Isoflurane reduces pain and inhibits apoptosis of myocardial cells through the phosphoinositide 3-kinase/protein kinase B signaling pathway in mice during cardiac surgery

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Abstract. Heart bypass surgery is the most common treatment for myocardial ischemia. Clinical investigations have revealed that isoflurane anesthesia is efficient to alleviate pain during cardiac surgery, including heart bypass surgery. Previous studies have revealed the protective effects of isoflurane on myocardial cells with myocardial ischemia during the perioperative period. The present study aimed to investigate the mechanism underlying the protective effects of isoflurane on myocardial cells in mice with myocardial ischemia. ELISA, flow cytometry, immunofluorescence and western blotting were used to analyze the effects of isoflurane anesthesia on myocardial cells. Briefly, myocardial cell apoptosis and viability, pain, phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway expression and the pharmacodynamics of isoflurane were studied in mice treated with isoflurane for heart bypass surgery. The results demonstrated that isoflurane anesthesia efficiently attenuated pain in mice during surgery. Viability and apoptosis of myocardial cells was also improved by isoflurane in vitro and in vivo. The PI3K/AKT pathway was upregulated in myocardial cells on day 3 post-operation. Mechanistically, isoflurane promoted PI3K/AKT activation, upregulated B-cell lymphoma 2 (Bcl-2)-associated X protein and Bcl-2 expression levels, and reduced the expression levels of caspase-3 and caspase-8 in myocardial cells. In conclusion, the findings indicated that isoflurane is beneficial for pain attenuation and inhibits apoptosis of myocardial cells via the PI3K/AKT signaling pathway in mice during cardiac surgery.

Introduction

Myocardial ischemia-reperfusion injury has been reported to be associated with severe secondary cardiac injury and the danger of myocardial ischemia-reperfusion injury has been emphasized by medical professionals worldwide (1). Myocardial cell apoptosis is associated with unsatisfactory recovery following the treatment of ischemic heart disease (2,3). Heart bypass surgery is clinically the most common treatment for myocardial ischemia (4,5). A previous study indicated that administering anesthesia to patients with myocardial ischemia undergoing heart bypass surgery can reduce pain, thus increasing surgical success (6). The efficacy of volatile general anesthetics has been investigated in patients with myocardial ischemia who have undergone heart bypass surgery; as such anesthesia is commonly administered (7,8). Anesthetic considerations for adult heart bypass surgery have also been created for clinicians (9).

Isoflurane is a volatile general anesthetic that can reduce behavioral responsiveness in animals (10). In addition, pretreatment with isoflurane exerts protective effects on rats with focal cerebral ischemia; the mechanism underlying these effects has been reported to involve the downregulation of toll-like receptor 4, myeloid differentiation primary response 88 and nuclear factor-kB expression (11). It has also been suggested that isoflurane activates the serine/threonine-protein kinase-11-p53-p21 signaling pathway, thereby suppressing self-renewal of normal mouse neural stem cells (12). Furthermore, the cell cycle and respiration of human bronchial epithelial cells can be inhibited in a p53-dependent manner via emulsified isoflurane (13). These data suggest that isoflurane may regulate various signaling pathways in the perioperative period.

The PI3K/AKT signaling pathway serves an essential role in cell growth, proliferation and survival under physiological conditions (14). To date, few studies have reported the relationship between the PI3K/AKT signaling pathway and myocardial ischemia. Recently, animal models have been used to investigate isoflurane-induced neuroapoptosis mediated by the PI3K/AKT pathway; results have demonstrated that neuroapoptotic activity is affected by PI3K and AKT expression levels (15). In addition, it has been reported that the
PI3K/AKT/glycogen synthase-3β (GSK-3β) pathway mediates the antioxidant, anti-inflammatory and anti-apoptotic effects exhibited by isoflurane anesthesia (16). Furthermore, the PI3K/AKT/GSK-3β signaling pathway and mitochondrial ATP-sensitive potassium channels can regulate the protective effects exerted by proanthocyanidins on anoxia-reoxygenation-induced myocardial cell injury (17,18). The present study demonstrated that isoflurane upregulated the PI3K/AKT signaling pathway, which contributed to reduced apoptosis of myocardial cells during the perioperative period.

