의학박사 학위논문

Sevoflurane Postconditioning Reduces Apoptosis by Activating the JAK-STAT Pathway After Transient Global Cerebral Ischemia in Rats

백서의 일시적 전뇌허혈 모델에서 sevoflurane 후처치의 뇌보호 효과: JAK/STAT 세포신호 전달 경로 활성화에 의한 세포자멸사의 감소

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김 현 창

Sevoflurane Postconditioning Reduces Apoptosis by Activating the JAK-STAT Pathway After Transient Global Cerebral Ischemia in Rats

by

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A Thesis submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medicine (Anesthesiology) at Seoul National University College of Medicine

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백서의 일시적 전뇌허혈 모델에서 **sevoflurane** 후처치의 뇌보호 효과:
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위 원 장 임 영 진 (인)

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위 원 김 상 정 (인)

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Abstract

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Objective: The antiapoptotic effects of sevoflurane postconditioning are responsible for neuroprotection against cerebral ischemia-reperfusion injury. Phosphorylation of the Janus family tyrosine kinases (JAK) 2-signal transducers and activators of transcription (STAT) 3 pathway is linked to antiapoptosis. Here, we determined whether the antiapoptotic effects of sevoflurane postconditioning are associated with activation of the JAK2-STAT3 pathway after global transient cerebral ischemia in rats.

Methods: Forty-five rats were randomly assigned to five groups: sham (n=5), control (10 min of ischemia, n=10), sevoflurane postconditioning (2 periods of sevoflurane inhalation after ischemia for 10 min, n=10), AG490 (a JAK2 selective inhibitor, intravenous administration of 40 mg kg^{-1} before ischemia, n=10), and sevoflurane postconditioning plus AG490 group (n=10). The number of apoptotic cells as well as the expression of JAK2, phosphorylated JAK2 (P-

JAK2), STAT3, phosphorylated STAT3 (P-STAT3), Bcl-2 (antiapoptotic protein), and Bax (proapoptotic protein) were evaluated three days after ischemia.

Results: The apoptotic cell count was significantly lower in the sevoflurane postconditioning group than in the control, AG490 and sevoflurane postconditioning plus AG490 groups. JAK2 and STAT3 levels were comparable among all five groups. P-JAK2, P-STAT3 and Bcl-2 levels were higher and Bax levels were lower in the sevoflurane postconditioning group relative to the control, AG490 and sevoflurane postconditioning plus AG490 groups.

Conclusions: Sevoflurane postconditioning reduced apoptosis by increasing P-JAK and P-STAT expression after transient global ischemia in rats, and AG490 reversed the beneficial antiapoptotic effects of sevoflurane postconditioning, suggesting that the JAK-STAT pathway may be involved in the antiapoptotic mechanism of sevoflurane postconditioning.

Key words: apoptosis; brain; ischemia; JAK/STAT signaling pathway; sevoflurane

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Introduction

Global cerebral ischemia-reperfusion (I/R) injury, which can result in permanent neurological sequelae and brain death, often occurs during major bleeding, severe hypotension and cardiac arrest. Numerous investigations have been carried out to identify effective agents to prevent the brain from I/R injury.

Sevoflurane, a kind of volatile anesthetic agents, exerts neuroprotective effects by decreasing apoptosis.[1-4] After focal or global cerebral ischemia, sevoflurane postconditioning demonstrates beneficial effects by regulating the expression of apoptosis-related proteins such as Bcl-2 and Bax.[2, 3] However, the mechanism of sevoflurane postconditioning-induced antiapoptosis remains unclear.

The Janus family tyrosine kinases (JAK)-signal transducers and activators of transcription (STAT) pathway is an important cell signaling pathway (Fig. 1).[5] The JAK-STAT pathway is known to involve cellular processes such as inflammation, cell-cycle control and development and apoptosis.[6] JAK proteins are cytosolic tyrosine kinases associated with the intracellular domain of membrain-bound receptors.[7] JAK proteins become activated after extracellular ligand binding to membrane receptors. JAK kinases phosphorylates STAT protein family after stimulation of several membrane receptors.[8] STATs are intracellular proteins which function as signal transducers and transcription activators. Phosphorylated STAT proteins translocate to the nucleus and promote gene transcription by binding DNA.[9] There are four types of JAK and seven types of STAT proteins.[10] Among them, the JAK2-STAT3 pathway, a survival signaling pathway, is linked to apoptosis. A previous investigation indicated that ischemic postconditioning significantly reduced myocardial apoptosis after I/R injury *via* the JAK2-STAT3-Bcl-2 pathway.[11] Another previous investigation showed that sevoflurane postconditioning reduced apoptosis following myocardial I/R injury *via* STAT3 activation in non-diabetic rats.[12] However, no previous study has evaluated whether the antiapoptotic effects of sevoflurane postconditioning are associated with the JAK2-STAT3 pathway in a model of cerebral I/R injury.

In this study, we determined whether the JAK2-STAT3 pathway is involved in a neuroprotective mechanism of sevoflurane postconditioning, especially in terms of its antiapoptotic effects, in a rat model of transient global cerebral ischemia. We hypothesized that sevoflurane postconditioning would enhance JAK2-STAT3 expression and reduce apoptosis by upregulating Bcl-2 and downregulating Bax.

Material and Methods

The present investigation was in accordance with national guidelines and relevant ARRIVE guidelines. The experimental protocol was approved by the Seoul National University Institutional Animal Care and Use Committee (No. 14-0014). Male Sprague-Dawley rats weighing 350-380 g were used.[2, 4] Rats were housed in an animal room under a 12 h day-night cycle at 20°C. The fasting period was 12-16 h before the experiments and water was freely accessed.

A total of 45 rats were randomly allocated to one of five groups (Fig.2): (i) the sham group (n = 5) received no treatment, (ii) the control group (n = 10) received global cerebral ischemia for 10 min, (iii) the sevoflurane postconditioning group (n = 10) received two periods of inhalation of 2% vol. sevoflurane for 10 min, folllowed by a washout period of 10 min after ischemia,[4] (iv) the AG490 group (n = 10) intraperitoneally received 40 mg/kg of AG490 (Sigma-Aldrich, St. Louis, MO, USA), which is a JAK2 selective inhibitor, 30 min before ischemia, and (v) the sevoflurane postconditioning plus AG490 group (n = 10) received 40 mg/kg of AG490 before ischemia and two periods of inhalation of 2% vol. sevoflurane for 10 min, followed by a washout period of 10 min after ischemia.

Anesthesia was induced with intraperitoneal injection of 20 mg/kg zoletil and 5 mg/kg xylazine. Tracheal intubation was performed and mechanical ventilation was provided with 40% oxygen and 60% nitrogen. Minute ventilation was adjusted to maintain normocarbia. Zoletil (10 mg/kg/h) was infused continuously and xylazine (5 mg/kg) was administrated intermittently *via* the femoral venous catheter to maintain anesthesia. Sevoflurane was not used as a maintenance agent to exclude sevoflurane-induced protective effect. To achieve an adequate depth of anesthesia, we intermittently monitored the corneal and pedal reflexes and response to tail pinch during the surgical procedure.[13] Supplementary xylazine (5 mg/kg) and zoletil (10 mg/kg) were injected intravenously when such reflexes or the response to tail pinch was found or increased systolic arterial pressure or heart rate (> 20% of the baseline value) was detected during surgery. Continuous arterial pressure monitoring and

measurements of arterial blood gases, glucose and hemoglobin were conducted *via* the femoral artery catheterization. Rectal temperature was maintained at 37.0 ± 0.1°C using a heating pad. A subcutaneous 22-gauge needle thermistor was inserted beneath the right temporalis muscle adjacent to the skull to monitor pericranial temperature (model TCAT-2 Temperature Controller; Harvard Apparatus, Holliston, MA, USA), and the pericranial temperature was maintained at 37.5 ± 0.1°C using a heating lamp. We isolated both CCAs and the right internal jugular vein after a midline incision between the neck and sternum. Cerebral blood flow (CBF) was monitored using a laser Doppler monitoring system (Moor Instruments VMS-LDF2, Axminster, UK) on the right or left side of the brain during the procedure. A laser Doppler sensor was placed 4-5 mm lateral and 1-2 mm posterior to the bregma on the skull hemisphere after a midline skin incision. After all surgical procedures were done, we allowed a 30-min physiological stabilization period. Mean arterial pressure (MAP) and heart rate were measured using a bedside monitor (Ultraview SL, Spacelabs, Washington, DC, USA). Temperatures at the rectum and temporalis muscle were recorded. Arterial blood gases (pH, $PaCO_2$, and PaO_2) and hemoglobin were measured using a blood gas analyzer (Stat Profile pHOx Plus L, Nova Biomedical, MA, USA). Glucose was measured with a glucometer (One Touch Ultra, Lifescan, Milpitas, CA, USA). All physiological variables were measured at 30 min of stabilization (baseline), after 8 min of ischemia (ischemia) and after 30 min of reperfusion (recovery).

