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Effect of isoflurane on myocardial ischemia-reperfusion injury through the p38 MAPK signaling pathway

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Abstract. – OBJECTIVE: To investigate the effect of isoflurane on myocardial ischemia-reperfusion injury through the p38 mitogen-activated protein kinase (MAPK) signaling pathway.

MATERIALS AND METHODS: A total of 36 specific-pathogen-free (SPF) Sprague-Dawley rats were randomly divided into sham group (n=12), model group (n=12) and isoflurane group (n=12). In model group and isoflurane group, the myocardial ischemia-reperfusion injury model was established via the ligation of left anterior descending coronary artery (ischemia for 30 min and reperfusion for 3 h). In sham group, the left anterior descending coronary artery was not ligated, but the chest was opened and threaded using the same method. After ischemia, the rats in isoflurane group were inhaled with isoflurane. The cardiac function of rats in each group was detected before ischemia (T0) and once every 2 h after reperfusion (T1-T4) for a total of 5 times, and the cardiac function indexes included ejection fraction (EF), fractional shortening (FS), left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP). After the rats were executed, the myocardial infarction tissues were taken for hematoxylin-eosin (HE) staining and 2,3,5-triphenyltetrazolium chloride (TTC) staining to observe the morphological changes in tissues and the degrees of myocardial ischemia and infarction. The malondialdehyde (MDA) content and superoxide dismutase (SOD) activity in myocardial cells in the infarction site in each group were detected using the MDA and SOD kits. Moreover, the expression levels of related proteins in the p38 MAPK signaling pathway in myocardial cells in the infarction site were detected via Western blotting.

RESULTS: In model group, the cardiac function was significantly damaged ($p<0.01$), there was significant pathological damage in the myocardium, the area of myocardial infarction was significantly increased ($p<0.01$), the MDA content was significantly increased ($p<0.01$), the SOD activity declined obviously ($p<0.01$), and the expression levels of p-p38 and p-tau protein were significantly increased ($p<0.01$) compared with those in control group. After intervention with isoflurane, the cardiac function of rats was significantly improved ($p<0.01$), the pathological damage in myocardial tissues was alleviated, the area of myocardial infarction was reduced ($p<0.01$), the MDA content declined ($p<0.01$), the SOD activity was increased ($p<0.01$), and the expression levels of p-p38 and p-tau protein were decreased ($p<0.01$).

CONCLUSIONS: Isoflurane can, through inhibiting the p38 MAPK signaling pathway, effectively protect the cardiac function of rats from myocardial ischemia-reperfusion injury, reduce the area of myocardial infarction, alleviate the pathological damage in myocardial cells and reduce the oxidative stress response.

Key Words:
p38 MAPK signaling pathway, Isoflurane, Ischemia-reperfusion injury, SD rats.

Introduction

Cardiovascular diseases are common diseases affecting the public health, which has not only a high morbidity rate but also a high mortality rate, second only to the tumor[1]. Despite of the rapid development of medical technology currently and the continuous efforts made by medical staffs, such a problem is still not well solved. The cardiovascular diseases, including coronary heart disease and myocardial infarction, have acute onset, so they will seriously threaten the life and health of patients if not treated in time and ef-