The anti-apoptotic effects of isoflurane on myocardial cells in mice with myocardial ischemia were investigated during the perioperative period in the present study. The results of the present study suggested that isoflurane may significantly improve the viability and reduce apoptosis of myocardial cells via regulation of the PI3K/AKT signaling pathway in mice with myocardial ischemia. In conclusion, these results indicated that isoflurane anesthesia may inhibit myocardial cell apoptosis through upregulation of the PI3K/AKT signaling pathway during the perioperative period.

Materials and methods

Ethics statement. The animal study was implemented according to the Guide for the Care and Use of Laboratory Animals (19) and was approved by the Department of Anesthesiology of the First Affiliated Hospital of Soochow University (Suzhou, China). All surgical operations and euthanasia were performed to minimize suffering.

Animal study. A total of 20 male C57BL/6 mice (age, 8 weeks; weight, 25-30 g) were purchased from the Jackson Laboratory (Ben Harbor, ME, USA) and were housed under a 12-h artificial light/dark cycle at 23±1°C with a relative humidity of 50±5%. All mice were given free access to food and water. A mouse model of myocardial ischemia was established according to a previous study (20). Mice with myocardial ischemia were then divided into two groups and were prepared for heart bypass surgery. The experimental mice received 0.5% isoflurane (0.2 mg/kg), whereas mice in the control group were anesthetized with 35 mg isoflurane (0.2 mg) (21). The present study suggested that isoflurane may significantly improve the viability and reduce apoptosis of myocardial cells via regulation of the PI3K/AKT signaling pathway in mice with myocardial ischemia. In conclusion, these results indicated that isoflurane anesthesia may inhibit myocardial cell apoptosis through upregulation of the PI3K/AKT signaling pathway during the perioperative period.

Pain assessment. Isoflurane efficacy for postoperative pain in mice with myocardial ischemia that underwent heart bypass surgery was determined via general appearance parameters (GAP) scores on day 3 after surgery. GAP scores were determined on the basis of previously published parameters, including posture, coat condition, activity, breathing pattern and interactions with other mice (22).

Evaluation of toxicity. The toxicity of isoflurane was assessed using the National Congenital Hypothyroidism Institute Common Toxicity Criteria (23). Blood pressure measurements and urinalysis were performed on day 3 after surgery. Electrocardiograms and biochemical detection were performed every 3 days. Toxicity was defined as the presence of any drug-related toxicities, as described in a previous study (24).

Cell culture and regents. Myocardial cells were isolated from experimental mice and cultured in minimum essential medium (MEM) (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Myocardial cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Myocardial cells were treated with PI3K inhibitor LY294002 (1 mg/ml, TargetMol, Boston, MA, USA) or PBS for 12 h at 37°C for further analysis.

Proliferation assay. Myocardial cells (1x10⁵/well) isolated from isoflurane-treated or placebo-treated mice were digested and seeded in 96-well plates for 12 h at 37°C. The Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to detect cell growth according to the manufacturer's protocol.

