Sevoflurane Postconditioning Upregulates HIF-1α Pathway Enhances BNIP3 Mediated Mitochondrial Autophagy in Myocardium of Aged Mice

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Abstract

**BACKGROUND:** Diminished mitochondrial autophagy in aged myocardium may be due to impaired HIF-1α protein expression. Previous studies confirmed that upregulation of HIF-1α expression protects myocardial tissue from ischemia/reperfusion (I/R) injury and found that sevoflurane post-conditioning (SpostC) mediated mitochondrial autophagy plays a significant role in myocardial protection. However, the protective mechanism of SpostC in aged myocardium is unclear. This study aimed to investigate whether SpostC regulates BNIP3 - mediated mitochondrial autophagy by upregulating HIF-1α expression, thus alleviating myocardial I/R injury in aged mice.

**Methods:** An in vivo mouse model of myocardial I/R injury was established and treated with sevoflurane at the time of reperfusion, and at the end of reperfusion, echocardiographic changes, myocardial infarct size, mitochondrial ultrastructure, and autophagosomes were measured, mitochondrial respiratory function and enzyme activity were detected, serum LDH, CKM, CK-MB, TNNT2, IL-6 levels were determined, and Western blot was used to examine the expression levels of phosphorylated HIF-1α, LC3-II, BNIP3, Beclin1, TLR9, and IL-6 protein in myocardial tissue.

**RESULTS:** In young, healthy myocardium, SpostC upregulated the expression of HIF-1α, activated the downstream target gene BNIP3 protein, and upregulated the expression levels of autophagy essential proteins LC3-II, Beclin-1, and TLR9, attenuated myocardial oxidative stress injury, stabilized mitochondrial ultrastructure, inhibited cardiomyocyte apoptosis, and ultimately reduced myocardial infarct size. In aged myocardium, SpostC also played an excellent myocardial protective role.

**CONCLUSION:** SpostC was able to upregulate HIF-1α expression, promote BNIP3-mediated mitochondrial autophagy, reduce myocardial infarct size, and alleviate myocardial I/R injury in aged mice.

Background

The prevalence and mortality of cardiovascular disease in China are on a continuous rise. According to ‘Report on Cardiovascular Health and Diseases in China 2019: an Updated Summary’, the number of people suffering from cardiovascular disease is about 330 million. Among deaths due to cardiovascular diseases, the mortality of acute myocardial infarction (AMI) showed a rapid upward trend, and the hospitalization rate of non-ST-segment elevation myocardial infarction (STEMI) increased by three times\(^1\). The pathophysiological basis is ischemia/reperfusion (I/R) injury caused by myocardial ischemia followed by reperfusion. With the acceleration of population aging in China, the prevalence of AMI in the elderly is increasing, age is a strong predictor of adverse outcomes in AMI, and approximately 60% of AMI deaths occur in patients over 75 years of age\(^2\). Therefore, it is an important scientific issue to be solved in many disciplines to reduce the incidence and mortality of perioperative cardiac adverse events in elderly patients and prevent myocardial ischemia and hypoxia injury.

Ischemic post-conditioning produces a good myocardial protection effect in healthy myocardium and is one of the most promising and important measures against myocardial ischemic injury in the perioperative period\(^3\). However, unfortunately, the protective effect of ischemic post-conditioning in aged myocardium is missing \(^4\). Abnormal energy metabolism is the most fundamental pathophysiological change in aged myocardium, and the reason for this abnormal energy metabolism is that the myocardial hypoxia-inducible factors α (HIF-1α) signaling pathway is damaged\(^5\). The lack of protective effect of aged myocardium is mainly due to the impaired HIF-1α signaling pathway. Effective measures to improve or restore the impaired HIF-1α signaling pathway in the aged myocardium are urgently needed to reverse this negative pattern and thus improve the safety of perioperative anesthesia and surgery safety.
Sevoflurane post-conditioning (SpostC) produces myocardial protective effects similar to ischemic/hypoxic pre-conditioning and is a promising anti-myocardial injury treatment for I/R injury occurring in healthy myocardium\cite{6}. Sevoflurane has been shown to reduce perioperative mortality and morbidity in clinical studies\cite{7}. Moreover, using sevoflurane pre-conditioning or post-conditioning in liver surgery can save patients' costs during hospitalization by reducing the incidence of adverse events\cite{8}. However, the mechanism of SpostC in the aged myocardium remains unclear.

