



# Effect of moxibustion preconditioning on autophagy-related proteins in rats with myocardial ischemia reperfusion injury

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**Background:** Autophagy has increasingly been recognized as playing an essential role in the pathogenesis of myocardial ischemia reperfusion injury (MIRI). Moxibustion, a form of heat therapy commonly used in traditional Chinese medicine (TCM), has been shown to exhibit cardioprotective effects. However, whether the cardioprotective effect of moxibustion is related to the regulation of autophagy remains unknown. This study aimed to investigate the possible mechanism underlying the cardioprotective effect of moxibustion preconditioning at PC6 on MIRI by measuring the expressions of proteins involved in the regulation of autophagy.

**Methods:** Male Sprague-Dawley rats were randomly divided to receive moxibustion preconditioning or autophagy inhibitor 3-Methyladenine (3-MA) intervention. Then the MIRI model was established by ligating the left anterior descending (LAD) coronary artery for 30 minutes followed by reperfusion for 4 hours. After 4 hours of reperfusion, the myocardial infarction area was assessed using Evans blue and TTC staining, and cTnT and lactate dehydrogenase (LDH) levels in the serum were determined by ELISA. Hematoxylin and eosin (H&E) staining was performed for morphological evaluation of ventricular tissues. Expressions of autophagy components Beclin 1, Bcl-2, and Akt were assessed using quantitative real-time PCR, immunohistochemistry (IHC) and western blot.

**Results:** Moxibustion preconditioning significantly reduced the necrotic area and the levels of cTnT and LDH were similar to the 3-MA intervention, also attenuated morphological alterations were induced by MIRI. Simultaneously, the mRNA and protein expressions of Beclin 1 and Akt were up-regulated, while those of Bcl-2 were down-regulated by MIRI. Moxibustion preconditioning and 3-MA intervention reversed MIRI-induced changes in Beclin 1, Akt, and Bcl-2 expressions.

**Conclusions:** Moxibustion preconditioning at PC6 can attenuate myocardial injury for MIRI in a similar way to 3-MA intervention. This cardioprotective effect of moxibustion preconditioning may be mediated by modulating autophagy via regulation of Beclin 1, Bcl-2 and Akt.

**Keywords:** Myocardial ischemia reperfusion injury (MIRI); moxibustion preconditioning; Beclin 1; Bcl-2; Akt

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## Introduction

Myocardial infarction (MI) is one of the leading causes of global mortality and morbidity (1). It is caused by an obstruction of the coronary artery, and the primary treatment method is to restore blood flow to the heart through surgical procedures, such as percutaneous coronary angioplasty (PCI) and coronary artery bypass grafting (2). Although these interventions have improved post-MI survival rates and clinical outcomes, the process of restoring blood and oxygen induces various pathological changes within the myocardium, in a phenomenon known as myocardial ischemia reperfusion injury (MIRI) (3). Research shows that MIRI negatively affects myocardial functions, metabolic processes, and myocardium ultrastructure, increasing the likelihood of adverse ventricular remodeling and chronic heart failure (4). Despite ongoing efforts, there is yet no effective therapy for preventing and treating MIRI. Therefore, there has been a growing public interest in finding safe and effective complementary treatments and increasing scientific inquiry in investigating the effects of these treatments for MIRI.

The pathogenesis of MIRI is associated with increased oxidative stress and inflammation, mitochondrial damage, calcium overload, apoptosis, and autophagy (5-9). Among these possible factors, there has been a growing interest in investigating the role of autophagy in MIRI. Autophagy is an intracellular catabolic mechanism involving sequestration and degradation of damaged proteins and other dysfunctional cellular components (9). Under basal conditions, it serves as a housekeeping mechanism for maintaining cellular homeostasis. During times of energy stress or nutrient starvation, it promotes cellular survival by promoting ATP production and reducing energy crisis. However, when autophagy is excessive and sustained, it can detrimentally activate apoptosis and aggravate cellular injury (10). MIRI research has shown that autophagy activation is prolonged, maladaptive, and damaging to the heart (11). Therefore, the modulation of autophagy represents a novel therapeutic approach to attenuating MIRI and warrants further study (12).

Research has shown that there is a close relationship between Akt, Bcl-2, Beclin 1 and autophagy. Increasing evidence shows that Akt activation plays a critical role in cardioprotection after ischemia/reperfusion (I/R) (13) because Akt signaling and Akt lysosomal co-localization with Phafin2 complex is vital for autophagy induction (14,15).

Moreover, the process of autophagy involves a complex network of intersecting proteins, namely Beclin 1 and Bcl-2. Beclin 1 is one of the key players in the regulation of autophagy. It plays a central role in the formation of a membrane structure called autophagosome, which is responsible for engulfing damaged cellular components tagged for degradation (16). Beclin 1, in turn, is regulated by its interactions with Bcl-2, an anti-apoptotic protein that inhibits autophagy by binding to the BH3 domain of Beclin 1. In brief, research demonstrates that they all play essential roles in the regulation of autophagy.

