Is longer sevoflurane preconditioning neuroprotective in permanent focal cerebral ischemia?

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Research Highlights

1. In the current study, a rat model of permanent focal cerebral ischemia was used for the first time to demonstrate that 60-minute sevoflurane preconditioning has neuroprotective effects in rats with permanent cerebral ischemia.
2. Long time (120 minutes) sevoflurane preconditioning had no obvious neuroprotective effects in rats with permanent cerebral ischemia.
3. Sixty-minute sevoflurane preconditioning exerts the best neuroprotective effects in rats with permanent cerebral ischemia by inhibiting apoptosis.

Abstract

Sevoflurane preconditioning has neuroprotective effects in the cerebral ischemia/reperfusion model. However, its influence on permanent cerebral ischemia remains unclear. In the present study, the rats were exposed to sevoflurane for 15, 30, 60, and 120 minutes, followed by induction of permanent cerebral ischemia. Results demonstrated that 30- and 60-minute sevoflurane preconditioning significantly reduced the infarct volume at 24 hours after cerebral ischemia, and 60-minute sevoflurane preconditioning additionally reduced the number of TUNEL- and caspase-3-positive cells in the ischemic penumbra. However, 120-minute sevoflurane preconditioning did not show evident neuroprotective effects. Moreover, 60-minute sevoflurane preconditioning significantly attenuated neurological deficits and infarct volume in rats at 4 days after cerebral ischemia. These findings indicated that 60-minute sevoflurane preconditioning can induce the best neuroprotective effects in rats with permanent cerebral ischemia through the inhibition of apoptosis.

Key Words
neural regeneration; brain injury; anesthesia; sevoflurane; preconditioning; cerebral ischemia; apoptosis; caspase-3; neuroregeneration
INTRODUCTION

Up to now, intravenous thrombolysis with tissue plasminogen activator has been the most effective treatment for acute ischemic stroke, but it has to be administered within 3 hours after vessel occlusion because the risk of hemorrhage increases with time[3]. Because of the narrow time window, this treatment is only applicable for less than 5% of patients with acute ischemic stroke in the United States (American Stroke Association). Most patients encounter permanent focal cerebral ischemia, which is more complicated in the pathological process and more difficult to treat than cerebral ischemia/reperfusion. Thus, a treatment that is able to confer neuroprotection in both transient and permanent brain ischemia may be beneficial.

Sevoflurane belongs to volatile anesthetics, and preconditioning with sevoflurane has been shown to exert protective effects against ischemic injury in the brain, heart[25-28], liver[29-30], and kidney[31]. In cerebral ischemia models, some studies reported that a higher concentration of sevoflurane pretreatment resulted in better neuroprotective effects than a lower concentration; however, others hold the opposite opinion[32-34]. The neuroprotection of sevoflurane preconditioning may be associated with antioxidant enzymes activation, reduced expression of apoptosis-regulating proteins, inhibition of apoptosis, activation of the extracellular signal-regulated protein kinase, anti-inflammation, mitoK (ATP) channel opening, protein kinase C-ε activation, and p38 MAPK phosphorylation[9, 12-26]. Although studies have shown the neuroprotective effects of sevoflurane preconditioning in the transient cerebral ischemia model, its effect in the permanent focal cerebral ischemia model remains unclear.

In the ischemic penumbra, apoptotic cell death is a common way of neuronal death[27]. Caspase-3, an apoptotic executor, plays an important role in ischemic neuronal injury. Increased caspase-3 expression has been reported in transient and permanent focal cerebral ischemia model[28-30]. It remains unknown whether caspase-3 expression and apoptosis may be inhibited by sevoflurane preconditioning in the permanent middle cerebral artery occlusion (MCAO) model, although sevoflurane preconditioning can attenuate apoptosis and caspase-3 expression in the transient MCAO model in rats[13].

Based on these results, we hypothesized that sevoflurane preconditioning could improve neurological function in the permanent MCAO model, reduce infarct volume, and that the protective effects of sevoflurane preconditioning might be associated with the reduction of caspase-3 expression and apoptosis. The aim of the current study was to verify this hypothesis.