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fectively[2,3]. The coronary atherosclerosis leads to ischemia and hypoxia in the corresponding myocardial region, resulting in myocardial apoptosis and damaging the myocardial tissues, ultimately causing irreversible damage to the heart[4]. At present, coronary atherosclerosis is usually treated with coronary artery bypass grafting[5]. Jones et al[6] studied and found that myocardial ischemia-reperfusion injury is a key factor leading to myocardial apoptosis, as well as an important cause affecting the prognosis of patients. Reducing the damage of ischemia-reperfusion to myocardial cells and lowering the degree of myocardial infarction are important measures to enhance the therapeutic effect on patients with myocardial ischemia and improve the prognosis of patients[7]. P38 mitogen-activated protein kinase (MAPK) plays an important role in ventricular remodeling and myocardial apoptosis. Inhibiting the p38 MAPK signaling pathway using specific inhibitors can significantly reduce ventricular remodeling and myocardial apoptosis[8]. Zhang et al[9] studied and found that the p38 MAPK signaling pathway is involved in regulating the myocardial apoptosis, and blocking the p38 MAPK signaling pathway, and lowering the p-tau protein expression can effectively reduce the myocardial injury caused by ischemia. Isoflurane is a kind of commonly-used anesthetic in clinic. Jeong et al[10] studied and found that the intervention with isoflurane in the rat model of middle cerebral artery occlusion in advance can effectively reduce the area of cerebral infarction. It remains unclear whether isoflurane can reduce myocardial ischemia-reperfusion injury in myocardial cells and whether the p38 MAPK signaling pathway is involved in regulating this process. In this study, the rat model of myocardial ischemia-reperfusion injury was established via ligation of coronary artery, and isoflurane was used for intervention, so as to explore the effect of isoflurane on myocardial ischemia-reperfusion injury and clarify whether the p38 MAPK signaling pathway is involved.

Materials and Methods

Main Instruments and Reagents

Isoflurane vapourizer (Dräger Vapor 2000, Telford, PA, USA), 5415R high-speed low-temperature desk centrifuge Eppendorf (EP, Hamburg, Germany), electrocardiograph (Shanghai Alcott Biotech Co., Ltd., Shanghai, China), electronic analytical balance (made by Mettler-Toledo group, Columbus, OH, USA), BL-420S biological function experiment system (Hefei Yuanming Science and Technology Co., Ltd., Hefei, China), Bio-Pro 200E gel imaging system (Hefei Yuanming Science & Technology Co., Ltd., Hefei, China), HX-100E small-animal ventilator (Wuhu Yifan Medical Equipment, Wuhu, China), microtome (Retsch, Haan, Germany), optical microscope and photography system (Germany CCD system), malondialdehyde (MDA) and superoxide dismutase (SOD) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China), hypersensitive enhancedchemiluminescence (ECL) kit (Beyotime, Shanghai, China), nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), bovine serum albumin (BSA) (Roche, Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), hematoxylin and eosin (HE) (Beijing Solarbio Science and Technology Co., Ltd. , Beijing, China), monoclonal p-p38, p38, p-tau and tau primary antibodies (purchased from BioWorld, Louis Park, MN, USA), and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1.0 g/L) (Shenzhen Jingmei Biotech Co., Ltd., Shenzhen, China).

Animal Selection and Grouping

A total of 36 healthy male adult specific pathogen-free (SPF) Sprague-Dawley rats weighing 240-260 g were purchased from the Laboratory Animal Center of Guangdong Province (Laboratory Animal Production License No.: SCXK (Guangdong, China) 2015-0002). They were fed adaptively for 1 week in the SPF animal house under the temperature of 24-26°C and humidity of 40-70%, and had free access to the water and food. The above rats were randomly divided into sham group (n=12), model group (n=12) and isoflurane group (n=12). In model group and isoflurane group, the chest was opened, the left anterior descending coronary artery (LAD) was ligated to block the myocardial blood supply, and the ligated coronary artery was loosened after a period of time to recover the blood supply, thus establishing the myocardial ischemia-reperfusion injury model (ischemia for 30 min and reperfusion for 3 h). In sham group, the LAD was not ligated, but the chest was opened and threaded using the same method. After modeling, the rats in isoflurane group were inhaled with isoflurane for 30 min, and isoflurane was then discharged for 15 min. This study was approved by the Animal Ethics Committee of Guangdong Medical College Animal Center.

