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Damage-associated molecular patterns as a mechanism of sevoflurane-induced neuroinflammation in neonatal rodents

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Background: General anesthesia is inevitable for pediatric patients undergoing surgery, though volatile anesthetic agents may cause neuroinflammation and neurodevelopmental impairment; however, the underlying pathophysiology remains unclear. We aimed to investigate the neuroinflammation mechanism in developing rat brains associated with sevoflurane exposure time, by identifying the specific damage-associated molecular patterns (DAMPs) pathway and evaluating the effects of non-steroidal anti-inflammatory drugs (NSAIDs) in alleviating neuroinflammation.

Methods: A three-step experiment was conducted to investigate neuroinflammation induced by sevoflurane. First, the exposure time required for sevoflurane to cause neuroinflammation was determined. Next, the specific pathways of DAMPs involved in neuroinflammation by sevoflurane were identified. Finally, the effects of NSAIDs on sevoflurane-induced neuroinflammation were investigated. The expression of various molecules in the rat brain were assessed using immunohistochemistry, immunofluorescence, quantitative real-time polymerase chain reaction, western blot analysis, and enzyme-linked immunosorbent assay.

Results: In total, 112 rats (aged 7 days) were used, of which six rats expired during the experiment (mortality rate, 5.3%). Expression of CD68, HMGB-1, galectin-3, TLR4, TLR9, and phosphorylated NF-κB was significantly increased upon 6 h of sevoflurane exposure. Conversely, transcriptional levels of TNF-α and IL-6 significantly increased and IFN-γ significantly decreased after 6 h of sevoflurane exposure. Co-administration of NSAIDs with sevoflurane anesthesia significantly attenuated TNF-α and IL-6 levels and restored IFN-γ levels.

Conclusions: In conclusion, 6 h of sevoflurane exposure induces neuroinflammation through the DAMPs pathway, HMGB-1, and galectin-3. Co-administration of ibuprofen reduced sevoflurane-induced neuroinflammation.

Keywords: Alarmins; Damage-associated molecular pattern molecules; Galectin 3; HMGB1 protein; Neonate; Non-steroidal anti-inflammatory agents; Rats; Sevoflurane.

Introduction

Every year, tens of thousands of children worldwide require surgery to both save and enhance the quality of their lives. Increases in maternal ages and rates of premature and multi-fetal births have led to a rise in the number of neonates requiring surgery for congenital diseases or conditions. Many pediatric patients also require multiple surgeries to

improve their health.

General anesthesia is essential for surgical procedures; however, the findings of several animal studies have raised concerns that almost all anesthetic drugs currently used have been linked to unfavorable effects on the developing brain or neuronal system, such as apoptosis of neuronal cells, inappropriate synaptic formation, changes in dendritic architecture, inhibition of myelination, and faulty axonal targeting [1–6]. Moreover, early lifetime exposure to anesthetic drugs has also been associated with decreased long-term memory and cognitive impairment [7]. Although these laboratory findings do not entirely correlate with clinical findings, some studies report that children who have received multiple administrations of anesthesia experience a higher incidence of conditions such as attention-deficit/hyperactivity disorder, learning disabilities, and autism [8–12]. However, the exact mechanism for this adverse effect of anesthetic drugs on developing brains has not yet been fully understood, and this remains a topic of interest.

In recent studies in adults, neuroinflammation is proposed as the main mechanism for neurologic diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis and cognitive dysfunction induced by sevoflurane [13–15]. Neuroinflammation is associated with an increase in proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) that are regulated by microglial cells [16,17]. Microglial cells are resident macrophages present in the central nervous system and involved in immune surveillance through micro-environmental sampling. Microglial cells play a role in maintaining brain homeostasis and tissue regeneration [18]. Though not fully understood, microglial activation is thought to be initiated by removing the inhibitory neuronal signaling and activating pattern-recognition receptors by exogenous pathogen-associated molecular pattern molecules (PAMPs) and/or endogenous damage-associated molecular patterns (DAMPs) [18]. As the anesthetic is not a direct toxin, neuroinflammation caused by anesthetics can be presumed to be induced by DAMPs rather than PAMPs.

Sevoflurane is the most used inhalational anesthetic in pediatric surgery, and several studies have also suggested neuroinflammation as a mechanism of anesthetic-induced brain damage in preclinical neonatal research [14,19–21]. However, the exact cascade of neuroinflammation remains unelucidated [20]. Thus, we aimed to identify the specific mechanism of sevoflurane-induced neuroinflammation in the developing rat brain. We hypothesize that DAMPs are the primary mechanism of neuroinflammation induced by sevoflurane. Among DAMPs, high mobility group box-1 (HMGB-1) and galectin-3 are candidates of critical factors involved in neuroinflammation caused by anesthetic agents. Additionally, we evaluated whether non-steroidal anti-inflammatory drugs (NSAIDs) can reduce sevoflurane-induced neuroinflammation. These findings may provide valuable insights into the causes of brain damage caused by anesthetic drugs in human patients, particularly in the developing brains of neonates and pediatric patients.

