Effects of Sevoflurane Pretreatment on Myocardial Ischemia-Reperfusion Injury Through the Akt/Hypoxia-Inducible Factor 1-alpha (HIF-1α)/Vascular Endothelial Growth Factor (VEGF) Signaling Pathway

Jingwei Dong
Mingjun Xu
Wenyu Zhang
Xiangming Che

Corresponding Author: Xiangming Che, e-mail: chexming@hotmail.com
Source of support: Departmental sources

Background: The aim of this study was to investigate the effects of sevoflurane (SEV) on myocardial ischemia/reperfusion (I/R) injury in rats and its mechanism.

Material/Methods: Sixty male Sprague-Dawley rats were randomly divided into 3 groups: Sham group (n=20), I/R group (n=20) and I/R+SEV group (n=20). The I/R model was established by ligating and recanalizing the left anterior descending coronary artery (LAD). Triphenyl tetrazolium chloride (TTC) test and echocardiography (ECG) were used for analysis. Hematoxylin and eosin (H&E) staining was applied to detect the morphological changes. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was conducted to detect the apoptosis levels. The expression level of superoxide dismutase 2 (SOD2) was measured. Finally, the effect of SEV on the protein kinase B (Akt)/hypoxia-inducible factor 1-alpha (HIF-1α)/vascular endothelial growth factor (VEGF) signaling pathway was detected via western blotting.

Results: SEV could significantly improve I/R-induced cardiac insufficiency, inhibit cardiac infarction, and as well as reduce the infarction area from 53.21±2.11% to 32.33±3.49% (P<0.05). Compared with rats in I/R group, the cardiac myofilament was better in alignment, degradation and necrosis were milder, and cell edema was notably reduced in the I/R+SEV group. Thus, SEV could significantly reverse the decreased expression of SOD2 caused by I/R and reduce oxidative stress in the heart (P<0.05). According to the western blotting results, SEV was capable of obviously activating the expressions of phosphorylated-Akt (p-Akt), HIF-1α, and VEGF.

Conclusions: SEV can significantly improve myocardial injury caused by I/R in rats, and its mechanism might be related to the activation of the Akt/HIF-1α/VEGF signaling pathway.

MeSH Keywords: Hypoxia-Inducible Factor 1, alpha Subunit • Proto-Oncogene Proteins c-akt • Receptors, Vascular Endothelial Growth Factor • Reperfusion Injury

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/914265
Background

Ischemic heart disease is the main cause of death and also a major public health problem worldwide [1]. Reperfusion is an important strategy for the heart to rescue the irreversible injury of myocardial tissues. However, reperfusion after ischemia can usually cause certain damage to the myocardium, which is called myocardial ischemia/reperfusion (I/R) injury [2]. I/R injury is an inevitable pathophysiological phenomenon in the treatment of ischemic heart disease and heart surgery in patients with thoracotomy, which can lead to reperfusion arrhythmia, temporary mechanical dysfunction, myocardial stunning, and other pathological changes [3,4]. Therefore, inhibiting myocardial I/R injury is of great significance to the prevention and treatment of ischemic cardiomyopathy.

Studies have shown that myocardial I/R injury is closely related to many factors, such as the generation of large amounts of oxidized free radicals, changes in cardiac hemodynamics, calcium overload in myocardial cells, inflammation, necrosis and apoptosis of myocardial cells [5,6]. Moreover, a large number of animal experiments and clinical studies have confirmed that abnormal inhibition of the protein kinase B (Akt)/hypoxia-inducible factor 1-alpha (HIF-1α) signaling pathway is also an important cause of I/R injury. HIF-1α, as a protective factor against hypoxia, can stimulate transcription of multiple protective genes, such as vascular growth factors (VEGFs), and can promote myocardial cell survival during I/R [7]. In addition, the activation of HIF-1α can transform oxidative phosphorylation into glycolysis in cardiomyocytes under hypoxic conditions, thus inhibiting excessive accumulation of reactive oxygen species (ROS) in myocardial cells [8]. However, the activation of HIF-1α can be regulated by the Akt signal, so activating the Akt/HIF-1α/VEGF axis can effectively inhibit I/R injury [9].