Western blot analysis. Myocardial cells were isolated from experimental mice, homogenized in lysis buffer containing protease-inhibitor (M-PER reagent for cells; Thermo Fisher Scientific, Inc.) and were centrifuged at 5,700 x g at 4°C for 10 min. The supernatant was used to analyze protein expression. Briefly, SDS-PAGE assays were performed as previously described (25). For western blotting, the following primary antibodies: Anti-binding immunoglobulin protein (BIP, cat. no. ab108615), anti-CCAT-enhancer-binding protein homologous protein (CHOP, cat. no. ab179823), anti-superoxide dismutase (SOD, cat. no. ab13533), anti-proto-oncogene tyrosine-protein kinase ROS (ROS, cat. no. ab5512), anti-glutathione (GSH, cat. no. ab26255), anti-GAPDH (cat. no. ab8245), anti-B-cell lymphoma 2 (Bcl-2, cat. no. ab692), anti-Bcl-2-associated X protein (Bax, cat. no. ab53154), anti-caspase-3 (cat. no. ab2302), anti-caspase-8 (cat. no. ab25901), anti-PI3K (cat. no. ab86714), anti-AKT (cat. no. ab8805) and anti-phosphorylated (p)-AKT (cat. no. ab105731) (all 1:1,000 dilutions; Abcam, Shanghai, China), were added for 12 h at 4°C after blocking (5% skimmed milk) for 60 min at 37°C. Following three washes with PBS, horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) secondary antibodies (1:5,000; cat. no. ab108615), anti-CCAAT-enhancer-binding protein homologous protein (CHOP, cat. no. ab179823), anti-superoxide dismutase (SOD, cat. no. ab13533), anti-proto-oncogene tyrosine-protein kinase ROS (ROS, cat. no. ab5512), anti-glutathione (GSH, cat. no. ab26255), anti-GAPDH (cat. no. ab8245), anti-B-cell lymphoma 2 (Bcl-2, cat. no. ab692), anti-Bcl-2-associated X protein (Bax, cat. no. ab53154), anti-caspase-3 (cat. no. ab2302), anti-caspase-8 (cat. no. ab25901), anti-PI3K (cat. no. ab86714), anti-AKT (cat. no. ab8805) and anti-phosphorylated (p)-AKT (cat. no. ab105731) (all 1:1,000 dilutions; Abcam, Shanghai, China), were added for 12 h at 4°C after blocking (5% skimmed milk) for 60 min at 37°C. Following three washes with PBS, horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) secondary antibodies (1:5,000; cat. no. ab6728; Abcam) and anti-rabbit IgG secondary antibodies (1:5,000; cat. no. ab6721; Abcam) were added to the membranes for 2 h at 37°C, in order to detect proteins of interest. The results were visualized using a chemiluminescence detection system (Roche Diagnostics, Indianapolis, IN, USA). The blots were analyzed using ImageJ software version 1.2 (National Institutes of Health, Bethesda, MD, USA).

MTT assay. Myocardial cells (1x10³ cells/well) were isolated from control mice and were then incubated with 1 mg/ml isoflurane in 96-well plates for 72 h at 37°C, each condition was tested in triplicate; PBS was added instead of isoflurane as a control. At each time point (12, 24, 36, 48, 60 and 72 h), 20 µl MTT (5 mg/ml) in PBS was added to each well, and the plates were incubated for a further 4 h. Subsequently, the majority of the medium was removed and 100 µl dimethyl sulfoxide was added to the wells to solubilize the crystals. The optical density was measured using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.
Apoptosis assay. Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) and flow cytometry were used to analyze the apoptotic rate of myocardial cells obtained from mice with myocardial ischemia treated with isoflurane. Myocardial cells were isolated from experimental mice, and were trypsinized and collected. The cells were then washed in cold PBS, adjusted to 1x10^6 cells/ml with PBS, and were labeled with Annexin V-fluorescein isothiocyanate (V-FITC) and propidium iodide-phycocyanin (Annexin V-FITC kit; BD Biosciences, San Diego, CA, USA). Apoptosis was analyzed using a FACScan flow cytometer (BD Biosciences) and calculated using Expo32-ADC v. 1.2B software (Beckman Coulter, Inc., Brea, CA, USA). The experiment was performed according to a previous study (26).

Cell cycle analysis. The effects of isoflurane were determined on the cell cycle progression of myocardial cells obtained from isoflurane-treated mice with myocardial ischemia. Cell cycle analysis was determined using the Cell Cycle Analysis kit (cat. no. PK-CA577-K920; PromoCell GmbH, Heidelberg, Germany). The number of myocardial cells in S, G2 and M phases were analyzed according to a previously published study (27).

Drug pharmacodynamics. The serum concentration of isoflurane, and the Cmax concentrations of isoflurane (0-0.40 mg/kg) were investigated in mice with myocardial ischemia that underwent heart bypass surgery following isoflurane treatment. These analyses were conducted as described in a previous study (28).