Transient global cerebral ischemia was performed by bilateral common carotid arteries (CCAs) occlusion with systemic hypotension.[2, 4] Heparin of 50 U was administrated i.v. 5 min before cerebral ischemia. After heparin injection, blood was withdrawn from the right internal jugular vein to induce hypotension. When MAP reached the target pressure (26-30 mmHg) and the regional CBF was reduced to 50% of baseline, both CCAs were clamped with surgical clips. The MAP of 26-30 mmHg and regional CBF less than 10% of baseline were maintained during the induced cerebral ischemia period. After 10 min of ischemia, the CCAs were declamped, and withdrawn blood was infused slowly

while monitoring hemodynamic parameters. After 45 min of reperfusion, arterial blood gases, hemogloblin and MAP were measured again.

After completion of the experiment, all catheters were removed and 0.5% bupivacaine was administered subcutaneously around the wound to reduce postoperative pain. The incision sites were sutured. The rats were then placed back in a cage and observed at room temperature until complete recovery from anesthesia.

At three days after cerebral ischemic insult, a histopathological assessment was performed. Rats were injected i.p. with zoletil (20 mg/kg) and then decapitated. Brains were cut transversely into two parts using a slice matrix. The anterior part was stored in liquid nitrogen at -80 °C for Western blot analysis. The posterior part (formalin-fixed paraffin-embedded) was cut coronally (5-µm thick) and stained with hematoxylin and eosin (H&E). Necrotic neurones were identified by karyolytic or pyknotic nuclei and cytoplasmic shrinkage. To assess DNA fragmentation, terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) staining was performed using an Apoptag Peroxidase In Situ Apoptosis Detection Kit S7101 (Millipore Corp., Billerica, MA, USA). TUNEL-positive cells with blue-stained apoptotic bodies were considered as apoptotic cells. An investigator blinded to the group assignment assessed two brain tissue slides (H&E and TUNEL staining) per rat using microscopy. For each rat, six optical fields (left: three, right: three) in the hippocampal CA1 area were evaluated at a magnification (x400). CA1 region was examined because CCA occlusion produces reliable neuronal loss in CA1 area compared to CA2, CA3 and CA4 area.[14] The total number of cells and the number of necrotic and apoptotic cells were counted manually in each field of view using an imaging software. The percentage of necrotic or apoptotic cells was calculated as the ratio of the number of necrotic or apoptotic cells to the total cell number in each field, respectively.

For Western blotting, the transversely excised brain tissue, which includes the anterior part of hippocampus, was cut into 20-100 mg into small pieces. The extracts were washed with ice-cold PBS and centrifuged at 500 × g for 5 min. Cytosolic and nuclear proteins were extracted seperately using a

NE-PER® Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Waltham, MA, USA). We homogenized tissue using a tissue grinder in the appropriate volume of cytoplasmic extraction reagent (CER) I. We vortexed the tube vigorously and incubated the tube on ice. Ice-cold CER II was added to the tube. The tube was vortexed and incubated. We centrifuged the tube for 5 min at 1600 × g. We transferred the supernatant (cytoplasmic extract). The remaining insoluble fraction containing nuclei was suspended in ice-cold NER. We vortexed it for 40 min and centrifuged it at 16000 × g for 10 min. The supernatant (nuclear extract) was transferred to a clean pre-chilled tube. Extracts were stored at -80 °C until use. Western blotting of Bcl-2, Bax, JAK2, phosphorylated JAK2 (P-JAK2), STAT3, and phosphorylated STAT3 (P-STAT3) was performed with antibodies against Bcl-2 (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA), Bax (1:1000 dilution; Cell Signaling Technology), JAK2 (1:1000 dilution; Sigma-Aldrich), P-JAK2 (1:1000 dilution; Sigma-Aldrich), STAT3 (1:2000 dilution; Sigma-Aldrich), and P-STAT3 (1:1000 dilution; Sigma-Aldrich) respectively. Peroxidase-conjugated antimouse immunoglobulin (Sigma-Aldrich) was used as secondary antibody for antigens of β-actin. Peroxidase-conjugated antirabbit immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the secondary antibody for the other proteins including Bcl-2, Bax, JAK2, STAT3, P-JAK2, and P-STAT3. Each protein was detected using enhanced chemiluminescence (Amersham, UK), and the band intensities were measured by densitometry. β-Actin (42 kDa) and histidine H3 (17 kDa) intensity levels were used as the control values for cytoplasmic proteins (Bcl-2 [26 kDa], Bax [20 kDa], JAK2 [130 kDa], P-JAK2 [125 kDa], and STAT3 [92 kDa]) and nucleic protein (P-STAT3 [88 kDa]), respectively.

Statistical analyses

Jeon et al.[2] showed that the percentage of apoptotic cells in TUNEL-staining was 49 ± 14 % in the hippocampal CA1 following transient global cerebral ischemia in rats. We assumed that a 40% decrease in the percentage of TUNEL-positive cells in the sevoflurane postconditioning group would

be considered significant. At the type I error of 0.05 and with a power of 80%, 10 rats in each group were necessary.

All values are presented as the median and interquartile range (IQR). Physiological variables were analyzed by repeated-measures ANOVA. The mean percentages of necrotic and apoptotic cells in the hippocampal CA1 and relative protein levels of Bcl-2, Bax, JAK2, P-JAK2, STAT3, and P-STAT3 were analyzed using the Kruskal–Wallis test followed by the Mann-Whitney *U* test. SPSS software (version 18.0, SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. *P*-values of 0.05 were considered statistically significant.

Results

A total of 45 rats were used. One rat in the control group was excluded due to survival failure. The mean blood pressure was lower during ischemia compared with baseline values in all treatment groups ($P < 0.01$, Table 1). The mean blood pressure during ischemia was higher in the sham group than in the other four groups ($P < 0.01$). Otherwise, there were no significant differences in physiological data among the five groups.

The percentage of necrotic cells in the hippocampal CA1 showed a difference among groups ($P < 0.001$). The median (IQR) percentage of necrotic cells was significantly lower in the sevoflurane postconditioning group than the control and AG490 groups (20 [15-24]% *vs.* 52 [45-57]%, and 57 [46-65]%, $P < 0.001$ and $P < 0.001$, respectively, Fig. 3). The percentage of necrotic cells was lower in the sevoflurane postconditioning plus AG490 group than in the control group (25 [14-31]% *vs.* 52 [45-57]%, $P < 0.001$).

The percentage of apoptotic cells in the hippocampal CA1 showed a difference among groups ($P < 0.001$). The median (IQR) percentage of apoptotic cells in the hippocampal CA1 was significantly lower in the sevoflurane postconditioning group than the control and AG490 groups (24 [16-34]% *vs.* 39 [34-47]%, and 54 [40-62]%, $P < 0.001$ and $P < 0.001$, respectively, Fig. 4). The percentage of apoptotic cells was lower in the sevoflurane postconditioning plus AG490 group than in the control group (28 [21-41]% *vs.* 39 [34-47]%, $P < 0.001$).

The cytosolic JAK2 level was comparable among the five groups ($P = 0.632$). The cytosolic P-JAK2 level demonstrated a difference among groups ($P = 0.036$). The cytosolic P-JAK2 level was higher in the sevoflurane postconditioning group than in the AG490 group ($P = 0.019$, Fig. 5). The cytosolic STAT3 level was comparable among the five groups ($P = 0.927$). P-STAT3 expression in the nucleus was barely detectable in the AG490 and sham groups. The nuclear P-STAT3 level demonstrated a difference among groups ($P < 0.001$). Nuclear P-STAT3 levels were higher in the

sevoflurane postconditioning group than in the control, AG490, sevoflurane postconditioning plus AG490, and sham groups ($P = 0.002$, $P < 0.001$, and $P = 0.007$, Fig. 6).

The cytosolic Bax level demonstrated a difference among groups ($P < 0.001$). Cytosolic Bax levels were lower in the sevoflurane postconditioning group than in the control and AG490 group ($P = 0.002$ and $P < 0.001$, Fig. 7). Cytosolic Bax levels were lower in the sevoflurane postconditioning plus AG490 group than in the AG490 group ($P = 0.014$). The cytosolic Bcl-2 levels demonstrated a difference among groups ($P < 0.001$). Cytosolic Bcl-2 levels were higher in the sevoflurane postconditioning group than in the control and AG490 group ($P = 0.006$ and $P < 0.001$ respectively).

