Sevoflurane preconditioning protects blood-brain-barrier against brain ischemia

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1. ABSTRACT

Sevoflurane preconditioning has recently been demonstrated to protect ischemic brain *in vivo* and *in vitro*. However, mechanisms underlying this neuroprotection have not been delineated. We therefore assessed the hypothesis that sevoflurane pretreatment protected blood-brain-barrier (BBB) *via* suppression of cell adhesion molecules (CAMs) and matrix metalloproteinases (MMPs) after ischemia. Repeated sevoflurane preconditioning was administered 24 hours before transient middle cerebral artery occlusion (MCAO). Neurologic deficits and expression of CAMs, MMPs and occludin were examined up to 3 days after ischemia. Evans blue (EB) extravasation and electron microscopy was detected at 2 days after ischemia. The data showed that sevoflurane pretreatment markedly improved BBB integrity and neurological outcomes after ischemia, robustly suppressed ischemia-induced decreases of occludin and increases of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), MMP-2, MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1). Sevoflurane pretreatment also suppressed the activation of astrocytes and microglias in ipsilateral cortex and corpus callosum. In conclusion, repeated sevoflurane preconditioning confered potent protection against brain ischemia, partly by improving BBB integrity.

2. INTRODUCTION

Inhalational anesthetics have been reported to effectively precondition the brain against experimental ischemic injuries *in vivo* (1, 2) and *in vitro* (3, 4). The molecular and cellular mechanisms have been implicated to include electrophysiological and metabolic modulation, reduction of glutamate-mediated excitotoxicity, antioxidant and anti-apoptosis mechanisms (5). However, impact on blood-brain-barrier (BBB) function by inhalational anesthetics pretreatment in stroke remains unknown.

During ischemia-reperfusion injuries, up-regulated cellular adhesion molecules (CAMs) and matrix metalloproteinases (MMPs) have been known to mediate the firm adhesion of leukocytes to sites of inflammation and degrading neurovascular matrix, such as tight-junctions (TJs). Disruption of BBB triggers inflammatory cascades, leading to further brain injuries (6). We therefore assessed the hypothesis that sevoflurane preconditioning provided neuroprotection by attenuation of BBB leakage *via* suppression of CAMs and MMPs against transient focal cerebral ischemia in rats.

3. MATERIALS AND METHODS

3.1. Experimental groups and sevoflurane preconditioning

Adult male Sprague-Dawley rats (250-280g, Shanghai SLAC Laboratory Animal Co. Ltd.) were randomly allocated into three groups: sham (neither exposed to anesthetic pretreatment, nor ischemia induced), vehicle, and sevoflurane preconditioning (sevo-pre) groups. In the sevo-pre group, rats were exposed for 30 min on 4 consecutive days to 0.5 minimum alveolar concentration (MAC) sevoflurane (1.2% sevoflurane in air, Baxter) in an anesthetic chamber. Then, the rats were subjected to transient MCAO 24 hours later.

3.1. Animal model of transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by left MCAO for 60 min as previously described (7). Briefly, rats were anesthetized, intubated and mechanically ventilated with 1.5% isoflurane (Abbott) in air. The carotid arteries were exposed and a 4-0 nylon monofilament coated with a silicone tip (0.4 - 0.45 cm diameter) was introduced into the external carotid artery and advanced 1.9-2.0 cm along the internal carotid artery until occluding the origin of the MCA. After 60 min, the filament was withdrawn to establish reperfusion and the wound sutured. Rectal temperature was maintained at 370°C with a temperature servo-system perioperatively. The left femoral artery and vein were cannulated for blood pressure monitoring, blood gas analysis and fluid administration. To confirm the induction of ischemia and successful reperfusion, changes in regional cerebral blood flow (rCBF) before, during, and after MCAO were evaluated by laser Doppler flowmetry.

3.2. Measurement of evans blue extravasation

BBB permeability was determined by measuring evans blue (EB) extravasation. 2.5% EB dye (4 ml/kg, Sigma) was slowly administered i.v. at 48 hours after reperfusion. After circulating for 3 hours, the rat was perfused with 300 ml saline and then decapitated for brain. The brain was dissected into sections of 2 mm thickness and soaked in methanamide for 48 hours. To pellet the brain tissue, the sample was centrifuged for 30 min at 14000 rpm. The absorption of the supernatant was measured at 632 nm with a spectrophotometer (Bio-Rad). The content of EB was valued as μg/g of brain tissue by using a standardized curve. n = 8/group.

3.3. Electron microscopy

BBB ultrastructure was detected by transmission electron microscopy at 48 hours after ischemia. Proximal MCA cortical tissues were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 12 hours and 1% osmium tetroxide for 1 hour. After dehydration in alcohol series, the tissues were embedded in 618# resin. The ultrathin sections were prepared using a Reichert ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined under a CM120 electron microscope at 80kv. n = 4/group.

3.4. Assessment of neurologic deficits

Neurologic deficits scores were assessed up to 3 days after ischemia as previously described (7). The five-point behavioral rating scale included: 0, no deficit; 1, forelimb weakness, and torso turning to the ipsilateral side when held by tail; 2, circling to the affected side; 3, unable to bear weight on the affected side; and 4, no spontaneous locomotor activity or barrel rolling. Any animal without a deficit was excluded from the study. A single observer blinded to group assignment performed neurological testing. n = 8/group.

3.5. Protein sample preparation

Proximal MCA cortical tissues (n=4 per condition) were harvested at 6, 24, 48, 72 hours after ischemia and stored at -80°C. The tissues were homogenized in cell lysis buffer (Cell Signaling Technology) added with protease inhibitor cocktails (Roche) on ice. Whole cell lysates were sonicated for 30 seconds, centrifuged at 14000× g for 30 min at 4°C. The supernatants were collected as protein samples. Protein concentrations were determined using Bradford assay and stored at -80°C awaiting western blot and gelatin zymography analysis.