Statistical analysis. All data are presented as the means ± standard error of the mean of triplicate experiments. Statistical analysis was performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences between two experimental groups were analyzed by Student’s t-test. Comparisons of data between multiple groups were performed using one-way analysis of variance, followed by Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Isoflurane attenuates pain and endoplasmic reticulum stress in mice with myocardial ischemia during surgery. Initially, pain was analyzed to examine the anesthetic effects of isoflurane on mice with myocardial ischemia during heart bypass surgery. The results demonstrated that pretreatment with isoflurane significantly attenuated pain in mice undergoing heart bypass surgery (Fig. 1). To investigate the efficacy of heart bypass surgery, heart rate and mean arterial blood pressure were recorded between isoflurane and placebo (pentobarbital) groups (Figs. 2 and 3). Heart rate and mean arterial blood pressure were recovered to normal levels following heart bypass surgery in the isoflurane group. Endoplasmic reticulum stress of myocardial cells was analyzed in mice following treatment with isoflurane. The key markers of continuous endoplasmic reticulum stress, BIP and CHOP, were downregulated within myocardial cells obtained from isoflurane-treated mice compared with the control (Fig. 4). The results also indicated that SOD, ROS and GSH expression levels were downregulated in myocardial cells obtained from isoflurane-treated mice compared with the placebo (Fig. 5). Taken together, these results suggested that isoflurane may attenuate pain, improve heart rate and mean arterial blood pressure, and reduce endoplasmic reticulum stress in mice with myocardial ischemia during surgery.

Isoflurane improves viability and the G2/M transition of myocardial cells obtained from experimental mice. The effects of isoflurane on viability, cytotoxicity and cell cycle progression of myocardial cells were analyzed. As illustrated in Fig. 6, isoflurane markedly improved myocardial cell viability compared with in the control group. In addition, isoflurane had reduced cytotoxic effects compared with the
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Analysis of cell cycle progression of myocardial cells revealed that isoflurane promoted the transition from G2 phase to M phase, thereby enhancing myocardial cell proliferation (Fig. 8). Isoflurane increased the number of myocardial cells in S phase and increased the number of cells in G2/M phase (Fig. 9). Furthermore, isoflurane significantly promoted proliferation of myocardial cells compared with the placebo, as determined using the MTT assay (Fig. 10). Taken together, these data suggested that isoflurane may exert beneficial effects on the viability and the transition of cells from G2 to M phase.

Figure 4. Protein expression levels of BIP and CHOP in myocardial cells obtained from mice with myocardial ischemia following heart bypass surgery. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01 vs. the placebo group. BIP, binding immunoglobulin protein; CHOP, CCAAT-enhancer-binding protein homologous protein.

Figure 5. Protein expression levels of SOD, ROS and GSH in myocardial cells from mice with myocardial ischemia following heart bypass surgery. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01 vs. the placebo group. GSH, glutathione; ROS, proto-oncogene tyrosine-protein kinase ROS; SOD, superoxide dismutase.

Figure 6. Viability of myocardial cells from mice with myocardial ischemia following heart bypass surgery and isoflurane treatment. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01 vs. the placebo group.

Figure 7. Cytotoxicity of isoflurane on myocardial cells from mice with myocardial ischemia following heart bypass surgery. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01 vs. the placebo group.

Figure 8. Effects of isoflurane on the number of myocardial cells in G2 and M phases. Cells were isolated from mice with myocardial ischemia following heart bypass surgery and isoflurane treatment. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01 vs. the placebo group.
Isoflurane inhibits myocardial cell apoptosis. To investigate the benefits of isoflurane on myocardial cells, myocardial cell apoptosis and survival were analyzed. As shown in Fig. 11, the results of the present study demonstrated that isoflurane treatment increased the survival of myocardial cells obtained from experimental mice, as determined using the Cell Counting kit-8 assay. A TUNEL assay demonstrated that the rate of myocardial cell apoptosis was decreased in cells obtained from isoflurane-treated mice compared with the control group (Fig. 12). In addition, the expression levels of Bcl-2, Bax, cleaved caspase-3 and cleaved caspase-8 were analyzed in myocardial cells. The results demonstrated that the expression levels of Bcl-2 and Bax were upregulated, whereas the levels of cleaved caspase-3 and cleaved caspase-8 were downregulated in myocardial cells obtained from isoflurane-treated mice (Fig. 13). These results suggested that isoflurane may increase survival rate and inhibit heart bypass surgery-induced apoptosis of myocardial cells.