HIF-1\(\alpha\) is a key regulatory molecule that triggers the trigger in myocardial protective effects. Previous studies have shown that upregulation of HIF-1\(\alpha\) expression in rat myocardium has significant advantages in improving myocardial structure and cardiac function\cite{9}. BNIP3 is a key protein downstream of the HIF-1\(\alpha\) signaling pathway that regulates mitochondrial autophagy, can be transcriptionally activated by HIF-1\(\alpha\) during hypoxia, resulting in upregulation of BNIP3 expression, which plays a vital role in apoptosis and necrosis of the mitochondrial pathway. Phosphorylation of Serines 17 and 24 of BNIP3 directly promotes its binding to Bcl-xL, and directly recruit LC3 - II to promote the initiation of mitochondrial autophagy flow. At the same time, Beclin1 and TLR9 proteins play an important role in HIF-1\(\alpha\)-mediated mitophagy\cite{10}. However, it is unclear whether BNIP3 is a key hypoxia-sensitive gene involved in HIF-1\(\alpha\)-mediated mitophagy and aging myocardial metabolic disorders.

Therefore, in this study, we proposed to use an in vivo myocardial ischemia/reperfusion injury model to detect myocardial mitochondrial ultrastructure, mitochondrial respiratory function and enzyme activity, myocardial infarct size, and protein expression levels, expecting to improve cardiac function after myocardial ischemia in aged mice by upregulating HIF-1\(\alpha\) and stabilizing its function to promote mitochondrial autophagy, and to further explore the role of HIF-1\(\alpha\)/BNIP3-mediated mitochondrial autophagy in SpostC in attenuating ischemia-reperfusion injury in aged myocardium.

**Methods And Materials**

**2.1 Animal selection:** 18-month-old male C57BL/6J mice and 8-week-old male C57BL/6J mice were selected for the study. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (the USA, Revised 1996).

**2.2 Setting:** Tertiary teaching hospital, Urumqi, Xinjiang, PR China. The study was conducted from April 2019 to April 2021.

**2.3 Ethics:** Ethical approval for this study (Ethics Committee: First Affiliated Hospital of Xinjiang Medical University: IACUC-20170214025) was provided by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, Xinjiang, China (Chairperson: Jian Liu) on 16 February 2017.

**2.4 In Vivo myocardial I/R model establishment:** Mice fasted overnight, and pentobarbital was injected intraperitoneally for anesthesia. After anesthesia, the mice were fixed on the experimental table, endotracheal intubation was performed, and a small animal ventilator was connected. The right femoral vein was cannulated to inject the drug. An incision was made 2-3 mm along the left edge of the sternum. After separating the pectoral muscle layer by layer, the ribs were cut at the fourth intercostal space to entirely expose the left auricle and left ventricle. Torn open the pericardium, and a small non-invasive circular needle was threaded through a 6-0 silk thread, entered 2-3 mm below the lower edge of the left auricle, passed through the superficial layer of the myocardium below the left descending branch of the coronary artery. Led both ends of the thread out of the silicone sleeve (one side of the
silicone sleeve was close to the left ventricular wall), tightened the ligature thread, and the successful ligation was marked by a significant elevation of the ST segment on the electrocardiogram.

2.5 Grouping: 8-week-old male C57BL/6J mice were randomly divided into four groups (n=10) to verify whether upregulation of myocardial HIF-1α protein enhances BNIP3-mediated mitochondrial autophagy: (1) sham-operated group (C-sham group); (2) control group (C-I/R group); (3) I/R+sevoflurane post-conditioning group (C-SpostC group); (4) I/R+sevoflurane post-conditioning group+2ME2(HIF-1α specific blocker) group (C-SpostC+2ME2 group). 18-month-old male C57BL/6J mice were randomly divided into four groups (n=10) to verify the effect of decreased HIF-1α protein expression in aged myocardium on mitochondrial autophagy: (1) sham-operated group (L-sham group); (2) control group (L-I/R group); (3) I/R+sevoflurane post-conditioning group (L-SpostC group); and (4) I/R+sevoflurane post-conditioning group+2ME2 group (L-SpostC+2ME2 group).

2.6 Oxidative stress in myocardial tissue: Serum LDH, CKM, CK-MB, TNNT2, and IL-6 levels were determined by ELISA.

2.7 Echocardiographic evaluation: At the end of 24 hours of reperfusion, small animal echocardiography (Vevo 2100 imaging system) was performed to evaluate cardiac function. All cardiac functional parameters in five consecutive cardiac cycles were obtained by M-mode ultrasound. A licensed ultrasound inspector performed ultrasound measurements.

2.8 Myocardial structure: Paraffin-embedded sections of mouse myocardial tissue were used to observe the structural changes of myocardial cells by HE (Hemalum) staining.

2.9 Arrangement and morphological changes of myocardial mitochondria under electron microscope: After reperfusion, the heart was excised, and the specimen was taken in the free wall of the left ventricle using a double-sided blade. The ultrastructure and autophagosome of cardiomyocytes were observed under transmission electron microscope (TEM).

2.10 Expression of HIF-1α subunit and hypoxia-sensitive genes: Real-time PCR and Western Blot were used to determine the expression of HIF-1α subunit, BNIP3 (p-BNIP3), LC3-II, Beclin1, and TLR9 protein in myocardial tissue.

