Effect of anesthetic post-treatment on blood-brain barrier integrity and cerebral edema after transient cerebral ischemia in rats

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This certifies that the Doctoral Dissertation of Jae Hoon Lee is approved.

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Jae Hoon Lee

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ABSTRACT

Effect of Anesthetic Post-treatment on Blood-Brain Barrier Integrity and Cerebral Edema after Transient Cerebral Ischemia in Rats

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(Directed by Professor Bon-Nyeo Koo)

Although anesthetics, such as propofol and isoflurane, have been reported to offer neuroprotection against cerebral ischemia injury, their impact on cerebral edema following ischemia is not clear. The objective of this investigation is to evaluate the effects of anesthetic post-treatment on blood-brain barrier (BBB) integrity and cerebral edema after transient cerebral ischemia and its mechanism of action, focusing on modulation of aquaporins (AQPs), matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF)-1α.

Cerebral ischemia was induced in male Sprague-Dawley rats (n=103) by occlusion of the right middle cerebral artery for 1 h. For post-treatment with propofol (n=37), 1 mg/kg/min of propofol was administered for 1 h from the start of reperfusion, and for post-treatment with isoflurane (n=25), 2.1 vol% of isoflurane was administered for 1 h from the start of reperfusion. Saline-treated controls received 0.9% normal saline at the rate of 0.1 ml/kg/min for 1 h from the start of reperfusion (n=41). Nineteen rats undergoing sham surgery were also included in the investigation. Edema and BBB integrity were assessed by quantification of cerebral water content and extravasation of Evans blue, respectively, following 24 h of reperfusion. In addition, the expression of AQP-1, AQP-4, MMP-2, MMP-9, and VEGF was determined 24 h after

reperfusion and the expression of HIF-1α was determined 8 h after reperfusion.

Propofol or isoflurane post-treatment significantly reduced cerebral edema ($P<0.05$) and BBB disruption ($P<0.05$) compared with the saline-treated control. Furthermore, post-treatment with propofol or isoflurane reduced the expression of AQP-4 and MMP-2, compared to the saline-treated control ($P<0.05$). However, there were no differences in cerebral edema, BBB disruption, and expression of AQP-4 and MMP-2 between propofol and isoflurane post-treatment. In further evaluation of the factors related to the formation of cerebral edema, the expression of AQP-1, AQP-4, MMP-2, MMP-9, and VEGF at 24 h and of HIF-1α at 8 h following ischemia/reperfusion was significantly suppressed in the propofol post-treatment group ($P<0.05$).

In conclusion, propofol post-treatment attenuated cerebral edema and BBB disruption after transient cerebral ischemia and the effects of propofol post-treatment on cerebral edema and BBB integrity were not different from those of isoflurane post-treatment. Additionally, alleviation of cerebral edema by propofol post-treatment was shown to be associated with reduced expression of AQP-1, AQP-4, MMP-2, MMP-9, VEGF and HIF-1α.

Key words: blood-brain barrier, cerebral edema, cerebral ischemia, isoflurane, propofol

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I. INTRODUCTION

Stroke is a major cause of death in most developed countries and its incidence is slowly increasing.[1] Cerebral edema occurs frequently following ischemic stroke. Edema, defined as an abnormal increase in water content, has a crucial effect on morbidity and mortality after stroke because the swelling of cerebral tissue caused by edema can increase intracranial pressure, favor herniations, and contribute to ongoing ischemic injury.[2] Furthermore, cerebral ischemia has been reported to damage the integrity and permeability of the blood-brain barrier (BBB).[3,4]

Aquaporins (AQPs) are water channels that allow bidirectional water flux through cell membranes and thus facilitate water transport to and from the central nervous system.[5] Among three AQPs identified in the rodent brain, AQP-1 and AQP-4 are permeable only to water and are presumed to participate in cerebrospinal fluid formation and brain water homeostasis.[2] Furthermore, previous reports have shown that AQP-1 and AQP-4 are involved in the formation of cerebral edema after cerebral ischemia.[6,7] Matrix metalloproteinases (MMPs) are secreted enzymes that can degrade all protein constituents of the extracellular matrix. Abnormal expression and activation of MMPs leads to breakdown of the extracellular matrix and tight junctions, resulting in opening of the BBB and vasogenic edema.[8] In particular, expression of MMP-2 and MMP-9 increases after

transient cerebral ischemia.[9]

Hypoxia inducible factor (HIF)-1 is a transcription factor composed of an oxygen-regulated subunit, HIF-1α, and a constitutively expressed subunit, HIF-1β. HIF-1 activity is determined by the availability and activity of the HIF-1α subunit.[10] HIF-1 exerts its various activities through proteins encoded by its downstream genes such as vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporter.[11] HIF-1α was also reported to regulate AQP-4 expression in ischemic cerebral injury.[12] In addition, VEGF, a downstream product of HIF-1, can induce vascular leak[13] and MMP-9 activation.[14] Therefore, HIF-1 is closely associated with cerebral edema.

Anesthetic agents, such as isoflurane and propofol, have been shown to have neuroprotective effects against cerebral ischemia.[15] General anesthesia, consisting of multiple components, including hypnosis, immobility, amnesia, and analgesia, can be achieved via interactions with multiple molecular targets.[16] Intravenous anesthetics such as propofol are reported to mainly bind to and act through γ-aminobutyric $acid_A$ ($GABA_A$) receptors meanwhile, volatile anesthetics such as isoflurane activate $GABA_A$ and glycine receptors and inhibit N-methyl-D-aspartate (NMDA) receptors.[16,17] As the inhibitory or anti-excitatory effects of anesthetic agents can reduce cerebral metabolic rates and excitotoxicity after cerebral ischemia, the neuroprotective effects of anesthetic agents in cerebral ischemia have been investigated.[15] In particular, previous studies have demonstrated that post-treatment with isoflurane or propofol after transient cerebral ischemia has a neuroprotective effect against cerebral ischemia reperfusion injury.[18,19] However, the effect of isoflurane or propofol post-treatment on cerebral edema and BBB integrity after cerebral ischemia reperfusion injury has not been clarified. Although isoflurane and propofol share a common characteristic in that they can reduce cerebral metabolic rate, isoflurane may increase cerebral blood flow in a dose-dependent manner due to a cerebral vasodilatory effect, whereas propofol may decrease cerebral blood flow in proportion to reductions in cerebral metabolic rate.[20] Therefore, isoflurane may increase intracranial pressure, while propofol may reduce intracranial pressure.[20]

Furthermore, isoflurane was reported to increase the permeability of the BBB at low concentration and break down the BBB at high doses,[21,22] implying that isoflurane may aggravate or cause cerebral edema. On the other hand, previous reports have demonstrated that pre-treatment with propofol could reduce cerebral edema after ischemia.[7] Taken together, isoflurane and propofol may affect the formation of cerebral edema induced by ischemia.

The present study examined the effects of post-treatment of propofol or isoflurane on cerebral edema and permeability of the BBB following transient cerebral ischemia. Moreover, by evaluating the protein expression patterns of AQP-4 and MMP-2 in ischemia-reperfusion injury, this study compared the mechanisms by which the anesthetic agents propofol and isoflurane affect the development of cerebral edema after ischemia. Next, this investigation determined the extent to which the expression of MMPs and AQPs is ameliorated by propofol post-treatment. Finally, this study also assessed the effects of propofol post-treatment on the expression level of VEGF and HIF-1α.