Isoflurane exerts beneficial effects on myocardial cells via the PI3K/AKT signaling pathway. The PI3K/AKT signaling pathway was examined in myocardial cells to aid understanding of the molecular mechanism underlying isoflurane-mediated signal transduction. The present study reported that the expression levels of PI3K and AKT were increased within myocardial cells obtained from isoflurane-treated
mice (Fig. 14). AKT phosphorylation was also upregulated in myocardial cells obtained from isoflurane-treated mice compared to placebo-treated mice (Fig. 15). In addition, treatment with the PI3K inhibitor LY294002 (PI3KIR) reduced isoflurane-induced anti-apoptotic effects within myocardial cells (Fig. 16). Furthermore, PI3KIR treatment abolished isoflurane-stimulated promotion of myocardial cell survival (Fig. 17). Taken together, these results suggested that myocardial cells benefited from isoflurane via the PI3K/AKT signaling pathway.

Pharmacodynamics of isoflurane in mice with myocardial ischemia during the perioperative period. Following analysis of the isoflurane-mediated signaling pathway in myocardial cells, the pharmacodynamics of isoflurane in mice with myocardial ischemia during the perioperative period were investigated (28). As shown in Fig. 18, serum concentration of isoflurane peaked 60 min post-treatment. The C_{max} concentration of isoflurane increased linearly with increasing dose (0-0.40 mg/kg) (Fig. 19). Drug accumulation was not observed in experimental mice. These data suggested that isoflurane may protect myocardial contractility.

Discussion

Myocardial ischemia-reperfusion injury is the most common complication of myocardial infarction, cardiopulmonary bypass surgery, heart attack, heart transplantation and other cardiovascular diseases, which ultimately results in irreversible injury and even mortality (28). Myocardial ischemia is also associated with the highest incidence of disability worldwide and is closely associated with myocardial infarction (29). Surgical treatments can efficiently alleviate cardiac failure and suppress other metabolic diseases induced by cardiovascular disease. Anesthesia is an important intervention that may reduce pain, and is widely used for heart bypass surgery in clinical settings. The results of the present study indicated that isoflurane anesthesia may significantly attenuate the pain of mice with myocardial ischemia that underwent heart bypass surgery.

Isoflurane is a volatile general anesthetic that is used to abolish behavioral responsiveness in animals, in order to attenuate pain and facilitate surgery (30,31). The mechanism underlying isoflurane anesthesia may be associated with the action of the human glycine receptor (32). In recent years, additional functions of isoflurane have been reported in various types of disease (33-35). In the present study, the additional functions of isoflurane as an anesthetic for mice with myocardial ischemia during heart bypass surgery were analyzed. Heart rate and arterial blood pressure were increased following heart bypass surgery in the isoflurane group compared with in the placebo group. Notably, isoflurane markedly improved the viability and survival of myocardial cells during the perioperative period. Furthermore, the apoptotic rate of myocardial cells was inhibited following isoflurane anesthesia during the perioperative period. In the present study, pentobarbital group was used as a control group, in order to confirm that isoflurane, which is the most commonly used volatile anesthetic, would be more effective at protecting mice against ischemia-reperfusion injury.