2.11 Cardiomyocyte apoptosis measurement: TUNEL (TdT-mediated dUTP Nick-End Labeling) was used to determine cardiomyocyte apoptosis.

2.12 Infarct size determination: At the end of reperfusion, the heart was excised immediately and frozen in a -80°C refrigerator for 7 min, then stained with Evans Blue, and incubated in 1%TTC (PH=7.4) for 25 min(37°C). Sliced the heart along the apex to the bottom and then incubated in 10% formaldehyde overnight. Photographs were taken with a digital camera, and the infarct size was analyzed by Image J software.

2.13 Statistical analysis: Measurement data were presented as mean ± standard deviation(±s), and GraphPad Prism 5.0 statistical software was used for data analysis, and one-way ANOVA and Newman-Keuls method were used for significance test among groups, and a value of P less than 0.05 was considered statistically significant.

Results

3.1 SpostC upregulates HIF-1α signaling pathway in healthy myocardium and enhances BNIP3-mediated mitochondrial autophagy.

Oxidative stress in myocardial tissue: The level of myocardial oxidative stress was determined after the application of HIF-1α specific blocker 2ME2. At the end of 2 hours of reperfusion, serum LDH, CKM, CK-MB, TNNT2, and IL-6 levels were significantly increased in the C-I/R and C-SpostC+2ME2 groups compared with the C-sham group (P<0.05). And compared with the C-I/R group, serum LDH, CKM, CK-MB, TNNT2, and IL-6 levels changed significantly in the C-SpostC and C-I/R+2ME2 groups (P<0.05), confirming that 2ME2 abrogated the above effects of SpostC (P<0.05). (Table1, Fig. 1)
**Echocardiographic assessment**: Compared with the C-sham group, the C-I/R group had significantly lower stroke volume (SV)%, ejection fraction (EF)%, and fraction shortening (FS)% due to dysfunction of the left ventricular wall (P<0.05). In addition, the diastolic left ventricular diameter (LVIDd) and systolic left ventricular diameter (LVIDs) in C-I/R group were significantly increased. At the same time, the end-diastolic left ventricular anterior wall thickness (LVAWd), systolic left ventricular anterior wall thickness (LVAWs), diastolic left ventricular posterior wall thickness (LVPWd), Systolic left ventricular posterior wall thickness (LVPWs) were significantly decreased (P<0.05). SpostC could improve the above cardiac function indexes, making it significantly better than the C-I/R group, but the HIF-1α blocker completely abrogated the ability of SpostC to improve cardiac function (P<0.05). There was no significant difference in cardiac function indexes between the C-I/R and C-SpostC+2ME2 groups (P>0.05). (Table2, Fig. 2)

**HE staining of cardiomyocytes**: Microscopic observation showed that the myocardial tissue was more regularly arranged in the C-sham group, with only focal disorganization, a small number of cardiomyocytes were swollen, and myocardial fibers were fractured; myocardial tissue was arranged irregularly in the C-I/R group, cardiomyocytes were swollen and vacuolar degeneration, some myocardial fibers were fractured, and a small number of chronic inflammatory cells infiltrated; compared with the C-I/R group, the disorder of myocardial arrangement, cardiomyocytes swelling, myocardial fiber fracture and inflammatory cell infiltration in the C-SpostC group were alleviated. There was no significant difference in pathological changes between the C-SpostC+2ME2 group and the C-I/R group, but the pathological changes were more severe in the C-SpostC+2ME2 group. (Fig. 3)

**Myocardial mitochondrial ultrastructure**: Myocardial mitochondrial ultrastructure was observed by TEM. C-sham group: myocardial cell nucleus, myocardial fiber, and mitochondrial structure are intact. C-I/R group: myocardial structure was severely damaged, myofibrils were basically aligned, and myotome was slightly shortened segmentally. Mitochondria were obviously swollen, and mitochondrial cristae were extensively dissolved into flocculent, outer membrane segmentally dissolved, matrix slightly dissolved, visible autophagosomes, more lipid droplets. C-SpostC group: myocardial structure was clear, myofibrils were basically aligned, myotome length was uniform; mitochondrial cristae were extensively dissolved into flocculent, outer membrane segmentally dissolved, autophagosomes were visible. C-SpostC+2ME2 group: myocardial structure was damaged, matrix dissolved; mitochondria were obviously swollen, similar to C-I/R group. (Fig. 4)

**Changes in HIF-1α subunit and hypoxia-sensitive gene expression**: HIF-1α subunit and hypoxia-sensitive genes are crucial in resisting myocardial I/R injury and regulating mitochondrial function. It was found that SpostC was able to upregulate HIF-1α and downstream LC3-II, BNIP3, Beclin1, and TLR9 levels after I/R injury (P<0.05, Table2-1, Table2-2, Fig. 2), but the use of 2ME2 abrogated the above effects of SpostC. (P<0.05). (Table3, 4, Fig. 5)

