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Alamandine protects rat from myocardial ischemia-reperfusion injury by activating JNK and inhibiting NF-κB

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether alamandine plays a protective role in myocardial ischemia-reperfusion injury (IRI) by activating C-Jun N-terminal kinase (JNK) and inhibiting the expression of key proteins in nuclear factor-kappa B (NF-κB) pathway.

MATERIALS AND METHODS: Twenty-four Sprague Dawley (SD) rats were numbered and divided into three groups using a random number table, including sham operation group (Sham group), myocardial ischemia-reperfusion injury model group (IRI group), and alamandine pretreatment and myocardial IRI treatment (alamandine group), with 8 SD rats per group. Myocardial tissues were collected from each group. The damage of myocardial tissue was detected using hematoxylin-eosin (H&E) and Masson staining. Finally, the expression levels of JNK and NF-κB pathway-related proteins in myocardial tissue of each group were detected by Western blot.

RESULTS: Compared with the IRI group, the alamandine treatment significantly improved cardiac function indicators induced by myocardial IRI in rats, including HR, MAP, LVESP, LVEDP, LVdp/dtmax, and -LVdp/dtmax. In addition, the pathological changes and cell damage of myocardium after alamandine pretreatment were significantly alleviated. At the same time, alamandine can significantly reduce the levels of TNF-α, IL-1β, IL-6, and NO. Finally, Western blot analysis showed that alamandine pre-treatment can protect cardiomyocytes from myocardial IRI by activating JNK phosphorylation and inhibiting the expression of related proteins in the NF-κB signaling pathway.

CONCLUSIONS: Alamandine can protect rat from myocardial IRI via activating JNK phosphorylation and inhibiting NF-κB signaling pathway to reduce the inflammatory response.

Key Words
Alamandine, NF-κB/TNF-α signaling pathway, Myocardial ischemia-reperfusion injury.

Introduction

With the changes in people's lifestyle and diet, the incidence of coronary atherosclerotic heart disease (CHD) is increasing, becoming one of the most serious diseases that endanger human health[1-4]. Currently, more than 7 million people die of ischemic heart disease worldwide each year, and nearly half die of acute myocardial infarction (AMI)[5,6]. The incidence of AMI is increasing year by year, which seriously endangers health. It is an important public health problem at present, and its prevention and treatment is one of the most difficult tasks in front of medical personnel[6,7]. The current major intervention for the treatment of acute myocardial infarction is the rapid opening of infarct-related arterial reperfusion myocardium by percutaneous coronary intervention (PCI), drug thrombolysis or coronary artery bypass grafting (CABG)[8,9]. However, in clinical practice, we found that in the treatment of acute ischemic myocardial reperfusion, these treatments are accompanied by ischemia-reperfusion (IR) damage, which may affect the therapeutic effect, and may have serious adverse consequences. Therefore, it is quite urgent to reduce myocardial IR injury in the clinical treatment strategy[10,11]. Effective application of drug pretreatment or post-reperfusion drug intervention to effectively reduce ischemia-reperfusion injury is a hot drama in cardiovascular research at home and abroad[12].

JNK, also known as C-Jun N-terminal kinase or stress-activated protein kinase (SAPK), is a member of the mitogen-activated protein kinase (MAPK) superfamily. JNK is a serine/threonine kinase located in the cytoplasm of cells with a specific functional region (Thr-Pro-Tyr). It could encode JNK1, JNK2, and JNK3. JNKI and JNK2 are present in various tissues of the body, while JNK3 is mainly found in

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tissues such as myocardium, heart, and testis[13,14]. Nuclear factor-kappa B (NF-κB) is a protein that specifically binds to the κB sequence on many gene promoters to initiate transcriptional function[15-17]. It plays an important role in immune response, inflammatory response, cell differentiation and growth, cell adhesion and apoptosis, so NF-κB is involved in the initiation of inflammatory response[17].

Alamandine is an oral administration of DPP-4 inhibitor, which can enhance the secretion of insulin by promoting islet B cells viability and inhibit the secretion of glucagon, meanwhile it could reduce the degradation of GLP-1, thereby improving the blood glucose of T2DM patients and reducing the occurrence of hypoglycemia without weight gain[18,19]. In recent years, the influence of alamandine on the occurrence and development of organ damage caused by human poisons and ischemia has attracted the attention of scholars in various fields at home and abroad[19,20]. Therefore, this work intends to observe the effect of alamandine on myocardial IR through administration in a rat model of myocardial ischemia-reperfusion injury, and to explore the intervention mechanism of alamandine on the ischemia-reperfusion injury, hoping to provide an experimental basis for anti-myocardial ischemia-reperfusion injury of alamandine.