RESULTS

Quantitative analysis of experimental animals

Ninety-two Sprague-Dawley rats were used. This study consisted of four parts. In the first part, the effects of 120-minute sevoflurane anesthesia on physiological parameters were assessed. Sixteen rats were randomly assigned to pentobarbital control and sevoflurane groups (n = 8 per group). The pentobarbital control group rats were anesthetized with pentobarbital and allowed in-halation of 30% oxygen. In the second part of the study, the protective effects of sevoflurane preconditioning on infarct volume and neurological deficits at 24 hours after MCAO were evaluated. Forty rats were randomly assigned to five groups: ischemia, 15-, 30-, 60-, and 120-minute sevoflurane preconditioning groups (n = 8 per group). The ischemia group was subjected to permanent MCAO alone. In the third part, the long-term neuroprotective effects of sevoflurane preconditioning on infarct volume and neurological deficits were investigated. Sixteen rats were randomly divided into ischemia and 60-minute sevoflurane preconditioning groups (n = 8 per group), and were sacrificed 4 days after stroke. Finally, we examined the anti-apoptotic effects of sevoflurane preconditioning. Fourteen ani-
mals were assigned to sham-surgery (n = 2), ischemia (n = 4), and 60- and 120-minute sevoflurane preconditioning groups (n = 4 per group). The sham-surgery group was subjected to a neck incision and craniotomy without artery occlusion. In the ischemia group, four rats (two from the ischemia group, and two from the 120-minute sevoflurane preconditioning group) died within 24 hours, and two rats in the 120-minute sevoflurane preconditioning group died within 4 days. Behavior test results included data collected from the rats that died.

Plasma glucose concentration increased following sevoflurane preconditioning
The physiological values in the period of sevoflurane anesthesia are presented in supplementary Table 1 online. During preconditioning, arterial oxygen tension (Pao\_2) was not different between the sevoflurane and pentobarbital control groups (P > 0.05). At 60 and 120 minutes after sevoflurane anesthesia, potential hydrogen (pH) value was significantly higher (P < 0.01), while arterial carbon dioxide tension (PaCO\_2) was significantly lower than in the pentobarbital control group (P < 0.01). However, the fluctuation of PaCO\_2 and pH was within the physiological range. At 120 minutes after sevoflurane anesthesia, mean arterial pressure was significantly lower (P < 0.01) and plasma glucose concentration was significantly higher compared with the animals in the pentobarbital control group (P < 0.01).

Effects of sevoflurane preconditioning on neurological deficits and infarct volume
At 24 hours after MCAO, 30- and 60-minute sevoflurane preconditioning significantly reduced infarct volume compared with the ischemia group (P < 0.05, P < 0.01; Figure 1); however, sevoflurane preconditioning did not attenuate neurological deficits (P > 0.05; Figure 2).

At 4 days after MCAO, 60-minute sevoflurane preconditioning significantly attenuated neurological deficits (P < 0.01; Figure 3) and reduced infarct volume compared with the ischemia group (P < 0.05; Figure 4).

Sevoflurane preconditioning reduced cell apoptosis
At 24 hours after MCAO, TUNEL-positive cells were only found in the ischemic core and penumbra. The number of TUNEL-positive cells in the penumbra was significantly lower in the 60-minute sevoflurane preconditioning group compared with the ischemia group and the 120-minute sevoflurane preconditioning group (P < 0.01; Figure 5). Caspase-3-positive cells were only found in the penumbra, and the number of the cells was significantly lower in the 60-minute sevoflurane preconditioning group compared with the ischemia group and the 120-minute sevoflurane preconditioning group (P < 0.01; Figure 6).

DISCUSSION
The current study demonstrated that 60-minute sevoflurane preconditioning significantly reduced the infarct volume and the number of TUNEL-positive and caspase-3-positive cells in the penumbra at 24 hours after permanent MCAO. In addition, 60-minute sevoflurane preconditioning also attenuated neurological deficits and infarct volume at 4 days after MCAO.
of sevoflurane preconditioning was 60 minutes, at which infarct volume and apoptosis were significantly attenuated.

![Figure 3](image1)

Figure 3  Sevoflurane preconditioning (SP) reduced infarct volume at 4 days after middle cerebral artery occlusion.
(A) Infarct volume at 4 days after middle cerebral artery occlusion (n = 8 per group). Data are presented as mean ± SD and were analyzed by two-sample t-test. *P < 0.05, vs. ischemia group.
(B) Representative 2,3,5-triphenyltetrazolium chloride staining of the infarct volume at 4 days after middle cerebral artery occlusion. White part is the cerebral infarction lesion. Infarct animal (a), 60-minute (min) SP animal (b).