3.6. Western blotting

Western blot analyses were performed as previously described (8). The blots were semi-quantified using gel densitometry with Quantity One software (Bio-Rad). The primary antibodies used in this study were rabbit anti-MMP-9 monoclonal antibody (Abcam), rabbit anti-MMP-2 monoclonal antibody (Abcam), rabbit anti-TIMP-1 polyclonal antibody (Millipore), rabbit anti-occludin polyclonal antibody (Abcam), rabbit anti-ICAM-1 polyclonal antibody (Santa Cruz) and rabbit anti-VCAM-1 polyclonal antibody (Santa Cruz). To detect multiple signals on a single membrane, the membrane was incubated in Restore Plus western blot stripping buffer (Pierce Biotechnology) for 5–15 min at room temperature between the various labeling procedures. n = 4/group.Three independent experiments were performed in duplicate.

3.7. Gelatin zymography

Zymography was carried out in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 0.1% gelatin. 20μg protein samples, mixed with 2 × nonreducing sample buffer (20% glycerol, 2% SDS, 0.04% bromophenol blue), were subjected to electrophoresis by a mini-gel apparatus (Bio-Rad). Following electrophoresis, the gels were washed twice with 2.5% Triton X-100 for 1 hour to remove the SDS and incubated 40 hours at 370°C in digestion buffer (50 mM Tris–HCl, 50 mM NaCl, 5 mM $CaCl_2$, 2 μM $ZnCl_2$, 0.02% Brij-35, pH 7.6). After incubation, the gels were stained for 3 hours with 0.5% Coomassie blue and destained in the buffer containing 30% methanol and 10% glacial acetic acid. The bands of gelatinolytic activity were scaned and analyzed by Quantity One software (Bio-Rad). n = 4/group.Three independent experiments were performed in duplicate.

3.8. Immunofluorescence staining

The immunofluorescence staining was taken at 48 hours after reperfusion (n=4/group). Rabbit anti-MMP-9 polyclonal antibody (Abcam), rabbit anti-MMP-2 polyclonal antibody (Abcam), rabbit anti-TIMP-1

Table 1. Physiological variables during sevoflurane preconditioning

Group	pH	PaCO$_2$(mmHg)	PaO$_2$ (mmHg)	Temperature (°C)
control	7.34±0.03	41.2±2.3	96.8±1.8	37.4±0.1
sevo-pre	7.33±0.02	42.1±1.1	96.5±3.7	37.2±0.2

Data are mean±SEM. n = 8/group

polyclonal antibody (Millipore), rabbit anti-occludin polyclonal antibody (Abcam), rabbit Iba-1 antibody (Wako chemical), mouse monoclonal anti-NeuN antibody (Millipore), mouse polyclonal anti-VEGF antibody (Santa cruz) and mouse monoclonal anti-GFAP antibody (Cell Signaling Technology) were used as primary antibodies. Sections were incubated with primary antibodies for 1 hour at 37°C and then at 4°C overnight, followed by incubation for 1 hour at 37°C with DyLight^{TM} 488-conjugated goat anti-mouse and DyLight^{TM} 594-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories). Sections were then stained with DAPI (Thermo Scientific) for 2 min at room temperature for counterstaining.

3.9. Statistical analysis

All data was expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with analysis of variance (ANOVA) followed by *post hoc* Fisher's PLSD tests. *P*-values less than 0.05 were considered statistically significant. "n" refers to the number of rats.

4. RESULTS

4.1. Physiological variables

Physiological variables of anesthetic pretreatment and perioperative period were detected as previously described. There were no significant differences in physiological values between control and sevo-pre groups (table 1). Before, during and after ischemia, no significant differences in physiological variables were detected between sham, vehicle, and sevo-pre groups as shown in table 2.

4.2. Sevoflurane preconditioning improved BBB integrity after MCAO

EB extravasation and electron microscopy was detected for BBB integrity. The results showed that EB stained tissues were mostly located in ipsilateral cortex and striatum, total EB stained volume was less in sevo-pre group (112.2±37.8 mm^3) than in vehicle group (325.8±23.0 mm^3, *P*<0.01), and total EB content was lower in sevo-pre group (3.16±0.33 μg/g) than in vehicle group (7.41±1.19 μg/g, *P*<0.01). rCBF change was monitored to rule out the possibility that sevoflurane might have altered rCBF and therefore BBB integrity. The data showed that there was no difference in vehicle and sevo-pre groups during ischemia or reperfusion (Figure 1 A-E). Furthermore, sevoflurane preconditioning markedly alleviated ischemia induced neurologic deficits (*P*< 0.05) (Figure 1 F).

Transmission electron microscopy also showed that the BBB ultra-structures of stroke rats in vehicle group were severely disrupted after cerebral ischemia, including shrinking of blood vessels, swelling of astrocytic perivascular processes, and disruption of basement membranes, decreased but intact tight-junctions. In the sevo-pre group, we observed that endothelial cells were slightly shrunken, and tight-junctions and basement membranes were intact. (Figure 2).

4.3. Sevoflurane preconditioning inhibited the activation of astrocytes and microglia cells

Astrocytes and microglia cells have been demonstrated to transform into an activatied form and produce inflammatory factors, when stimulated by pathogen-derived molecules or other microglial activators. Our immunofluorescence data showed that, in vehicle group, typically branched astrocytes increased in ipsilateral cortex and corpus callosum 48 hours after ischemia. Sevoflurane preconditioning significantly suppressed ischemia-induced proliferation of GFAP-positive astrocytic cells (Figure 3A). Ramified resting microglia cells (in sham group), with long branching processes and small cellular bodies, were replaced by quantity of ameboid activated microglia cells (in vehicle group) in ipsilateral cortex and striatum post-injury. The inreases of activated microglia cells were markedly inhibited in sevo-pre group (Figure 3B). The results indicated that activation and proliferation of these glia cells could be suppressed by sevoflurane preconditioning.

4.4. Sevoflurane preconditioning suppressed up-regulation of cell adhesion molecules

Upregulated CAMs, especially ICAM-1 and VCAM-1, mediate adhesion of leukocytes to sites of inflammation after ischemia. Figure 4 shows western blot analyses of ICAM-1 and VCAM-1 in cortex tissues at various time points after MCAO. Compared with sham group, ICAM-1 significantly increased and peaked at 48 hours after ischemia, and VCAM-1 was gradually up-regulated till to 72 hours post-injury. Sevoflurane preconditioning effectively suppressed increases in ICAM-1 and VCAM-1 at 24, 48 and 72 hours after MCAO.