Materials and Methods

Reagents and Instruments

Alamandine was obtained from Shanghai Pharmaceutical Co., Ltd. (Shanghai, China). Hematoxylin-eosin (H&E) and Masson dyes were products of Nanjing Jiancheng Co., Ltd. (Nanjing, China). Ordinary optical microscope was from Nikon Corporation (Tokyo, Japan). Computer with Image J software was provided by Lenovo (Beijing, China). Paraffin-embedded slicer was obtained from Shenzhen Huiwo Technology Co., Ltd. (Shenzhen, China). The digital gel imaging system was from Bio-Rad (Hercules, CA, USA).

Animals and Experimental Protocol

This investigation was approved by the Animal Ethics Committee of Xi'an Medical University Animal Center. 24 clean-grade Sprague Dawley (SD) male rats weighing 180-220 g were housed in a temperature control room with 12 hours on day and 12 hours at night, and were free to eat and drink. Rats were housed one week before modeling. They were fasted overnight before the experiment and were free to drink water. The experimental animals were anesthetized with 20% urethane at a dose of 1 g/kg and were then fixed, with the skin prepared in the neck and chest. The skin of the neck was pulled and cut; then, the trachea was separated, opened and inserted with a tube connected to the animal ventilator. The skin was cut along the midline of the sternum from the flat line of the sterno-lock joint to the top of the xiphoid. The chest muscles were bluntly separated, and the 2nd, 3rd, and 4th ribs of the experimental animals were cut at the left edge of the sternum. The incision was opened with a chest sac, and the heart was cut and exposed. A needle holder was used to hold a small curved needle to wear a 2/0-T line about 2 mm below the left circumflex for ligation. After surgically ligating the coronary artery, the ST-segment of the electrocardiogram was markedly elevated or depressed, suggesting that the experimental model was successful. During myocardial reperfusion, tissue hyperemia occurred in the local myocardium, and ST segment fell back, which was a successful reperfusion. SD rats were randomly divided into sham operation group (Sham group), myocardial ischemia-reperfusion injury group (IRI group), and Alamandin treatment group (alamandine group), with 8 rats in each group.

1) Sham operation group (Sham group): the experimental animals were only threaded around the root of the left-handed branch of the coronary artery about 2 mm, but not ligated.
2) Myocardial ischemia-reperfusion injury group (IRI group): the experimental animals were threaded around the root of the left-lateral branch of the coronary artery about 2 mm, and after ligation for 30 min, reperfusion for 60 min.
3) Myocardial ischemia-reperfusion injury and alamandine treatment group (alamandine group): the experimental animals were threaded around the root of the left circumflex artery of the coronary artery about 2 mm. After ligation for 30 min, reperfusion for 60 min, 20 mg/kg alamandine was administered intravenously.

Before and after the establishment of the IRI model, echocardiography was used to detect left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS). All rats were sacrificed by anesthesia after taking blood from the internal iliac vein. The changes in body mass and daily activities of the rats were observed throughout the administration period. Thereafter, blood samples were taken from the abdominal aorta at 72 hours after surgery, and the animals

were sacrificed. Heart tissue (apical and interventricular septum) was taken for the correlation test.

Histological Examination

The transverse paraffin sections of SD rats were made, and H&E staining was performed. At the 72 h postoperatively, the rats in each treatment group were sacrificed by carbon dioxide asphyxiation, and the heart was washed with phosphate buffer to cut transversely in the middle of the largest infarct area. It was fixed overnight with 4% paraformaldehyde solution. On the second day after fixation, the heart was dehydrated and longitudinally paraffin-embedded. After the embedding was completed, each specimen was cut into sections of about 4 um thickness for H&E staining. After the staining was completed, the sheets were preserved, and the myocardial structure and fibrosis range were observed by ordinary light microscopy and photographed. Histological changes were assessed by semi-quantitative detection of myocardial tissue damage necrosis. The evaluator randomly took 5 fields of view for each specimen in a blinded manner and averaged the damage score for each specimen.

TUNEL (Terminal-Deoxynucleotidyl Transferase Mediated Nick End Labeling) Assay

The apoptosis of rat cardiomyocytes was determined using the TUNEL method. The rat heart was washed with phosphate buffer, fixed in 4% paraformaldehyde solution overnight, embedded in paraffin and sectioned by 4 μm. DUN staining was used to observe the nuclear morphology of the section, and fluorescein isothiocyanate (FITC)-labeled Tunel positive cells were observed under a fluorescence microscope. The five-point sampling method captured 5 high power microscopes (400 times) and counted green cells (Tunel apoptotic cells) and blue 4',6-diamidino-2-phenylindole (DAPI) total cells. The number of green cells/blue cells was the apoptotic index. The final apoptotic index is the average of 5 fields of view.