![Figure 4](image2)

Figure 4  Sevoflurane preconditioning (SP) attenuated neurological deficit scores at 4 days after middle cerebral artery occlusion.
Data are expressed as median (horizontal bars) with the individual values of infarct animals (filled circles) and SP animals (opened circles). n = 8–10 per group. Neurological deficit scores were analyzed using Kruskal-Wallis nonparametric analysis of variance followed by Mann-Whitney test for analysis of individual differences. *P < 0.05, vs. ischemia group.

The ischemic penumbra is an area directly in contact with the ischemic core where cells receive cerebral blood flow at a perfusion pressure of 18–23 mmHg, enough to survive but not to be functional. The blood flow in the area is so low that the Na⁺/K⁺ pump activity is inhibited because of the reduced adenosine 5' triphosphate formation, which terminally induces caspases activation and apoptosis through the glutamate pathway. These cells may survive and restore function if the apoptotic process is prevented. Sevoflurane pretreatment has been shown to have neuroprotective effects by inhibiting caspase-3 activation and apoptosis in the penumbra in the transient MCAO model[19]. Our results also demonstrated that 60-minute sevoflurane preconditioning significantly reduced infarct volume and the density of TUNEL-positive and caspase-3-positive cells in the penumbra, supporting the hypothesis that the neuroprotection of sevoflurane preconditioning might be associated not only with the reduction of infarct size, but also with apoptosis attenuation and caspase-3 expression reduction in the permanent MCAO model.

![Figure 5](image3)

Figure 5  Sevoflurane preconditioning (SP) reduced TUNEL-positive cells 24 hours after middle cerebral artery occlusion.
(A) TUNEL-positive cells (TUNEL staining, × 400). Sham-surgery group (a) did not show TUNEL-positive cells. A large number of TUNEL-positive cells (red; marked with black arrows) were seen in the ischemia group (b) and the 120-min SP group (d). The number of TUNEL-positive cells was significantly lower in the 60-min SP group (c). Scale bar: 100 µm.
(B) Quantification of TUNEL-positive cells. The number of TUNEL-positive cells was significantly lower in 60-minute (min) SP animals. Data are presented as mean ± SD (n = 4 per group) and were analyzed using one-way analysis of variance followed by Bonferroni post-hoc test. *P < 0.01, vs. ischemia group; *P < 0.01, vs. 120-min SP group.

In our study, we found that the optimal duration of sevoflurane preconditioning was 60 minutes, which significantly reduced infarct volume, apoptosis, and caspase-3
expression. The mechanism through which the prolonged exposure abolishes the protective effect of sevoflurane preconditioning remains unclear.

Figure 6. Sevoflurane preconditioning (SP) reduced caspase-3-positive cells 24 hours after middle cerebral artery occlusion (n = 4 per group).  
(A) Caspase-3-positive cells (immunohistochemical staining, × 400). Sham-surgery group (a) did not show caspase-3-positive cells. A large number of caspase-3-positive cells (yellow brown; marked with black arrowheads) were seen in the ischemia group (b) and the 120-minute (min) SP group (d). The number of caspase-3-positive cells was significantly lower in the 60-min SP group (c). Scale bar: 100 µm.  
(B) Quantification of caspase-3-positive cells. The number of caspase-3-positive cells was significantly lower in 60-min SP animals. Data are presented as mean ± SD and were analyzed using one-way analysis of variance followed by Bonferroni post-hoc test. *P < 0.01, vs. ischemia group; **P < 0.01, vs. 120-min SP group.