Moxibustion is an integral part of traditional Chinese medicine (TCM) with a wide range of therapeutic benefits. It is a form of heat therapy and involves the burning of mugwort herb (*Artemisia vulgaris*) directly or indirectly over specific acupuncture points (17). Previous studies have also shown that moxibustion preconditioning has a protective effect on the myocardium (18). In both research and clinical settings, Neiguan (PC6) is a commonly selected acupuncture point for the treatment of heart-related conditions, including hypertension, cardiac hypertrophy, and MIRI (19,20). We previously showed that moxibustion at PC6 could effectively alleviate myocardial ischemia in MIRI rats (21). However, the mechanisms of how moxibustion preconditioning on PC6 modulates MIRI and affects autophagy are still mostly unknown. Therefore, this study aimed to examine the effects of moxibustion preconditioning on MIRI and autophagy and to explore whether the cardioprotective effects of moxibustion preconditioning are mediated by regulation of autophagy-related proteins Beclin 1, Bcl-2 and Akt.

## Methods

### *Experimental animals*

A total of 60 male Sprague Dawley rats (2 months old, 250±20 g) were purchased from Shanghai Sippr-BK Laboratory Animal Co. Ltd. For the week preceding the experiment, the rats were acclimatized to a 12-hour light/dark cycle in a controlled environment with a temperature of approximately 25 °C and a relative humidity of 50%. They had free access to standard mouse chow and tap water. This study was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine Laboratory Animal Center and was conducted in accordance to the National Institutes of Health's Guide for the Care and Use

of Laboratory Animals.

### *Animal grouping and experimental design*

After one week of acclimatization, the rats were randomly divided into four groups which are outlined below: (I) in the sham operation group (Sham, n=15), the rats were held down by the technician, with gentle but without moxibustion for 5 minutes a day for 4 consecutive days. On the fifth day, they underwent a sham operation, consisting of left thoracotomy but without the ligation of the left anterior descending (LAD) coronary artery; (II) in the MIRI model group (MIR, n=15), the rats were also held down for 5 minutes a day for 4 consecutive days without moxibustion. However, on the fifth day, MIRI was induced by ligating the LAD for 30 minutes, followed by 4 hours of reperfusion; (III) in the moxibustion pretreatment plus MIRI group (Moxi, n=15), the rats were held down gently and received moxibustion at bilateral PC6 with 7 small moxibustion cones a day for 4 consecutive days, and on the fifth day, MIRI was induced; (IV) in the 3-MA plus MIRI group (3-MA, n=15), the rats were held down for 5 minutes a day for 4 consecutive days without moxibustion, and on the 5th day, MIRI was induced. However, 10 minutes before the ligation of LAD, the rats were administered a tail vein injection of an autophagy inhibitor 3-methyladenine (3-MA, dissolved in saline, 15 mg/kg) (AAB, USA) (22).

### *MIRI model establishment and moxibustion intervention*

The MIRI model was established as previously described (21). The rats were anesthetized with an injection of pentobarbitone sodium (50 mg/kg) into their abdominal cavities. They were then placed supine on a temperature-controlled experimental board set at  $37\pm 3$  °C and intubated with a small animal ventilator (R407, RWD, China) set at a respiratory rate of 60–70 breaths per minute. After disinfecting the surgical area, the left chest was opened to expose the heart at the 3rd intercostal space. The pericardium was separated, the heart was exteriorized, and the LAD was quickly ligated with 6.0 prolene suture, approximately 2 mm in width and depth in order to induce MI (23). The appearance of a paler color below the ligation area and ST-segment elevation on ECG (PowerLab System, AD Instruments, USA) were used to confirm a successful occlusion of the LAD. After 30 minutes of ischemia, the suture was removed to allow reperfusion for 4 hours.

Only the rats in the Moxi group received moxibustion

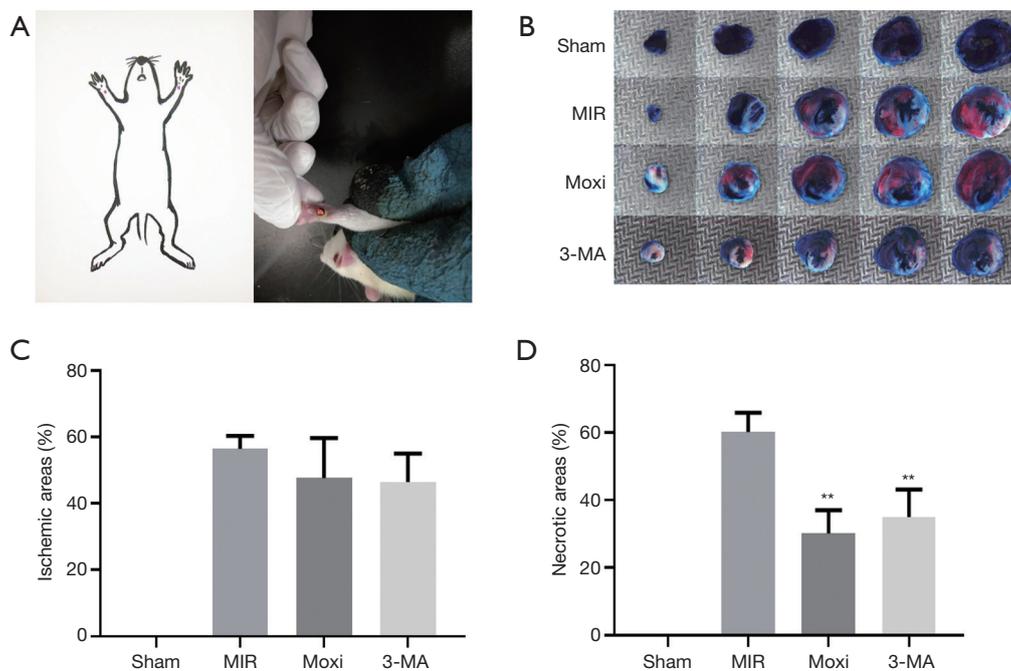
pretreatment before MIRI induction. Moxibustion was performed as previously described (21) by gently restraining the rats and applying a total of 7 small moxibustion cones (5 mg/cone) on PC6 for 40–45 seconds per cone and the cone was moved when it burned to a third of its size (*Figure 1A*). A 3-year 1:40 golden moxa, obtained from Nanyang Wolong Han medicine moxa factory (Hanan, China), was used for the experiment.