4.5. Sevoflurane preconditioning inhibited up-regulation of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases

MMPs, especially MMP-2 and MMP-9, play a critical role in degrading neurovascular matrix after acute ischemia. To determine the effect of sevoflurane preconditioning on MMPs, western blot and gelatin zymography analyses were taken to detect protein expression and activity of MMP-2 and MMP-9 (Figure 5). The data showed that MMP-2 and MMP-9 significantly increased after ischemia, both of which were reduced by sevoflurane preconditioning at 24, 48 and 72 hours after ischemia. Double-labelled immunofluorescent staining at 48 hours after MCAO showed that increases of MMP-2 and MMP-9 in neurons and astrocytes were inhibited by sevoflurane pretreatment in ipsilateral cortex, striatum and corpus callosum (Figure 6).
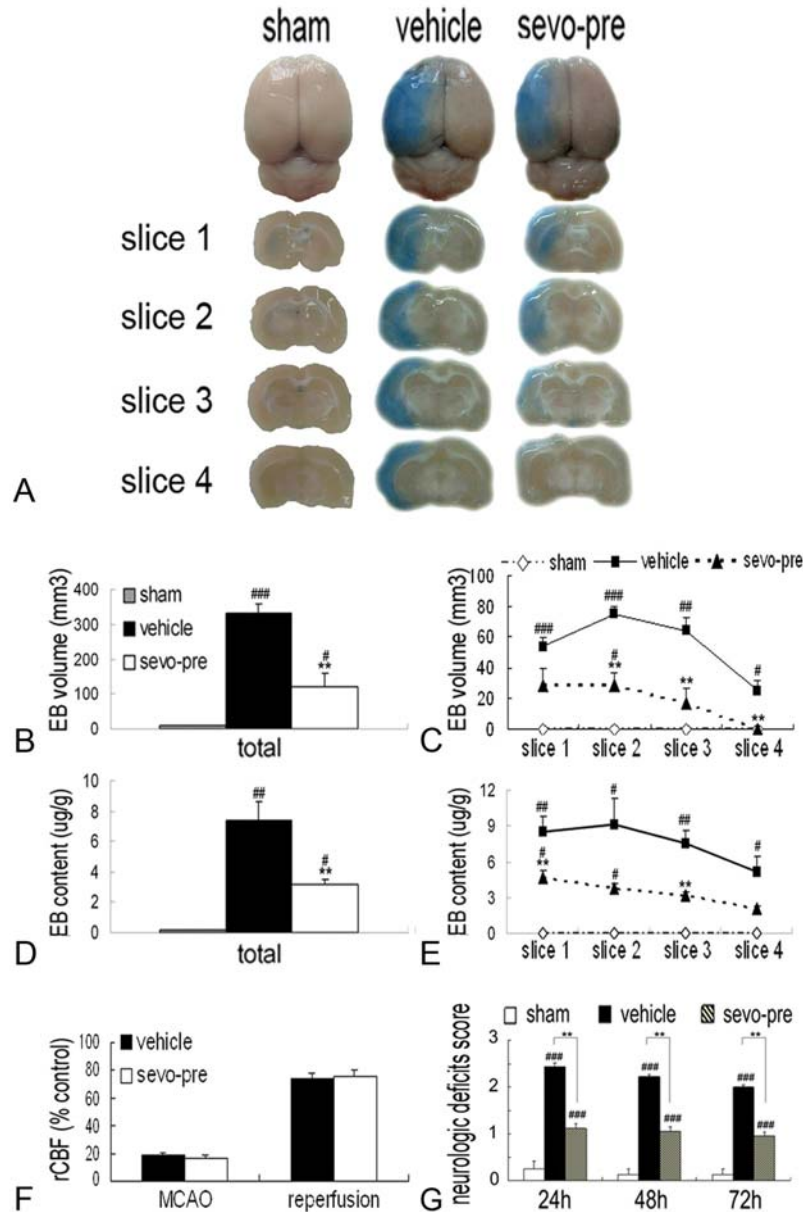
Figure 1. Effect of sevoflurane preconditioning on evans blue (EB) extravasation (n = 8/group) and neurologic outcomes (n = 8/group) after ischemia. A. Representative images of EB-stained brains 48 hours after MCAO. Each section was 2 mm thick. B. and C. Effects of sevoflurane preconditioning on EB-stained volume of brain tissues (mm^3). D and E. Effects of sevoflurane preconditioning on EB content of brain tissues (μg/g). F. Changes of rCBF were not different between vehicle and sevo-pre group at the ischemia and reperfusion. G. Effect of sevoflurane preconditioning on neurologic deficits scores up to 3 days after ischemia. Compared with vehicle group, **p<0.01. Compared with sham group, #p<0.05, ##p<0.01, ###p<0.001.

Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) is a specific inhibitor of MMP-9. Disbalance between MMP-9 and TIMP-1 could induce disruption of BBB. Western blot analysis showed that TIMP-1 increased and peaked at 48 hours in vehicle group after ischemia, which was parallel with MMP-9 (Figure 7A and 7B). Sevoflurane pretreatment significantly inhibited TIMP-1 expression at 48 hours after ischemia (*P*<0.01). Double-labelled immunofluorescent staining at 48 hours after MCAO confirmed that increases of TIMP-1 in neurons and

astrocytes were suppressed by sevoflurane pretreatment in ipsilateral cortex, striatum and corpus callosum (Figure 7C and 7D).