Inhaling certain amounts of anesthetics before ischemia or before reperfusion can inhibit I/R-induced injury. Sevoflurane (SEV), as a common anesthetic, has advantages such as short induction and recovery time, less stimulation, high safety and (SEV), as a common anesthetic, has advantages such as short induction and recovery time, less stimulation, high safety and (SEV), as a common anesthetic, has advantages such as short induction and recovery time, less stimulation, high safety and (SEV), as a common anesthetic, has advantages such as short induction and recovery time, less stimulation, high safety and prevention of I/R injury in a clinic setting by constructing an I/R injury model in rats, detecting the role of SEV in I/R injury and exploring its molecular mechanism.

Material and Methods

Laboratory animal grouping and processing

A total of 60 male Sprague-Dawley rats weighing 285.61±10.66 g were divided into 3 groups by random number table. The 3 groups were: the sham operation group (Sham group, n=20), the I/R group (n=20), and the IR+SEV group (n=20). There were no statistical differences in basic data such as weeks of age and body weight among the 3 groups of rats. Rats in each group were placed in an organic glass anesthesia room with good air tightness, and the calibrated evaporator was connected to the anesthesia room where 0% gas (Sham group and I/R group) or 2.5% SEV gas (I/R+SEV group) was delivered. After 15 minutes, the rats in each group were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital, and then the cannula was inserted into the left carotid artery to measure the blood pressure of the study rats. Two-lead electrocardiography (ECG) of the limb was used to detect the heart rate. The thorax in the fourth intercostal space was opened, and the pericardium was cut off to expose the heart. The left anterior descending coronary artery (LAD) was ligated at 2 mm above the left atrial appendage by the 6-0 silk thread to induce ischemia. After ischemia for 30 minutes, the silk thread was loosened, followed by reperfusion for 2 hours. Rats in the Sham group underwent the same procedure, but the silk thread was not ligated. After reperfusion, the rats were sacrificed, and the myocardial tissue in the anterior wall of the left ventricle of the rats was removed, rinsed with normal saline to remove blood, and then placed in a refrigerator at –80°C (Figure 1). This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center.

Echocardiography detection

In order to detect the cardiac function of each group of rats, MyLab 30CV ultrasound system (Esaote, S.P.A, Genea, Italy) and 10-MHz linear ultrasonic transducers were used to detect the echocardiogram of rats in each group. After shaving the
hair in the anterior thoracic region of the study rats and anesthesia, the rats were placed on a heating plate at 37°C with the left side facing up. Parameters including ejection fraction (EF), fraction shortening (FS) and heart rate were detected.

**Triphenyl tetrazolium chloride (TTC) staining**

Fresh heart tissue samples were put into a rat heart slice grinder and frozen in a refrigerator for 30 minutes at –20°C for slicing. The heart tissue was cut into slices with a thickness of about 2 mm along the short axis of the heart from apical segments, with 3 to 4 slices per heart. The cut slices were placed in fresh TTC solution (2%) and fully contacted the TTC solution for an incubation time of not less than 0.5 hours. After 0.5 hours, the slices were taken out and fixed with 4% paraformaldehyde, followed by photography.

**Immunohistochemical staining**

The cut myocardial tissue sections were baked in an oven at 60°C for 30 minutes and then dewaxed with xylene (5 minutes, 3 times), followed by dehydration with 100%, 95%, and 70% ethanol, respectively, for 3 times. The endogenous peroxidase activity was inhibited by 3% hydrogen peroxide methanol, and then the tissues was sealed with sheep serum for 1 hour. Antibodies against superoxide dismutase 2 (SOD2) were diluted at 1: 200 with phosphate-buffered saline (PBS) and incubated at 4°C overnight, followed by washing with PBS for 4 times in a shaker. After the second antibody was added, the color was developed with diaminobenzidine. After the completion of color development, 6 samples were randomly selected from each group, and 5 fields of view were randomly selected from each sample, followed by photographing under 200× and 400× optical microscopes.