### *Collection of tissue and serum samples*

After 4 hours of reperfusion, the rats were anesthetized with pentobarbitone sodium (80 mg/kg). Six rats from each group were used to assess the myocardial infarction area using Evans blue and TTC staining (Sigma, USA). For the remaining 9 rats in each group, blood samples and left ventricular tissues were collected for further analysis. Blood was withdrawn from the abdominal aorta and centrifuged for 10 minutes at  $3,500\times g$  to obtain the serum, which was then stored at  $-80$  °C. Three of the nine heart tissues were placed in 4% paraformaldehyde for morphological evaluation and immunohistochemistry (IHC), and the other 6 were stored in  $-80$  °C freezer for western blot and real-time PCR analysis.

### *Assessment of myocardial infarction area*

Evans Blue and TTC staining were used to visualize the area of myocardial infarction (24). After anesthesia, Evans blue (2%, dissolved in PBS) was injected into the apex cordis, at the caudal end of the rats' hearts. The hearts were then quickly harvested and frozen at  $-80$  °C. The frozen myocardial tissue below the ligation site was sliced into 5 sections of approximately 1–2 mm each and placed in 2% TTC (dissolved in PBS) for 10 minutes at 37 °C in a dark incubator. The stained heart slices were fixed in 4% paraformaldehyde for 24 hours and then photographed (Nikon-TV, Japan) and analyzed using image analysis software Image-ProPlus. There were 3 main zones in the stained myocardial tissue: the infarct tissue appeared white, the at-risk tissue for infarction appeared red, and the normal tissue with no infarction appeared blue. In this study, the severity of myocardial infarction was assessed by estimating the percentage of total ischemic area and the percentage of necrotic areas using the following formulae: percentage of total ischemic areas = (infarct plus at-risk areas)/total myocardial areas; percentage of necrotic areas = infarct areas/(infarct plus at-risk areas).



**Figure 1** Moxibustion preconditioning decreased necrotic areas of MIRI (n=6 each group). (A) The location of PC6 and moxibustion intervention; (B) representative photographs of ventricular tissue sections with Evans blue-TTC staining, showing 3 main zones: infarction (white), at-risk for infarction (red), and no infarction (blue); (C,D) averaged percentages of ischemic and necrotic areas, respectively. There was no significant difference in the ischemic area among the 3 groups, but the necrotic area was significantly smaller in the 3-MA and Moxi groups compared to the MIR group. All data are expressed as mean  $\pm$  SD. \*\*,  $P < 0.01$  vs. MIR group. MIRI, myocardial ischemia reperfusion injury.

#### Enzyme-linked immunosorbent assay (ELISA)

The serum levels of cTnT and lactate dehydrogenase (LDH) were determined using cTnT ELISA kit and LDH ELISA kit respectively (Yifeixue Biotech, China), according to the manufacturer's protocol.

#### Hematoxylin & eosin (H&E) staining

H&E staining was used for morphological evaluation. The ventricular tissues fixed in 4% paraformaldehyde were dehydrated, paraffin embedded, and sliced into 4  $\mu$ m thick sections. The sections were then stained with hematoxylin and eosin. After H&E staining, the sections were dehydrated and observed under an optical microscope. For each tissue section, 6 random fields were photographed, analyzed, and averaged by an investigator blinded to the

group assignment.

#### Quantitative real-time polymerase chain reaction (RT-qPCR)

RT-qPCR was used to measure the mRNA expressions of Beclin 1, Bcl-2, and Akt. After extracting total RNA in myocardial tissues with TRIzol (Abcam, USA), cDNA was synthesized by reverse transcription and then amplified by a real-time fluorescence quantitative PCR system (IQ5TM, Bio-Rad, USA). Amplification was carried out by denaturing at 94  $^{\circ}$ C for 30 seconds, annealing at 55  $^{\circ}$ C for 45 seconds, and extending at 72  $^{\circ}$ C for 30 seconds for 40 cycles. The primer sequences are listed in *Table 1*. GAPDH was used as the reference gene for all PCR experiments. Each sample was analyzed 3 times and the relative expressions of genes were determined using the  $2^{-\Delta\Delta CT}$  method.