II. MATERIALS AND METHODS

1. Animal preparation

All animal procedures were performed according to a protocol that was approved by the Yonsei University Animal Care and Use Committee and was in accordance with National Institutes of Health guidelines for care and use of laboratory animals. A total of 122 adult male Sprague-Dawley rats aged 8-10 weeks, weighing 280-320 g were obtained from Orientbio Inc. (Seongnam, Korea) for this study. Rats were allowed free access to food and water before and after surgery.

Anesthesia was induced with intraperitoneal injection of a mixture of 30 mg kg^{-1} zoletil (Virbac Lab., Carros, France) and 10 mg kg^{-1} xylazine (Bayer Korea Ltd., Seoul, Korea). Zoletil is a combination of a dissociative anesthetic, tiletamine hypochloride, and a benzodiazepine, zolazepam hypochloride. Tiletamine is a non-competitive antagonist at the phencyclidine site of the N-methyl-d-aspartate receptor. Xylazine is an alpha-2 adrenergic agonist and exerts effects on presynaptic and postsynaptic receptors of the central and peripheral nervous systems. After tracheal intubation, the lungs were mechanically ventilated with 50% oxygen to achieve normocapnia. Rats were placed in the supine position on a heated pad, with body temperature maintained at 37 ± 0.5℃ according to a rectal thermometer. Polyethylene catheters were inserted into the right femoral artery for blood pressure measurement and sampling for arterial blood gas evaluation.

2. Middle cerebral artery occlusion model and grouping

The experimental middle cerebral artery occlusion (MCAO) model was generated as previously described.[7] Under an operating microscope, the right common carotid artery, external carotid artery (EC), and internal carotid artery (IC) were exposed through a midline incision. The EC was ligated, coagulated, and cut down just proximal to the lingual and maxillary artery branches. All other branches of the EC were coagulated and transected. The IC was then isolated from the vagus nerve to avoid damage. A 4-0 monofilament nylon suture (Dermalone, United States Surgical, CT, USA) with a flame-rounded head was inserted through

the IC via a small incision in the EC stump. The distance from bifurcation of the common carotid artery to the tip of the suture was approximately 18.5 mm in all rats, which is consistent with published descriptions of the MCAO model. Cerebral blood flow was monitored by laser Doppler flowmetry (LDF, Omega flow, FLO-C1, Neuroscience, Tokyo, Japan) using a flexible probe placed in cortical areas supplied by the MCA (2 mm posterior and 6 mm lateral to the bregma). When the MCA was occluded by insertion of the thread, rats that did not show a cerebral blood flow reduction of at least 70% were excluded from the experiment.[23] After 60 min of occlusion, the suture was withdrawn, the skin was sutured, and the rats were allowed to recover. All rats were sacrificed 8 h or 24 h after reperfusion.

Rats were divided randomly into four groups. The sham group (n=19) underwent sham surgery and received 0.9% saline for 1 h after surgery. Sham surgery was similar to the MCAO modeling but without nylon suture occlusion and reperfusion. The other three groups underwent MCAO/reperfusion as described earlier. The experimental control (EC) group received 0.9% saline at the rate of 0.1 ml/kg/min intravenously for 1 h from the start of reperfusion (n=41), the propofol (Pro) group received 1% propofol at the rate of 0.1 ml/kg/min intravenously for 1 h from the start of reperfusion (n=37), and the isoflurane (Iso) group received 2.1 vol% [1.5 minimum alveolar concentration (MAC)] of isoflurane for 1 hr from the start of reperfusion (n=25).

3. Neurobehavioral assessment

Animals were examined for neurological deficits 24 h after reperfusion by an investigator who was blind to the identity of the groups using a 5-point neurological function score as described previously: 0=no deficit; 1=failure to extend left forepaw fully; 2=circling to the left; 3=failing to the left; 4=unable to walk spontaneously.[24] The rota-rod test was used to assess the recovery of impaired motor function after MCAO (n=15 in each group). The accelerating rota-rod test (ENV-577, Med Associates Inc., Geordia, VT, USA) was carried out as described by Hunter et al. with a slight modification.[25] The exercise time was

measured as the time the animal remained on an accelerating rota-rod cylinder. The speed was increased from 4 to 40 rpm within 5 min. The trial ended if the animal fell off the rungs or gripped the device and spun around for two consecutive revolutions without attempting to walk on the rungs. The exercise time on the rota-rod for each rat was measured 24 h after reperfusion.

4. Measurement of infarct volume, edema volume, and water content

At 24 h after reperfusion, animals were anesthetized and decapitated. The brains were quickly isolated and sectioned into serial 2-mm-thick coronal slices (n=5 in each group). The brain slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Aldrich, St Louis, MO, USA) in the dark at 37℃ for 30 min and fixed with 4% paraformaldehyde (PFA, Sigma Aldrich) overnight. The posterior surface of each slice was photographed and analyzed using a computer-assisted image analysis system (Optimas ver 6.1, Optimas, Bothell, WA, USA). The lesion volume was calculated by multiplying the area by the thickness of slices. This investigation adopted a previously described method to eliminate the contribution of edema to the ischemic lesion using the following formula: Corrected infarct volume = Contralateral hemisphere volume – (Ipsilateral hemisphere volume – Measured infarct volume).[26] The edema volume was calculated by subtracting the volume of the contralateral hemisphere from that of the ipsilateral hemisphere.

To quantify water content in the ischemic hemisphere, the wet-dry weight method was used. Brains (n=5 in each group) were divided into ipsilateral and contralateral hemispheres. The ipsilateral hemisphere tissue was placed on a piece of aluminum foil. After the wet weight of the hemisphere was measured, the tissue was dried at 110℃ for 24 h to obtain the dry weight. The brain water content in the ischemic hemisphere was calculated by using the following equation: %water = [(wet weight – dry weight) / wet weight] × 100%.

5. Blood-brain barrier disruption

The integrity of the BBB was investigated using Evans blue extravasation.[27]

Immediately after reperfusion 4 ml kg^{-1} of 2% Evans blue (Sigma Aldrich) in normal saline was injected into the tail vein (5 rats in each group). The animals were killed 24 h after reperfusion. For quantitative measurements, the ischemic hemisphere was homogenized in 3 mL of N,N-dimethylformamide (Sigma Aldrich), incubated for 18 h at 55℃, and centrifuged. The supernatants were analyzed at 620 nm by spectrophotometry. The tissue content of Evans blue dye was quantified from a linear standard curve derived from known amounts of the dye and was expressed as micrograms per gram of tissue.