Some studies have shown that the prolonged exposure to sevoflurane causes neurotoxicity in naive mice. A study reported that 2.5% sevoflurane for 120 minutes induced caspase-3 activation and apoptosis in the naive mouse brain; another study showed that 3% sevoflurane for 6 hours induced a degree of active caspase-3-positive and TUNEL-positive cells in the brain tissue of neonatal naive mice. Feng's study in "adolescent" rats suggests that the neuronal loss caused by sevoflurane may be associated with decreased neuronal nitric oxide synthase. Although there is no evidence to prove that the long duration of sevoflurane inhalation causes obvious neuronal apoptosis or loss in adult rats, we cannot rule out the possibility that the prolonged exposure time may cause damage to the ultrastructure of neurons and gradually counteract the protective effects induced by sevoflurane. We also noticed that the mean arterial pressure was significantly lower at 120 minutes after sevoflurane anesthesia, but not at 60 minutes. As far as we know, hypotension leads to a reduced oxygen supply to the brain. Moreover, the plasma glucose concentration was significantly higher at 120 minutes after sevoflurane anesthesia, which involves decreasing insulin secretion and impairing glucose use. It is also possible that the lack of oxygen-glucose is involved in the loss of the neuroprotective effects of 120-minute sevoflurane preconditioning.

Codaccioni et al. reported that sevoflurane preconditioning reduced the infarct volume at 3 days post stroke, but not at 7 days, in the transient MCAO model. Similarly, we found that 60 minutes of 1 minimum alveolar concentration (MAC) sevoflurane preconditioning conferred neuroprotective effects 4 days after permanent MCAO. However, there is no evidence for growth of the ischemic lesion beyond 4 days after MCAO in rats, although infarcts mature 2 days after stroke in rats. Thus, we speculated that sevoflurane preconditioning-induced neuroprotection might be no longer detectable beyond 4 days; even so, sevoflurane preconditioning is still beneficial in stroke therapy. Furthermore, a previous study reported that 24 hours before MCAO, 3 hours of 1 minimum alveolar concentration isoflurane induced neuroprotection at 4 days after permanent MCAO; in contrast to isoflurane preconditioning, sevoflurane preconditioning had a shorter induction duration and shorter interval between pretreatment and MCAO. In our preliminary study, the interval was set at 1.5, 6, 12, and 24 hours, while we found that only 1.5 hours had neuroprotective effects (data not shown).

In the present study, we used 2.4% sevoflurane based on the concentration-response relationship of sevoflurane. Additionally, some reports showed that 4% sevoflurane provided better neuroprotection than 2% in the transient focal cerebral ischemia model and the global cerebral ischemia/reperfusion model. Conversely, others showed that 2.8% sevoflurane caused severe hippocampal injury compared with 1.4% in the model of bilateral common carotid artery occlusion. Thus, to investigate the neuroprotective effects of sevoflurane preconditioning, we used 2.4% sevoflurane to
prevent injury resulting from a high concentration of sevoflurane.

In conclusion, pretreatment with 2.4% sevoflurane for 60 minutes (the optimal duration) induced neuroprotective effects by attenuating cell apoptosis and caspase-3 expression in the permanent MCAO model in rats. In contrast to 60-minute sevoflurane preconditioning, the prolonged duration (120 minutes) of pretreatment had no neuroprotective effects. However, further studies are needed prior to clinical application to determine the optimal protocol for sevoflurane pretreatment.

MATERIALS AND METHODS

Design
A randomized, controlled animal study.

Time and setting
The experiments were conducted in the Neuroscience Research Center of Sichuan University, China from July 2011 to March 2012.

Materials

Animals
A total of 92 male specific pathogen-free Sprague-Dawley rats aged 2 months and weighing 250–300 g were provided by the Animal Centre of Sichuan Province (License No. SCXK (Chuan) 2008-24). Animals were raised in a room maintained at 24–26°C, and allowed free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University and carried out in accordance with the Guidelines laid down by the NIH in the US regarding the care and use of animals for experimental procedures.

Drugs
Sevoflurane (C₃H₆F₃O) was purchased from Shanghai Hengui, Shanghai, China.

Methods
Sevoflurane preconditioning
In sevoflurane-preconditioned animals, anesthesia was induced with 6% sevoflurane in a box, and maintained through a mask at 2.4% concentration (1 minimal alveolar concentration) in a gas mixture of 30% oxygen and 70% nitrogen with a constant flow of 500 mL/minute[37]. The rectal temperature of each animal was maintained at 37 ± 0.5°C with a heated electric blanket. Plasma glucose concentration was measured using ACCU-CHEK Active (Roche, Basel, Switzerland) at three time points: immediately, 60 and 120 minutes after anesthesia. At 60 and 120 minutes after anesthesia, blood samples were collected from the right femoral artery for the measurement of pH, PaCO₂, and PaO₂ using ABLE800 FLEX Blood Gas Analyzer (Radiometer America, Westlake, OH, USA). Mean arterial pressure was measured with a physiologic recorder (Biolap 420E+, Taimeng, Chengdu, China).