**Table 1** Primers sequence of genes

Gene name	Forward	Reverse
<i>Akt</i>	5'-GTGCTGGAGGACAATGACTACGG-3'	5'-AGCAGCCCTGAAAGCAAGGA-3'
<i>Bcl 2</i>	5'-GGTGAAGCTGGGGGAGGATTG-3'	5'-GCATGCTGGGGCCATATAGT-3'
<i>Beclin 1</i>	5'-GAGAGGAGCCATTATTGAAAC-3'	5'-CTCCCAATCAGAGTGAAGC-3'
<i>GAPDH</i>	5'-GCACCGTCAAGGCTGGAGAAC-3'	5'-GGTGAAGACGCCAGTGA-3'

### IHC

IHC was performed to assess the expressions of Beclin 1, Bcl-2, and Akt. Ventricular tissue sections placed on slides were deparaffinized in xylene for 20 minutes and rehydrated through a graded concentration series of ethanol. The sections were then placed in citrate buffer (10 mM, pH 6.0) and heated to 60 °C for 5–10 minutes for antigen retrieval. Following this, sections were incubated in 3% hydrogen peroxide in methanol for 10–15 minutes to block endogenous peroxidase activity. After washing in distilled water, the sections were blocked with 5% BSA blocking buffer for 20 minutes at room temperature and incubated with primary antibodies for Beclin 1, Bcl-2, and Akt (diluted 1:200; catalogue number respectively: 29,281, 40,415, 40,576; SAB, USA) for 1–2 hours at 37 °C and overnight at 4 °C. After washing in PBS, they were incubated with secondary antibodies (diluted 1:10,000) for 30 minutes at 37 °C. The sections were then incubated with SABC for 20 minutes, stained with DAB solution, and observed under a light microscope. The absorbance of each image was analyzed using image analytic system. The positive expressions of total-Akt and Beclin 1 were identified in the cell nucleus and cytoplasm, while that of Bcl-2 was identified in the cell cytoplasm. A semi-quantitative evaluation of total-Akt, Bcl-2, Beclin 1 was performed using a method described in the literature (25).

### Western blot analysis

In addition to IHC, the levels of autophagy-related proteins Beclin 1, Bcl-2, and Akt were evaluated with western blot. Frozen myocardial tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer at a ratio of 100 mg: 1 mL and centrifuged at 12,000 ×g for 10 minutes at 4 °C. BCA protein assay (Pierce, USA) was performed using the supernatants to determine protein concentration.

The proteins were then loaded and separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by a transfer to PVDF membrane. Following this, the membranes were blocked with 5% nonfat milk for 2 hours and then incubated with primary antibodies for Beclin 1, Bcl-2, and Akt (diluted 1:1,000; catalogue number respectively: 29,281, 40,415, 40,576; SAB, USA) overnight at 4 °C. After washing 3 times for 10 minutes each, the membranes were incubated with secondary antibodies (diluted 1:10,000) for 2 hours. Anti-β-actin (diluted 1:1,000) was used as the loading control protein. The bands were imaged and analyzed using the Bio-Imaging system.

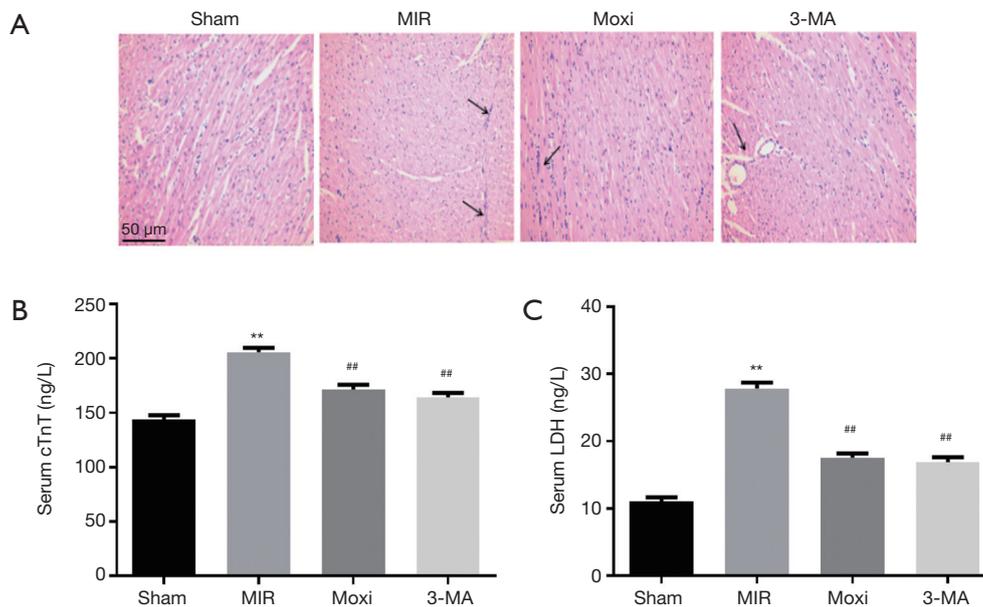
### Statistical analysis

All data are presented as mean ± standard deviation and were analyzed with SPSS 21.0. The normality of data distribution was tested with the Shapiro-Wilk test. If the data were normally distributed, groups were compared by one-way analysis of variance (ANOVA), followed by the Fisher's LSD method. If the data were not normally distributed, the Dunnett's T3 method was used for group comparisons. A value of  $P \leq 0.05$  was considered statistically significant.