Table 1. Physiological variables

Group		Control	Sevo postC	AG490	Sevo postC + AG490	Sham
		(n = 9)	(n = 10)	(n = 10)	(n = 10)	(n = 5)
	Time					
pH	Baseline	7.42 (7.39-7.45)	7.41 (7.40-7.45)	7.44 (7.38-7.45)	7.42 (7.41-7.44)	7.44 (7.42-7.48)
	Ischemia	7.50 (7.48-7.52)	7.46 (7.42-7.52)	7.39 (7.36-7.52)	7.47 (7.41-7.52)	7.44 (7.43-7.50)
	Recovery	7.41 (7.37-7.46)	7.40 (7.37-7.43)	7.43 (7.39-7.46)	7.41 (7.38-7.45)	7.45 (7.43-7.49)
$PaCO_2$	Baseline	35 (31-39)	37 (33-42)	28 (27-37)	36 (34-38)	33 (26-36)
(mmHg)	Ischemia	27 (23-30)	28 (24-37)	30 (24-39)	29 (22-38)	35 (31-38)
	Recovery	39 (35-42)	38 (37-41)	35 (32-37)	36 (33-40)	35 (32-38)
PaO_2	Baseline	258 (242-274)	275 (229-280)	264 (239-283)	266 (235-287)	298 (274-306)
(mmHg)	Ischemia	272 (255-282)	271 (250-276)	262 (238-264)	268 (241-273)	272 (252-297)
	Recovery	248 (228-259)	245 (222-264)	248 (217-277)	243 (197-250)	273 (250-296)
Hb	Baseline	12.9 (12.6-14.0)	14.1 (13.9-14.6)	13.1 (10.8-15.1)	14.2 (13.4-14.6)	13.1 (11.8-14.0)
(g/dL)	Ischemia	12.4 (11.2-12.9)	12.3 (12.0-14.4)	12.7 (11.7-13.7)	13.2 (12.4-13.4)	14.2 (13.5-14.8)
	Recovery	13.8 (13.2-14.1)	14.1 (14.0-14.4)	14.5 (14.0-14.8)	14.3 (13.8-14.8)	13.1 (11.8-14.0)
MAP	Baseline	88 (80-97)	100 (77-116)	96 (76-108)	83 (76-95)	84 (80-97)
(mmHg)	Ischemia	28 (27-30)*	28 (27-29)*	28 (27-28)*	28 (27-29)*	79 (73-82)†
	Recovery	104 (100-126)	107 (88-128)	90 (83-125)	108 (94-116)	86 (72-93)
Glucose	Baseline	160 (149-239)	217 (173-322)	167 (148-197)	185 (139-296)	192 (177-252)
(mg/dL)	Ischemia	177 (162-215)	252 (228-324)	172 (147-193)	222 (142-399)	195 (176-260)
	Recovery	201 (183-266)	249 (211-335)	170 (160-212)	249 (175-265)	197 (177-244)
Laser	Baseline	73 (66-82)	77 (69-80)	64 (63-70)	88 (80-88)	78 (73-81)
Doppler	Ischemia	24 (18-28)*	19 (11-23)*	17 (17-25)*	14 (12-21)*	81 (74-93)†
(PU)	Recovery	102 (85-137)	118 (87-133)	102 (90-107)	106 (102-117)	81 (76-97)

Values are are median (IQR).

All physiological variables were measured at 30 min of stabilization (baseline), after 8 min of ischemia (ischemia) and after 30 min of reperfusion (recovery).

* $P < 0.05$, statistically significant compared with baseline within the same group.

† $P < 0.05$, statistically significant compared with versus other groups.

Sevo, sevoflurane; postC, postconditioning; Hb, hemoglobin; MAP, mean arterial blood pressure; PU, perfusion units.

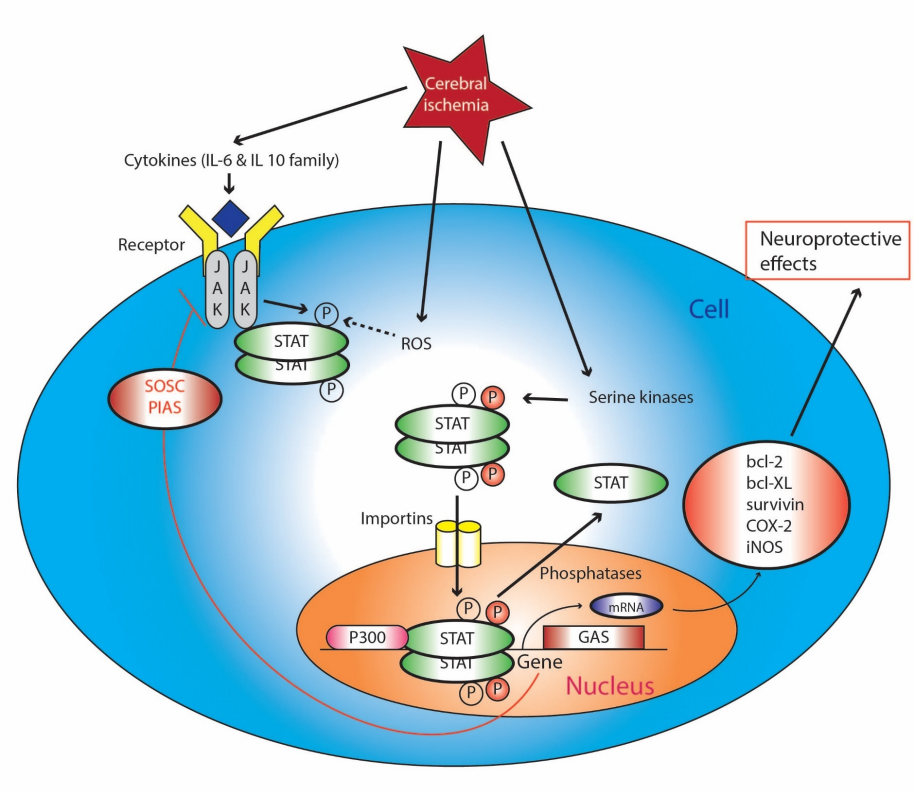

Figure 1 The JAK-STAT signal transduction pathway. IL, interleukin; JAK, Janus family tyrosine kinases; STAT, signal transducers and activators of transcription; SOSC, suppressor of cytokines signaling genes; PIAS, protein inhibitors; GAS, interferon-γ activated sequence; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase.

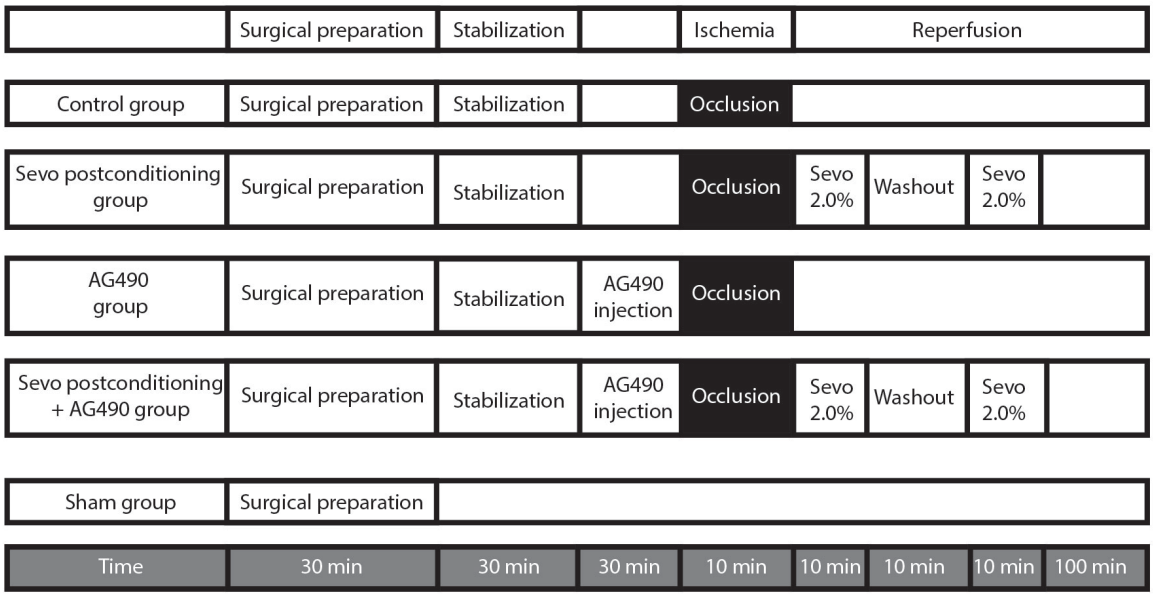

Figure 2 Experimental protocol for transient global ischemia/reperfusion injury of rats. The sevoflurane postconditioning group and the sevoflurane postconditioning plus AG490 group received two periods of inhalation of 2% vol. sevoflurane for 10 min, followed by a washout period of 10 min after ischemia. Sevo, Sevoflurane.