4.6. Sevoflurane preconditioning suppressed decreases in occludin

Occludin is one of important tight-junction proteins to maintain BBB integrity. Semi-quantitative analysis by western blot showed that ischemia-induced decreases in occludin were significantly inhibited by

Table 2. Physiological variables during focal cerebral ischemia

| Group	pH	PaCO$_2$ (mmHg)	PaO$_2$ (mmHg)	MABP (mm Hg)	Temperature (°C)
Sham	7.36±0.02	44.3±1.8	95.8±2.8	96.1±3.7	37.3±0.1
vehicle					
pre-ischemia	7.35±0.07	46.6±2.7	96.0±3.0	84.0±3.6	37.3±0.2
peri-ischemia	7.33±0.03	46.9±1.4	86.7±3.8	79.6±3.5	37.6±0.3
post-ischemia	7.33±0.03	40.9±2.9	93.7±4.9	87.8±4.3	37.7±0.4
sevo-pre					
pre-ischemia	7.30±0.05	44.5±2.3	89.7±2.9	84.0±4.5	37.4±0.3
peri-ischemia	7.32±0.07	49.7±1.9	85.3±3.3	75.4±3.4	37.6±0.2
post-ischemia	7.29±0.05	48.1±3.4	88.5±3.4	76.2±4.0	37.4±0.3

Data are mean±SEM. n = 8/group
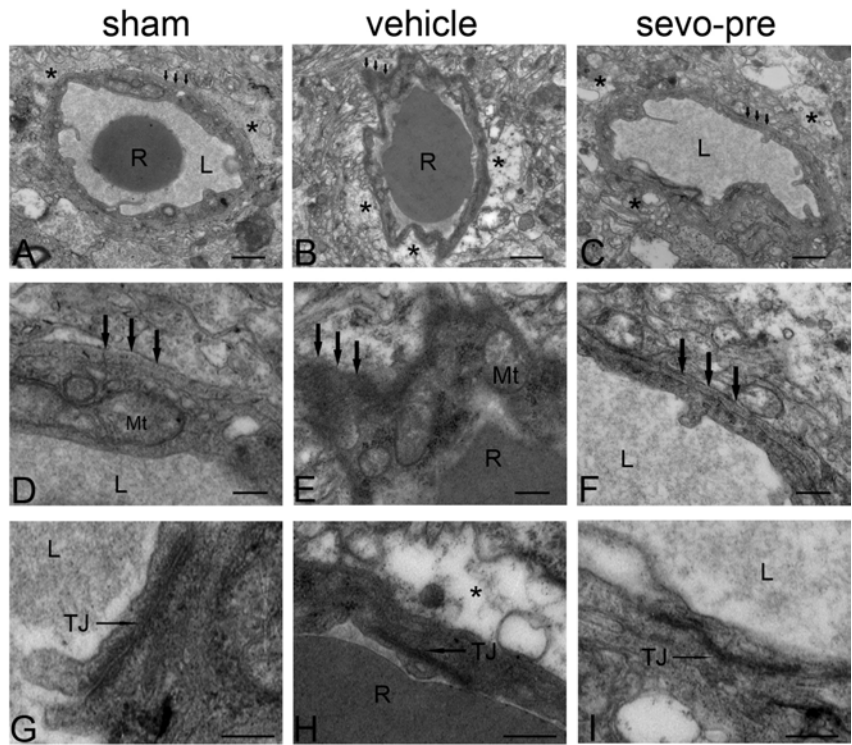
Figure 2. Effect of sevoflurane preconditioning on ultra-structure of BBB in ipsilateral cortex by using transmission electron microscopy at 48 hours after MCAO (n = 4/group). A, D and G: In sham group, tight-junctions (TJs) and base membranes (three arrows) were intact. B, E and H: In vehicle group, endothelial cells were severely shrunken, vacuoles were present in the mitochondria of endothelial cells, base membranes were incomplete, the feet of perivascular astrocytes (asterisk) were swollen, with reduced electron density. Decreased TJs were intact. C, F and I: In sevo-pre group, blood vessels were slightly shrunken, TJs and base membranes were intact, slightly swelling of astrocytic perivascular processes were observed. TJ: tight junction, L: capillary lumen, R: a red cell. Scale bar: A-C is 1μm; D-F is 0.25μm; G-I is 0.2 μm.

sevoflurane preconditioning at 48 and 72 hours after ischemia (P<0.01) (Figure 8A and 8B). Immunofluorescent staining at 48 hours after MCAO showed that occludin positive protein arranged along with microvessels continuously (labelled with anti-VEGF antibody) in sham group (Figure 8C). Ischemic injuries led to decreases and discontinuous arrangement of occludin positive protein, which can be suppressed by sevoflurane pretreatment.

5. DISCUSSION

BBB is recognized as a dynamic "barrier" between brain and blood that, by regulating the exchange of substances, maintains optimal conditions for neuronal and glial functions. The unique selective barrier consists of brain capillary endothelial cells, basal lamina, pericytes and astrocytic end-feet (9). During ischemia and reperfusion, vasogenic edema, resulting from dysfunction of BBB, allows increased passage of plasma proteins, water and invading proinflammatory factors into the extracellular compartment, triggers secondary neuro-inflammatory cascades, and leads to further extensive damage of brain parenchyma (10). Based on previous results (11) that sevoflurane pretreatment inhibits neuro-inflammation *via* suppressing ischemia-induced up-regulation of interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-alpha), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX$_2$), we hypotheses that sevoflurane preconditioning could confer neuroprotection by restoring
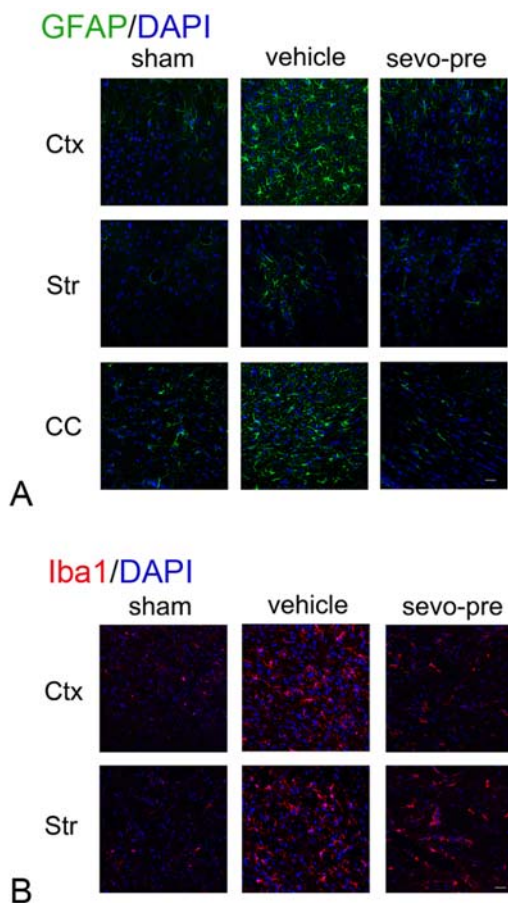
Figure 3. Effect of sevoflurane preconditioning on the activation of astrocytes and microglial cells in cerebral cortex, striatum and corpus callosum at 48 hours after reperfusion. n = 4/group. Ctx = cortex, Str = striatum, CC = corpus callosum. Scale bar = 10 μm.
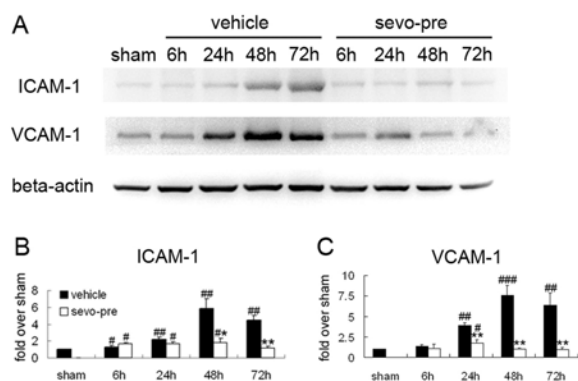