6. Immunoblot analysis of AQPs, MMPs, occludin, VEGF, and HIF-1α

Five rats from each group were sacrificed 8 h or 24 h after MCAO. The brain was quickly removed and the ipsilateral hemisphere was dissected. All procedures were performed on ice. The dissected tissues were either frozen or immediately used. Tissues were homogenized in modified RIPA-buffer [50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, complete protease inhibitor cocktail (Sigma Aldrich)] and incubated for 20 min on ice. After 15 min centrifugation (13,000 rpm, 4℃), protein concentration of the supernatant was determined using a BCA-kit (Pierce, Rockford, IL, USA). The sample was mixed with Laemmli sample buffer and boiled for 5 min. The samples (50 μg protein/well) were separated on 8%, 10%, or 12% SDS-PAGE and electro-transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat skim milk dissolved in TBS with 0.1% Tween20 (TBST) for 1 h at room temperature and then probed overnight at 4℃ using six different primary antibodies: monoclonal mouse anti-AQP-1 (1:1000 Abcam, Cambridge, UK), monoclonal mouse anti-AQP-4 (1:500, Abcam), monoclonal mouse anti-MMP-2 (1:1000, Abcam), polyclonal goat anti-MMP-9 (1:200, Santa Cruz Biotechnology, CA, USA), polyclonal rabbit anti-occludin (1:250, Abcam), monoclonal mouse anti-HIF-1α (1:1000, Chemicon, Temecula, CA, USA), polyclonal rabbit anti-VEGF (1:2000, Abcam), polyclonal mouse anti-β-actin (1:4000, Abcam), and polyclonal rabbit anti-GAPDH (1:3000, Cell Signaling Technology, Inc, MA, USA). The membranes were extensively washed

in TBS-T and incubated with peroxidase-conjugated anti-rabbit IgG (1:3000) or anti-mouse IgG (1:3000) for 2 h at room temperature. The blots were developed with an ECL detection kit (Pierce) using LAS4000 (Fuji PhotoFilm, Tokyo, Japan). Optical density of the bands was quantified in a computer-assisted image analysis system (NIH Image J) and normalized with respect to β-actin or GAPDH.

7. Immunohistochemical analysis

At 24 h after MCAO, some animals in each group were anesthetized and transcardially perfused with 0.9% saline followed by 4% PFA. Brains were isolated, cut into 2 mm thick slices, post-fixed in PFA overnight at 4℃, and processed for paraffin embedding. For cryosectioning, tissues were soaked in 30% sucrose solution at 4℃ overnight, embedded in OCT-medium (Tissue-Tek, Sakura, Japan) and frozen at -80°C. Cryosections (30 μm) were generated using a Leica CM 1850 Cryostat (Leica instruments GmbH, Nussloch, Germany), collected on Muto Silane coated microscope slides, dried at room temperature for 2-3 h, and refrigerated until use. For AQP-1, AQP-4, and GFAP immunohistochemistry, cryosections were fixed for 10 min with 4% PFA at 4℃, subjected to microwave oven antigen retrieval with sodium citrate buffer (10 mmol/L, 0.05% Tween20, pH 6.0), and washed with PBS (5×3 min). After 1 h incubation with 10% normal donkey serum (Jackson ImmunoResearch Laboratories), slides were incubated overnight at 4℃ with polyclonal rabbit anti-AQP-1 (1:500, Abcam), monoclonal mouse anti-AQP-4 (1:100, Abcam), or polyclonal goat anti-GFAP (1:500, Abcam) antibody. Slides were washed with PBS (5×3 min), and then incubated for 1 h at room temperature with rhodamine-conjugated donkey anti-mouse IgG, rhodamine-conjugated donkey anti-rabbit IgG, or FITC-conjugated donkey anti-goat IgG secondary antibody. Slides were washed with PBS (5×3 min), mounted, and visualized using a confocal laser scanning microscope (LSM 700, Carl Zeiss, Germany).

For MMP-2 or MMP-9 immunohistochemistry, 5-μm paraffin sectioned slides were deparaffinized in xylene and rehydrated with PBS (pH 7.4). Endogenous peroxidase in the slides was blocked using 0.1% H_2O_2 in PBS for 30 min. The

sections were pre-incubated in 5% normal goat serum in PBS for 30 min and incubated overnight at 4℃ with polyclonal rabbit anti-MMP-2 (1:50, Santa Cruz Biotechnology) or polyclonal goat anti-MMP-9 (1:50, Santa Cruz Biotechnology). The antigens were detected with 3,3-diaminobenzidine (DAB) using an Elite ABC kit (Vector, Burlingame, CA, USA) and counterstained with hematoxylin. The sections were dehydrated through graded ethanol, mounted, and cover slipped before viewing.

8. Statistical analysis

Data were presented as mean ± SD or mean ± SEM. One-way ANOVA with Tukey post-hoc tests was used for comparisons among the groups. Statistical analyses were performed with PASW statistics 18 (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

III. RESULTS

1. Mortality and exclusion

A total of seven rats (two rats allocated to the EC group, three in the Pro group, and two in the Iso group) were excluded from the experiment due to insufficient reduction of cerebral blood flow after MCAO. In addition, 10 rats in the EC group (25.6%), five in the Pro group (14.7%), and three in the Iso group (13.6%) died before assessment. No mortality occurred in the Sham group. Finally, 29 rats in the EC group and the Pro group and 20 rats in the Iso group completed the experiment and were included in the analysis. All rats allocated to the Sham group (n=19) also finished the experiment and were used in the analysis

2. Physiological data

Mean arterial pressure (MAP) and blood gas variables are shown in Table 1. The MAPs of the Pro group and the Iso group 30 min after reperfusion were significantly lower than that of the Sham group at the same time point and that of the same group at the other time points. Other variables were similar among the groups and time points.

Table 1. Physiologic variables

	MAP	pH	PaO_2	$PaCO_2$
Before MCAO				
Sham	101.3 ± 5.3	7.35 ± 0.03	266.8 ± 38.5	36.7 ± 1.1
EC	95.0 ± 8.9	7.37 ± 0.02	256.0 ± 42.6	35.9 ± 0.8
Pro	100.3 ± 9.6	7.34 ± 0.03	260.7 ± 62.1	41.2 ± 6.7
Iso	108.3 ± 14.8	7.32 ± 0.03	281.7 ± 40.5	37.8 ± 4.0
At 30 min after MCAO				
Sham	101.0 ± 5.5	7.40 ± 0.06	258.0 ± 24.7	35.8 ± 1.6
EC	94.2 ± 8.7	7.38 ± 0.02	260.3 ± 38.2	35.7 ± 1.3
Pro	97.8 ± 8.7	7.40 ± 0.05	278.3 ± 72.9	38.5 ± 3.7
Iso	106.0 ± 15.7	7.36 ± 0.05	302.3 ± 52.8	38.3 ± 4.2
At 30 min after reperfusion				
Sham	104.0 ± 5.1	7.40 ± 0.04	280.2 ± 30.4	35.5 ± 0.7
EC	93.8 ± 10.2	7.36 ± 0.03	272.0 ± 51.8	35.9 ± 1.9
Pro	78.1 ± 7.8*†	7.37 ± 0.04	275.2 ± 65.1	37.3 ± 2.8
Iso	75.0 ± 11.7*‡	7.39 ± 0.05	306. 3 ± 29.9	39.3 ± 5.4

Sham group (n=19); EC and Pro group (n=29 in each group); Iso group (n=20). MAP, mean arterial pressure; MCAO, middle cerebral artery occlusion; EC, experimental control; Pro, propofol; Iso, isoflurane. Values are means ± SD. $^{*}P<0.05$ vs. Sham group at 30 min after reperfusion. $^{\dagger}P<0.05$ vs. Pro group before MCAO and at 30 min after MCAO. ‡$P<0.05$ vs. Iso group before MCAO and at 30 min after MCAO.