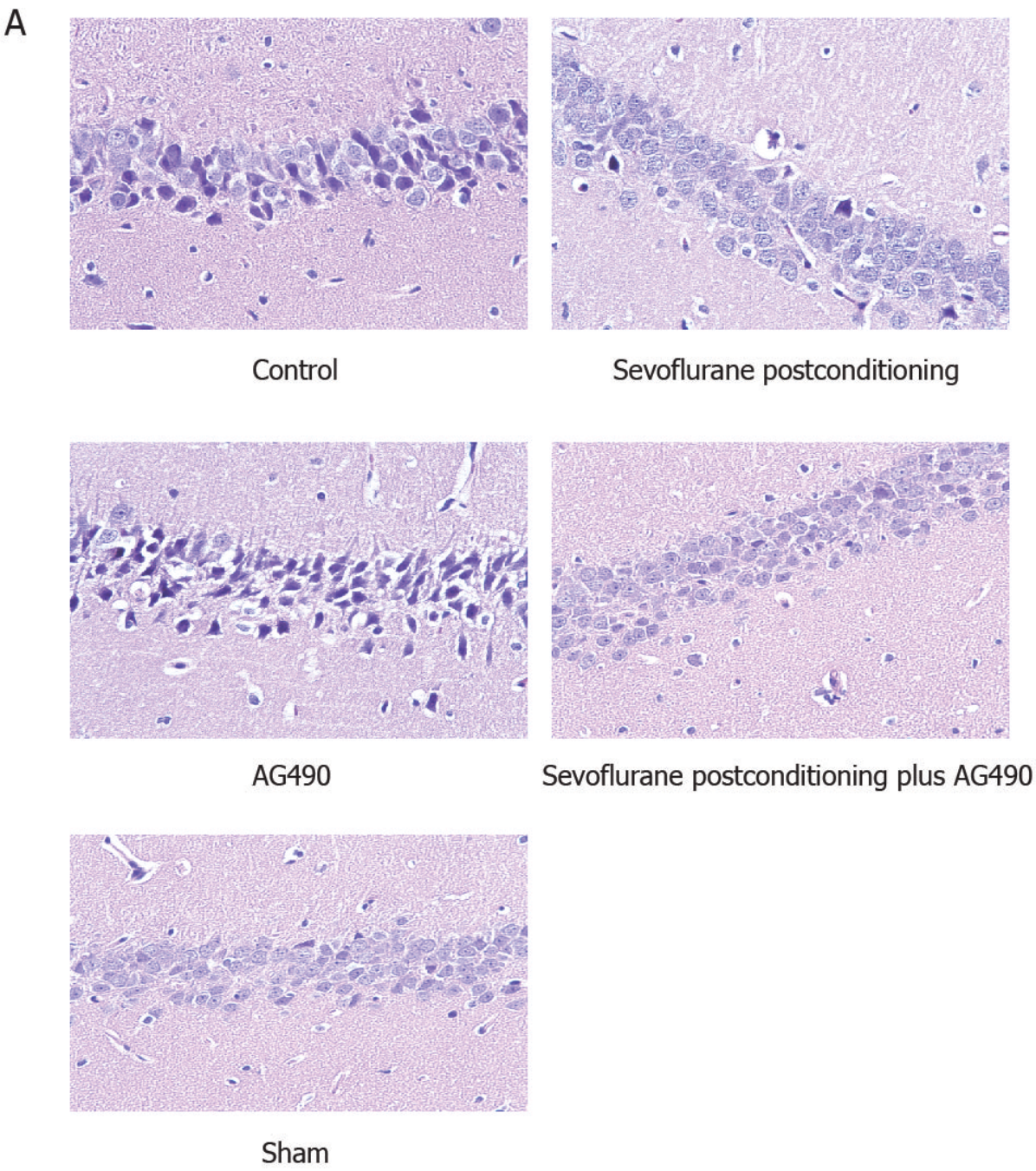

Figure 3 (A) Representative photomicrographs from a single rat with H&E staining in the hippocampal CA1 regions three days after transient global cerebral ischemia.

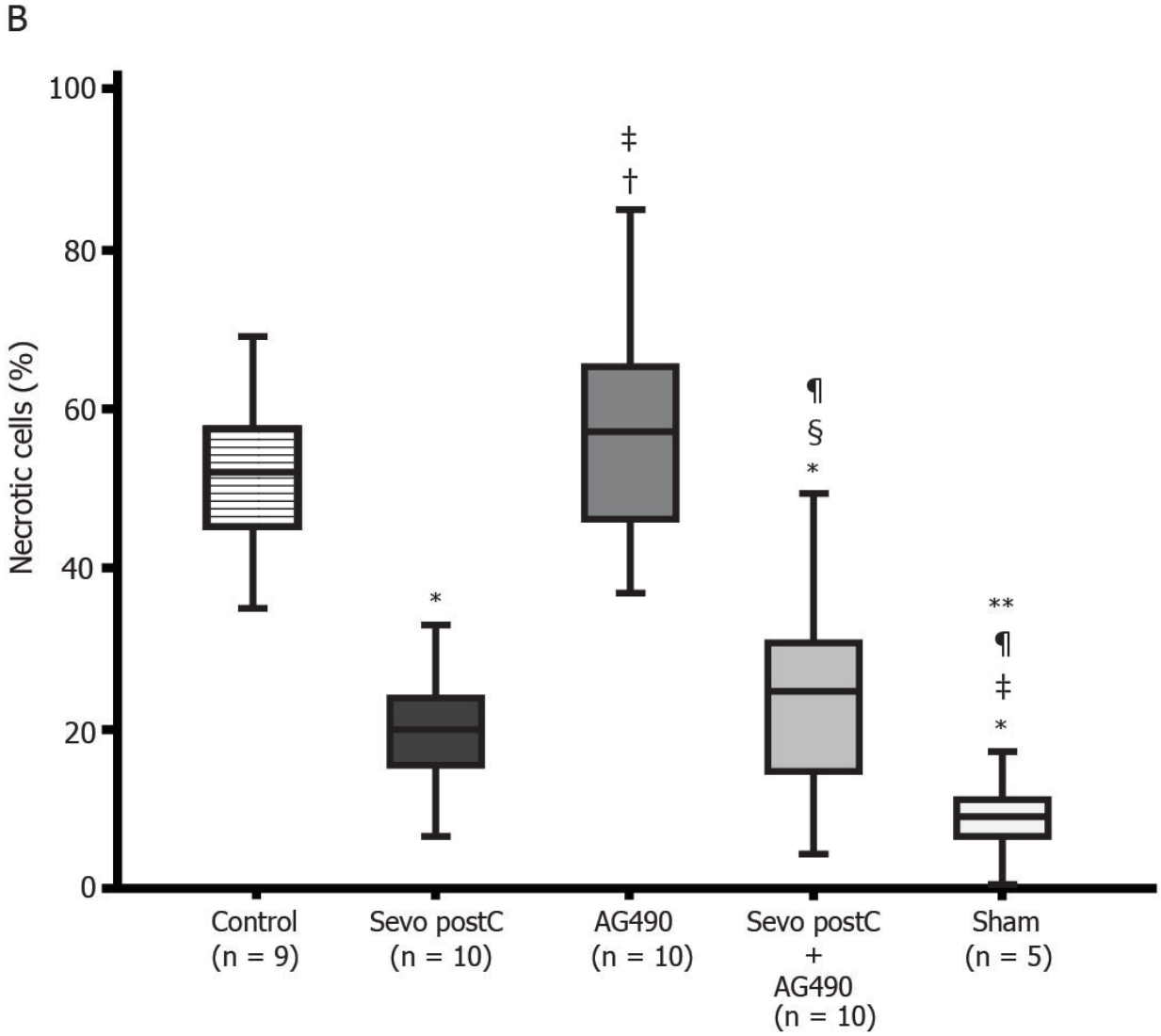

Figure 3 (B) Percentages of necrotic cells in the hippocampal CA1 region three days after ischemia (H&E staining). Necrotic neurones are identified by karyolytic or pyknotic nuclei and cytoplasmic shrinkage. The total number of cells and the number of necrotic cells are counted manually in each field of view using an imaging software by an investigator blinded to the group assignments. The percentage of necrotic cells in the hippocampal CA1 shows a difference among groups ($P < 0.001$). Results are presented as the median (bar in the box), interquartile (box) and full ranges (upper and lower bars).

*P < 0.01 *vs.* the control group. †P < 0.05 *vs.* the control group. ‡P < 0.01 *vs.* the sevoflurane postconditioning group. §P < 0.05 *vs.* the sevoflurane postconditioning group. ¶P < 0.01 *vs.* the AG490 group. **P < 0.01 *vs.* the sevoflurane postconditioning plus AG490 group. H&E, hematoxylin and eosin; CA1, Cornu Ammonis area 1; Sevo, sevoflurane; postC, postconditioning.

