aerobic exercise treatment in rats alleviates myocardial I/R injury via SIRT3/SOD2/NF-κB pathway. SIRT3 is well known as a deacetylase highly express in mitochondrial content and involved in mitochondrial oxidative stress. The positive feedback of NAD+/NADH ratio directly regulates SIRT3 activity（22）. Activation of SIRT3 diminished the phosphorylation and acetylation of FOXO3a protein then promote the deacetylation of SOD2 via SIRT3/FOXO3a/SOD pathway（23）. Besides, SIRT3 can deacetylate SOD2 directly（24）. Then, the activation of SOD2 accelerates to clear ROS contributes to the protection of mitochondrial integrity（25）. Thus, the transition of mitochondrial permeability transition pore(mPTP) to irreversible opening is reduced, which results in less ROS releases into cytoplasm and oxidative stress（26）. This results in TLR4/NF-κB pathway is inhibited. In turn, inflammatory factors released by the activation of TLR4/NF-κB pathway are reduced as well as ROS production[22]. Eventually, the inflammatory response is suppressed, myocardial cell autophagy and necrosis are reduced, and myocardial I/R inju ry is effectively alleviated.

In this study,animal experiments found that Aerobic exercise treatment alleviates myocardial I/R injury significantly. HE staining was applied to observe the histological morphology in rats of each group. The results revealed that Aerobic exercise could reduce histological changes in myocardia. Then, the biological analysis of cTnI、CK-MB and BNP were measured to detect the injury of myocardial tissues. The results revealed that Aerobic exercise could suppress the level of cTnI、CK-MB and BNP, which demonstrated that Aerobic exercise could alleviate myocardial I/R injury. Moreover, immunohistochemical assay revealed that Aerobic exercise could decrease the expression of Beclin-1 、LC3II/Iratio 、TLR4 and p65 and increase the expression of SOD2. Further, it was found that the expression of SITR3 was enhanced and the expression of phosphorylated p65 was inhibited by Aerobic exercise treatment using Western blotting. Above all, results showed that Aerobic exercise could regulate SIRT3/SOD2/NF-κB pathway to alleviate myocardial I/R injury. The current research

has some limitations. In the next step, we will use transgenic mice to further verify this experiment from cell experiments.

Declarations

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

CLR design the research and revised the article; YY and ZLL drafted the manuscript; CLR and YY collected the data and analyzed the data. CLR 、 YY and ZLL did the statistical analysis.

Ethical approval and consent to participate

This study was approved by the ethics committee of Yan'an University and in accordance with Tianjin City Construction University .

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

None

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Figure Legends

Figure.1 Representive photos of histologcal character detected by HE staining of each group (Magnification ×40, n=10).Sham operation group; I/R group; Aerobic exercise; Pro DTC group.

Figure.2 The expression level of cTnI、CK-MB and BNP were detected by biological analysis of each group (n=10). Sham operation group; I/R group; Aerobic exercise; Pro DTC group.Compared with Sham operation group,$^{\#}$ P<0.05, ▲P>0.05.compared with I/R group, *P<0.05.

Figure.3 The expression level of SOD2, TLR4 and p65 detected by Immunohistochemical Assay of each group(Magnification ×400,n=10). Sham operation group; I/R group; Aerobic exercise; Pro DTC group.Compared with Sham operation group,$^{\#}$ P<0.05, ▲P>0.05.compared with I/R group, *P<0.05.

Figure.4 The expression level of SIRT3, TLR4 and phosphorylated p65 detected by Western blotting Assay of each group. Sham operation group; I/R group; Aerobic exercise; Pro DTC group.Compared with Sham operation group,$^{\#}$ P<0.05, ▲P>0.05.compared with I/R group, *P<0.05.

Figure.5 Hypothetic diagram of aerobic exercise regulating SIRT3/SOD2/NF-κB pathway