3. Effect of post-treatment with propofol or isoflurane on infarct volume and neurobehavioral function after ischemia-reperfusion

To investigate the effect of post-treatment with propofol or isoflurane on ischemic damage, brain infarct size and neurobehavioral function were assessed. The infarct volume in the ipsilateral hemisphere of the EC group was greater than those of the Pro group ($P<0.001$) and the Iso group [$P<0.001$, Fig.1 (A), (B)]. The EC group showed a shorter duration of rota-rod test [$P<0.001$, Fig.1 (C)] and higher neurologic score [$P<0.001$, Fig.1 (D)] than the Pro group and the Iso group. However, there were no differences in infarct volume and neurobehavior function between the Pro group and the Iso group.

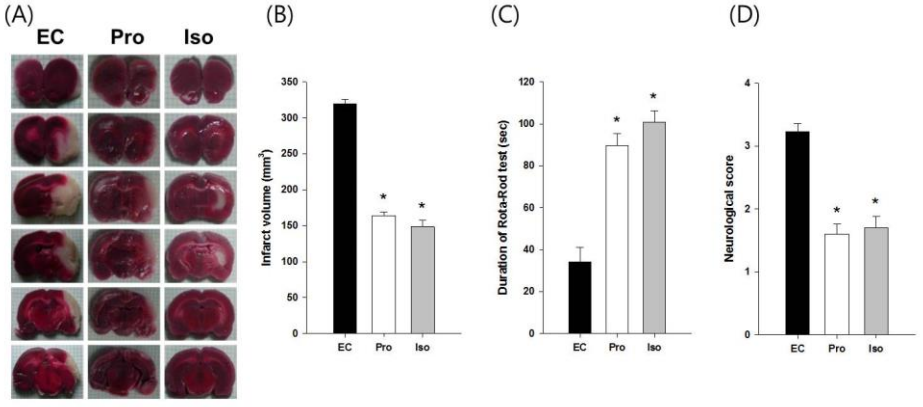

Figure 1. Post-treatment with propofol or isoflurane reduces infarct volume and ameliorates neurobehavioral function. Brain damage was estimated by TTC staining after 1 h ischemia and 24 h reperfusion. Animals in the Pro group received 1mg/kg/min of propofol from the onset of reperfusion for 1 h. Animals in the Iso group received 2.1 vol% of isoflurane for 1 hr from the start of reperfusion. Neurobehavioral function was assessed by rota-rod test and 5-point neurological function score. (A) Representative TTC staining images of brain sections of a MCAO rat. (B) Quantification of infarct volume (n=5 in each group). (C) Duration of Rota-Rod test (n=15 in each group). (D) Neurologic function score (n=15 in each group). Values are means ± SEM. *$P<0.001$ vs. EC group.

4. Effect of post-treatment with propofol or isoflurane on brain edema after ischemia-reperfusion

To determine the effect of post-treatment with propofol or isoflurane on brain edema after cerebral ischemia-reperfusion injury, brain edema volume and the content of cerebral water of the ipsilateral hemisphere were assessed 24 h after MCAO. Both brain edema volume and brain water content were greater in the EC group than the Pro group and the Iso group [$P<0.05$, Fig.2 (A), (B)]. However, there were no differences in brain edema volume and brain water content between the Pro group and the Iso group.

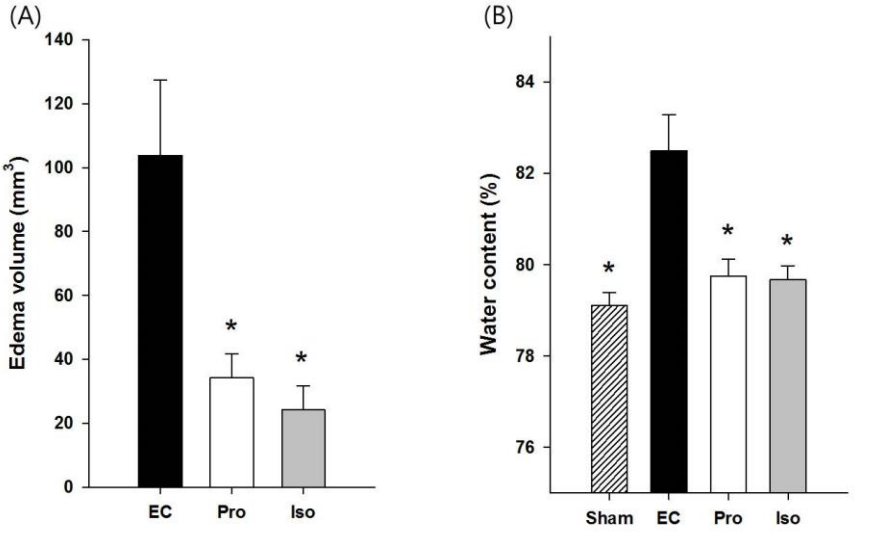

Figure 2. Post-treatment with propofol or isoflurane reduces brain edema. Brain edema was assessed by edema volume estimation and water content of brain tissues. (A) Quantification of brain edema volume (n=5 in each group). (B) Water content in ischemic hemisphere tissues (n=5 in each group). All values are means ± SEM. *$P<0.05$ vs. EC group.

5. Effect of post-treatment with propofol or isoflurane on blood-brain barrier integrity after ischemia-reperfusion

Evans blue dye was used as a marker of albumin extravasation to evaluate the effect of propofol or isoflurane post-treatment on BBB permeability. Representative images of Evans blue dye in brain tissues are shown in Fig.3 (A). Evans blue leakage increased significantly in ipsilateral hemispheres of the EC group compared with those of the Pro group and the Iso group [$P<0.05$, Fig.3 (B)]. In addition, the integrity of tight junctions was evaluated by assessing the expression of occludin, a tight junction protein. The expression of occludin in the EC group decreased significantly at 24 h after MCAO compared to the Sham group [$P<0.05$, Fig.3 (C)]. Post-treatment with propofol or isoflurane preserved occludin expression compared to the EC group ($P<0.05$). However, there were no differences in Evans blue leakage or occludin expression between the Pro group and Iso group.

These data demonstrate that ischemia and subsequent reperfusion for 1 h induce edema in cerebral tissue and impair the integrity of the BBB. Nevertheless, post-treatment with propofol or isoflurane alleviated both cerebral edema and BBB damage.

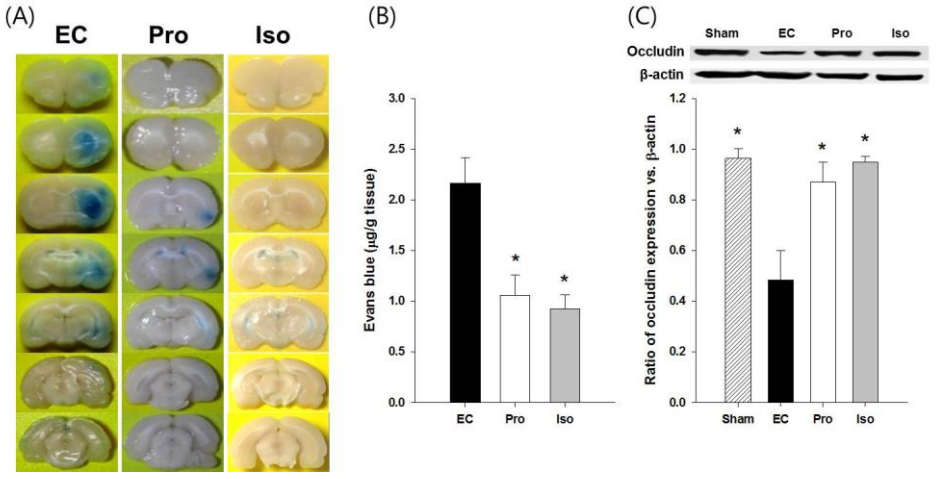

Figure 3. Post-treatment with propofol or isoflurane preserves blood-brain barrier integrity. BBB permeability was estimated by Evans blue leakage. The integrity of tight junctions was evaluated by the expression of occludin, a tight junction protein. (A) Representative images of Evans blue extravasation in coronal sections. (B) Quantification of Evans blue leakage in ischemic hemispheres (n=5 in each group). (C) Quantification of occludin expression in ischemic hemispheres (n=5 in each group). The expression of occludin was assessed by immunoblotting and normalized to β-actin expression. All values represent means ± SEM. *P<0.05 vs. EC group.