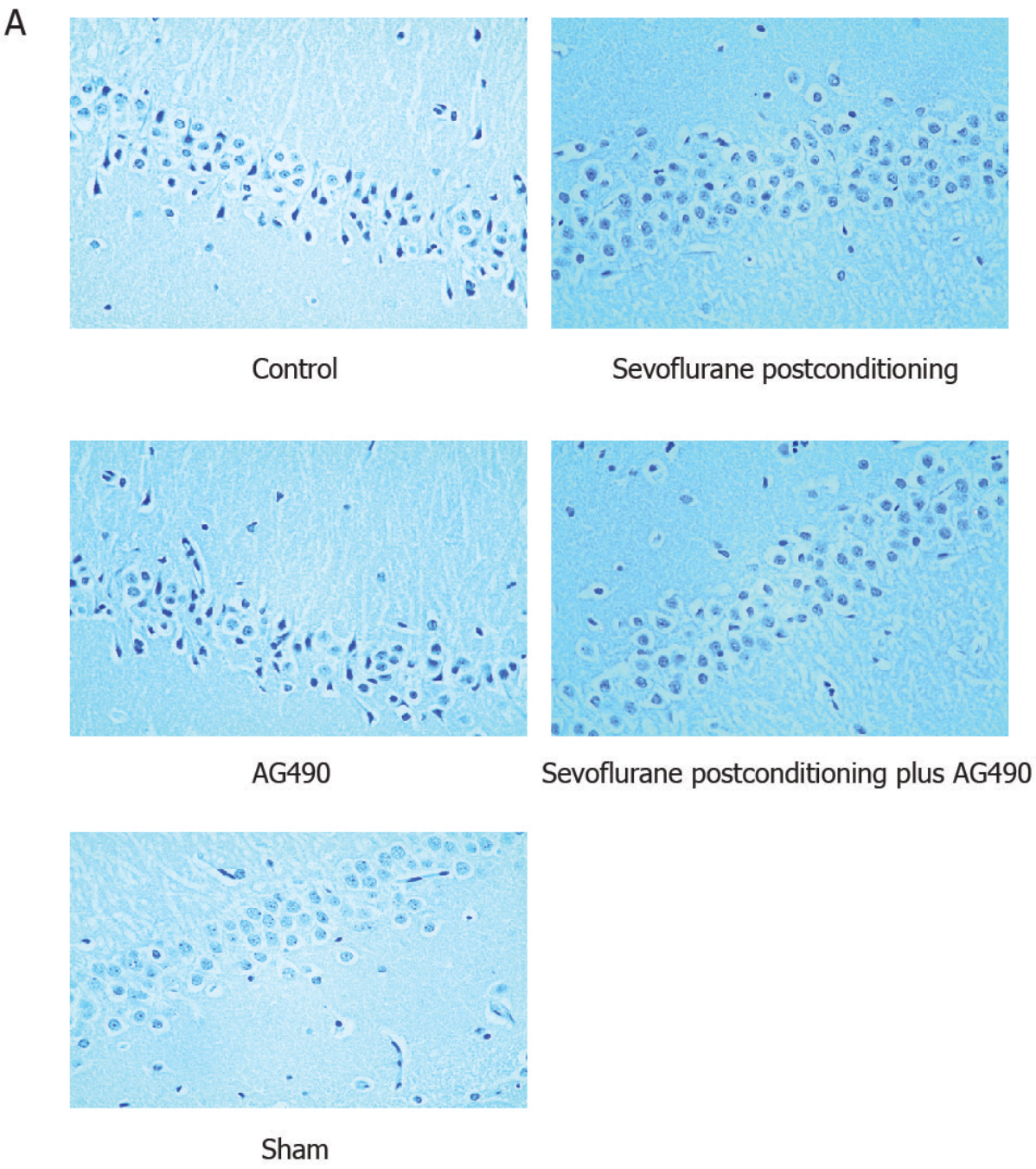

Figure 4 (A) Representative photomicrographs from a single rat with TUNEL staining showing apoptotic cells in the hippocampal CA1 region three days after transient global cerebral ischemia.

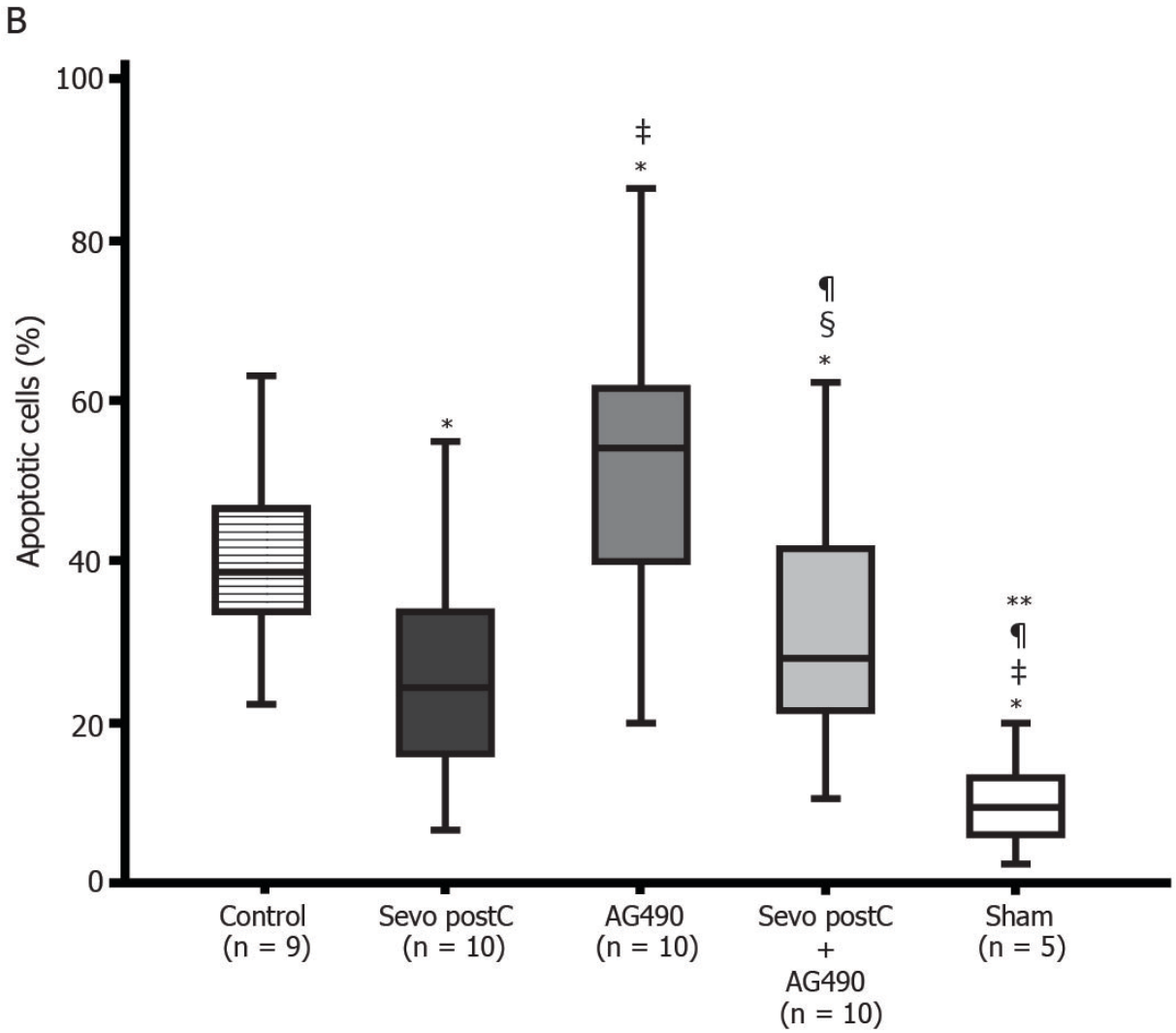

Figure 4 (B) Percentages of apoptotic cells in the hippocampal CA1 region three days after ischemia (TUNEL staining). TUNEL-positive cells with blue-stained apoptotic bodies are considered as apoptotic cells. The total number of cells and the number of apoptotic cells are counted manually in each field of view using an imaging software by an investigator blinded to the group assignments. The percentage of apototic cells in the hippocampal CA1 shows a difference among groups ($P < 0.001$). Results are presented as the median (bar in the box), interquartile (box) and full ranges (upper and lower bars).

*P < 0.01 *vs.* the control group. ‡P < 0.01 *vs.* the sevoflurane postconditioning group. §P < 0.05 *vs.* the sevoflurane postconditioning group. ¶P < 0.01 *vs.* the AG490 group. **P < 0.01 *vs.* the sevoflurane postconditioning plus AG490 group. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end-labelling; CA1, Cornu Ammonis area 1; Sevo, sevoflurane; postC, postconditioning.