**Myocardial apoptosis levels**: When I/R injury occurred in healthy myocardium, the apoptosis rate increased, and the survival rate decreased. SPostC reduced the apoptosis rate compared with the C-I/R group (P<0.05). C-SpostC+2ME2 significantly increased the apoptosis rate after I/R injury compared with the C-SPostC group (C-SPostC group: 20.881±5.477% vs C-SpostC+2ME2 group: 44.566±7.179%, P<0.05) (Fig. 6)

**Myocardial infarct size**: SpostC significantly reduced myocardial infarct size after I/R injury in healthy myocardium. Infarct size (IS)/ischemic myocardial area (Area at risk, AAR) (19.37±2.97% in C-SpostC group vs. 49.56±1.77% in C-I/R group, P<0.05), but the myocardial infarct size increased to 53.23±2.84% after application of 2ME2. (Fig. 7)

### 3.2 Impaired HIF-1α in Aged Myocardium Leads to diminished BNIP3-Mediated Mitochondrial Autophagy, but SpostC Still Plays an Excellent Myocardial Protective Role
Oxidative stress in myocardial tissue: At the end of 2 hours of reperfusion, serum LDH, CKM, CK-MB, TNNT2, and IL-6 levels were significantly increased in the L-I/R and L-SpostC+2ME2 groups compared with the L-sham group (P<0.05). And SpostC significantly decreased serum LDH, CKM, CK-MB, TNNT2, and IL-6 levels compared with the L-I/R group (P<0.05). (Table 5, Fig. 8)

Echocardiographic assessment: Compared with the L-sham group, the L-I/R group had significantly lower stroke volume (SV)%, EF%, and FS% due to left ventricular ischemia-reperfusion injury (P<0.05). In addition, LVIDd and LVIDs in the L-I/R group were significantly increased, whereas LVAWd, LVAWs, LVPWd, and LVPWs were significantly decreased (P<0.05). SpostC could improve the above cardiac function indexes in aged heart, making it significantly better than those in the L-I/R group, but the ability of SpostC to improve cardiac function was completely abrogated by the HIF-1α blocker 2ME2 (P<0.05). there was no significant difference in cardiac function indexes between the L-I/R and L-SpostC+2ME2 groups (P>0.05). (Table 6, Fig. 9)

HE staining of cardiomyocytes: Microscopic observation showed that the structure of myocardial tissue was basically normal in L-sham group, with only focal cardiomyocyte swelling and a small amount of chronic inflammatory cell infiltration; myocardial tissue was arranged irregularly in the L-I/R group, cardiomyocytes were swollen and vacuolar degeneration, some myocardial fibers were fractured, more chronic inflammatory cells were infiltrated, and local necrosis was observed. Compared with the L-I/R group, the disorder of myocardial arrangement, myocardial cell swelling, myocardial fiber fracture, and inflammatory cell infiltration were reduced in the L-SpostC group. Compared with the L-I/R group, the pathological changes were more severe in the L-SpostC+2ME2 group. (Fig. 10)

Myocardial mitochondrial ultrastructure: L-sham group: myofibrils were basically aligned, myotome length was basically uniform; mitochondrial cristae were extensively dissolved into flocculent, outer membrane segmentally dissolved, matrix segmentally dissolved. L-I/R group: mitochondria were obviously swollen, and mitochondrial cristae were extensively dissolved into flocculent, outer membrane segmentally dissolved, matrix obviously dissolved, autophagosomes were visible. However, the mitochondrial morphology of the L-SpostC group was still good, only some mitochondria were slightly swollen, and no noticeable dissolution of myofilament was observed. The damage of myocardial ultrastructure in L-SpostC+2ME2 group was similar to that in L-I/R group. (Fig. 11)

Changes in HIF-1α subunit and hypoxia-sensitive gene expression: We further determined the expression of HIF-1α and downstream LC3-II, BNIP3, Beclin1, TLR9, and IL-6 proteins. The results showed that the expression of HIF-1α and downstream LC3-II, BNIP3, Beclin1, and TLR9 was significantly increased in the L-SpostC group, whereas IL-6 protein expression was significantly decreased (P<0.05 compared with L-I/R and L-SpostC+2ME2 groups). However, the protective effect of the L-SpostC+2ME2 group on the above proteins was completely reversed after the administration of 2ME2, in which LC3-II protein levels were not significantly different in the L-I/R and L-SpostC+2ME2 groups (P>0.05). (Table 7, 8, Fig. 12)