**Hematoxylin and eosin (H&E) staining**

The hearts obtained in each group were placed in 10% formalin overnight, then dehydrated and embedded in wax blocks. Subsequently, all myocardial tissues were cut into thin slices with a thickness of 5 μm, fixed on glass slides and dried for staining. According to the instructions, they were soaked in xylene, ethanol at gradient concentration and hematoxylin, and then sealed with resin. After drying in the air, the slices were stained with hematoxylin, the cells were photographed and counted under the optical microscope.

**Western blotting detection**

After heart tissues of rats in each group were fully ground in lysis buffer, they were ultrasonically lysed, and the lysis buffer was centrifuged to extract supernatant, which was successively split into Eppendorf tubes. The protein concentration was measured via the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and the ultraviolet spectrophotometry, and the protein volume of all samples was set constant to equal concentration. After subpackaging, the slices were placed in the refrigerator at –80°C. After the total protein was extracted, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed. Then the protein in the gel was transferred to cellulose acetate (polyvinylidene fluoride) membrane (Roche, Basel, Switzerland), incubated in the primary antibody at 4°C overnight, and incubated in the goat anti-rabbit secondary antibody for 1 hour away from light. Protein bands were scanned and quantified using an Odyssey membrane sweeper, and glyceraldehyde 3-phosphate dehydrogenase was used to correct the level of proteins to be tested.

**Statistical analysis**

All the data were analyzed by Statistical Product and Service Solutions 22.0 software (IBM, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation, and the comparisons of data between 2 groups were conducted using the t-test. P<0.05 represented that the difference was statistically significant.

**Results**

**Effects of SEV pretreatment on cardiac function of rats in each group**

As shown in Figure 2, the ECG results manifested that there were no statistical differences in the heart rate among the 3 groups of rats, so it could be excluded that the difference between EF (%) and FS (%) in each group was caused by the
difference in the heart rate. Compared with rats in the control group, I/R rats had enlarged ventricular cavity and thinned cardiac wall, and SEV pretreatment could notably improve the abnormal changes in the cardiac structure caused by I/R. Furthermore, the levels of FS (%) and EF (%) in each group of rats were detected, which revealed that SEV pretreatment markedly reversed the decreases in FS (%) and EF (%) in I/R group ($P < 0.05$). The aforementioned results indicated that SEV pretreatment can improve the level of cardiac function caused by reperfusion in rats.

**SEV pretreatment reduced the heart infarction area of rats caused by I/R**

The heart infarction area in each group was evaluated by TTC staining. The results demonstrated that the infarction area of the 3 groups of rats were $1.03\pm0.02$ versus $54.82\pm2.34$ versus $34.92\pm2.98$, with statistically significant differences $P<0.05$, suggesting that that the inhalation of 2.5% SEV effectively reduces the myocardial infarction area in I/R rats (Figure 3).
H&E staining results of the heart of rats in each group

In order to evaluate the changes in the myocardial cell microstructure in the heart cross section of rats in each group, H&E staining was performed for the myocardial tissue. According to the results, myocardial cells in I/R group showed obvious edema, disordered myofilament arrangement, degradation, and necrosis to varying degrees. However, after SEV pretreatment, the edema of the myocardial tissue in rats was obviously reduced, and the abnormality of myofilament was also significantly improved (Figure 4). These results denoted that SEV can alleviate I/R-induced myocardial injury.

Effect of SEV pretreatment on myocardial cell apoptosis in rats

Through TUNEL staining, the apoptosis level of myocardial cells in the 3 groups of rats was detected. The results revealed that after I/R injury in rats, the number of apoptotic myocardial cells was evidently increased ($P<0.05$), about 72.12±1.22 times as much as that in the control group, while after SEV pretreatment, the number of apoptotic myocardial cells was decreased to 23.12±2.83 times as much as that in the control group ($P<0.05$) (Figure 5). These results indicated that SEV pretreatment can significantly inhibit the apoptosis of myocardial cells of rats.