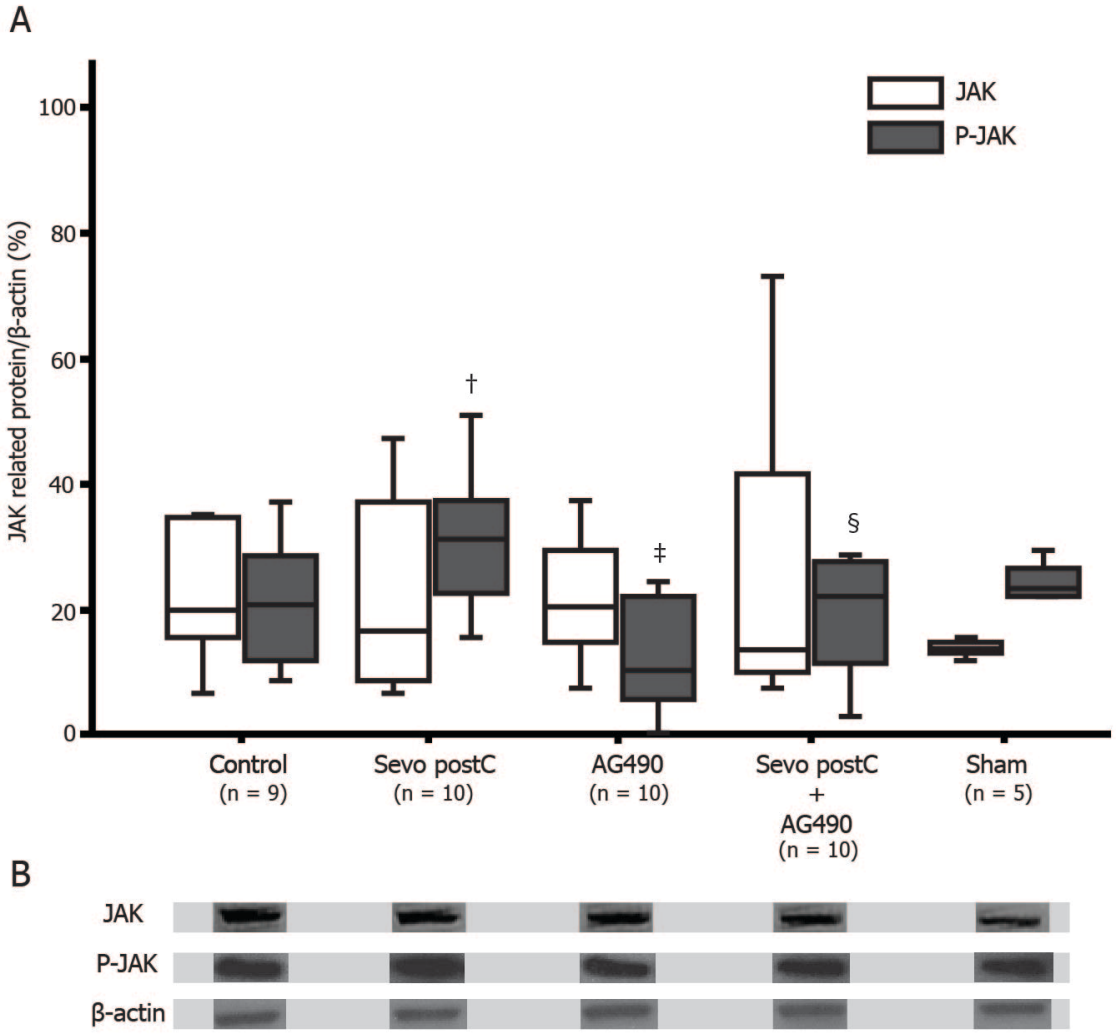

Figure 5 (A) Densitometric evaluation of JAK2 and P-JAK2 in the cytoplasm. (B) Representative Western blot of JAK2 and P-JAK2 from a single animal three days after transient global cerebral ischemia in rats. The cytosolic JAK2 level is comparable among the five groups ($P = 0.632$). The cytosolic P-JAK2 level demonstrates a difference among groups ($P = 0.036$). Results are presented as the median (bar in the box), interquartile (box) and full ranges (upper and lower bars). β-Actin is used as a control for JAK2 and P-JAK2.

†$P < 0.05$ *vs.* the control group. ‡$P < 0.01$ *vs.* the sevoflurane postconditioning group. §$P < 0.05$ *vs.* the sevoflurane postconditioning group. Sevo, sevoflurane; postC, postconditioning; P-JAK, phosphorylated Janus family tyrosine kinases.

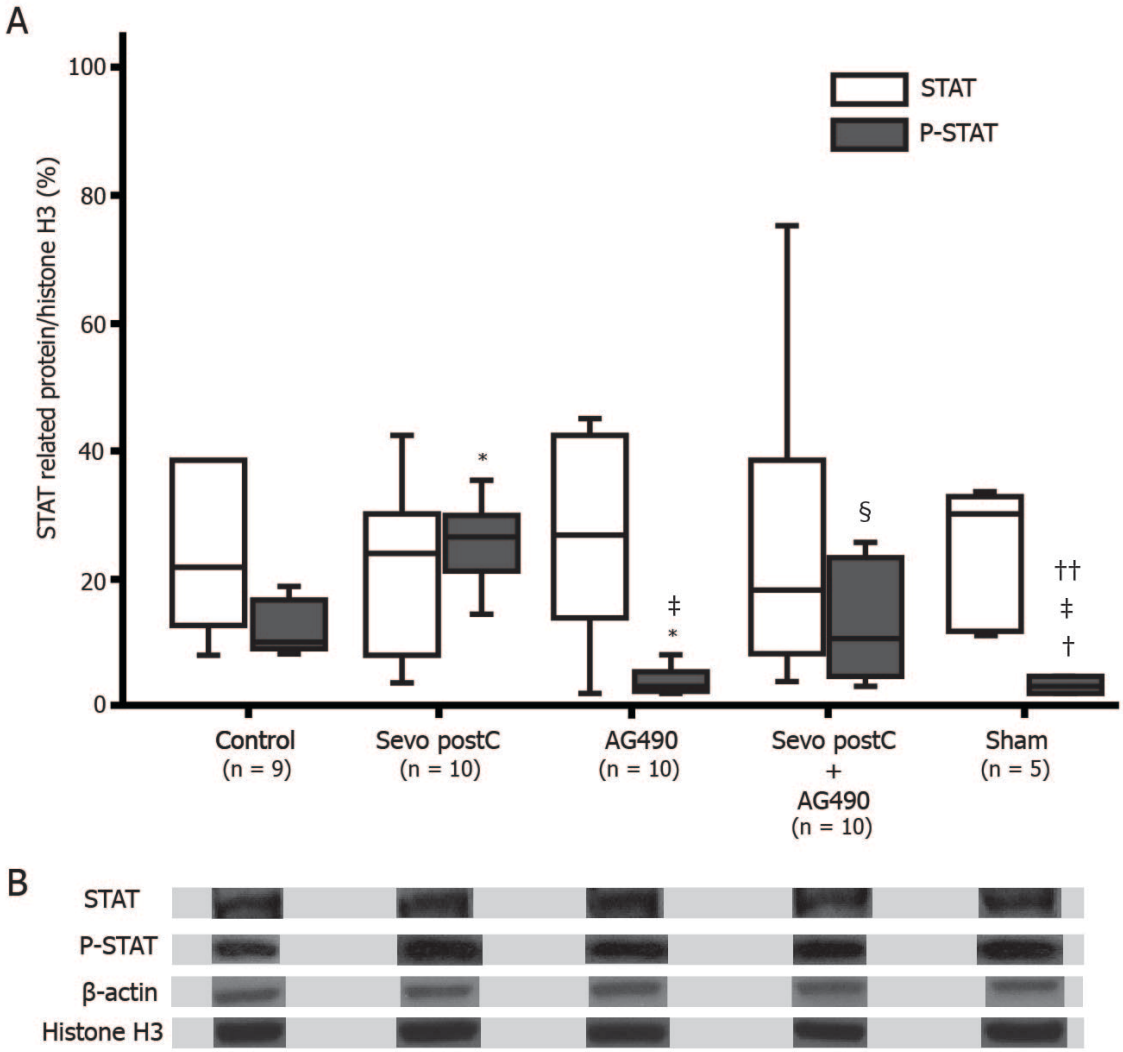

Figure 6 (A) Densitometric evaluation of STAT3 in the cytoplasm and P-STAT3 in the nucleus. (B) Representative Western blot analysis of STAT3 and P-STAT3 from a single rat three days after transient global cerebral ischemia in rats. Cytosolic STAT3 levels are comparable among the five groups ($P = 0.927$). The nuclear P-STAT3 level demonstrates a difference between groups ($P < 0.001$). Results are presented as the median (bar in the box), interquartile (box), and full ranges (upper and lower bars). β-Actin and histone H3 are used as controls for STAT3 and P-STAT3, respectively.