## Results

### *Moxibustion preconditioning decreased necrotic areas caused by MIRI*

The protective effect of moxibustion preconditioning against MIRI was assessed by determining myocardial infarction sizes (*Figure 1B,C,D*). The percentage of the necrotic area was significantly smaller for the Moxi and 3-MA groups compared to the MIR group ( $P < 0.01$ ). However, there was no significant difference in the percentage of ischemia area among the MIR, 3-MA, and



**Figure 2** Moxibustion preconditioning attenuated MIRI-induced pathological morphological changes in the ventricular tissues (n=3 each group) and reduced MIRI-induced myocardial enzyme levels (n=9 each group). (A) The arrows indicate areas of inflammatory cell infiltration (HE, ×400); (B,C) levels of cTnT and LDH in the serum of rats with sham operation (Sham), myocardial ischemia reperfusion (MIR), moxibustion pretreatment plus MIRI (Moxi), and autophagy inhibitor plus MIRI (3-MA). All data are expressed as mean ± SD. \*\*, P<0.01 vs. Sham group; ##, P<0.01 vs. MIR group. MIRI, myocardial ischemia reperfusion injury; LDH, lactate dehydrogenase.

Moxi groups.

#### ***Moxibustion preconditioning attenuated MIRI-induced morphological alterations and myocardial enzyme levels***

H&E staining was performed to observe the morphological changes in myocardial tissue below the ligation site (Figure 2A). MIRI resulted in myocardial cellular loss, myofibrillar degeneration, and inflammatory cell infiltration. These MIRI-induced morphological changes were attenuated by the administration of moxibustion preconditioning and 3-MA. ELISA was performed to test the levels of cTnT and LDH in serum (Figure 2B,C). The levels of cTnT and LDH were significantly higher for the MIR group compared to the Sham group. Both moxibustion preconditioning and 3-MA attenuated these MIRI-induced changes in cTnT and LDH levels (P<0.01).

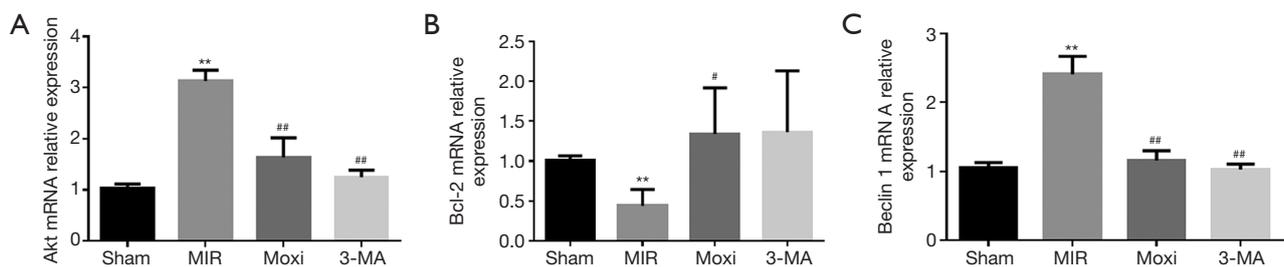
#### ***Moxibustion preconditioning modulated myocardial autophagy-related proteins***