Establishment of Myocardial Ischemia-Reperfusion Injury Model

The myocardial ischemia-reperfusion injury model of rats was established via the ligation of LAD. Before operation, urethane at a concentration of 20% was injected into rats (4 mL/kg) for general anesthesia, and then the rats were fixed on the anatomy plate. The body temperature was controlled using a thermostatic controller, the neck skin was cut, and the tracheal cannula was inserted and connected to the biological function experiment system to monitor the cardiac function. The chest skin was cut, and the chest wall and pericardium were also cut from the bottom up from the 5th intercostal space along the anterior median line and left mid-clavicular line to expose the heart. The LAD was threaded with the 6-0 nondestructive sutures at the lower edge of left auricle, and then ligated after resting for 30 min. After that, myocardial ischemia occurred, the epicardium turned grey white and there was ST-segment arch elevation in the electrocardiogram. The blood supply was restored after 30 min. In sham group, the chest was opened and threaded but not ligated. After operation, the chest cavity was closed, the skin was sutured, and the rats were placed on the thermal blanket and treated with anti-infective therapy using penicillin. The rats in isoflurane group were inhaled with isoflurane [1.0 MAC (1.38%)] for 30 min, and isoflurane was then discharged for 15 min.

Observation Indexes

Cardiac Function Detection

The cardiac function was monitored using the biological functional system before ligation (T0) and at 0 min (T1), 60 min (T2), 120 min (T3) and 180 min (T4) after reperfusion, and the indexes included ejection fraction (EF), fractional shortening (FS), left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP).

Detection of Area of Myocardial Infarction

Large-dose urethane was quickly intraperitoneally injected to kill the rats, and the heart was quickly taken and rinsed with normal saline. The left ventricle was separated, cut into 5 pieces perpendicularly to the longitudinal axis of the heart, counterstained with 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min and quickly fixed with 4% neutral paraformaldehyde, followed by photography using the high-definition camera. Next, the photos were imported into the computer and imported into Image Pro Plus 6.0 software, and the area of myocardial infarction was calculated. The normal region was blue, the infarction region was white, and the ischemic region was red. The infarction degree = infarction area/normal area, and the ischemia degree = ischemia area/normal area.

Myocardial HE staining

After the rats were executed, the heart was quickly taken and rinsed with normal saline. The left ventricle was separated, fixed with 4% neutral paraformaldehyde and washed with running water for 5 min after 48 h. Then the myocardial tissues were dehydrated with gradient alcohol, transparentized, soaked in paraffin and embedded to be prepared into paraffin block. Each paraffin block was sliced into 3-4 sections (5 μm thick) using the paraffin slicing machine, and baked at 70°C for 60 min, followed by deparaffinization, hydration, HE staining, dehydration, transparentization and sealing with neutral balsam. After staining, the sections were observed under an optical microscope (200×) and the lesion site was photographed using the photography system.

Determination of MDA Content and SOD Activity

After the rats were executed, the heart was quickly taken and rinsed with normal saline. The left ventricle was separated and added with pre-cooled normal saline (9 times in volume), followed by homogenization on an ice box using an ultrasonic homogenizer until there were no visible tissue fragments to the naked eyes. After the parameters of thermostatic centrifuge were adjusted, the tissues were centrifuged at 12000 rpm and 4°C for 10 min. Then, the supernatant was taken to detect the MDA content and SOD activity in heart tissues in each group using the MDA content and SOD activity kits strictly according to the instructions.

Detection of Expression of Related Proteins in Myocardial Tissues via Western Blotting

After the rats were executed, the heart was quickly taken and rinsed with normal saline. The left ventricle was separated and added with pre-cooled cell lysis buffer (9 times in volume), followed by homogenization on the ice box using the ultrasonic homogenizer until there were no visible tissue fragments to the naked eyes. After the

parameters of thermostatic centrifuge were adjusted, the tissues were centrifuged at 12000 rpm and 4°C for 10 min. The supernatant was taken as the total protein. After the protein was quantified using the BCA kit and the loading buffer system at an equal concentration was prepared, the protein was inactivated at 95°C, subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Then the target protein band was cut, sealed with 5% skim milk powder for 1 h, incubated with the corresponding primary antibodies (rabbit anti-rat p38, rabbit anti-rat p-p38, rabbit anti-rat tau, rabbit anti-rat p-tau and rabbit anti-rat GAPDH) at 4°C overnight, washed with TBST for 3 times, incubated again with the goat anti-rabbit secondary antibody at room temperature for 2 h and washed again with Tris-buffered saline and Tween-20 (TBST) for 3 times, followed by ECL color development and Kodak film exposure. Finally, the gray value was read and analyzed.