*P < 0.01 *vs.* the control group. †P < 0.05 *vs.* the control group. ‡P < 0.01 *vs.* the sevoflurane postconditioning group. §P < 0.05 *vs.* the sevoflurane postconditioning group. ††P < 0.05 *vs.* the sevoflurane postconditioning plus AG490 group. Sevo, sevoflurane; postC, postconditioning; P-STAT, phosphorylated signal transducers and activators of transcription.

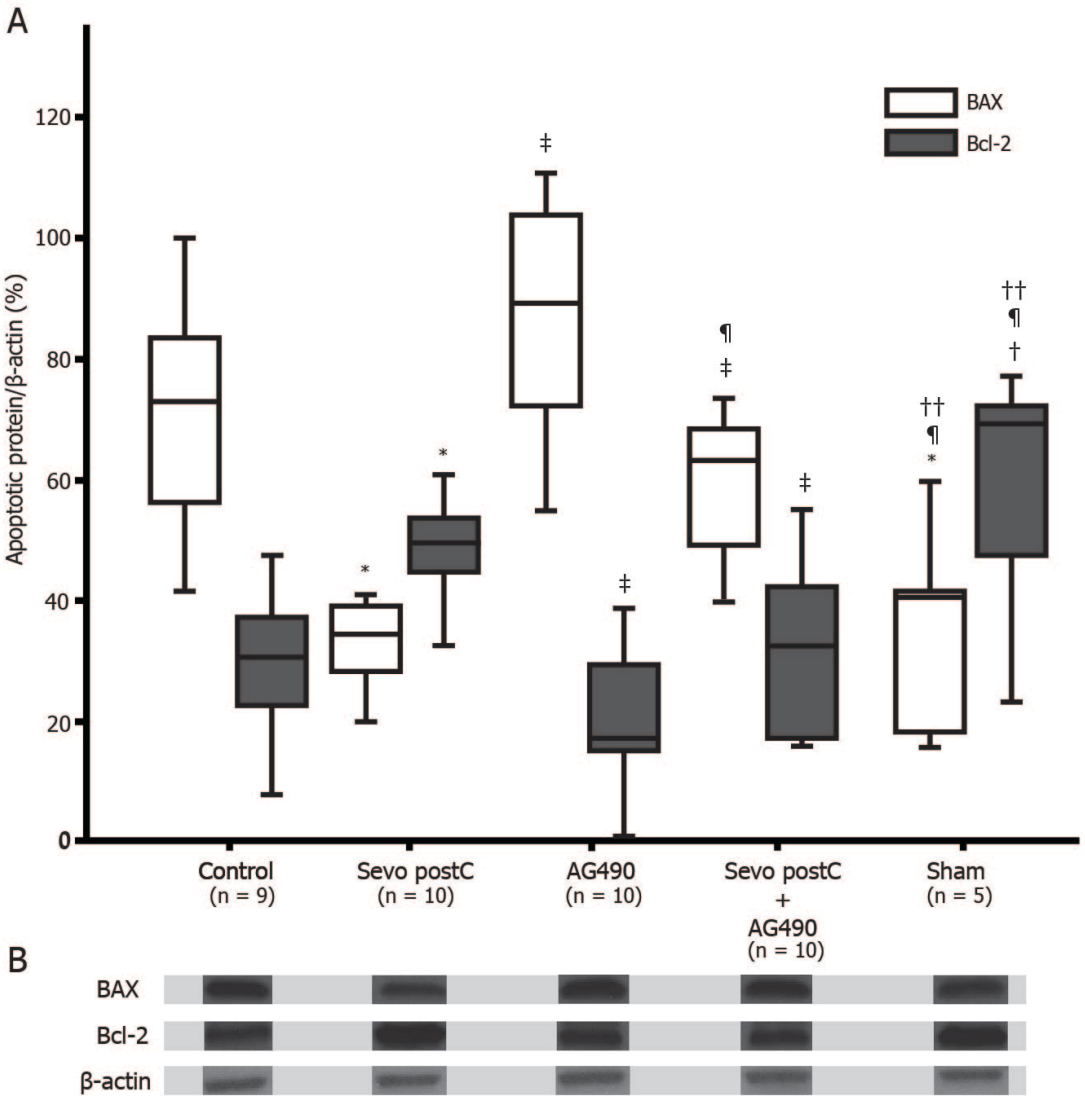

Figure 7 (A) Densitometric evaluation of BAX and Bcl-2 in the cytoplasm. (B) Representative Western blot analysis of BAX and Bcl-2 from a single rat three days after transient global cerebral ischemia in rats. The cytosolic Bax level demonstrates a difference among groups ($P < 0.001$). The cytosolic Bcl-2 level demonstrates a difference among groups ($P < 0.001$). Results are presented as the median (bar in the box), interquartile (box) and full ranges (upper and lower bars). β-Actin is used as a control.

*P < 0.01 *vs.* the control group. †P < 0.05 *vs.* the control group. ‡P < 0.01 *vs.* the sevoflurane postconditioning group. ¶P < 0.01 *vs.* the AG490 group. ††P < 0.05 *vs.* the sevoflurane postconditioning plus AG490 group. Sevo, sevoflurane; postC, postconditioning.

Discussion

This study demonstrated that sevoflurane postconditioning injcreased the expression of cytoplasmic P-JAK2 and nuclear P-STAT3, and reduced apoptosis by increasing Bcl-2 expression and decreasing Bax expression in a rat model of transient global cerebral I/R injury.

The role of the JAK2-STAT3 pathway in I/R injury has been studied in many organs such as the heart and brain.[6, 15-18] JAK2 protein functions in the transduction of signals from cytokines and growth factors to the nucleus.[6] STAT3 phosphorylation by JAK2 protein results in increased expression in genes related to cell survival.[19] STAT3 activation is related to antioxidant effects such as increased expression of free radical scavengers,[20] the antiinflammatory effects,[21] the survivor activating factor enhancement pathway,[22] inhibition of mitochondrial permeability transition pore opening[23] and the reperfusion injury salvage kinase pathway.[24]

The effect of JAK-STAT pathway activation on neuronal protection against cerebral I/R insult has shown contradictory results. Although few studies determined that blockage of the JAK2-STAT3 pathway leads to improved neurological outcomes and decreased infarct size in a transient focal cerebral I/R injury model of rats,[25, 26] most previous studies reported the beneficial effects of the JAK2-STAT3 pathway after cerebral I/R injury.[17, 27, 28] Zhou and colleagues showed that the antiapoptotic effects of recombinant human erythropoietin were abolished by the use of AG490, a specific JAK2 inhibitor, in a model of septic encephalopathy.[29] Zhu and colleagues demonstrated that AG490 reversed the beneficial effects of SMND-309, a novel derivate of salvianolic acid B, on infarcted cerebral volume and neurological function in a model of transient focal cerebral ischemia.[30] Suzuki and colleagues demonstrated that STAT3 is activated in neurons of the peri-infarct region during cerebral I/R injury.[31] Yan and colleagues reported that cardioprotection by emulsified isoflurane postconditioning is partly associated with activation of the JAK-STAT pathway. Our study demonstrated that sevoflurane postconditioning-induced neuroprotection (a significant reduction in

the number of apoptotic and necrotic cells) was abrogated when AG490 was administered before the induction of ischemia. Such findings suggest that the JAK-STAT pathway may be involved in the mechanism of sevoflurane postconditioning-induced neuroprotection in a rat model of global cerebral ischemia.

Apoptosis in cerebral I/R injury is a major mechanism of increased cellular death and is considered the target for treatment.[32] Mitochondrial release of apoptotic factor is a key factor of apoptosis, which is regulated by antiapoptotic proteins such as Bcl-2 and apoptotic proteins such as Bax.[33] In the previous report, sevoflurane postconditioning reduced apoptosis by regulating the expression of apoptotic and antiapoptotic proteins.[2] In accordance with previous investigations, our investigation demonstrated that sevoflurane postconditioning resulted in increased Bcl-2 expression, decreased Bax expression, and fewer apoptotic cells compared with the control group.

The relationship between the JAK2-STAT3 pathway and apoptosis was investigated in several studies.[34, 35] Genes of antiapoptotic proteins such as Bcl-2 and Bcl-xl are targets of STAT3.[36] Inhibition of the JAK2-STAT3 pathway by AG490 causes decreased Bcl-2 expression, increased Bax expression, and the induction of apoptosis in tumor cells.[16] Previous reports showed that the JAK2-STAT3-Bcl-2 pathway, which is activated by ischemic postconditioning and melatonin, played an essential role in reducing myocardial I/R injury.[16] A previous experimental report using a model of transient focal cerebral ischemia in mice demonstrated that recombinant human granulocyte colony-stimulating factor had neuroprotective effects *via* the JAK2-STAT3-Bcl-2 pathway.[28] In this study, sevoflurane postconditioning reduced apoptosis by increasing the expression of P-JAK2, P-STAT3 and Bcl-2, and decreasing that of Bax in a rat model of transient global cerebral ischemia. Administration of AG490 reversed the beneficial effects of sevoflurane postconditioning on apoptosis and the JAK2-STAT3 pathway. This finding suggests that expression of the JAK2-STAT3 pathway may be associated with the antiapoptotic effects of sevoflurane postconditioning.