6. Effects of post-treatment of propofol or isoflurane on the expression of AQP-4 and MMP-2

AQP-4 is associated with the formation of cerebral edema by acting as a transmembrane water channel,[2] and MMP-2 may participate in the disruption of the BBB through its extracellular matrix degrading activity.[8] Expression of AQP-4 and MMP-2 in the EC group increased significantly at 24 h after MCAO compared with the Sham group ($P<0.05$, Fig.4). Post-treatment with propofol or isoflurane reduced the expression of AQP-4 and MMP-2 compared with the EC group ($P<0.05$). However, the patterns of AQP-4 and MMP-2 expression were not different between the Pro group and the Iso group.

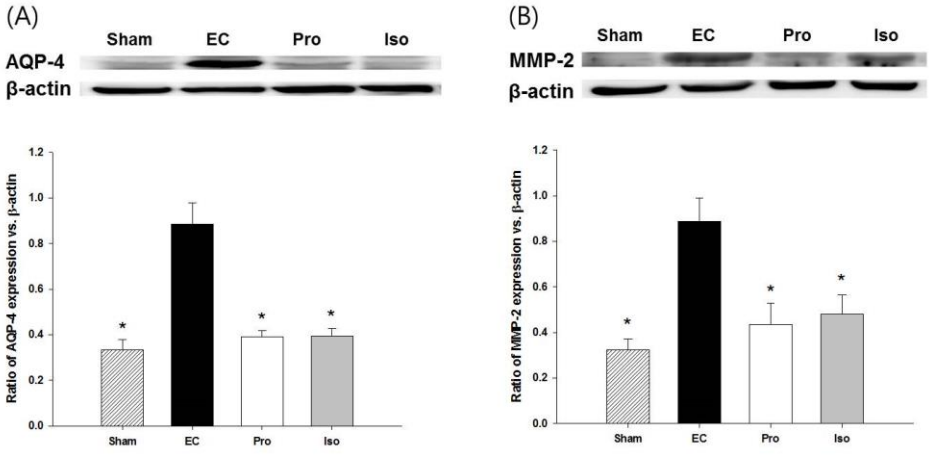

Figure 4. Post-treatment with propofol or isoflurane reduced the expression of AQP-4 (transmembrane water channel) and MMP-2 (extracellular matrix degrading enzyme) after ischemia-reperfusion. The expression of AQP-4 and MMP-2 was assessed by immunoblotting. (A) Quantification of AQP-4 expression in ischemic hemispheres (n=5 in each group). (B) Quantification of MMP-2 expression in ischemic hemispheres (n=5 in each group). Quantification values of AQP-4 and MMP-2 expression were normalized to β-actin and presented as means ± SEM. *$P<0.05$ vs. EC group.

7. Post-treatment with propofol reduced the expression of AQP-1 and -4 in astrocytes after ischemia-reperfusion

Further investigation was focused on the effects of propofol post-treatment after cerebral ischemia on factors associated with cerebral edema and BBB disruption. Next, the expression of AQP-1 after cerebral ischemia reperfusion injury was assessed. The immunoblotting results in Fig.5 (A) show significantly lower expression of AQP-1 in the Pro group compared with the EC group ($P<0.05$). Under normal conditions AQP-1 was expressed only in epithelial cells of choroid plexus. However, after cerebral ischemia reperfusion injury, astrocytes located in the ischemic penumbra area expressed AQP-1, as shown in Fig.5 (C). Furthermore, post-treatment with propofol reduced the expression of AQP-1 in the astrocytes.

The expression of AQP-4 was also significantly reduced in the Pro group compared with the EC group, as shown in Fig.4 (A) ($P<0.05$). AQP-4 is expressed in astrocytes under normal conditions. AQP-4 expression in the astrocytes around the area of ischemic penumbra was greater in the EC group than in the Sham group and Post-treatment with propofol reduced the expression of AQP-4 in the astrocytes [Fig.5 (D)].

Our data indicate that 1 h of ischemia and subsequent reperfusion increased the expression of AQP-1 and AQP-4 in astrocytes. However, post-treatment with propofol decreased the expression of AQP-1 and AQP-4 after the insult.