Figure 4. Sevoflurane preconditioning suppressed the up-regulation of ICAM-1 and VCAM-1 after ischemia. A. Representative Western blot of ICAM-1 and VCAM-1 in ipsilateral cortex at various time points after reperfusion. B and C, semi-quantitative analysis of ICAM-1 and VCAM-1. n = 4/group. Compared with vehicle group, *$p < 0.05$, **$p < 0.01$; compared with sham group, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$.

BBB integrity against focal cerebral ischemia. In the present study, we detected BBB integrity by using EB extravasation measurement and transmission electron microscopy. We found that BBB leakage significantly increased at 48 hours after MCAO and the disruption could be reversed by sevoflurane preconditioning. Furthermore, improvement of neurologic deficits after reperfusion confirmed the neuroprotective effects of sevoflurane preconditioning.

Astrocytes and microglia cells are inflammatory cells in the central nervous system, by synthesizing and releasing cytokines, chemokines, CAMs and growth factors in the pathogenesis of neuro-inflammatory and neurodegenerative disorders. Suppression of hyperactivation of astrocytes and microglia cells has been demonstrated to induce neuroprotection against cerebral ischemic insults (12). Our results showed that sevoflurane preconditioning significantly inhibited activation and proliferation of these two glial cells in ipsilateral cortex, striatum and corpus callosum at 48 hours after ischemia. The findings is consistent with Karwacki's results that sevoflurane could suppress the activation of microglia in a model of intracerebral haemorrhage (ICH) (13).

The expression of adhesion molecules on capillary endothelial cells and neutrophils, in particular ICAM-1 and VCAM-1, is a major event to trigger secondary neuro-inflammation by facilitating the adhesion and transendothelial migration of neutrophils and macrophages. Kitagawa *et al.* reported that ICAM-1 knockout mice developed smaller ischemic infarction size (14). Our results showed that sevoflurane pretreatment suppressed the elevation of ICAM-1 and VCAM-1 after MCAO, supporting the beneficial effect of sevoflurane on ischemic brain. NFkappaB regulates the expression of ICAM-1 and VCAM-1 on endothelial cells, promoting the adherence of inflammatory cells. Based on our previous study (11), we infer that sevoflurane pretreatment may reduce the elevation of CAMs at the transcriptional level *via* suppression of NFkappaB.