Myocardial apoptosis levels: In the aged myocardium, SPostC reduced the apoptosis rate after I/R injury from 40.05±5.51% to 25.52±8.50% (P<0.05). In contrast, the apoptosis rate after I/R injury was significantly increased in the L-SpostC+2ME2 group after the use of HIF-1α blocker 2ME2 (L-SpostC group: 25.52±8.50 vs. L-SpostC+2ME2 group: 53.98±7.37, P<0.05). (Fig. 13)

Myocardial infarction size: We used 2ME2 to block HIF-1α signaling pathway in the aged myocardium and measured infarct size changes. SpostC significantly reduced myocardial infarct size in aged myocardium after I/R. (31.06±3.20% in C-SpostC group vs. 57.54±3.50% in C-I/R group, P<0.05). After administration of 2ME2, myocardial infarct size was significantly increased in the L-SpostC+2ME2 group (59.63±5.71%, P<0.05 compared with the L-
I/R+SpostC group), confirming that the myocardial protective effect of SpostC was abrogated after the use of HIF-1α-specific blocker. (Fig. 14)

**Discussion**

In recent years, the incidence of perioperative cardiovascular disease in elderly patients has been steeply rising, and increasing attention has been paid to this phenomenon. The present study confirmed that the HIF-1α signaling pathway is a crucial molecule in aged myocardium against ischemic-hypoxic injury. First, in an in vivo I/R model of young, healthy mice, HIF-1α upregulation was able to activate downstream target gene BNIP3 protein expression while also increasing the expression levels of essential autophagy proteins LC3-II, Beclin-1, and TLR9, which attenuated myocardial oxidative stress injury, stabilized mitochondrial ultrastructure, and ultimately reduced myocardial infarct size. Secondly, in an in vivo I/R injury model of aged mice, the expression of HIF-1α protein was decreased, and the expression levels of essential autophagy proteins BNIP3, LC3-II, Beclin-1, and TLR9 were reduced, which ultimately affected the morphology and function of mitochondria and increased the sensitivity of myocardium to ischemia-reperfusion injury. We demonstrated that SpostC could upregulate HIF-1α to enhance BNIP3-mediated mitochondrial autophagy, reduce the myocardial infarct size and attenuate the severity of myocardial I/R injury in aged mice. However, this protective effect was abrogated after application of the HIF-1α blocker 2ME2.

In cardiomyocytes, oxygen acts as a terminal electron acceptor during mitochondrial oxidative phosphorylation in target organs, and changes in intracellular oxygen concentration will profoundly affect energy metabolism. At the same time, the ability of cells to respond to changes in oxygen concentration is an effective way to prevent energy deprivation and cell death. In healthy hearts, the reduction in intracellular oxygen concentration is mediated by an evolutionarily conserved nuclear transcription factor, HIF-1α[11], and the HIF-1α signaling pathway plays a crucial role in glucose metabolism and microangiogenesis and cell proliferation[12]. Based on the important role of HIF-1α signaling pathway in myocardial protection, effective activation of HIF-1α signaling pathway may be a potential target for regulating energy metabolism in aged myocardium. In this study, we found that the structure and function of mitochondria in aged cardiomyocytes changed after I/R injury, which affected the energy metabolism of aged cardiomyocytes, SpostC could stabilize the mitochondrial structure and avoid the imbalance of energy metabolism.

Hypoxic response may be one of the causes of the accelerated aging process, which is regulated by the HIF-1α signaling pathway, while the mechanism of the HIF-1α signaling pathway with silencing regulatory proteins, AMP-activated protein kinase (AMPK), rapamycin complex 1 (mTORC1), and nuclear factor κB (NFκB) signaling pathways are all associated with aging[13, 14]. SpostC can produce myocardial protective effects similar to ischemic/hypoxic preconditioning. This study also confirmed that SpostC has a good myocardial protection effect for young, healthy myocardium with I/R injury[15]. Unlike other myocardial protection strategies, the ability of inhaled anesthetics such as sevoflurane to effectively decrease the mitochondrial membrane potential (△Ψm) is thought to be an important feature of its myocardial protective effect and an important mechanism for its ability to alleviate mitochondrial injury[16, 17]. Our study confirmed that the I/R injury of aged myocardium increased the level of myocardial oxidative stress, destroyed the cell structure, reduced the expression of HIF-1α protein, and weakened the autophagy mediated by BNIP3. SpostC could increase the expression of HIF-1α signaling pathway, stabilize the structure and function of mitochondria, and reduce the size of myocardial infarction. However, this protective effect was also abrogated after the application of HIF-1α signaling pathway blocker 2ME2, indicating that activation of HIF-1α pathway maintains mitochondrial integrity and stabilizes mitochondrial autophagy.