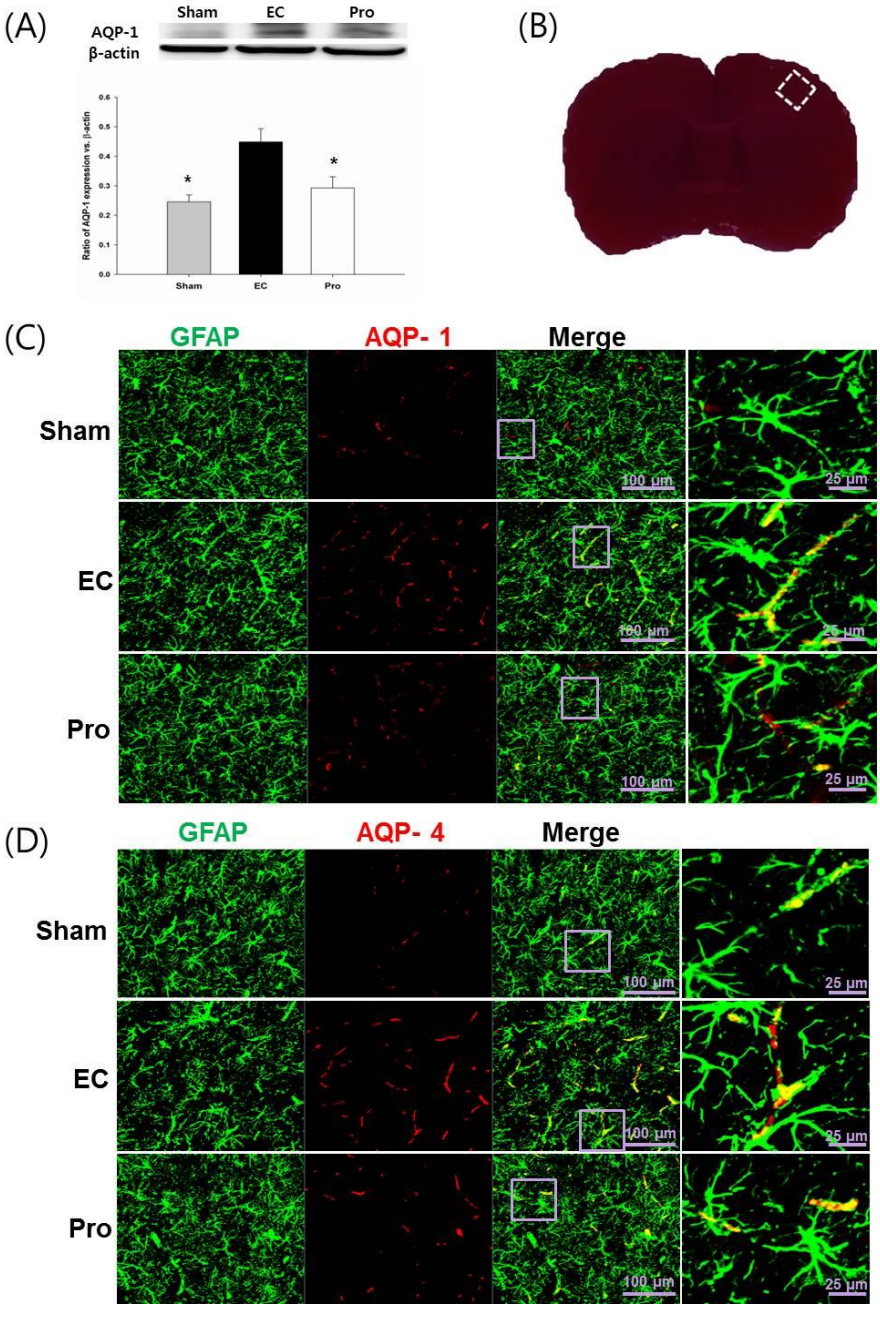

Figure 5. Effect of post-treatment with propofol on the expression of AQP-1 and AQP-4 in astrocytes after ischemia-reperfusion. The expression of AQP-1 was assessed by immunoblotting. (A) Quantification of AQP-1 expression in

ischemic hemispheres (n=5 in each group). Quantification values of AQP-1 expression were normalized to β-actin and presented as means ± SEM. *P<0.05 vs. EC group. (B) Coronal sections of TTC-stained rat brain. The labeled square areas represent the locations of ischemic penumbra where the following immunostaining images were taken: (C) Double immunostaining of AQP-1 (red) and GFAP (green). (D) Double immunostaining of AQP-4 (red) and GFAP (green).

8. Post-treatment with propofol reduced the expression of MMP-2 and -9 after ischemia-reperfusion

Our immunoblotting data showed that the expression of MMP-2 and -9 in the EC group was significantly greater than that in the Sham group [P<0.05, Fig.4 (B), Fig. 6 (A)]. However, post-treatment with propofol decreased the expression of MMP-2 and -9 after cerebral ischemia reperfusion injury (P<0.05). In addition, more MMP-2 or MMP-9-positive cells were detected in the area of ischemic penumbra in the EC group than in the Pro or Sham groups [Fig.6 (B)].

These studies show that the expression of MMP-2 and MMP-9 increased after 1 h of ischemia and subsequent reperfusion. However, post-treatment with propofol decreased the levels of MMP-2 and -9 expression after the insult.

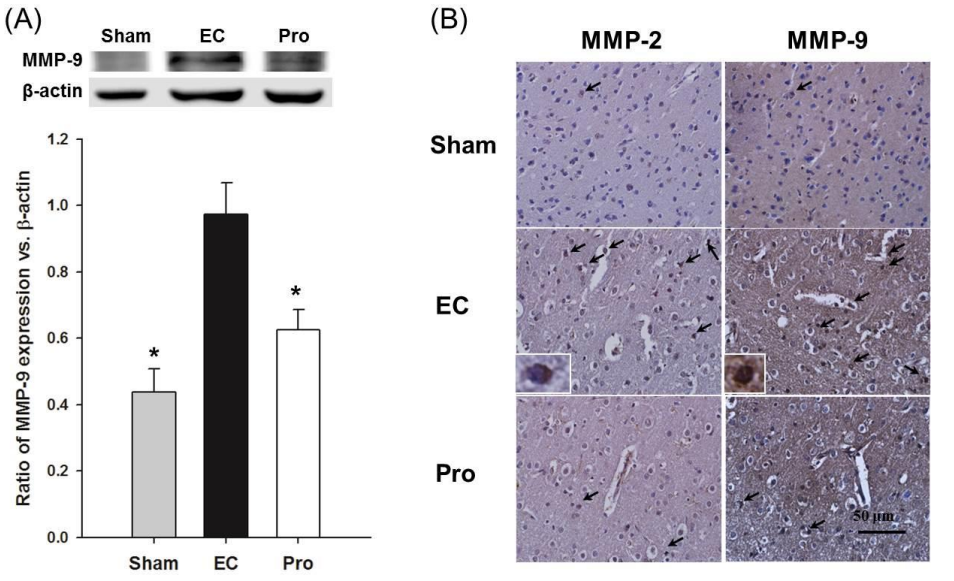

Figure 6. Post-treatment with propofol down-regulates the expression of MMP-2 and MMP-9 after ischemia-reperfusion. The expression of MMP-9 was assessed by immunoblotting. In addition, immunoreactivity for MMP-2 and -9 was evaluated in the ischemic penumbra. (A) Quantification of MMP-9 expression in ischemic hemispheres (n=5 in each group). (B) Cells that were positive for MMP-2 and MMP-9 were distinctly stained a dark brown color (in magnified box). The Sham group showed low immunoreactivity whereas strong MMP-2 and -9 immunoreactivity in cell nuclei and cytoplasm was observed in the EC group (arrows). Post-treatment with propofol reduced the immunoreactivity for MMP-2 and -9 in the Pro group. Quantification of MMP-9 expression was normalized to β-actin and presented as means ±SEM. *P<0.05 vs. EC group.

9. Post-treatment with propofol reduced the expression of VEGF and HIF-1α after ischemia-reperfusion

Finally, the expression of VEGF was assessed at 24 h after MCAO. Additionally, I attempted to assess the expression of HIF-1α in order to demonstrate its role as an upstream regulator of VEGF. As a previous study showed that neuroprotection was achieved only if selective inhibition of HIF-1α was performed within 12 h after MCAO,[28] the expression of HIF-1α was evaluated at 8 h after MCAO. At 8 h after MCAO, the expression of HIF-1α in the EC group was significantly greater than that in the Sham group [$P<0.05$, Fig.7 (A)]. Furthermore, post-treatment with propofol significantly reduced the expression of HIF-1α compared with the EC group ($P<0.05$). The pattern of VEGF expression at 24 h after MCAO was similar to that of HIF-1α at 8 h after MCAO [Fig.7 (B)].

These data indicate that 1 h of ischemia and subsequent reperfusion activated the expression of VEGF and HIF-1α and that this increase in expression could be reduced by post-treatment with propofol.