MMPs, a family of zinc-dependent endopeptidases, are demonstrated to mediate BBB leakage, edema and hemorrhage by degrading neurovascular matrix after cerebral ischemia (15). MMP-9 and MMP-2 has been suggested to be spatio-temporally associated with BBB damage and neuronal injury following MCAO. Emerging evidence indicates that the early reversible opening of BBB is associated with an early transient elevation in MMP-2 (16, 17). During the second opening of BBB, there is marked increase in MMP-9, leading to extensive damage to the blood vessels (18). The treatment of MMP inhibitor or MMP gene knockout is associated with a reduction in infarction and attenuation of BBB opening after focal cerebral ischemia (14, 19). The sources of MMPs may include neuron, astrocyte, infiltrating neutrophil and reactive microglia (20). These results are partly consistent with our findings that MMP-9 and MMP-2 increased in ipsilateral neurons and astrocytes at 24-48 hours after ischemia and that up-regulation of MMP-9 and MMP-2 was significantly inhibited by sevoflurane preconditioning.
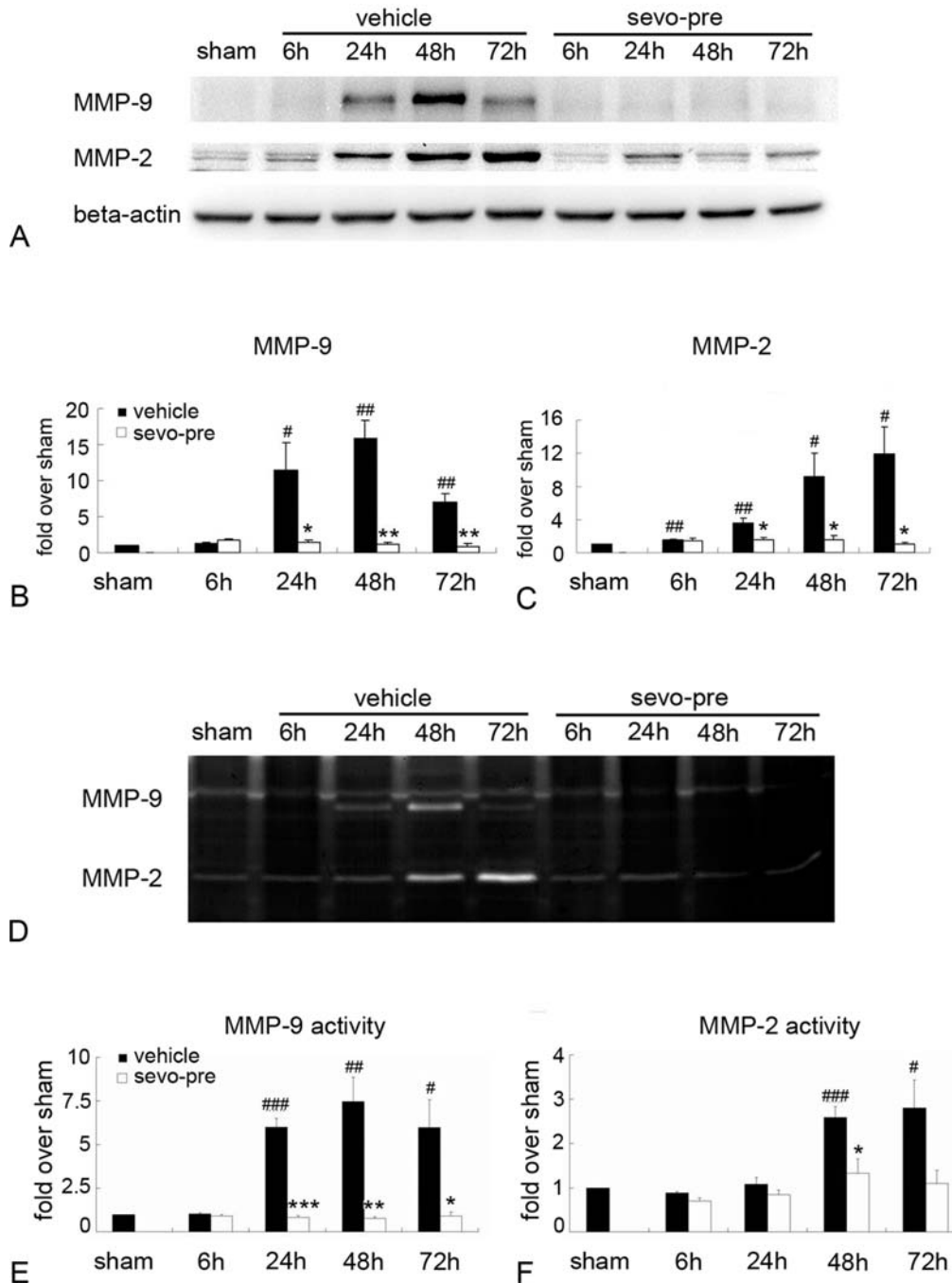
Figure 5. Sevoflurane preconditioning suppressed the up-regulation of MMP-9 and MMP-2 after ischemia. A. Representative western blot of MMP-9 and MMP-2 in ipsilateral cortex at various time points after reperfusion (n = 4/group). B and C. Semi-quantitative analysis of MMP-9 and MMP-2. D. Representative gelatin zymography of MMP-9 and MMP-2 in ipsilateral cortex at various time points after reperfusion (n = 4/group). E and F. Semi-quantitative analysis of MMP-9 and MMP-2. Compared with vehicle group, *p<0.05, **p<0.01, ***p<0.001; compared with sham group, #p<0.05, ##p<0.01, ###p<0.001.