Autophagy plays a dual regulatory role in I/R and is an important factor determining cell survival or apoptosis. Moderate ischemic stimulation can upregulate autophagy and provide energy to cells. However, overstimulation may
lead to excessive autophagy and autophagic cell death. It has been found that mitochondrial function decreased after myocardial I/R injury, as evidenced by reduced mitochondrial number, abnormal morphology, mitochondrial DNA mutations, reduced copy number, decreased mitochondrial protein expression, abnormal metabolism, oxidative stress, and abnormal mitochondrial signaling\(^{18}\). Currently, PTEN-induced putative kinase1 (PINK1)-Parkin pathway\(^{19}\) and receptor (BNIP3, NIX, FUNDC1)-mediated pathways are considered to be the main pathways mediating mitochondrial autophagy\(^{20}\). Among them, BNIP3, a downstream target gene of the HIF-1\(\alpha\) signaling pathway, is a key receptor mediating mitochondrial autophagy\(^ {21}\). The primary mechanism is that BNIP3 competes with Beclin-1 through the BH3 structure to bind to Bcl-2 or Bcl-XL, which induces the free Beclin-1 to participate in the formation of PI3K complex and activate the P13K/Akt pathway, thus activating mitochondrial autophagy\(^{22,23}\). BNIP3 can also directly bind to LC3 to promote mitochondrial autophagy\(^{24,25}\). A Study confirmed that SPostC promotes autophagy through NO-dependent mechanisms against myocardial I/R injury, but this study did not involve mitophagy and related receptors in aged myocardium\(^ {26}\). The results of the present study showed that compared with the I/R group, SpostC significantly decreased myocardial oxidative stress levels, stabilized mitochondrial morphology and structure, and increased the expression of mitochondrial autophagy-related signaling pathway HIF-1\(\alpha\) and downstream LC3-II, BNIP3, Beclin1 and TLR9 proteins in aged myocardium, thereby inhibiting cardiomyocyte apoptosis. The mechanism is related to the regulation of HIF-1\(\alpha\) signaling pathway activity by SpostC.

The present study still has some limitations. Energy metabolism (e.g., AMPK pathway and GLUT-related pathway, etc.) was not addressed in the study of HIF-1\(\alpha\) signaling pathway and downstream target proteins in aged myocardium.

**Conclusion**

In the myocardial I/R injury model of aged mice, SpostC can activate HIF-1\(\alpha\) pathway. The upregulation of HIF-1\(\alpha\) and its downstream LC3-II, BNIP3, Beclin1, and TLR9 proteins can promote mitochondrial autophagy, inhibit cell apoptosis, and stabilize mitochondria's structure and function, inhibit myocardial oxidative stress, reduce myocardial infarct size, and ultimately protect the damaged elderly myocardium. In addition, this study confirmed that mitochondrial autophagy of aged myocardium was closely related to HIF-1\(\alpha\) pathway, which would provide new strategies for perioperative myocardial protection in elderly patients.

**Abbreviations**
Declarations

Ethics approval and consent to participate: Ethics: Ethical approval for this study (Ethics Committee: First Affiliated Hospital of Xinjiang Medical University: IACUC-20170214025) was provided by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, Xinjiang, China (Chairperson: Jian Liu) on 16 February 2017. consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interestsThe authors declare that they have no competing interests.

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Authors’ contributions:

JW and JJW made significant contributions to the conception or design of the work and drafted the article, TT was responsible for data acquisition, MM analyzed and interpreted the data, and YDH and SYC substantially revised the article. TTZ and LY coordinated all the work.

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Assistance with the article: Conceived and designed the experiments: HZ, JW, and TY. Performed the experiments: JW, LY, JY, YM, HM, and PX. Analyzed the data: JW, LY, YY, HW, and PX. Wrote the paper: JW, PX, HZ, HW, and JW.
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Presentation: None

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**Tables**

Table 1 Content (activity) of indicators in the serum of non-aged mice

$x \pm s$
### Table 2 Cardiac function indicators in non-aged status

| Cardiac function | C-sham       | C-I/R      | C-SpostC   | C-SpostC+2ME2 |
|------------------|--------------|------------|------------|---------------|
| EF (%)           | 76.52±5.69   | 53.90±4.08*# | 70.87±5.25 | 52.97±5.66   |
| FS (%)           | 50.23±3.38   | 27.42±2.51*# | 46.44±3.46 | 26.85±3.14   |
| SV (μl)          | 41.52±3.12   | 30.93±4.16*# | 37.43±3.12 | 29.90±3.30   |
| LVIDd (mm)       | 3.12±0.30    | 4.29±0.24*#  | 3.15±0.22  | 4.32±0.27    |
| LVIDs (mm)       | 1.73±0.17    | 2.73±0.21*#  | 1.73±0.16  | 2.64±0.18    |
| LVAWd (mm)       | 0.70±0.03    | 0.50±0.02*#  | 0.65±0.05  | 0.50±0.03    |
| LVAWs (mm)       | 1.48±0.17    | 0.88±0.11*#  | 1.53±0.26  | 0.86±0.09    |
| LVPWd (mm)       | 1.12±0.06    | 0.76±0.10*#  | 1.06±0.12  | 0.77±0.09    |
| LVPWs (mm)       | 1.61±0.07    | 0.84±0.10*#  | 1.58±0.10  | 0.85±0.11    |

*P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group. EF: ejection fraction; FS: fractional shortening; SV: stroke volume; LVIDd: left ventricular internal diameter in diastole; LVIDs: left ventricular internal diameter in systole; LVAWd: left ventricular anterior wall in diastole; LVAWs: left ventricular anterior wall in systole; LVPWd: left ventricular posterior wall thickness in diastole; LVPWs: left ventricular posterior wall thickness in systole.