Establishment of the permanent MCAO model
After a 50-minute washout period (Figure 7), permanent MCAO model was established as previously described with minor modifications[38]. The animals were anesthetized with an intraperitoneal injection of pentobarbital (55–65 mg/kg). With the aid of an operating microscope, the right middle cerebral artery was cauterized using Sabre-2400 bipolar electrocauterizer (Conmed Co., Englewood, CO, USA) from the inferior margin of the olfactory tract to the inferior cerebral vein. The electric coagulation power was 3 W. The middle cerebral artery was cut below the rhinal fissure to avoid recanalization. The animals were allowed to recover at the ambient temperature of 24–26°C.

Figure 7  Schematic illustration of the experimental protocol.
The upper bar represents the ischemia group, the lower bar represents sevoflurane preconditioning animals. min: Minutes.

Neurological deficit score
Neurological deficit scoring was performed blindly 24 hours after MCAO according to the method proposed by Rogers[39]: 0, no apparent deficits; 1, failure to extend left forepaw fully; 2, decreased grip of the left forelimb when tail pulled; 3, spontaneous movement in all directions; left circling only if pulled by tail; 4, spontaneous left circling or walking; 5, walking only if stimulated; 6, no responsiveness to stimulation and with depressed level of consciousness; 7, dead (supplementary videos 1–3 online).

2,3,5-triphenyltetrazolium chloride staining for assessment of infarct volume
After scoring, animals were sacrificed under deep anesthesia to measure the infarct volume. The brains were removed and cut into six 2-mm-thick coronal slices using a Rat Brain Matrix (MyNeurolab, St. Louis, MO, USA). The sections were incubated in 2% phosphate-buffered...
2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at 37°C, followed by overnight immersion in 4% phosphate-buffered paraformaldehyde. After the brain slices were scanned, the images were analyzed blindly using Adobe Photoshop CS4 11.0.1 (Adobe Systems, San José, California, USA). The unstained area was defined as the infarction area. The infarct volume was calculated using Swanson’s[40] method to correct for edema. The corrected infarct volume (%) = (volume of unleisioned side – volume of normal brain on leisioned side)/volume of unleisioned side × 100%.

**Sample collection**

Animals were deeply anesthetized and transcardially perfused with ice-cold saline, followed by ice-cold 4% phosphate-buffered paraformaldehyde. The brains were removed and post-fixed in the same fixative at 4°C for 36 hours. The brains were embedded in paraffin, and then 6-µm-thick sections were cut approximately 1.3 mm rostral to the bregma for staining[13].

**TUNEL detection of cell apoptosis in brain tissues**

TUNEL staining was performed according to the manufacturer’s instructions (11684809910; Roche Diagnostics, Indianapolis, IN, USA). TUNEL-positive cells were counted in five to eight fields in the penumbra and were expressed as the average of all fields of each animal[13].

**Immunohistochemistry for detection of caspase-3 expression in brain tissues**

After deparaffinization, washing, incubation (3% H2O2 for 15 minutes) and antigen retrieval (95°C for 1 hour), the sections were incubated with the primary rabbit anti-caspase-3 polyclonal antibody (#9662, 1:800; Cell Signaling Technology, USA) overnight at 4°C. The sections were then incubated with the working fluid of the goat anti-rabbit secondary antibody (EnVision™ Detection System; K5007, Dako, Denmark) at 37°C for 1 hour. Caspase-3 staining was visualized using diaminobenzidine and sections were counterstained with hematoxylin. The penumbra was determined using a previously described method[41]. The sections were examined by light microscopy (Olympus). Caspase-3-positive cells were counted in five to eight fields in the penumbra and were expressed as the average of all fields of each animal[13].

**Statistical analysis**

Data were presented as mean ± SD. Physiological parameters and infarct volume were analyzed by two-sample t-test. The number of TUNEL-positive and caspase-3-positive cells was analyzed using one-way analysis of variance followed by Bonferroni post-hoc test. Neurological deficit scores were analyzed using Kruskal-Wallis nonparametric analysis of variance followed by Mann-Whitney test for analysis of individual differences. The value of P < 0.05 was considered statistically significant.

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