Hemodynamic Analyses

A 20-gram heparin-filled catheter (Spacelabs Medical, Inc., Redmond, WA, USA) was inserted from the right carotid artery into the left ventricle and connected to a pressure transducer (Biolap 420F, Taimeng, Chengdu, China) for hemodynamics parameter measurement. Heart rate (HR), mean arterial pressure (MAP), left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVEDP), and left ventricular pressure maximal increase or decrease rate (±dP/dtmax) were recorded for each treatment group.

Cytokine Measurement by Enzyme-Linked Immunosorbent Assay (ELISA)

After homogenization of the heart tissue of each treatment group, the levels of TNF-α, IL-1β, IL-6, and NO in the heart homogenate were determined using an ELISA kit according to the manufacturer's instructions (LifeSpan BioSciences, Inc., Seattle, WA, USA). The concentration of each group of cytokines was quantitatively determined with reference to a standard curve.

Western Blot

Cells were lysed by protein lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 x g for 15 minutes at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Il, USA). The extracted proteins were separated using a 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride membrane. Western blot analysis was performed according to standard procedures. The immunoblots were incubated with the primary antibodies p-p38, p38, p-ERK, ERK, p-JNK, JNK, NFATc1, NF-κB, and internal reference GAPDH, H3, and then incubated with the secondary antibodies anti-mouse and anti-rabbit, which were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

Data was processed using the Statistical Product and Service Solutions (SPSS) 22.0 program (IBM, Armonk, NY, USA) and were expressed as mean ± standard deviation. $p<0.05$ was considered to be statistically significant. The continuity variables were analyzed by the *t*-test, and the categorical variables were analyzed using the χ^2-test or Fisher's exact probability method.

Results

The Effect of Alamandine on the Electrocardiogram of the Model Rats Induced by Myocardium Ischemia-Reperfusion Injury

At the beginning of the experiment, there was no significant difference in heart rate, QRS complex voltage and Q-T interval between the groups.

Table I. The effect of alamandine on the electrocardiogram of the model rats induced by myocardium ischemia-reperfusion injury.

Group	Heart rate/min	QRS/mv	Q-T interval/ms
Sham	464.00±14.98	0.596±0.065	61.88±7.28
IRI	373.00±37.52*	0.418±0.075*	71.56±6.93*
Alamandine	399.80±26.46#	0.529±0.089#	64.84±3.98#

Data are expressed as mean±SEM. n=8 in each group.
*p<0.05 *vs.* Sham, #p<0.05 *vs.* IRI.

Compared with Sham group, the heart rate of the IRI model group was significantly slowed down, the QRS complex voltage was significantly decreased, and the Q-T interval was significantly prolonged (p<0.05). Under several cases, arrhythmias occurred. In addition, compared with the IRI group, the heart rate of the alamandine group and the Sham group increased, the QRS complex voltage increased significantly, and the QT interval was significantly shorter (p<0.05). Besides, the heart rate of the alamandine group increased more significantly. The increase of QRS complex voltage was more pronounced, and the QT interval shortened more significantly (Table I).

Alamandine Improved Hemodynamic Function in Ischemia-Reperfusion Injury

Table II showed the process of hemodynamics during each experimental period. At the beginning of the experiment, there was no significant difference in systemic hemodynamics between the groups before coronary occlusion (p>0.05). IRI resulted in decreased systolic function of LVSP and dP/dtmax, and diastolic dysfunction showed by LVEDP and -dP/dtmax, which was significantly different from baseline values in the IRI model group and the alamandine group (p<0.05). Therefore, cardiac function recovery in the alamandine group, including LVSP, dP/dtmax, and -dP/dtmax were higher than those in the IRI group.

Table II. Hemodynamics during the experiment investigating the effect of alamandine on myocardium ischemia-reperfusion injury.