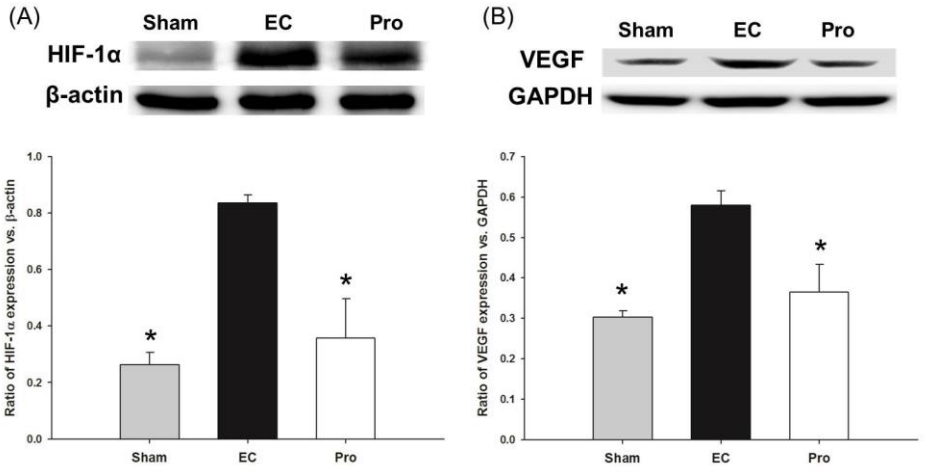

Figure 7. Effect of post-treatment with propofol on the expression of HIF-1α and VEGF after ischemia-reperfusion. The expression of HIF-1α and VEGF was assessed by immunoblotting. (A) Quantification of HIF-1α expression in ischemic hemispheres at 8 h after MCAO (n=5 in each group). Intensities of HIF-1α bands were normalized to β-actin. (B) Quantification of VEGF expression in ischemic hemispheres at 24 h after MCAO (n=5 in each group). Intensities of VEGF bands were normalized to GAPDH. All values are means ± SEM. *P<0.05 vs. EC group.

IV. DISCUSSION

The present study demonstrated that post-treatment with propofol or isoflurane reduces cerebral edema and the permeability of the BBB following cerebral ischemia-reperfusion injury. However, decreases in cerebral edema and Evans blue extravasation, as well as preservation of occludin were similar between propofol and isoflurane post-treatment. Furthermore, reductions in the expressions of AQP-4 and MMP-2 after transient cerebral ischemia were also comparable between propofol and isoflurane post-treatment. In further evaluation of the factors associated with the formation of cerebral edema and disruption of the BBB after cerebral ischemia, propofol post-treatment alleviated the increase in early expression of HIF-1α and later expression of AQP-4, AQP-9, MMP-2, MMP-9, and VEGF after ischemia-reperfusion injury.

In ischemic stroke, disruption of cerebral blood flow to brain tissue leads to insufficient Na^+/K^+ ATPase function and movement of osmotically active molecules (Na^+, Cl^-, and water), resulting in cellular swelling known as cytotoxic edema. Furthermore, injury from cerebral ischemia and subsequent reperfusion results in vasogenic edema due to changes in permeability or disruption of the blood-brain barrier (BBB).[2-4] Our results show that post-treatment of propofol or isoflurane reduce both water content in cerebral tissues and the disruption of the BBB after cerebral ischemia-reperfusion injury. Therefore, the post-treatment with propofol or isoflurane reduces cerebral edema after ischemia-reperfusion injury.

Contrary to propofol, isoflurane at high concentrations (>1.0 MAC) may increase ICP, as high concentrations of isoflurane predominantly exert a cerebral vasodilatory effect, which thus may increase cerebral blood volume and ICP.[20] Increased ICP may aggravate cerebral damage and edema after cerebral ischemia-reperfusion injury. Therefore, high concentrations of isoflurane may potentially lead to increases in cerebral edema after transient cerebral ischemia. Additionally, isoflurane was reported to increase BBB permeability[21,22] and break down the BBB.[22] Hence, this investigation attempted to demonstrate the effect of isoflurane post-treatment on the disruption of the BBB after transient cerebral

ischemia. However, our results showed that post-treatment with isoflurane reduces infarct size, edema formation, and disruption of the BBB after transient cerebral ischemia. Furthermore, isoflurane post-treatment decreased the expressions of AQP-4 and MMP-2 after cerebral ischemia-reperfusion injury: AQP-4 and MMP-2 are associated with water transport and BBB disruption after cerebral ischemic injury, respectively. These results imply that the previously reported characteristics of isoflurane might have little effect on the formation of cerebral edema and BBB disruption after transient ischemia because of the potent neuroprotective effects of isoflurane post-treatment.

AQP-4 is predominantly located in astrocytes and plays an important role in brain edema after ischemia.[2] In particular, it may be associated with cytotoxic edema because AQP-4-null mice are less prone to this condition.[29] In addition, previous reports have shown that transient cerebral ischemia and reperfusion increased the expression of AQP-4 and resulted in cerebral edema,[7,30] consistent with our results. Although AQP-4 expressed in astrocytes plays a major role in cerebral edema following ischemia, AQP-1 may also be associated with cerebral edema. In some mammalian species including human, AQP-1 is expressed only in the apical membrane of the choroid plexus epithelium in normal circumstances[5] but is strongly expressed in reactive astrocytes under pathological states such as subarachnoid haemorrhage,[31] traumatic brain injury,[32] and cerebral ischemia.[33] In our study, AQP-1 was rarely located in astrocytes in the absence of cerebral ischemia/reperfusion injury. However, transient ischemia with reperfusion up-regulated the expression of AQP-1, and AQP-1 expression in the EC group was located in astrocytes. Zheng et al.[7] and Zhu et al[34] demonstrated that propofol administered before cerebral ischemia suppressed the up-regulation of AQP-4 after cerebral ischemia and reperfusion injury through activation of the protein kinase C pathway, which could reduce cerebral edema. Our study showed that propofol administered after cerebral ischemia also could reduce the over-expression of AQP-1 and AQP-4 and thus decrease cerebral edema after transient cerebral ischemia.

In addition to inducing cytotoxic edema, cerebral ischemia/reperfusion injury

changes vascular permeability and induces disruption of the BBB, resulting in vasogenic edema. In cerebral ischemia and/or reperfusion injury, expression of VEGF is increased in various cells.[35,36] VEGF is a major regulator of normal and pathological blood vessel growth. However, VEGF also has the unique property of inducing vascular leak[13] through the generation of gaps between the endothelial cells lining small blood vessels.[37] Expression of MMP-2 and MMP-9 is also known to increase after transient cerebral ischemia.[9] These matrix metalloproteinases are secreted enzymes that are produced by several types of cell such as neurons, astrocytes, and infiltrating neutrophils[38] and can degrade all protein constituents of the extracellular matrix. Therefore, their abnormal expression and activation after ischemia leads to breakdown of the extracellular matrix and tight junctions, resulting in opening of the BBB.[39] Inhibition of MMPs results in decreased cerebral edema after focal cerebral ischemia[40] and MMP-9 knockout mice show a lower level of BBB disruption following focal cerebral ischemia.[41] Furthermore, VEGF can activate MMP-9[14] and neutralizing VEGF attenuates MMP-9 activation and cerebral edema after focal cerebral ischemia.[42] Therefore, propofol treatment might serve a role in relieving the disastrous effects of BBB disruption by lowering the levels of VEGF and MMPs. The decrease in BBB disruption by propofol post-treatment is supported by the preservation of occludin expression.