The effect of moxibustion preconditioning on MIRI-

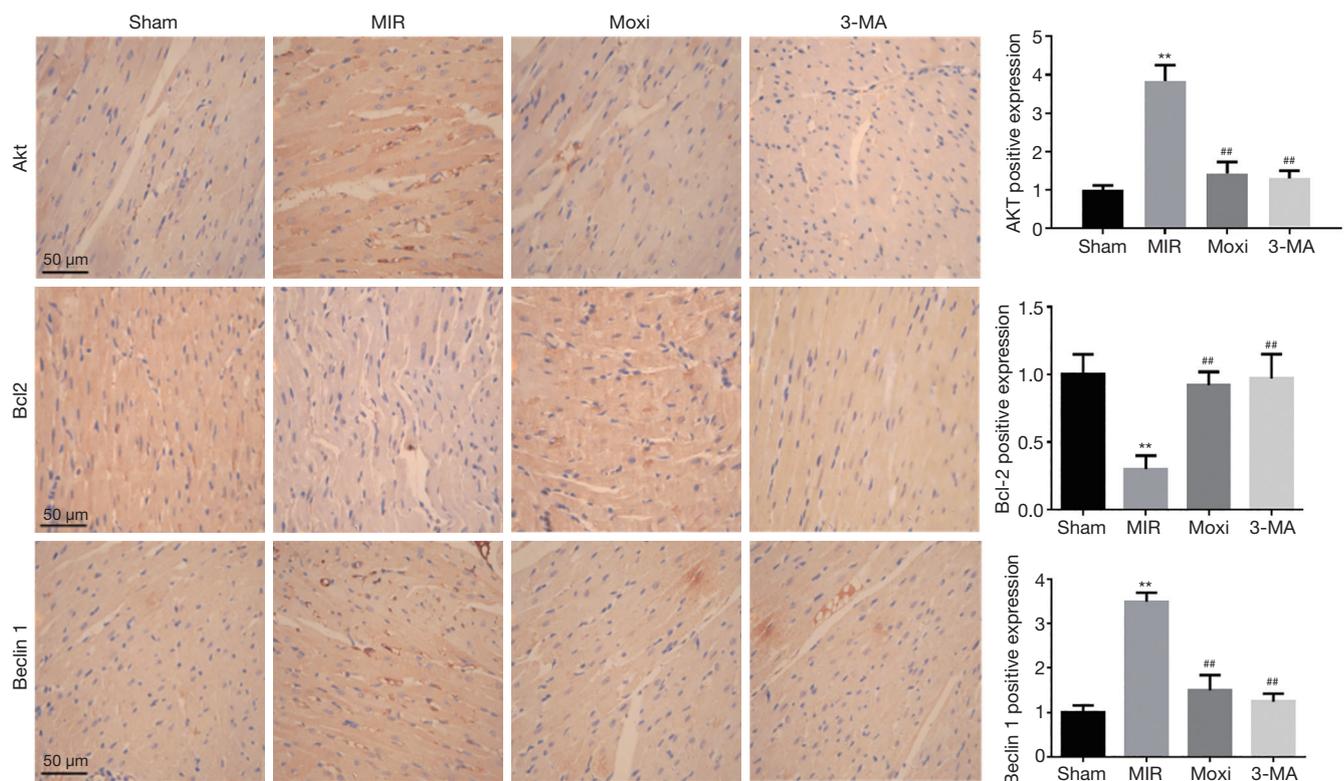
induced myocardial autophagy was evaluated by assessing the expressions of autophagy-related proteins Beclin 1, Bcl-2, and Akt. Gene expressions of Beclin 1, Bcl-2 and Akt were evaluated using RT-qPCR (Figure 3). The induction of MIRI caused a significant up-regulation of Beclin 1 and Akt mRNA expressions and a down-regulation of Bcl-2 (P<0.01). Compared to the MIR group, the mRNA expressions of Beclin 1 and Akt were significantly down-regulated by the application of moxibustion preconditioning and 3-MA (P<0.01), while that of Bcl-2 was up-regulated by moxibustion preconditioning (P=0.05).

According to the IHC data (Figure 4), the rats in the MIR group compared to the Sham group had increased expressions of Beclin 1 and Akt and lower expression of Bcl-2 (P<0.01). An injection of 3-MA, an autophagy inhibitor, normalized these changes in the MIR group by reducing Beclin 1 and Akt, and increasing Bcl-2 levels (P<0.01). Moxibustion preconditioning exerted similar normalizing effects on MIRI-induced changes, significantly reducing Beclin 1 and Akt and increasing Bcl-2 (P<0.01).

These IHC findings were consistent with the western blot data (Figure 5). Compared to the Sham group, the rats



**Figure 3** Detection of mRNA expression levels by RT-qPCR (n=6 each group). Relative mRNA expressions of (A) Akt, (B) Bcl-2, and (C) Beclin 1 in the ventricular tissues of rats with sham operation (Sham), myocardial ischemia reperfusion (MIR), moxibustion pretreatment plus MIRI (Moxi), and autophagy inhibitor plus MIRI (3-MA). All data are expressed as mean  $\pm$  SD. \*\*, P<0.01 vs. Sham group; #, P<0.05 vs. MIR group; ##, P<0.01 vs. MIR group. RT-qPCR, quantitative real-time polymerase chain reaction; MIRI, myocardial ischemia reperfusion injury.