Variable	Baseline	After reperfusion
LVSP (mmHg)		
IRI	132±3	111±5*
Alamandine	134±4	124±2#
LVEDP (mmHg)		
IRI	-12±4	1±3*
Alamandine	-13±2	-4±3
dp/dtmax (mmHg/s)		
IRI	4799±213	2510±279*
Alamandine	4694±231	3678±243#
-dp/dtmax (mmHg/s)		
IRI	-3888±219	-2734±179*
Alamandine	-3977±333	-3597±211#

Data are expressed as mean±SEM. LVSP=left ventricular systolic pressure; LVEDP=left ventricular end-diastolic pressure; ±dP/dtmax=maximum rate of increase/decrease in left ventricular pressure. n=8 in each group. *p<0.05 *vs.* baseline, #p<0.05 *vs.* after reperfusion.

Pretreatment With Alamandine Improved Myocardial Function in Ischemia-Reperfusion Injury

Quantitative analysis showed that compared with Sham group, the expression of CK-MB and LDH in peripheral blood of IRI rats was significantly increased (p<0.05), indicating a successful model had been constructed. In addition, compared with the IRI model, the above indicators of the alamandine protection group were significantly reduced. The alamandine protection group was statistically different from the other two groups; however, the expression level of the alamandine protection group did not return to normal levels (Figure 1A and 1B).

Alamandine Preserved Histologic Architecture and Mitigates Neutrophil Infiltration

The myocardial tissues of SD rats in each treatment group were stained by H&E and Masson. It was found that there were more mucus exudation and inflammatory cell infiltration in the IRI model group than in the Sham group, suggesting a more disordered state. However, alamandine treatment protected rats from myocardial damage than those in the IRI model group. The normal microstructure still existed, and few of the blood vessels showed pink protein mucus exudation, besides, only a small amount of inflammatory cell infiltration existed. Myocardial cell edema and infiltration of inflammatory cells were not evident (p<0.05) (Figure 2A, 2B, 2C). These results demonstrated that alamandine preserved histologic architecture and mitigates neutrophil infiltration after IRI.

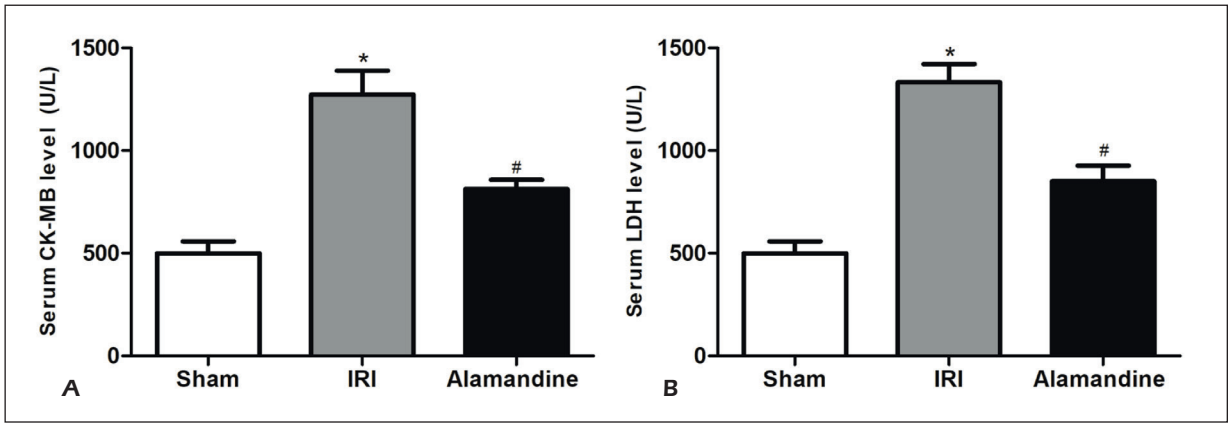

Figure 1. Alamandine pretreatment protects cardiomyocytes against myocardial ischemia-reperfusion injury. **A**, Serum CK-MB levels in the Sham group (n=8), IRI group (n=8), and alamandine treatment group (n=8). **B**, LDH expression level in serum samples collected from each treatment group. The data were expressed as mean ± SD, *indicates a significant difference compared with the Sham group (p<0.05), #indicates a significant difference compared with the IRI group (p<0.05).

Alamandine Decreased the Production of Apoptotic Cells

To assess whether alamandine can improve myocardial ischemia-reperfusion injury, we evaluated cell apoptosis by TUNEL staining and found that IRI significantly increased myocardial apoptosis, whereas alamandine administration significantly reduced apoptotic cell formation in myocardial injury (Figure 2A-2D).