Statistical Analysis

The data in this study were expressed as mean ± standard deviation. Statistical Product and Service Solutions (SPSS) 20.0 software (SPSS Inc., Chicago, IL, USA) was used for the data processing. Analysis of variance was used for the comparison among groups. Bonferroni's method was adopted for the pairwise comparison in the case of homogeneity of variance, while Welch's method was adopted in the case of heterogeneity of variance. $p<0.05$ suggested that the difference was statistically significant.

Results

Comparison of Cardiac Function

The cardiac function of rats in each group was recorded after myocardial ischemia-reperfusion injury. As shown in Table I, the cardiac function significantly declined, and LVEDP was significantly increased ($p<0.01$), while LVSP, FS and EF were significantly decreased ($p<0.01$) in model group compared with those in sham group. Compared with those in model group, the cardiac function in isoflurane group was significantly improved ($p<0.01$).

Comparison of Area of Myocardial Infarction

The area of myocardial infarction in each group was detected via TTC staining. As shown in Figure 1, the area of myocardial infarction was

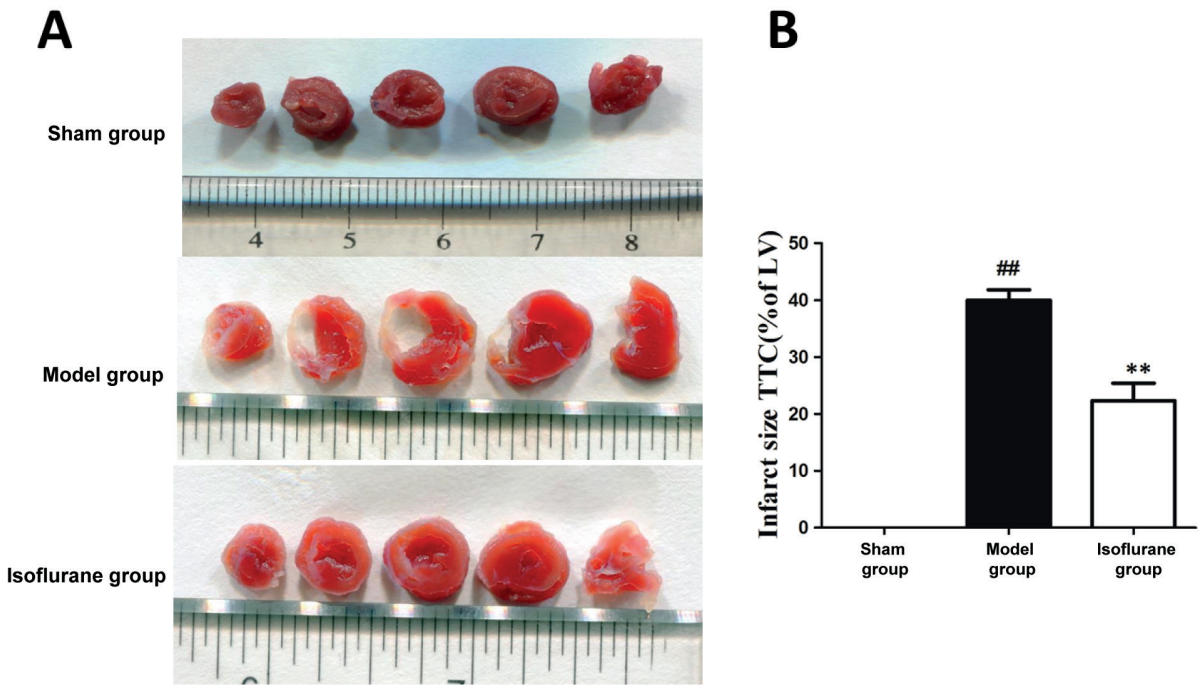

Figure 1. Comparison of area of myocardial infarction. A, TTC staining results, B, area of myocardial infarction, ##p<0.01 vs. sham group, **p<0.01 vs. model group.