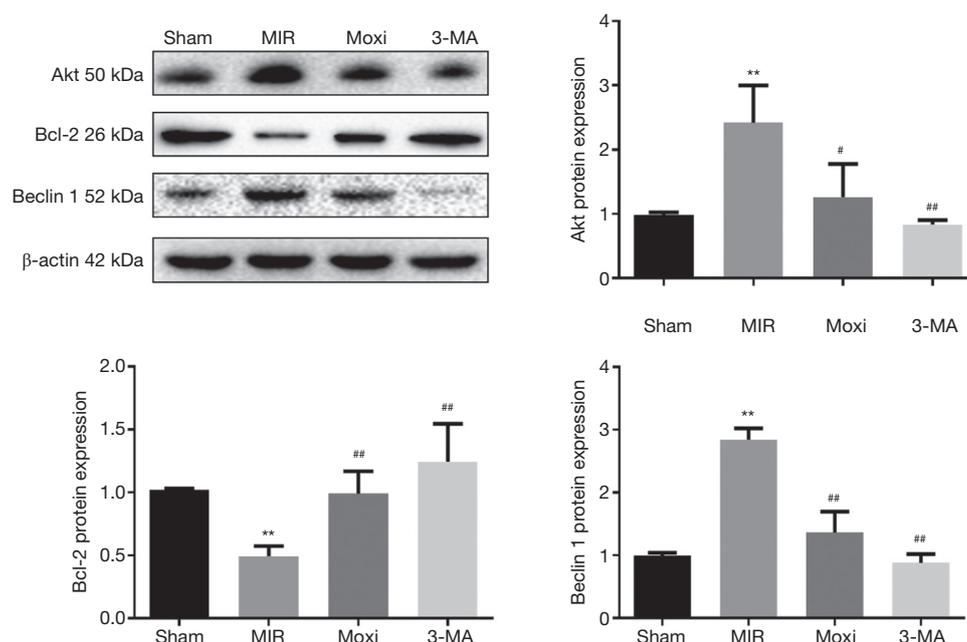


**Figure 4** Effect of moxibustion preconditioning on autophagy-related protein expressions by IHC (n=3 each group). Representative photographs of ventricular tissues with IHC staining of Akt, Bcl-2, and Beclin 1. Bar: 50  $\mu$ m. Statistical graphical representations of Akt, Bcl-2, and Beclin 1 are expressed as mean  $\pm$  SD. \*\*, P<0.01 vs. Sham group; ##, P<0.01 vs. MIR group. IHC, immunohistochemistry.

in the MIR group showed higher expressions of Beclin 1 and Akt (P<0.05) and a lower expression of Bcl-2 (P<0.01). These changes were normalized in both the 3-MA and Moxi groups; compared to the MIR group, the rats in the 3-MA and Moxi groups had significantly lower levels of Beclin 1 and Akt, and a higher level of Bcl-2 (P<0.01).

## Discussion

MIRI is a significant and paradoxical problem arising from the reperfusion of ischemic myocardium. While the timely restoration of blood is critical to reducing myocardial ischemic injury, the process of reperfusion can itself lead



**Figure 5** Detection of autophagy-related protein expressions by western blot (n=6 each group). Relative expressions of Akt, Bcl-2, and Beclin 1 in cardiac ventricular tissues of rats with sham operation (Sham), myocardial ischemia reperfusion (MIR), moxibustion pretreatment plus MIRI (Moxi), and autophagy inhibitor plus MIRI (3-MA) are expressed as mean  $\pm$  SD. \*\*, P<0.01 vs. Sham group; #, P<0.05 vs. MIR group; ##, P<0.01 vs. MIR group. MIRI, myocardial ischemia reperfusion injury.

to further myocardial damage and death. Moxibustion, an essential intervention in TCM, involves thermal stimulation of specific acupuncture points and is effective in treating various cardiovascular diseases (19). In this study, we found that moxibustion preconditioning at PC6 reduced myocardial necrotic area and myocardial enzyme levels. It also normalized myocardial cellular degeneration and myofibrillar disorganization induced by MIRI, indicating that moxibustion preconditioning has a cardioprotective effect against MIRI, which consistent with the previous (21).

There is a growing body of research demonstrating the complex role of autophagy in both physiological and pathological conditions. While autophagy plays an essential role in maintaining cellular homeostasis, research demonstrates that when it is excessive and sustained, such as in the case of MIRI, it can be maladaptive and damaging to the heart (26,27). Previous experimental studies have shown that myocardial injury could be mitigated by using autophagy inhibitors such as 3-MA (28). So, for our study, we used autophagy inhibitor 3-MA as a positive control drug to compare and confirm the cardioprotective effect of moxibustion preconditioning. We found that the administration of 3-MA decreased necrotic area and

myocardial enzyme levels while improving MIRI-induced morphological changes. Moxibustion preconditioning exerted similar cardioprotective effects on MIRI as the 3-MA intervention. Based on these findings, we hypothesized that moxibustion preconditioning protects the myocardium during MIRI by inhibiting autophagy in a similar way to 3-MA. 3-MA is a widely used autophagy inhibitor, and it suppresses autophagy by blocking the enzymatic action of class III phosphatidylinositol 3-kinase (PI3K), which plays a central role in the initiation and progression of autophagy (28,29). Once activated during MIRI, PI3K can phosphorylate and upregulate other key regulators of autophagy, including Akt (30,31). By inhibiting PI3K, we speculated that 3-MA would also downregulate Akt. Thus, autophagy-related proteins, Akt, Bcl-2, and Beclin 1 were examined.