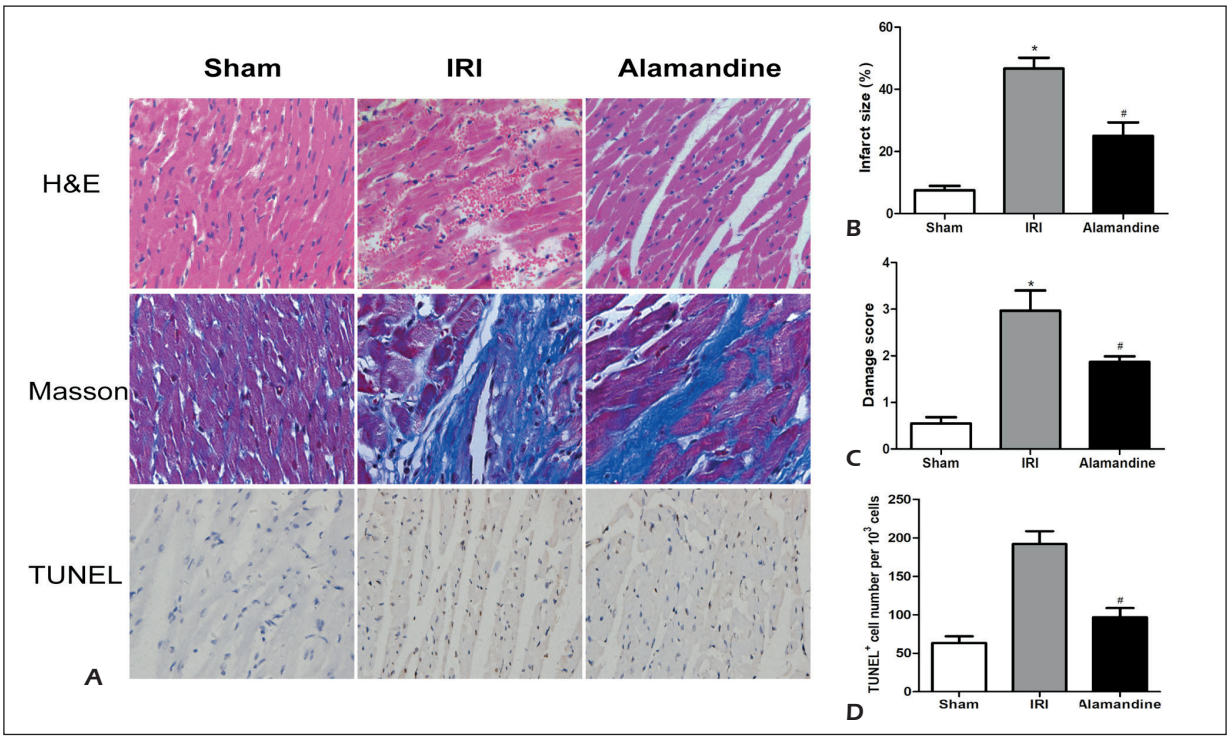

Figure 2. Alamandine protects the ischemic histological structure and reduces inflammatory infiltration. **A**, Myocardial sections of the Sham group, the IRI group, and the alamandine group were subjected to H&E staining, Masson staining, and TUNEL immunostaining (magnification × 200). **B**, Assessment of the lesion area of myocardial sections of each treatment group. **C**, Assessment of lesion scores of myocardial sections of each treatment group. **D**, TUNEL positive cells per 103 germ cells of testes in the three groups. The data were mean ± SD, *indicates a significant difference compared with the Sham group (p<0.05), #indicates a significant difference compared with the IRI group (p<0.05).

Alamandine Alleviated the Secretion of Inflammation Cytokines in Myocardium Ischemia-Reperfusion Injury

To investigate the effects of alamandine on myocardial ischemia-reperfusion inflammatory factors induced by IRI, the serum of rats after IRI and alamandine protection group were obtained, and the content of related cytokines including TNF-α, IL-1β, IL-6, and NO were detected. The results showed that alamandine significantly reduced the levels of TNF-α, IL-1β, IL-6, and NO (Figure 3A-3D).

Alamandine Activated JNK Signal Pathway Induced by the Myocardium Ischemia-Reperfusion Injury

To investigate whether alamandine induced myocardial ischemia-reperfusion injury caused by IRI through JNK signaling pathway, the myocardial tissues of rats in Sham group, IRI model and alamandine protection group were taken, and then the tissue proteins of each treatment group were extracted. As shown in Figure 4, Western Blot results indicated that alamandine pretreatment notably increased the expression of p-p38, p-ERK, and p-JNK.