### Table 3 Protein expression levels in non-aged myocardium

| Groups          | HIF-1α   | LC3-β    | Beclin1   |
|-----------------|----------|----------|-----------|
| C-sham          | 0.277±0.027 | 0.201±0.014 | 0.335±0.046 |
| C-I/R           | 0.413±0.035*# | 0.259±0.045*# | 0.608±0.071*# |
| C-SpostC        | 0.532±0.022 | 0.367±0.019 | 0.762±0.038 |
| C-SpostC+2ME2   | 0.236±0.046* | 0.221±0.031* | 0.362±0.076* |

*P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group. HIF-1α: hypoxia-inducible factors α; LC3-β: Microtubule-associated protein light chain 3; Beclin1.
Table 4 Protein expression levels in non-aged myocardium

| Groups   | BNIP3     | TLR9     | IL-6     |
|----------|-----------|----------|----------|
| C-sham   | 0.366±0.043 | 0.356±0.029 | 0.338±0.050 |
| C-I/R    | 0.566±0.048*# | 0.555±0.031*# | 0.521±0.058*# |
| C-SpostC | 0.724±0.053 | 0.766±0.084 | 0.336±0.071 |
| C-SpostC+2ME2 | 0.324±0.036# | 0.453±0.049# | 0.694±0.086# |

*P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group. BNIP3: BCL2 Interacting Protein 3; TLR9: Toll Like Receptor 9; IL-6: Interleukin 6.

Table 5 Content (activity) of indicators in the serum of aged mice

| Groups   | LDH(U/L)   | CKM(mU/ml) | CK-MB(mU/ml) | TNNT2(pg/ml) | IL-6(pg/ml) |
|----------|------------|------------|--------------|--------------|--------------|
| L-sham   | 75.054±29.139 | 5.193±2.106 | 4.661±1.616 | 52.516±18.757 | 15.122±4.257 |
| L-I/R    | 107.849±25.415*# | 7.839±2.683*# | 7.218±2.335*# | 79.945±30.773*# | 28.313±10.543*# |
| L-SpostC | 78.017±23.467 | 5.436±2.115 | 4.950±2.246 | 55.328±19.972 | 18.829±8.073 |
| L-SpostC+2ME2 | 142.339±35.935*# | 10.333±2.619*# | 9.802±2.534*# | 107.288±29.151*# | 37.678±11.201*# |

* P<0.05 vs L-sham group; # P<0.05 vs L-SpostC group. LDH: lactate dehydrogenase; CKM: Creatine Kinase; CK-MB: Creatine kinase isoenzyme MB; TNNT2: Troponin T type 2; IL-6: interleukin 6.

Table 6 Cardiac function indicators in aged status

| Cardiac function | L-sham | L-I/R  | L-SpostC | L-SpostC+2ME2 |
|------------------|--------|--------|----------|---------------|
| EF (%)           | 77.93±3.99 | 53.80±2.47*# | 70.73±3.68 | 49.71±3.22*# |
| FS (%)           | 48.16±2.40 | 29.43±2.40*# | 41.19±2.83 | 26.24±2.39*# |
| SV (µl)          | 44.29±2.63 | 29.92±1.93*# | 37.32±2.74 | 30.42±3.11*# |
| LVIDd (mm)       | 3.63±0.24 | 5.11±0.28*# | 3.79±0.25 | 5.14±0.64*# |
| LVIDs (mm)       | 1.84±0.14 | 2.90±0.22*# | 2.00±0.13 | 3.00±0.24*# |
| LVAWd (mm)       | 0.80±0.08 | 0.61±0.04*# | 0.80±0.12 | 0.61±0.06*# |
| LVAWs (mm)       | 1.53±0.15 | 0.91±0.23*# | 1.59±0.26 | 0.89±0.16*# |
| LVPWd (mm)       | 1.10±0.10 | 0.77±0.07*# | 1.01±0.10 | 0.77±0.08*# |
| LVPWs (mm)       | 1.62±0.09 | 0.82±0.08*# | 1.59±0.10 | 0.89±0.22*# |
*P<0.05 vs L-sham group; #P<0.05 vs L-SpostC group. EF: ejection fraction; FS: fractional shortening; SV: stroke volume; LVIDd: left ventricular internal diameter in diastole; LVIDs: left ventricular internal diameter in systole; LVAWd: left ventricular anterior wall in diastole; LVAWs: left ventricular anterior wall in systole; LVPWd: left ventricular posterior wall thickness in diastole; LVPWs: left ventricular posterior wall thickness in systole.