However, we did not detected transient elevation of MMP-2 after ischemia. The different results may be due to diverse experimental animals, various insults (ischemia, hypoxia, ICH, or oxygen-glucose deprivation), and different timing of measurements. Regulation of MMPs expression and activation is tightly controlled by TIMPs. Disbalance between MMPs and TIMPs contributes to disruption of BBB. In the present study, we found that TIMP-1, a specific endogenous inhibitor of MMP-9, increased with MMP-9 after ischemia, and that the parallel
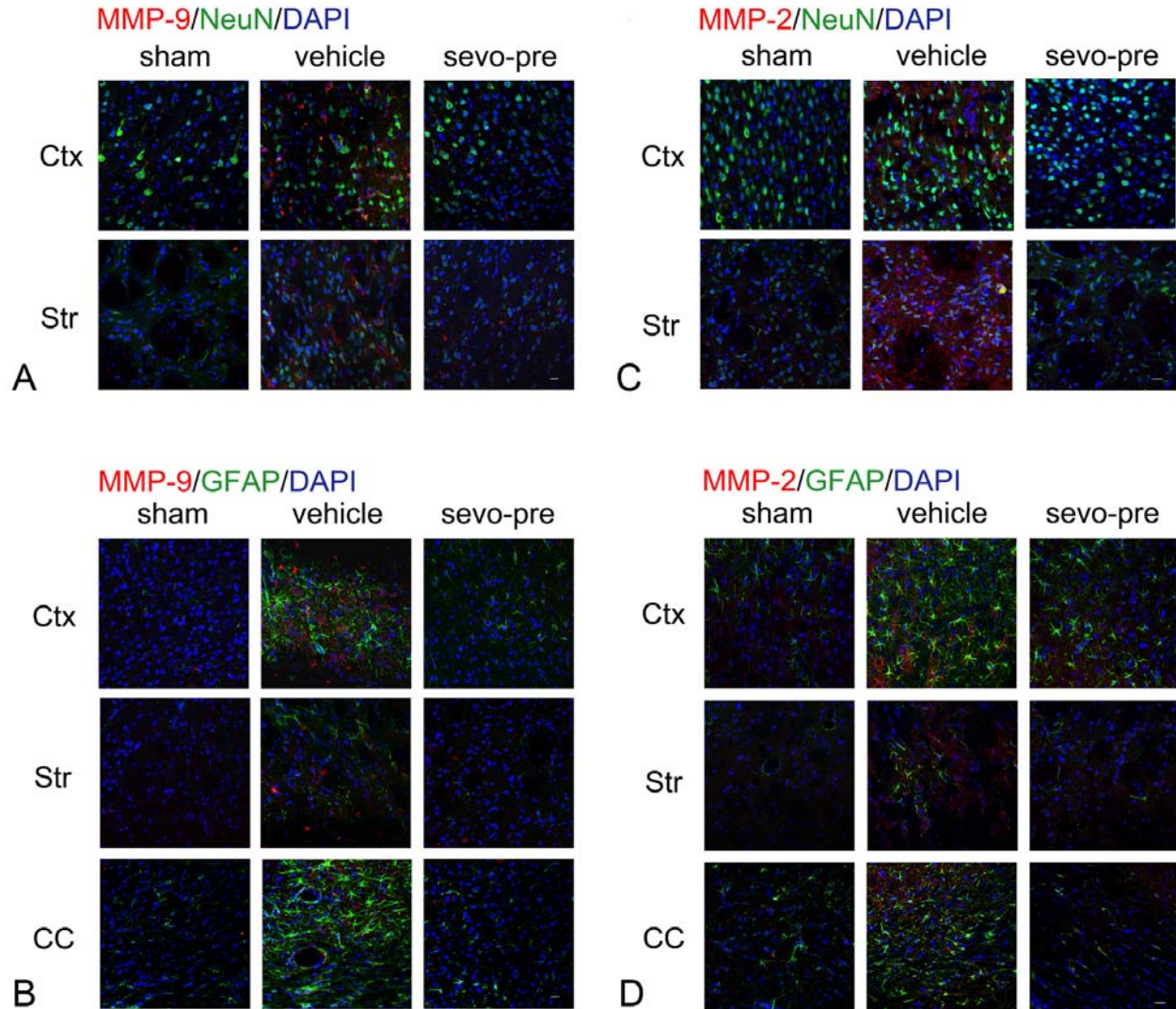
Figure 6. Sevoflurane preconditioning suppressed the up-regulation of MMP-9 and MMP-2 after ischemia. A and C. Immunofluorescence photomicrographs of MMP-9 / -2 (red) and NeuN (green) within the infarct at the cerebral cortex and striatum 48 hours after MCAO, showing that MMP-9 / -2 is expressed by NeuN+ neurons. B and D. Immunofluorescence photomicrographs of MMP-9 / -2 (red) and GFAP (green) within the infarct at the cerebral cortex, striatum and corpus callosum 48 hours after MCAO, showing that MMP-9 / -2 is expressed by GFAP+ astrocytes. n = 4/group. Ctx = cortex, Str = striatum, CC = corpus callosum. Scale bar = 10 μm.

elevation was suppressed by sevoflurane pretreatment. Woo *et al*. reported that inhibition of MMP-9 significantly suppressed the expression of iNOS, IL-1 beta and IL-6 at the transcriptional level through a mechanism involving suppression of NFkappaB, activator protein-1 (AP-1) and mitogen-activated protein kinases (MAPKs) in lipopolysaccharide-stimulated microglial cells (21). Our previous study showed that sevoflurane preconditioning suppressed inflammatory factors along with upstream NF-kappa B and p38 MAPK in rats exposed to focal cerebral ischemia (11). Therefore, we infer that sevoflurane pretreatment may inhibit ischemia-induced increase in MMP-9 at the transcriptional level *via* suppression of NFkappaB.