Alamandine Inhibited NF-ÐB Signal Pathway Induced by the Myocardium Ischemia-Reperfusion Injury

To investigate whether alamandine improved myocardial ischemia-reperfusion injury through NF-κB signaling pathway, the myocardial tissues of rats in Sham group, IRI model, and alamandine protection group were taken, and then, the nuclear and cytoplasmic proteins of each treatment group were extracted. As shown in Figure 5, Western blot results showed that the expression of NF-kB and NFATc1 in myocardial tissue of IRI was significantly higher than that in Sham group. However, the addition of alamandine significantly reduced NF-kB, NFATc1 nuclear protein expression levels; and its cytoplasmic protein changes were not evident. In sum, alamandine inhibited NF-κB signal pathway induced by the myocardium ischemia-reperfusion injury.

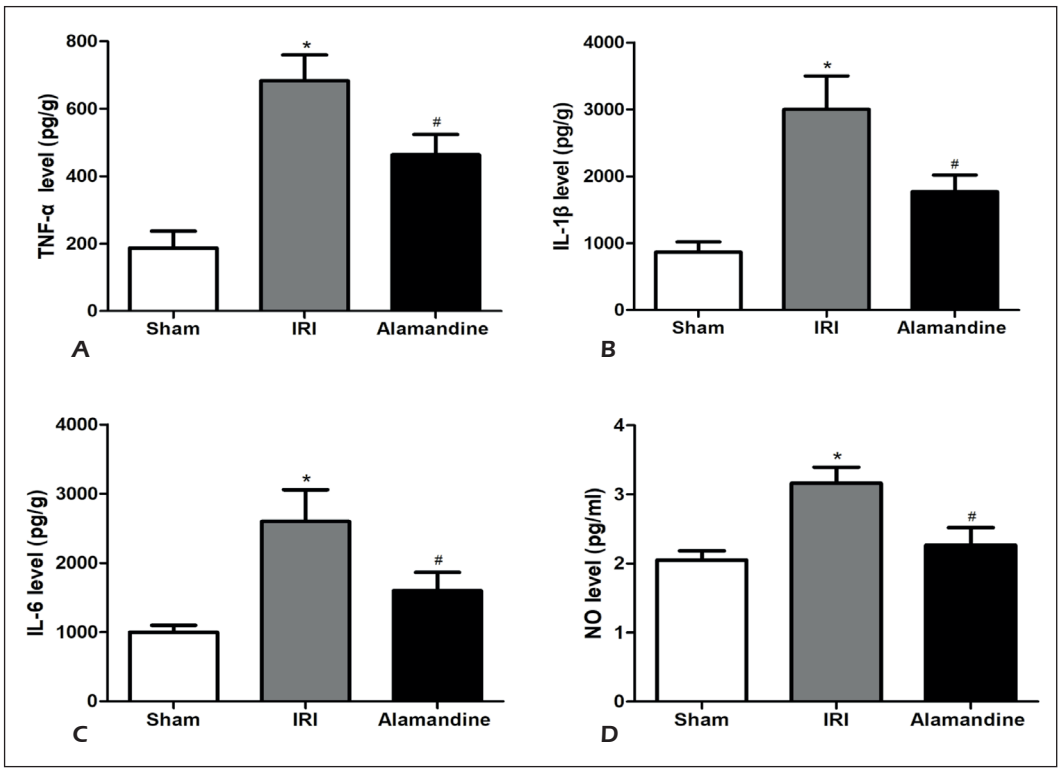

Figure 3. Alamandine reduces the secretion of inflammatory factors induced by myocardial ischemia-reperfusion injury. **A**, TNF-α content in myocardial tissue of each treatment group. **B**, IL-1β content in myocardial tissue of each treatment group. **C**, IL-6 content in myocardial tissue of each treatment group. **D**, NO content in myocardial tissue of each treatment group. The data were mean ± SD, *indicates a significant difference compared with the Sham group ($p<0.05$), #indicates a significant difference compared with the IRI group ($p<0.05$).