Accordingly, in our study, the upregulation of Akt in the MIRI group was normalized by 3-MA intervention. Similarly, moxibustion preconditioning also resulted in a decreased level of Akt expression. There was a significant difference in total Akt between the groups, so we did not further investigate the expression of phospho-Akt. However, the total Akt cannot reflect the level of phospho-Akt, so we

could not explore the role of Akt thoroughly; phospho-Akt should be detected in future studies. Previous studies have also demonstrated that Akt activation and Akt-mediated phosphorylation of Beclin 1 inhibit autophagy (32). While, in our study, Akt was associated with increased Beclin 1 and decreased Bcl-2 expressions, suggesting an upregulation of autophagy. Compared to the sham group, MIRI induction increased both Akt and Beclin 1 expressions, which were significantly attenuated by 3-MA and moxibustion preconditioning. These conflicting data might be explained by the complexity of PI3K/Akt signaling (29,33). Research shows that Akt is involved in both inhibition and activation of autophagy, depending on its interactions with either class I or class III PI3K, respectively (14,15,31). Class I PI3K/Akt signaling has been well-documented and is widely known to suppress autophagy and encourage cellular survival, growth, and proliferation (31). On the other hand, class III PI3K/Akt interaction promotes autophagy, with recent studies demonstrating the importance of Akt signaling and Akt lysosomal localization for autophagy induction (14,15). Furthermore, as reported in the literature (28), 3-MA works by suppressing the function of class III PI3K, and then downregulates proteins downstream of PI3K, including Akt. In our study, we only observed the expression of Akt, but not PI3K. Therefore, further studies are needed to clarify the relationships between PI3K/Akt signaling, moxibustion, and autophagy.

Beclin 1 plays a central role in the initial step of autophagy by promoting the formation and maturation of autophagosome, a double membrane vesicle that engulfs dysfunctional cellular components and fuses with lysosomes for degradation. Bcl-2 interacts with Beclin 1 and inhibits its autophagic activities. Cellular stress disrupts this Bcl-2/Beclin 1 interaction by phosphorylating ser70, ser87 and thr69 of Bcl-2 and displacing Beclin 1 from Bcl-2 (34). Research also shows that downregulation of Bcl-2 protein subsequently leads to Beclin 1 upregulation and autophagy induction (35). When there is cellular stress, however, Beclin 1 is displaced from Bcl-2 and autophagy is triggered (27). In our study, the induction of MIRI decreased the protein and mRNA expressions of Bcl-2 and increased that of Beclin 1 in the ventricular tissues, suggesting that MIRI acted as a stressor to promote autophagy. However, the administration of 3-MA and moxibustion preconditioning normalized these changes by increasing Bcl-2 expressions and decreasing Beclin 1, thereby reducing the level of autophagy.

In addition to autophagy itself, the interaction between autophagy and apoptosis mediated by Bcl-2/Beclin 1 also

plays a vital role in MIRI (36), so we took Bcl-2 and Beclin 1 as the focus of our study. Studies have shown that Bcl-2 can regulate apoptosis along with autophagy; specifically, Bcl-2 not only binds to Beclin 1 to suppress autophagy, but it also interacts with Bax/Bak to inhibit apoptosis. According to other recent research, acupuncture pretreatment at PC6 has been shown to exert a cardioprotective effect by modulating gene expressions and regulating various signaling pathways, including apoptosis (37,38). During cellular stress, Bcl-2 is displaced from both Beclin 1 and Bax/Bak, activating autophagy and apoptosis, respectively. Thus, upregulation of Bcl-2 is associated with modulation of both autophagy and apoptosis. In our study, moxibustion preconditioning upregulated Bcl-2 expression, indicating that it can promote cellular survival in MIRI through attenuation of both autophagy and apoptosis. In order to verify the hypothesis that moxibustion can inhibit myocardial apoptosis, further study is needed.

Autophagy is a lysosome-dependent cellular catabolic process. Besides Beclin 1, LC3, p62, and TFEB also play an essential role in autophagy (39). The transformation from LC3 I to LC3 II is the key step in autophagosome lipid membrane fusion which increases the volume of autophagosomes. P62 protein itself is one of the selective substrates of autophagy, which means when autophagy is activated, p62 will be degraded. TFEB is a member of the MiTF/TFE family, and the activation of TFEB increases the synthesis of lysosomal-associated membrane protein (LAMP) and accelerates the autophagy process. According to previous studies, there was an upregulation of LC3II/I and downregulation of p62 in MIRI, while moxibustion could reverse these changes (40). However, to completely demonstrate the involvement of autophagy, other autophagic markers apart from Beclin 1 should also be detected. Finally, in a previous study, moxibustion promoted autophagy against a lethal bacterial infection (41). Nevertheless, we observed the inhibition of autophagy by moxibustion. Combined, these data suggest that moxibustion might exert a dual effect of either activating or suppressing autophagy, depending on the autophagic activity level. Anyhow, more evidence is required to clarify the underlying mechanism and possible bi-directional modulatory effect of moxibustion preconditioning on autophagy.

## Conclusions

Autophagy during MIRI is characterized as excessive,

sustained, and maladaptive. Attenuating autophagy may present a novel therapeutic approach to reducing myocardial injury. This study showed that moxibustion preconditioning at PC6 could mitigate myocardial injury in MIRI. The cardio-protective effect of moxibustion preconditioning may be mediated by modulation of autophagy.

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### Footnote

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine Laboratory Animal Center and was conducted in accordance to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

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