Tight junctions (TJs) join endothelial cells together, restricting molecules from moving between the blood and the brain (22). Occludin, one of the main structural barrier proteins, is considered sensitive indicator of normal and disturbed functional state of the BBB (23). Our transmission electron microscopy showed that intact tight-junctions decreased by ischemic injury, which could be suppressed by sevoflurane pretreatment. We detected the expression of occludin after MCAO and also found that sevoflurane preconditioning significantly reduced the loss of occludin induced by ischemia and reperfusion. Yang *et al*. reported that up-regulation of MMP-9 degraded TJs, such as occludin and claudins, after cerebral focal ischemia (17). Thus, our results indicate that sevoflurane
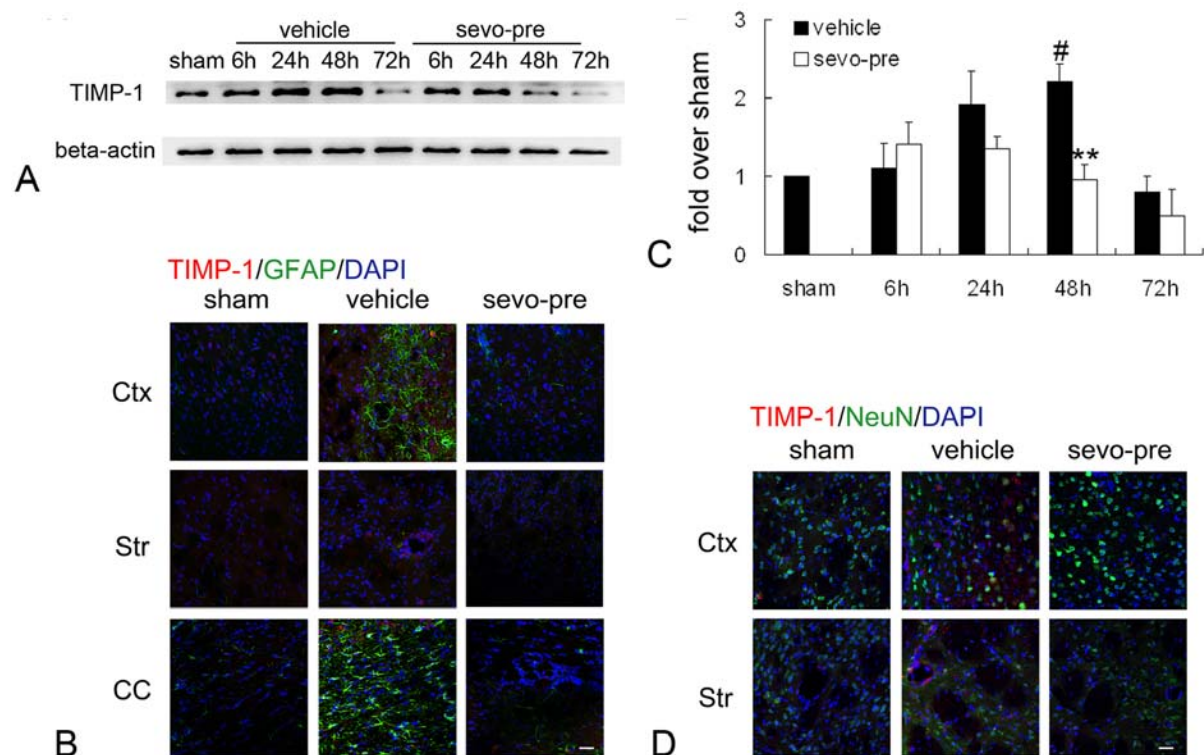
Figure 7. Sevoflurane preconditioning suppressed the up-regulation of TIMP-1 after ischemia. A. Representative western blot of TIMP-1 in ipsilateral cortex at various time points after reperfusion (n = 4/group). B. Immunofluorescence photomicrographs of TIMP-1 (red) and NeuN (green) within the infarct at the cerebral cortex and striatum 48 hours after MCAO, showing that TIMP-1 is expressed by NeuN+ neurons. C. Semi-quantitative analysis of TIMP-1. D. Immunofluorescence photomicrographs of TIMP-1 (red) and GFAP (green) within the infarct at the cerebral cortex, striatum and corpus callosum 48 hours after MCAO, showing that TIMP-1 is expressed by GFAP+ astrocytes. n = 4/group. Ctx = cortex, Str = striatum, CC = corpus callosum. Scale bar = 10μm. Compared with vehicle group, **p<0.01; compared with sham group, #p<0.05.
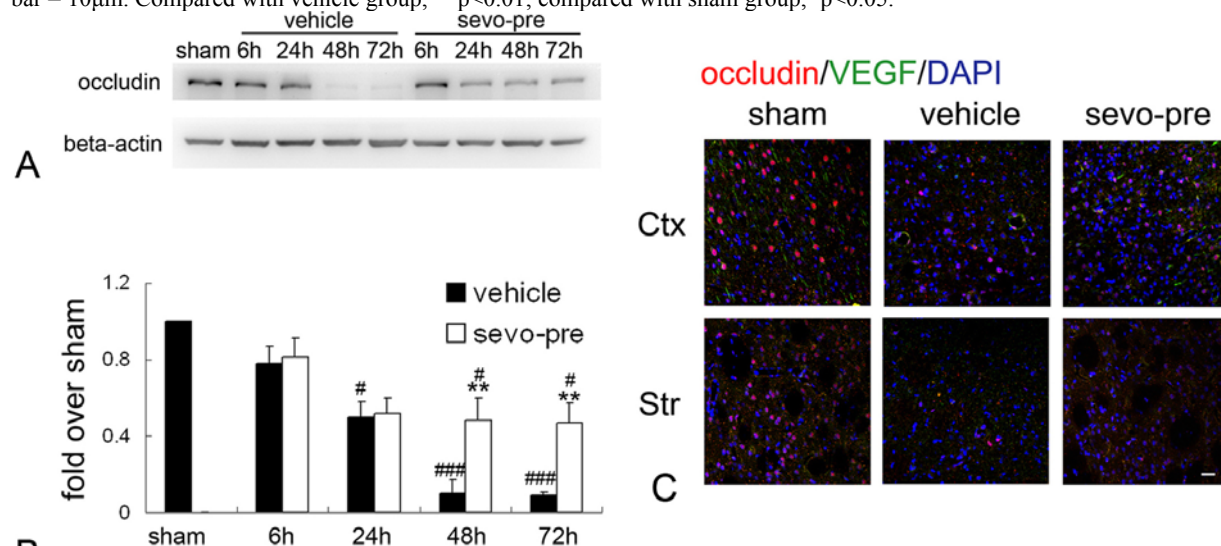

Figure 8. Sevoflurane preconditioning suppressed the decreases in occludin after ischemia. A. Representative western blot of occludin in ipsilateral cortex at various time points after reperfusion (n = 4/group). B. Semi-quantitative analysis of occludin. C. Immunofluorescence photomicrographs of occludin (red) and VEGF (green) within the infarct at the cerebral cortex and striatum 48 hours after MCAO, showing that occludin is expressed along with VEGF+ vessels. n = 4/group. Ctx = cortex, Str = striatum. Scale bar = 10μm. Compared with vehicle group, **p<0.01; compared with sham group, #p<0.05, ###p<0.001.

pretreatment reduces the loss of occludin *via* suppression of MMP-9, which improve the functional state of BBB after ischemia.

In conclusion, repeated preconditioning with sevoflurane confered potent neuroprotection against ischemic brain injury partly by improving BBB integrity. Sevoflurane pretreatment could suppress up-regulation of CAMs and MMPs, inhibit ischemia-induced decreases in occludin, and suppress the activation of astrocytes and microglia cells. Thus, sevoflurane pretreatment is a potential therapy for ischemic brain injury.

6. ACKNOWLEDGEMENTS

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Abbreviations: BBB: blood-brain-barrier; CAMs: cell adhesion molecules; MMPs: matrix metalloproteinases; MCAO: middle cerebral artery occlusion; rCBF: regional cerebral blood flow; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell molecule-1; TIMP-1: tissue inhibitor of matrix metalloproteinase-1; TJs: tight-junctions; MAC: minimum alveolar concentration; EB: Evans blue; iNOS: inducible nitric oxide synthase; COX2: cyclooxygenase-2; TNF-a: tumor necrosis factor-alpha; MAPK: mitogen activated protein kinase; AP-1: activator protein-1; ILs: interleukins; ICH: intracerebral haemorrhage; SDS: sodium dodecyl sulfate; SEM: standard error of the mean.

Key Words: Cerebral Ischemia, Volatile Anesthetic Preconditioning, Sevoflurane, Neuroprotection, Blood-Brain-Barrier, Matrix Metalloproteinases, Cell Adhesion Molecules, Tight-Junctions

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