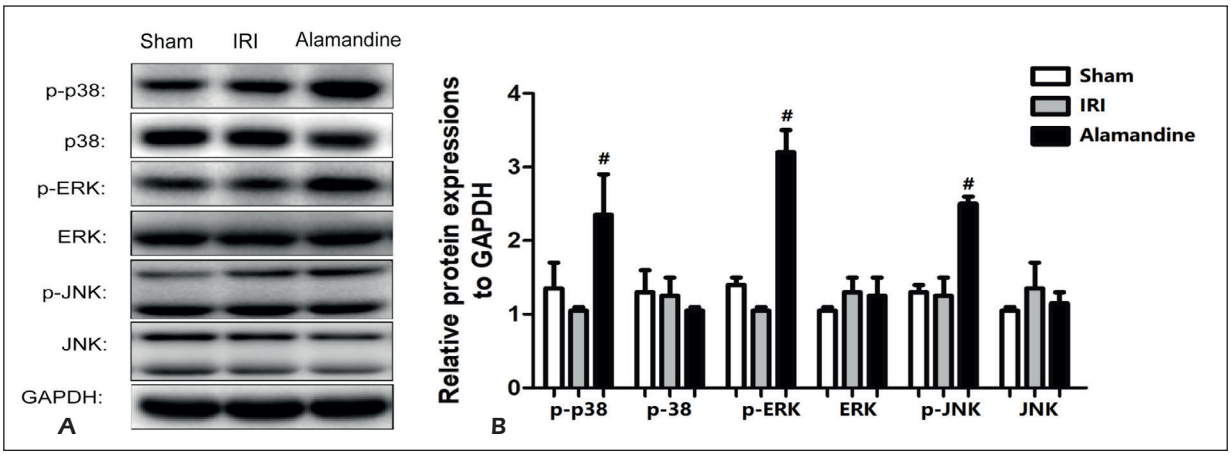

Figure 4. Alamandine activates changes in JNK signaling pathway-associated proteins during myocardial ischemia-reperfusion injury. **A**, Western blot was used to detect the expression levels of p-p38, p38, p-ERK, ERK, p-JNK, and JNK in myocardial tissue of Sham group, IRI group, and alamandine group. **B**, Quantitative determination of p-p38, p38, p-ERK, ERK, p-JNK, and JNK expression levels in myocardial tissue of each treatment group. The data were mean ± SD, * indicates a significant difference compared with the Sham group (p<0.05), #indicates a significant difference compared with the IRI group (p<0.05).

Discussion

Perioperative cardiac complications such as myocardial ischemia and infarction are the main causes of increased morbidity and mortality in non-cardiac surgery patients. After the occurrence of acute myocardial infarction, the blood supply to the ischemic area of the myocardium is restored as soon as possible. The myocardial damage in the ischemic area is worse than that of the ischemic period. This phenomenon is called ischemia-reperfusion injury (IRI)[9-11]. Myocardial ischemia-reperfusion injury is a common pathological process in clinical anesthesia, especially in cardiac surgery under cardiopulmonary bypass, thrombolytic therapy in patients with myocardi-