In our investigation, the sevoflurane postconditioning plus AG490 group showed fewer apoptotic cells compared with the control group and more apoptotic cells compared with the sevoflurane postconditioning group. Such results suggest that AG490 does not completely block neuroprotective effect of sevoflurane postconditioning and other pathways or mechanisms in addition to the JAK2-STAT3-Bcl-2 pathway may be involved in the neuroprotective mechanisms of sevoflurane postconditioning. A recent report showed that antioxidative effects by nuclear factor erythroid 2-related factor was involved in sevoflurane postconditioning-induced neuroprotection.[4] Volatile anesthetics may protect neurons by suppressing excitotoxicity *via* the supression of glutamate levels in the brain and reduction in N-methyl-D-aspartate receptor activation.[37] Preconditioning and postconditioning by sevoflurane reduces the inflammatory cytokines and improves neurological function after cerebral ischemia.[38] The mitochondrial adenosine triphosphate (ATP) potassium channel[39] is also responsible for sevoflurane postconditioning-induced neuroprotection.

In this study, the sevoflurane postconditioning plus AG490 group showed similar Bax and Bcl-2 expressions with the control group but higher Bax and lower Bcl-2 expressions than the sevoflurane postconditioning group, suggesting that the Bax and Bcl-2 levels may not give the full picture of apoptotic program in the cell. Indeed, there are other proteins in Bcl-2 family proteins such as Bcl-2-associated death promoter, Bcl-2 homologous antagonist killer, and Bcl-extra long (Bcl-xL).[40] STAT1 and STAT3 activation is related to Bcl-xL.[41] Cell survival is enhanced by increased nuclear factor erythroid 2-related factor and Bcl-xL levels.[42] The relationship of the JAK-STAT pathway with other Bcl-2 family proteins in elucidating the antiapoptotic mechanism of sevoflurane postconditioning needs to be determined in the future investigations.

We administered 40 mg/kg of AG490 intraperitoneally to block the JAK2-STAT3 pathway. Intraperitoneal injection of AG490 have been used in previous experimental investigations regarding traumatic brain injuy[43] and encephalomyelitis,[44] suggesting that systemic AG490 may cross the blood brain barrier. AG490 doses of 2-40 mg/kg have been administered intraperitoneally in many

previous experimental studies.[15, 18, 43-47] To eliminate the effects of JAK2 on STAT3 expression completely, the highest dose of AG490 in references was chosen in this study. AG490 is associated with potential febrile reaction, which can be a confounder in this study. A previous study showed that AG490 exacerbated fever and reduced the expression of interleukin-10 in a rat with brain inflammation induced by peripheral lipopolysaccharide stimulations.[48] AG490 also has anti-nociceptive effect, which may help to reduce postoperative pain in combination with bupivacaine infiltration around skin incisions. A recent study showed that AG490 attenuated thermal and mechanical hyperalgesia in a dose-dependent manner in a rodent model of inflammatory pain.[49]

In this study, histopathologic examination and Western blot analysis were performed three days after global cerebral I/R injury. Kawaguchi and colleagues found that the number of apoptotic cells in rats, which are anesthetized with isoflurane and subjected to focal cerebral ischemia, peaked four days after reperfusion and then decreased.[50] A previous investigation showed that P-STAT3 increased significantly 48 h after cerebral I/R injury.[51] In another investigation using a model of focal cerebral ischemic insult, STAT3 mRNA expression increased significantly from 6 h to 72 h of reperfusion.[25] Based on these previous reports, we decided that three days after cerebral I/R injury would be appropriate to evaluate both the degree of apoptosis and P-STAT3 expression.

There were some limitations in this investigation. First, the study period was limited to three days of post-ischemia. Therefore, the long-term effect of sevoflurane postconditioning on the JAK2-STAT3 pathway was not evaluated. Our previous studies, however, showed that sevoflurane postconditioning improved histopathologic and functional neurologic outcomes on postischemic 7 days.[2, 4] Second, there are four types of JAK and seven types of STAT proteins. We focused on the JAK2-STAT3 pathway among the many subtypes of the JAK-STAT family. To find the relationship between other subtypes of JAK-STAT family subtypes and sevoflurane postconditioning, further studies using other JAK inhibitors such as ZM-449829 (a JAK3 inhibitor),[52] or animals deficient in the JAK gene are needed. Third, neither interleukin-6 nor interleukin-10 levels in serum or brain tissue

were measured in this study. Such cytokines are known to induce JAK2-STAT3 expression.[6] Fourth, our study did not evaluate neuron-specific JAK2-STAT3 activation. Activation of the JAK-STAT pathway in atrocytes is observed after transient focal cerebral ischemia.[53] JAK2-STAT3 activation in glial cells may affect the result of our investigation. Fifth, apoptosis is regulated by the intrinsic and extrinsinc pathway, and the caspase-independent pathway. We evaluated the JAK2-STAT3-Bcl-2 pathway regarding the intrinsic signaling cascade of apoptosis after cerebral ischemia. Our investigation did not evaluate JAK-STAT pathway in the extrinsic and the caspase-independent pathway of apoptosis. Other type of STAT such as STAT1 is related to the extrinsic pathway,[54] and the caspase-independent pathway.[55] Sixth, we used relatively young animals in our experiment. Considering global cerebral ischemia is more common in senior patients, use of older animals may strengthen our results. Seventh, we did not perform a neuro-exmination. Although our study revealed histopathologic differences, adding the neuro-examination result may strengthen our results. Finally, all groups, although there is no statistical difference, showed hyperglycemia, which may be a confounding factor .

In conclusion, this study showed that sevoflurane postconditioning reduced apoptosis by increasing Bcl-2 and decreasing Bax expression *via* activation of the JAK2-STAT3 pathway in a rat model of transient global ischemia. Preischemic administration of AG490, a JAK2 selective inhibitor, reversed the beneficial antiapoptotic effects of sevoflurane postconditioning by inhibiting the JAK2-STAT3 pathway. These results suggest that the JAK2-STAT3 pathway may be involved in the antiapoptotic mechanism of sevoflurane postconditioning.

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국문 초록

목적: Sevoflurane 후처치의 반세포자멸사 효과는 뇌의 허혈-재관류 손상에 대한 신경보호에서 큰 역할을 하고 있다. Janus family tyrosine kinases (JAK) 2-signal transducers and activators of transcription (STAT) 3 경로의 인산화는 반세포자멸사와 연관이 되어 있다. 이에 쥐 모델을 이용한 일시적 전뇌허혈에서 sevoflurane 의 반세포자멸사 효과와 JAK2-STAT3 경로와의 연관성에 대하여 규명하고자 하였다.

방법: 45 마리의 쥐를 무작위로 5 군으로 나누었다: 겉보기수술군 (n=5), 대조군 (허혈 10 분, n=10), sevoflurane 후처치군 (허혈 10 분 후 2 회의 sevoflurane 흡입, n=10), AG490 군 (선택적 JAK2 억제제, 허혈 전 40 mg kg^{-1} 정맥 투여, n=10) 과 sevoflurane 후처치 및 AG490 군 (n=10). 허혈 3 일 후, 자멸세포수와 JAK2, 인산화 JAK2 (P-JAK2), STAT3, 인산화 STAT3 (P-STAT3), Bcl-2 (반세포자멸사 단백질), Bax (세포자멸사촉진 단백질)의 발현 정도를 평가하였다.

결과: 자멸세포수는 sevoflurane 후처치군에서 대조군, AG490 군, sevoflurane 후처치 및 AG490 군에 비해 유의하게 낮게 측정되었다. JAK2 와 STAT3 발현은 모든 군에서 유사하게 계측되었다. sevoflurane 후처치군에서 대조군, AG490 군, sevoflurane 후처치 및 AG490 군과 비교할 때, P-JAK2, P-STAT3, Bcl-2 발현은 높게 측정되었고, Bax 발현은 낮게 측정되었다.

결론: 쥐 모델을 이용한 일시적 전뇌허혈에서 sevoflurane 후처치는 P-JAK, P-STAT 증가시켰으며 세포자멸사를 감소시켰으며, AG490 은 sevoflurane 후처치로 인한

반세포자멸 효과를 역작용시켰다. JAK-STAT 경로는 sevoflurane 후처치로 인한 반세포자멸 기전과 관련될 수 있을 것이다.

주요어: 세포자멸사; 뇌; 허혈; JAK/STAT 경로; sevoflurane

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