As mentioned earlier, VEGF is an important gene downstream of HIF-1. Therefore, HIF-1 could increase vascular permeability via the regulation of both VEGF and MMP-9 expression. In addition, HIF-1α has been reported to be a regulator of AQP-4 expression in traumatic and ischemic cerebral injury.[12,43] Consequently, HIF-1α could contribute to cerebral edema following cerebral ischemia through regulation of VEGF, MMPs, and AQPs. However, the role of HIF-1α in cerebral ischemia is complex. While some previous studies suggested that HIF-1α plays a protective role,[44] other studies report that neuron-specific knockdown or pharmacological down-regulation of HIF-1α reduces damage after cerebral ischemia suggesting that HIF-1α exerts a detrimental effect.[36,45] Recent work suggests that timing distinguishes the two effects. HIF-1α expression show

two peaks, first at 4-8 h and a second peak 2-6 days after cerebral ischemia, with a nadir at 24 h.[28,44] Only early selective inhibition of HIF-1α expression provided neuroprotection after cerebral ischemia.[28] In addition, early inhibition of HIF-1α reduced BBB damage[37] and cerebral edema[12] after cerebral ischemia. To our knowledge, there are no reports on the association between propofol treatment and HIF-1α expression after cerebral ischemia. However, there is substantial evidence regarding the effect of propofol on HIF-1α expression. Propofol was reported to inhibit HIF-1 activity by suppressing HIF-1α protein synthesis or expression under hypoxic condition.[46,47] Furthermore, increased expression of HIF-1α induced by conditions other than hypoxic stimuli could also be suppressed by propofol treatment in macrophages and lung epithelial cells.[48,49] Therefore, propofol might reduce the expression of HIF-1α after transient ischemia in the brain. Our results show that propofol post-treatment reduced the early expression of HIF-1α leading to decreased expression of MMPs, AQPs, VEGF, and consequently reduced cerebral edema and BBB damage.

The present investigation has some limitations. First, this investigation cannot exclude the effect of cerebral ischemic injury on edema. Edema after cerebral ischemia may be associated with the severity of ischemic damage, and the anesthetics in this study reduced cerebral damage after transient ischemia. Therefore, both propofol and isoflurane may show comparable effects on cerebral edema because of their potent neuroprotective effects against cerebral ischemia. Nevertheless, this investigation further evaluated parameters related to the formation of cerebral edema and disruption of the BBB after cerebral ischemia. Second, this study evaluated cerebral edema and BBB integrity at 24 h after MCAO. Although the suppression of early expression of HIF-1α by propofol post-treatment reduced cerebral edema and BBB damage at 24 h after cerebral ischemia-reperfusion injury, the effects of propofol post-treatment on delayed expression of HIF-1α and the subsequent outcome after cerebral ischemia is not known. Additionally, AQPs may participate in the reabsorption of vasogenic edema in late ischemic stroke,[2] and MMPs and VEGF may be also associated with tissue regeneration through neurovascular remodeling and angiogenesis during

delayed phases after stroke.[35,39] Therefore, the long-term outcomes after transient cerebral ischemia should be clarified in future studies. Third, intralipid (the vehicle for propofol) was not administered as an experimental control in this study. However, previous studies showed that intralipid alone does not reduce injury or edema after transient cerebral ischemia.[7,18]

V. CONCLUSION

Propofol or isoflurane post-treatment attenuated cerebral edema and BBB disruption in focal cerebral ischemia-reperfusion injury in rats. The severity of cerebral edema and BBB disruption as well as the expressions of AQP-4 and MMP-2 after transient cerebral ischemia were comparable between propofol and isoflurane post-treatment. Finally, alleviation of cerebral edema following propofol post-treatment was associated with reductions in the expression of AQP-1, AQP-4, MMP-2, MMP-9, and VEGF, most likely due to suppression of HIF-1α.

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ABSTRACT (IN KOREAN)

백서에서 일시적 뇌허혈 손상 후의 마취제 처치가 혈액-뇌 장벽 및 뇌부종에 미치는 영향

<지도교수: 구본녀>

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이 재 훈

Propofol이나 isoflurane과 같은 마취제는 뇌의 허혈 손상에 대해 보호 효과가 있다고 알려져 있다. 하지만 마취제가 뇌허혈의 합병증인 뇌부종에 미치는 영향에 대한 연구는 부족한 실정이다. 이에 본 연구는 일시적 뇌허혈 손상 후에 마취제를 처치하였을 때, 혈액-뇌 장벽의 손상과 뇌부종 발생에 미치는 영향을 알아보고자 하였다. 또한 마취제 후처치가 혈액-뇌 장벽의 손상과 뇌부종 형성에 관여하는 인자들, 주로 aquaporin (AQP), matrix metalloproteinase (MMP), hypoxia-inducible factor (HIF) -1α 등의 조절에 미치는 영향에 대해서도 평가하였다.

총 103 마리의 숫컷 백서에서 1시간 동안 오른쪽 중뇌 동맥을 폐색시켜 뇌허혈을 유도하였다. Propofol 후처치는 중뇌동맥의 재관류와 동시에 propofol을 1 mg/kg/min으로 1시간 동안 투여하였고 (n=37), isoflurane 후처치는 동일 시점에 2.1 vol%의 isoflurane을 1시간 동안 투여하였다 (n=25). 생리식염수 투여 대조군은 동일 시점에 0.9% 생리식염수를 0.1 ml/kg/min으로 1시간 동안 투여 받았다 (n=41). 또 다른 19 마리의 백서에 대해 뇌허혈을 유도하지 않는 모의수술을 시행한 후 분석에 포함하였다. 뇌 부종과 혈액-뇌 장벽 손상은 각각 뇌

수분량 및 Evans blue 혈관외 누출을 뇌허혈 유도 24시간 후에 정량하여 평가하였다. 부가적으로 AQP-1, AQP-4, MMP-2, MMP-9, 그리고 VEGF의 발현을 역시 뇌허혈 유도 24시간 후에 측정하였고, HIF-1α의 발현은 뇌허혈 유도 8시간 후에 평가하였다.

생리식염수를 처치한 대조군에 비해 propofol 이나 isoflurane 후처치는 뇌부종과 Evans blue 혈관외 누출을 유의하게 감소시켰다 ($P<0.05$). 또한 propofol 이나 isoflurane 후처치군은 대조군에 비해 AQP-4와 MMP-2의 발현이 유의하게 낮았다 ($P<0.05$). 그러나 propofol 후처치군과 isoflurane 후처치군 간에 뇌부종이나 혈액-뇌 장벽 손상 정도, AQP-4와 MMP-2 발현 정도에는 차이가 없었다. 뇌부종 형성에 관여하는 인자에 관한 추가 실험 결과, 뇌허혈 유도 후 24시간에 평가한 AQP-1, AQP-4, MMP-2, MMP-9, 그리고 VEGF의 발현이 propofol을 후처치 하였을 때, 유의하게 감소하였다 ($P<0.05$). 뇌허혈 유도 8시간 후의 HIF-1α의 발현 역시 propofol을 투여하였을 때 생리식염수 투여군에 비해 유의하게 낮았다 ($P<0.05$).

결론적으로 일시적 뇌허혈 후의 propofol 처치는 뇌부종 및 혈액-뇌 손상을 줄여 줄 수 있으나, propofol 후처치의 뇌부종 및 혈액-뇌 손상에 미치는 영향은 isoflurane 후처치를 하였을 때와 차이가 없다. 또한 이러한 propofol 후처치 효과는 AQP-1, AQP-4, MMP-2, MMP-9, VEGF 그리고 HIF-1α 발현 감소와 관련되어 있다.

핵심되는 말: 뇌부종, 뇌허혈, 혈액-뇌 장벽, isoflurane, propofol

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