Effect of SEV pretreatment on oxidative stress in the heart of rats

Considering that oxidative stress is an important pathological change in ischemic cardiomyopathy, immunohistochemistry was adopted to detect the expression of antioxidant enzyme SOD2 in the heart of each group of rats. It was found that compared with that in the heart of rats in Sham group, the expression level of SOD2 in the heart of rats in I/R group was markedly decreased ($P<0.05$), but after SEV treatment, this level was significantly increased ($P<0.05$) (Figure 6). These results indicated that SEV pretreatment can reduce the oxidative stress level in the myocardial tissue of rats.

Expression level of the Akt/HIF-1α/VEGF signaling pathway in the myocardial tissue of rats in each group

To further explore the molecular mechanism of SEV on myocardial protection, the activation of the Akt/HIF-1α/VEGF pathway in myocardial cells was detected by immunohistochemistry. The results showed that the expression level of Akt in the myocardial tissue of rats in the I/R group was significantly decreased ($P<0.05$), while after SEV pretreatment, this level was increased ($P<0.05$) (Figure 7). These results indicated that SEV pretreatment can activate the Akt pathway in myocardial cells.
signaling pathway was examined. The results verified that the expressions of phosphorylated-Akt (p-Akt), HIF-1α, and VEGF in the myocardial tissue of rats in I/R group were inhibited (*P<0.05), while the expression levels of these 3 proteins were significantly increased after SEV pretreatment (*P<0.05) (Figure 7). Therefore, it is speculated that SEV may play a role in protecting the heart by activating the Akt/HIF-1α/VEGF signaling pathway.

Discussion

Rapid recovery of blood flow through occluded coronary arteries via mechanical or pharmaceutical intervention is the most effective strategy to limit myocardial infarction area and improve clinical prognosis after acute myocardial infarction [12]. However, I/R itself can also lead to additional myocardial cell death and increase the infarction area of the heart [13].
Factors leading to I/R injury mainly include oxidative stress, inflammation, and apoptosis. During myocardial ischemia, adenosine triphosphate consumption in myocardial cells decreases the uptake of Ca$^{2+}$ by the sarcoplasmic reticulum, resulting in a large accumulation of Ca$^{2+}$ in mitochondria [14]. During reperfusion, the re-entry of oxygen into myocardial cells will cause damage to the mitochondrial electron transport chain and increase the production of reactive oxygen species (ROS). Mitochondrial Ca$^{2+}$ overload and increased ROS production both promote the opening of mitochondrial membrane permeability transformation pores, resulting in cell energy disorder and eventually irreversible causing necrosis and apoptosis of cells. Therefore, inhibiting myocardial cell apoptosis, inflammation and oxidative stress during reperfusion can effectively improve cardiac dysfunction caused by I/R injury and reduce the infarction area of the heart [15,16].

SEV, as a new type of halogen inhalation anesthetic, has many clinical advantages, and thus it has been widely used in total anesthesia and anesthesia maintenance. In recent years, more and more studies have revealed that SEV has many other pharmacological effects besides anesthetic effects [17]. For example, 2.4% SEV pretreatment can reduce ischemic brain injury in rats by inhibiting neuronal cell apoptosis for 4 consecutive days (30 minutes per day) [18]. In addition, inhalation of 2% SEV for 2 consecutive hours can evidently improve I/R-induced brain injury in rats, which may be related to the activation of AKT phosphorylation and inhibition of the c-Jun NH(2)-terminal kinase 3/caspase-3 cascade by SEV [19]. SEV can inhibit glial cell migration and invasion by upregulating the micro RNA-637 (mir-637) level in the glioma U251 cell line [20]. In the liver I/R injury model, SEV pretreatment can also inhibit oxidative stress and apoptosis of hepatocytes, which might be associated with the inhibition of mir-200c by SEV [21]. However, this study confirmed for the first time that 2.5% SEV pretreatment could significantly improve cardiac insufficiency caused by I/R injury, alleviate myocardial injury, reduce oxidative stress level of myocardial cells, and inhibit the apoptosis of myocardial cells.