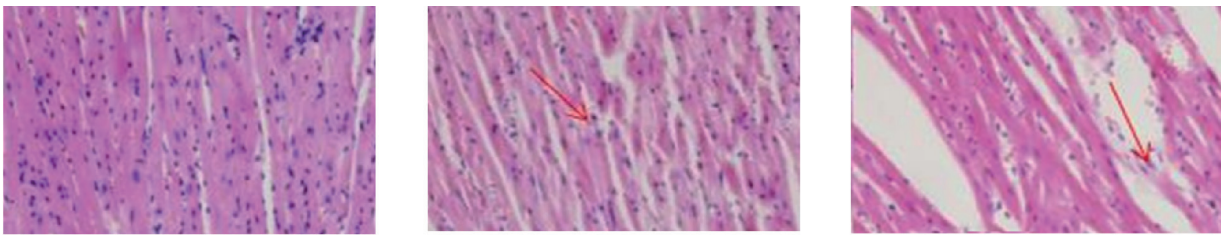

Figure 2. Morphological changes after myocardial ischemia-reperfusion injury in each group.

significantly increased after myocardial ischemia-reperfusion injury in model group compared with that in sham group (p<0.01), and it was significantly smaller in isoflurane group than that in model group (p<0.01).

Morphological Changes in Myocardial Tissues

After HE staining, the myocardial tissues were examined and photographed under the microscope (200×). The results revealed that in sham group, there were no abnormalities, the myocardial fibers were arranged orderly without fractures and enlarged necrotic gap, and the nucleus were fusiform or elliptical. In model group, the myocardial necrosis could be observed, and there were dissolution and fractures of myocardial fibers with obviously enlarged gap. In isoflurane group, the conditions were significantly improved, and there were occasionally fractures, dissolution and necrosis of myocardial fibers with enlarged gap (Figure 2).

Changes in MDA Content and SOD Activity in Myocardial Tissues

The changes in MDA content and SOD activity after myocardial ischemia-reperfusion injury were compared among groups. The results manifested that in model group, the SOD activity in myocardial tissues obviously declined (p<0.01), while the MDA content was obviously increased (p<0.01) compared with those in sham group. Compared with those in model group, the MDA content obviously declined (p<0.01), while the SOD activity was significantly enhanced in isoflurane group (p<0.01) (Table II).

Table I. Comparison of cardiac function of rats after ischemia-reperfusion injury.

Group	EF (%)	FS (%)	LVEDP (mmHg)	LVSP (mmHg)
Sham group	81.51±6.42	41.81±4.93	7.33±1.32	119.91±6.49
Model group	46.24±4.73%	22.81±1.43%	11.37±2.31%	102.71±4.42%
Isoflurane group	61.45±5.34#	34.89±3.56#	9.12±1.99#	113.76±5.78#
F	6.95-16.24	5.72-14.78	4.34-7.98	5.43-8.99
p	<0.01	<0.01	<0.01	<0.01

Note: %p<0.01 vs. sham group, #p<0.01 *vs.* model group.

Table II. Changes in MDA content and SOD activity in tissues after myocardial ischemia-reperfusion injury.

Group	MDA content (pg/mL)	SOD activity (U/mL)
Sham group	0.19±0.11	35.13±1.98
Model group	3.52±0.41%	11.65±0.48%
Isoflurane group	1.98±0.76#	23.68±1.13#
F	4.51-5.11	2.51-5.25
p	<0.01	<0.01