Table 7 Protein expression levels in aged myocardium

| Groups       | HIF-1α   | LC3-β   | Beclin1    |
|--------------|----------|---------|------------|
| L-sham       | 0.257±0.037 | 0.178±0.021 | 0.275±0.016 |
| L-I/R        | 0.389±0.057*# | 0.240±0.041*# | 0.524±0.040*# |
| L-SpostC     | 0.517±0.075 | 0.296±0.030 | 0.747±0.057 |
| L-SpostC+2ME2 | 0.269±0.040*# | 0.215±0.018*# | 0.336±0.018*# |

*P<0.05 vs L-sham group; #P<0.05 vs L-SpostC group. HIF-1α: hypoxia-inducible factors α; LC3-β: Microtubule-associated protein light chain 3-β.

Table 8 Protein expression levels in aged myocardium

| Groups       | BNIP3    | TLR9     | IL-6       |
|--------------|----------|----------|------------|
| L-sham       | 0.239±0.035 | 0.338±0.049 | 0.310±0.033 |
| L-I/R        | 0.482±0.072*# | 0.493±0.041*# | 0.529±0.042*# |
| L-SpostC     | 0.664±0.121 | 0.637±0.039 | 0.381±0.057 |
| L-SpostC+2ME2 | 0.315±0.050*# | 0.358±0.006*# | 0.693±0.027*# |

*P<0.05 vs L-sham group; #P<0.05 vs L-SpostC group. BNIP3: BCL2 Interacting Protein 3; TLR9: Toll Like Receptor 9; IL-6: Interleukin 6.

Figures
Figure 1

Content (activity) of indicators in the serum of non-aged mice. *P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group.
Figure 2

The cardiac function indicators of non-aged mice. (A) Cardiac ultrasound images. (b) Comparison of EF values among groups of non-aged mice. (c) Comparison of FS values among groups of non-aged mice. *P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group.
| Groups          | 40× | 100× | 200× | 400× |
|-----------------|-----|------|------|------|
| C-sham          | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| C-I/R           | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| C-SpostC        | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| C-SpostC+2ME2   | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |

**Figure 3**
Comparison of HE staining in non-aged mice.
Figure 4

Comparison of myocardial mitochondrial ultrastructure among groups of non-aged mice.
Figure 5

Changes in HIF-1α subunit and hypoxia-sensitive gene expression. (a) Comparison of protein expression levels among groups of non-aged mice.*P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group. (b) Protein bands diagram of non-aged mice.
|                | TUNEL | DAPI | Merge |
|----------------|-------|------|-------|
| **C-sham**     | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| **C-I/R**      | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| **C-SPostC**   | ![Image](image7) | ![Image](image8) | ![Image](image9) |
| **C-SPostC+2ME2** | ![Image](image10) | ![Image](image11) | ![Image](image12) |

**Figure 6**

Myocardial apoptosis levels. (a) Results of TUNEL staining. (b) Comparison of apoptosis among groups of non-aged mice. *P<0.05 vs C-sham group; #P<0.05 vs C-SPostC group.
Figure 7

Myocardial infarct size (a) Myocardial infarct area. (b) Comparison of myocardial infarct size among groups of non-aged mice. *P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group.
Figure 8

Content (activity) of indicators in the serum of aged mice. *P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group.
Figure 9

The cardiac function indicators of aged mice. (a) Cardiac ultrasound images. (b) Comparison of EF values among groups of aged mice. (c) Comparison of FS values among groups of aged mice. EF: ejection fraction; FS: fractional shortening; *P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group.
| Groups     | 40 × | 100 × | 200 × | 400 × |
|------------|------|-------|-------|-------|
| L-sham     | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| L-I/R      | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| L-SpostC   | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| L-SpostC+2ME2 | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |

**Figure 10**

Comparison of HE staining in aged mice.
Figure 11

Comparison of myocardial mitochondrial ultrastructure among groups of aged mice.
Figure 12

Changes in HIF-1α subunit and hypoxia-sensitive gene expression. (a) Comparison of protein expression levels among groups of aged mice.*P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group. (b) Protein bands diagram of aged mice.

Figure 13

Apoptosis ratio(%)
Myocardial apoptosis levels. (a) Results of TUNEL staining. (b) Comparison of apoptosis among groups of aged mice. *P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group.

Figure 14

Myocardial infarct size (a) Myocardial infarct area. (b) Comparison of myocardial infarct size among groups of aged mice. *P<0.05 vs C-sham group; #P<0.05 vs C-SpostC group.