Over the past few decades, studies have shown that HIF-1α plays an important regulatory role in the hypoxia response. As a transcription factor, HIF-1α can transcribe and activate various genes (including genes related to the oxidative stress, energy metabolism and angiogenesis), and the protein products of these genes can adapt to hypoxia stress by enhancing the oxygen transfer or promoting the metabolic reaction. HIF-1α has been proved to play a crucial role in the pathophysiological changes of embryonic angiogenesis, tumor angiogenesis and ischemic diseases [22,23]. During myocardial ischemia, the activation of HIF-1α can induce cell protection and metabolic reprogramming to cope with oxygen deprivation of myocardial cells. The upregulation of the HIF-1α level is one of the earliest adaptive responses at the molecular level of myocardial ischemia [24]. Experimental studies have demonstrated that HIF-1α can act as a mediator of ischemic pretreatment, so it can be an important target for inhibiting myocardial I/R injury. However, studies have shown that Akt phosphorylation is the initial signal of the HIF-1α activation. In hypoxia, Akt phosphorylation in myocardial cells is inhibited. Once Akt phosphorylation is activated, it can upregulate the expression of VEGF and finally activate various protective genes to combat I/R injury [25,26]. In this study, the expression of VEGF in the downstream of HIF-1α was detected. It was found that SEV pretreatment could increase the expression of VEGF, and VEGF was closely correlated with angiogenesis. In I/R injury, increasing angiogenesis could effectively improve hypoxia in the lesion area. Nevertheless, this study had some limitations: it was not verified by cell experiments; the pharmacological effects of SEV were not reversely verified with AKT inhibitors; and the related indicators were not detected and verified.

**Conclusions**

In conclusion, the results of this study revealed that SEV could effectively improve cardiac dysfunction, myocardial cell apoptosis, and oxidative stress caused by myocardial I/R injury in rats, and its mechanism might be related to the activation of the Akt/HIF-1α/VEGF signaling pathway.

**Conflict of interest**

None.