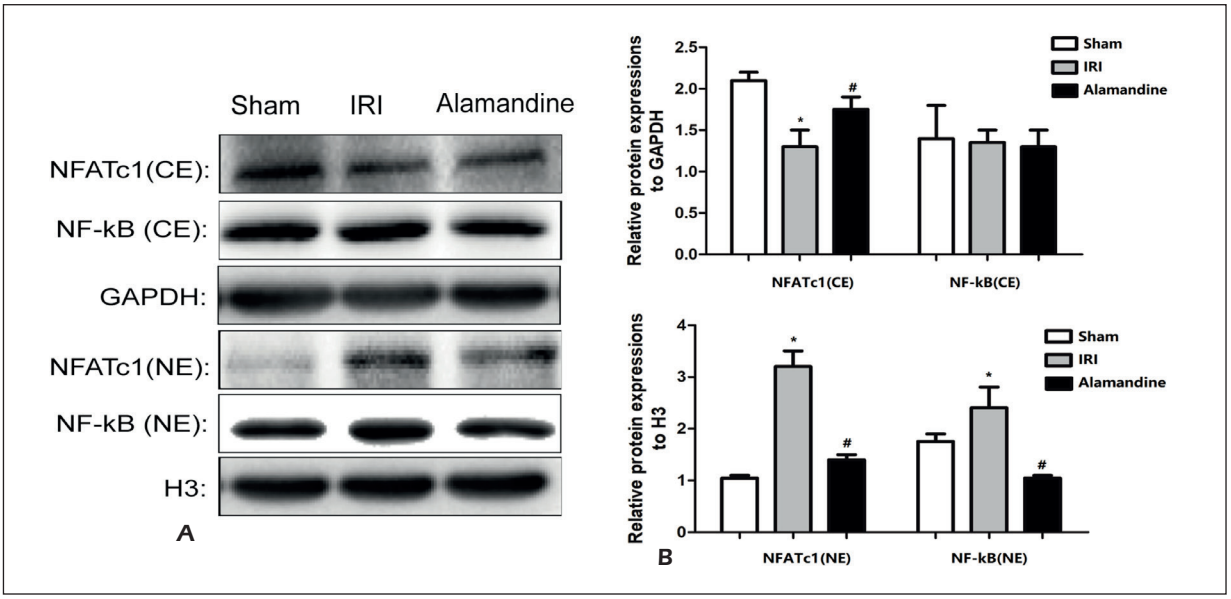

Figure 5. Alamandine inhibits changes in NF-κB signaling pathway-associated proteins during myocardial ischemia-reperfusion injury. **A**, Western blot was used to detect the expression levels of nuclear and cytoplasmic NFATc1 and NF-κB in myocardial tissue of Sham group, IRI group, and alamandine group. **B**, Quantitative determination of nuclear and cytoplasmic NFATc1 and NF-κB protein expression levels in myocardial tissue of each treatment group. The data were mean ± SD, *indicates a significant difference compared with the Sham group (p<0.05), #indicates a significant difference compared with the IRI group (p<0.05).

al infarction, and coronary heart disease patients undergoing non-cardiac surgery[10]. Therefore, how to prevent and treat acute myocardial IRI during the perioperative period and to save patients' lives has become a serious problem[11].

So far, the mechanism of IRI is not very clear, and it is generally recognized that the oxygen free radicals, the calcium overload, and the activated neutrophils are responsible[21-23]. The oxygen free radicals prove that a large number of free radicals in the ischemic area during ischemia-reperfusion is one of the most important factors for ischemia-reperfusion injury, and the application of free radical scavenger coenzyme can reduce the damage of cells in the ischemia-reperfusion zone[21]. The calcium overload during ischemia-reperfusion may activate ATPase to cause mitochondrial dysfunction caused by ATP depletion. ATP reduction causes excessively elevated Ca^{2+} concentration in the cytoplasm and activates Ca^{2+}-dependent phospholipase, which promotes hydrolysis of membrane phospholipids, thereby impairing cell membranes and organelle membranes, and promoting oxygen free radical production leading to myocardial damage. At the same time, due to the increase of Ca^{2+} influx in vascular smooth muscle during ischemia, it can cause vasoconstriction, and the increase of vascular resistance is unfavorable to the ischemic cycle, which makes the infarct enlargement[22]. Activated neutrophils produce cytokines, adhesion factors (intracellular adhesion molecule-1, L-selectin, and CD18), which bind neutrophils to vascular endothelial cells and release granular elastin, reactive oxygen species, lysosomal enzymes, cytokines, and other inflammatory mediators (such as platelet stimulating factor, thromboxane, leukotrienes, etc.); these substances can damage endothelial, vascular smooth muscle cells and cardiomyocytes[23,24]. In addition to these three mechanisms, mitochondrial damage and energy metabolism disorders are also important causes of myocardial IRI. The level of ATP in cells is the main factor determining cell apoptosis or necrosis[25].

Activation of JNK is achieved by phosphorylation of amino-terminal residues, and activation of JNK can be initiated by the stimulation of a variety of extracellular factors, including growth factors, cytokines, stress stimuli (ultraviolet light, high osmolality, IRI)[13]. Once JNK is activated, JNK will be transferred from the cytoplasm into the nucleus, which is widely involved in various biological reactions such as apoptosis, proliferation, metabolism and DNA damage repair, and its dysfunction can cause a variety of diseases such as diabetes, cancer, and ischemia-reperfusion injury[14]. In the inactive state, NF-κB binds non-covalently to IκB to form a complex, which exists as an inactive dimer in the cytosol[15,16]. When the activation signal is exposed, NF-κB dissociates from IκB, promotes NF-κB activation, activates NF-κB into the nucleus, binds to the DNA response element promoter, activates transcription of inflammatory genes, and induces cytokines and adhesion molecules. Upregulation of vasoregulatory factors further increases the concentration of downstream cytokines[15-17].

In this investigation, alamandine pretreatment significantly increased the levels of p-p38, p-ERK, and p-JNK while reduced the expression of nucleoproteins such as NF-kB and NFATc1. In addition, the pathological structure and apoptosis of myocardial cells in the alamandine administration group were significantly improved compared with the IRI group, indicating that inflammation induced by myocardial IRI can activate phosphorylation of JNK, while alamandine can attenuate this inflammatory response through inhibiting the activation of the NF-κB, which was consistent with the role of anti-inflammation of alamandine in other tissues.

Conclusions

We demonstrated that alamandine could significantly alleviate the pathological damage induced by myocardial ischemia-reperfusion injury and inhibit the inflammatory response. Its possible mechanism is through the activation of JNK phosphorylation and inhibition of NF-κB signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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