Note: %p<0.01 *vs.* sham group, #p<0.01 *vs.* model group

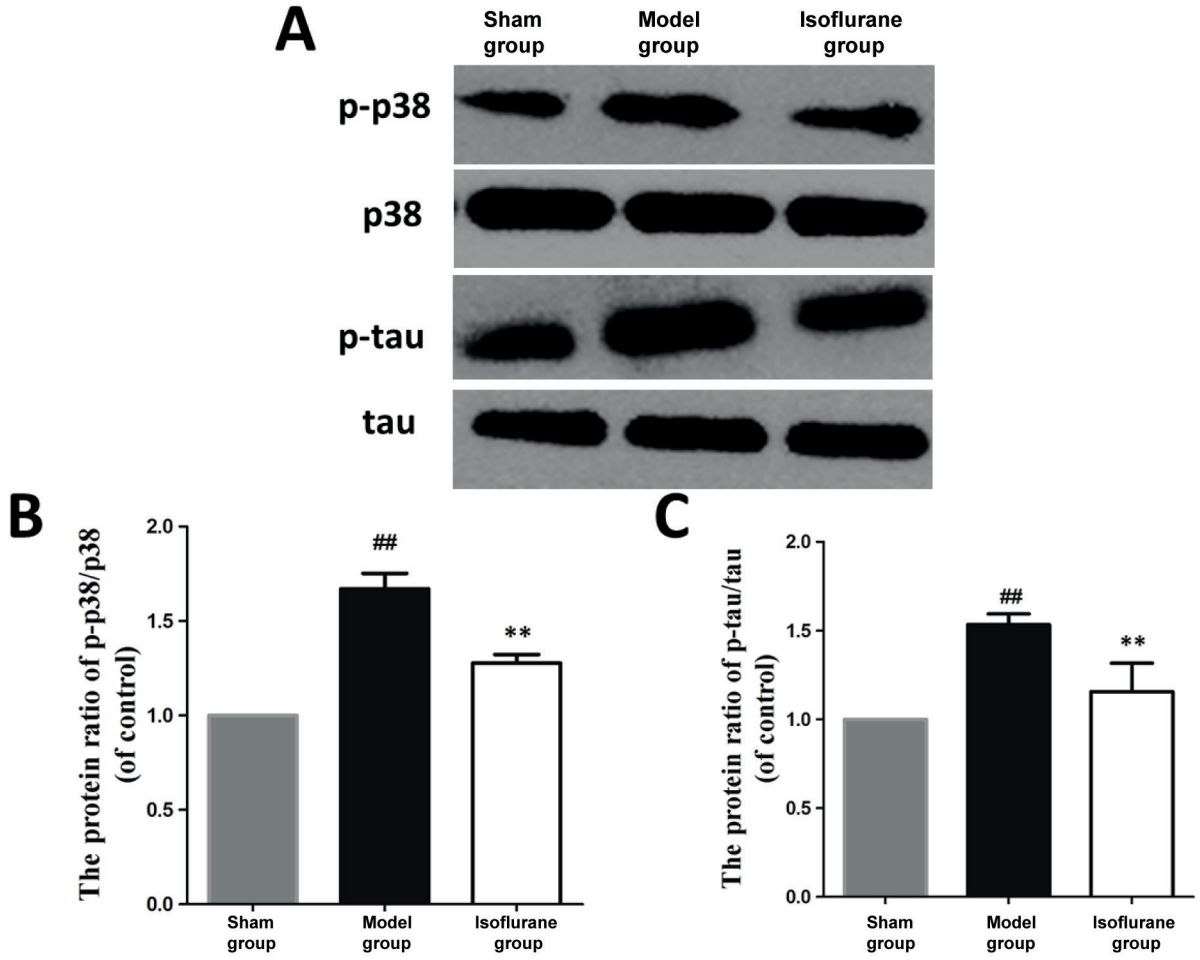

Figure 3. Changes in expression levels of p38 MAPK pathway-related proteins. ***A,*** Protein band, ***B,*** statistical graph of p-p38 protein, ***C,*** statistical graph of p-tau protein, $^{\#\#}p<0.01$ vs. sham group, $^{**}p<0.01$ vs. model group.

Changes in Expression Levels of p38 MAPK Pathway-Related Proteins

The changes in expression levels of p38 MAPK pathway-related proteins in myocardial tissues in each group were detected via Western blotting. As shown in Figure 3, the expression levels of p-p38 and p-tau in myocardial tissues were remarkably higher in model group than those in sham group ($p<0.01$), and they were remarkably lower in isoflurane group than those in model group ($p<0.01$).