Previous studies have suggested that cardiomyocyte apoptosis serves a crucial role in the initiation and progression of cardiovascular diseases (36-38). A previous study demonstrated that isoflurane anesthesia can attenuate activated microglial cytokine-induced apoptosis of ventral spinal cord 4.1 motoneuronal cells (39). Recently, research has reported that isoflurane may activate the caspase-induced apoptotic signaling pathway, which is consistent with the neuropathogenesis of senile dementia (40). However, in the present study, the expression levels of the apoptotic proteins cleaved caspase-3 and caspase-8 were downregulated. In addition, the expression levels of the proapoptotic protein Bax were increased in myocardial cells obtained from isoflurane-treated mice compared to placebo-treated mice, and the expression levels of the anti-apoptotic protein Bcl-2 were relatively higher in myocardial cells obtained from isoflurane-treated...
mice compared with in placebo-treated mice at the end of the perioperative period. These results suggested that isoflurane may exert beneficial anti-apoptotic effects on myocardial cells following heart bypass surgery. Notably, the results indicated that the PI3K/AKT signaling pathway may mediate the molecular mechanism underlying the effects of isoflurane on myocardial cells. Coincidentally, endoplasmic reticulum stress was also improved in myocardial cells from mice treated with isoflurane at the end of the perioperative period. However, the expression levels of the proapoptotic gene Bax were upregulated in cells from isoflurane-treated mice; this finding requires further analysis.

Mice administered isoflurane anesthesia exhibited increased PI3K and AKT expression in myocardial cells. Although previous reports have presented the safety profile of isoflurane, the isoflurane-mediated PI3K/AKT signaling pathway in myocardial cells has not been observed in previous studies (41,42). Jiang and Jiang (40) previously demonstrated that myocardial viability can be enhanced, oxidative stress can be reduced and adverse remodeling can be prevented in response to PI3K/AKT signaling activation following myocardial ischemia/reperfusion injury. In addition, Nagaoka et al (43) proposed a novel therapeutic modality for

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Figure 16. PI3KIR abolishes isoflurane-induced inhibition of apoptosis of myocardial cells obtained from experimental mice. Data are presented as the mean ± standard error of the mean of three independent experiments. "p<0.01. FITC, fluorescein isothiocyanate; PI3KIR, phosphoinositide 3-kinase inhibitor; PI-PE, propidium iodide-phycoerythrin.

Figure 17. PI3KIR reduces isoflurane-stimulated myocardial cell survival. Data are presented as the mean ± standard error of the mean of three independent experiments. "p<0.01. PI3KIR, phosphoinositide 3-kinase inhibitor.

Figure 18. Serum concentration of isoflurane in mice with myocardial ischemia that underwent heart bypass surgery following isoflurane treatment. Data are presented as the mean ± standard error of the mean of three independent experiments. AUC, area under the curve.
acute myocardial infarction via activation of the PI3K/AKT signaling pathway and reduced inflammation in a rat model. Guidetti et al (44) suggested that PI3K/AKT is stimulated by integrin engagement and further inhibits platelet activation in thrombus formation and stabilization; thus highlighting the potential effects of PI3K/AKT on venous thrombosis and anti-thrombotic therapeutic strategies. The results of the present study revealed that PI3K and AKT expression levels were upregulated in myocardial cells obtained from mice treated with isoflurane; conversely, PI3K inhibition suppressed PI3K and AKT expression levels, inhibited apoptosis and increased the apoptotic rate of myocardial cells induced by myocardial ischemia.

In conclusion, the findings of the present study provided an insight into the potential efficacy and preclinical mechanism of isoflurane in preoperative preparation and anesthesia. The data provided preclinical and experimental evidence to support the efficacy of isoflurane anesthesia. The present study also elaborated on the molecular mechanism underlying isoflurane-mediated protection of myocardial cells via the PI3K/AKT signaling pathway in mice that underwent heart bypass surgery, during the perioperative period. Taken together, these findings suggested that the isoflurane-mediated PI3K/AKT signaling pathway may contribute to the recovery of myocardial ischemia following heart bypass surgery in a clinical setting.

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

Authors' contributions
ZP performed the experiments. HL analyzed and interpreted the data from the experiments, and wrote the article. JY is the project leader and designed the experiments.

Ethics approval and consent to participate
The present study was approved by the Department of Anesthesiology of the First Affiliated Hospital of Soochow University (Suzhou, China).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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