---

**References:**

1. Li Y, Jiang Q, Ding Z et al: Identification of a common different gene expression signature in ischemic cardiomyopathy. Genes (Basel), 2018; 9: 56
2. Jin Q, Li R, Hu N et al: DUSP1 alleviates cardiac ischemia/reperfusion injury by suppressing the MIF-required mitochondrial fission and Bnip3-related mitophagy via the INK pathways. Redox Biol, 2018; 14: 576–87
3. Li L, Pan CS, Yan L et al: Ginsenoside Rg1 ameliorates rat myocardial ischemia-reperfusion injury by modulating energy metabolism pathways. Front Physiol, 2018; 9: 78
4. Li D, Wang X, Huang Q et al: Cardioprotection of CAPE-oNO2 against myocardial ischemia/reperfusion induced ROS generation via regulating the SIRT1/eNOS/NF-kappaB pathway in vivo. Redox Biol, 2018; 15: 62–73
5. Qiu Y, Wu Y, Meng M et al: GYY4137 protects against myocardial ischemia/reperfusion injury via activation of the PHLPP-1/Akt/Nrf2 signaling pathway in diabetic mice. J Surg Res, 2018; 225: 29–39
6. Cadenas S: ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection. Free Radic Biol Med, 2018; 117: 76–89
7. Luo SY, Xu QH, Peng G, Chen ZW: The protective effect of total flavones from Rhododendron simsii Planch. on myocardial ischemia/reperfusion injury and its underlying mechanism. Evid Based Complement Alternat Med, 2018; 2018: 6139372
8. Zhao D, Yang J, Yang L: Insights for oxidative stress and mTOR signaling in myocardial ischemia/reperfusion injury under diabetes. Oxid Med Cell Longev, 2017; 2017: 6437467
9. Shi J, Bei Y, Kong X et al: miR-17-3p contributes to exercise-induced cardiac growth and protects against myocardial ischemia-reperfusion injury. Theranostics, 2017; 7: 664–76
10. Tian HT, Duan XH, Yang YF et al: Effects of propofol or sevoflurane anesthesia on the perioperative inflammatory response, pulmonary function and cognitive function in patients receiving lung cancer resection. Eur Rev Med Pharmacol Sci, 2017; 21: 5515–22
11. Zhao J, Wang F, Zhang Y et al: Sevoflurane preconditioning attenuates myocardial ischemia/reperfusion injury via caveolin-3-dependent cyclooxygenase-2 inhibition. Circulation, 2013; 128: S121–29
12. Chen Y, Ba L, Huang W et al: Role of carvacrol in cardioprotection against myocardial ischemia/reperfusion injury in rats through activation of MAPK/ERK and Akt/eNOS signaling pathways. Eur J Pharmacol, 2017; 796: 90–100
13. Yu L, Gong B, Duan W et al: Melatonin ameliorates myocardial ischemia/reperfusion injury in type 1 diabetic rats by preserving mitochondrial function: role of AMPK-PGC-1alpha-SIRT3 signaling. Sci Rep, 2017; 7: 41337
14. Wang X, Yang L, Kang L et al: Metformin attenuates myocardial ischemia-reperfusion injury via up-regulation of antioxidant enzymes. PLoS One, 2017; 12: e182777
15. Zhang C, Tang Y, Li Y et al: Unfolded protein response plays a critical role in heart damage after myocardial ischemia/reperfusion in rats. PLoS One, 2017; 12: e179042
16. Timotin A, Pisarenko O, Sidorova M et al: Myocardial protection from ischemia/reperfusion injury by exogenous galanin fragment. Oncotarget, 2017; 8: 21241–52
17. Sobbeler FJ, Carrera I, Pasloske K et al: Effects of isoflurane, sevoflurane, propofol and alfaxalone on brain metabolism in dogs assessed by proton magnetic resonance spectroscopy (1H MRS). BMC Vet Res, 2018; 14: 69
18. Wang H, Shi H, Yu Q et al: Sevoflurane preconditioning confers neuroprotection via anti-apoptosis effects. Acta Neurochir Suppl, 2016; 121: 55–61
19. Shen XR, Fu YY, Liu HZ et al: Neuropeptidergic protection of sevoflurane against ischemia/reperfusion-induced brain injury through inhibiting JNK3/caspase-3 by enhancing Akt signaling pathway. Mol Neurobiol, 2016; 53: 1661–71
20. Yi W, Li D, Guo Y et al: Sevoflurane inhibits the migration and invasion of glioma cells by upregulating miRNA-637. Int J Mol Med, 2016; 38: 1857–63
21. Wu Y, Gu C, Huang X: Sevoflurane protects against hepatic ischemia/reperfusion injury by modulating microRNA-200c regulation in mice. Biomed Pharmacother, 2016; 84: 1126–36
22. Chin H, Arnijo BS, Miller E et al: Propranolol induces regression of hemicardia cells through HIF-1alpha-mediated inhibition of VEGF-A. Ann Surg, 2012; 256: 146–56
23. Watanabe Y, Murdoch CE, Sano S et al: Glutathione adducts induced by ischemia and deletion of glutaredoxin-1 stabilize HIF-1alpha and improve limb revascularization. Proc Natl Acad Sci USA, 2016; 113: 6011–16
24. Yang S, Yin J, Hou X: Inhibition of miR-135b by SP-1 promotes hypoxia-induced vascular endothelial cell injury via HIF-1alpha. Exp Cell Res, 2018; 370: 31–38
25. Shyu KG, Wang MT, Wang BW et al: Intramyocardial injection of naked DNA encoding HIF-1alpha/VP16 hybrid to enhance angiogenesis in an acute myocardial infarction model in the rat. Cardiovasc Res, 2002; 54: 576–83
26. Tasatargil A, Kuscu N, Dalakioglu S et al: Cardioprotective effect of nesfatin-1 against isoproterenol-induced myocardial infarction in rats: role of the Akt/GSK-3beta pathway. Peptides, 2017; 95: 1–9