Discussion

Currently, there are a number of studies on myocardial ischemia-reperfusion injury, but its mechanism remains unclear, and reliable research evidence is still lacked. According to the existing research results, the mechanism of myocardial ischemia-reperfusion injury is complicated, which is affected by a variety of factors, including metabolic disorders, release of a large number of oxygen free radical, inflammatory response and calcium overload. The cardiac function of patients will be greatly reduced and the clinical therapeutic effects (such as bypass surgery, interventional therapy and thrombolysis) will be limited once ischemia-reperfusion injury occurs[11]. The p38 MAPK signaling pathway in the phosphorylation cascade is involved in regulating a variety of biological behaviors, which involves various cytokines and enzymes[12]. Studies have demonstrated that the activation of this pathway will lead to myocardial apoptosis and necrosis, activate neutrophils, increase expression levels of cytokines and adhesion molecules, and phosphorylate cytoplasmic protein and reverse

transcription factors, thereby aggravating the myocardial ischemia-reperfusion injury[13]. Therefore, the activity of the p38 MAPK pathway can be reduced via appropriate intervention to inhibit or even reverse the myocardial ischemia-reperfusion injury, thus restoring the damaged cardiac function and healing the patients. It has been proved in studies that isoflurane can help restore the cardiac function of patients. In this study, the above viewpoint was demonstrated, and it was found that isoflurane could effectively restore the cardiac function of rats with ischemia-reperfusion injury, improve the pathological changes in myocardial cells, reduce the inflammatory response, lower the degrees of myocardial infarction and ischemia and improve the cardiac function indexes (EF, FS, LVSP and LVEDP). The pathological changes caused by myocardial ischemia-reperfusion injury mainly include the fracture, disordered arrangement and enlarged gap of myocardial fibers, and myocardial dissolution and necrosis, which can lead to heart failure, cardiac dysfunction and abnormal function of myocardial cells[14]. In this study, it was found that after inhalation of isoflurane after myocardial ischemia, the degrees of myocardial ischemia and infarction were lower than those in model group, indicating that the intervention with isoflurane can effectively inhibit the myocardial ischemia-reperfusion injury, and reduce the degree of myocardial infarction. MAPK existing in human body possesses important physiological effects and participates in a variety of physiological processes, including cell proliferation and gene expression, which is closely related to the cell apoptosis, survival, differentiation and growth, so it has attracted much attention of researchers[15]. MAPK can be activated by inflammatory factors, hypertonic environment, heat shock and ischemia-reperfusion injury, in which p-p38 and p38 are one of the important signaling pathways. Studies have demonstrated that the activation of this pathway can aggravate the body's damage[16]. However, a small number of studies also argue that the pathway has a certain protective effect[17,18]. The results of this study showed that the expression levels of p-p38 and p-tau in model group were significantly increased, while they declined significantly after treatment with isoflurane, suggesting that the intervention with isoflurane in myocardial cells during ischemia-reperfusion injury can effectively inhibit the p38 phosphorylation and protect the myocardium from injury. Studies have manifested that the oxidative stress response in the body has an inseparable relation with the ischemia-reperfusion injury. Ischemia-reperfusion injury can be aggravated by the oxidative stress response in the body, and the most commonly used and sensitive indexes reflecting the oxidative stress response are MDA and SOD[19]. The oxygen free radical scavenging in the body depends largely on SOD, and the SOD activity directly affects the free radical scavenging ability. Free radical scavenging in the body can help avoid oxidative damage in the body and aggravation of ischemia-reperfusion injury. In the case of oxidative stress injury in the body, the lipid peroxidation of membrane is also enhanced, further aggravating the injury. The end product of this process is MDA, whose content will increase under the oxidative stress injury in the body, thus seriously damaging cells and producing free radicals[20]. In this study, it was found that the intervention with isoflurane after myocardial ischemia significantly enhanced the SOD activity and reduced the MDA content, indicating that the application of isoflurane can not only enhance the free radical scavenging ability, but also inhibit the lipid peroxidation of membrane, lower the production of free radicals and effectively reduce the oxidative stress injury in the body, ultimately alleviating the ischemia-reperfusion injury.

Conclusions

We showed that, isoflurane can, through inhibiting the p38 MAPK signaling pathway, effectively protect the cardiac function of rats from myocardial ischemia-reperfusion injury, reduce the area of myocardial infarction, alleviate the pathological damage to myocardial cells and reduce the oxidative stress response.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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