Materials and Methods

Animal preparation

This animal trial was approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (IACUC No. 2017-0137; date of approval: September 28, 2017). All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and were approved by the Committee for the Care and Use of Laboratory Animals. All studies reported herein were conducted in accordance with Animal Research: Reporting of In Vivo Experiments guidelines (ARRIVE, https://arriveguidelines.org). A total of 23 gravid Wistar rats were obtained from the central laboratory of SLC Inc. (Hamamatsu). A total of 112 offspring Wistar rats obtained from gravida rats were used. They were housed in sterile cages under laminar airflow hoods in a local and specific pathogen-free experimental animal facility. The rats were maintained on a 12-h light/dark cycle at a controlled temperature of approximately 25°C and relative humidity of approximately 60%, with free access to food and water. After the date of birth (represented as P0), the offspring were kept with their parents until day seven of life (P7), and litter sizes ranged from 8 to 14 animals. At least one animal from each litter was assigned to each treatment group, and their body weights ranged from 9.5 g to 15.5 g. Animals of both sexes were used in the study.

Exposure protocol

We conducted three experiments to investigate the effects of sevoflurane on neuroinflammation. In the first experiment, we determined the exposure time of sevoflurane that causes neuroinflammation in 24 P7 rats. The rats were randomly assigned to one of the four groups: a control group without sevoflurane exposure (Con), a 2 h sevoflurane group (Sevo-2h), a 4 h sevoflurane group (Sevo-4h), and a 6 h sevoflurane group (Sevo-6h). The sevoflurane groups were exposed to 2.5% sevoflurane with 30% oxygen for the assigned time [21], and each group contained six rats. The second experiment, to identify the specific pathway of neuroinflammation, was repeated four times. We used 48 rats, with five in the Con group and seven in the Sevo-6h group in each set. As in

the first experiment, the Sevo-6h group was exposed to 2.5% sevoflurane with 30% oxygen for 6 h. Lastly, the third experiment was conducted twice to investigate the effects of NSAIDs on sevoflurane-induced neuroinflammation. We used a total of 40 rats, with six in the Con group, seven in the Sevo-6h group, and seven in the ibuprofen group (IBU) in each set. The Sevo-6h group was exposed to 2.5% sevofluran with 30% oxygen for 6 h, and the IBU group was administered 30 mg/kg of ibuprofen through intraperitoneal injection and then exposed to 2.5% sevoflurane with 30% oxygen for 6 h. The ibuprofen injection was prepared directly before the experiments by diluting it with preservative-free normal saline to a final concentration of 30 mg/kg to limit the maximum injection volume to 0.1 ml. The allocation of the number of used rats is summarized in Table 1.

Sevoflurane-anesthetized animals were briefly removed from the anesthetizing chamber for injections. All animals were kept in a 30% oxygen environment in an acrylic chamber within a heated incubator set at 34–38°C to maintain a rectal temperature of 36.0–37.5°C. After a 30-min recovery period from the inhaled anesthesia, intraperitoneal anesthesia was performed with ketamine (20 mg/kg), acepromazine (0.5 mg/kg), and xylazine (1 mg/kg). Following intraperitoneal anesthesia, the sternum was incised to extract blood serum from the heart prior to perfusion with phosphate-buffered saline (PBS). For assays, serum was separated from the blood samples by centrifugation at 4,000 rpm for 30 min at 4°C. These samples were preserved at −80°C until use. The rat was then perfused using a PBS solution, including heparin, and sacrificed. Brain tissue was obtained for analysis and stored at −80°C.

Immunohistochemistry (IHC)

The level of the cluster of differentiation 68 (CD68) expression in brain tissue was evaluated to assess the occurrence of neuroinflammation based on the duration of exposure to sevoflurane. Tissue samples for IHC examination were obtained from the brain, washed with physiologic saline, fixed in 10% buffered formalin, and embedded in paraffin. The tissue sections were stained with rabbit anti-CD68 (1:100; Abcam), anti-HMGB-1 (1:100; Cell Signaling Technologies), and anti-galectin-3 (1:500; LSBio). The staining was followed by treatment with 3,3′-diaminobenzidine as a chromogen (Abcam). The slides were then viewed using an Olympus™ IX73P2F microscope (Olympus America) equipped with an Olympus™ DP71 digital camera (20X magnification).

Immunofluorescence (IF)

Tissue samples for immunofluorescence analysis were taken from the brain, washed in physiologic saline, fixed in 10% buffered formalin, and embedded in paraffin. After blocking with PBS containing 5% normal goat serum (NGS) for 30 min, tissue sections were incubated with anti-toll-like receptor 2 (TLR2, 1:100; Santa Cruz Biotechnology, Inc.), anti-TLR4 (1:100; Santa Cruz Biotechnology, Inc.), anti-TLR9 (1:100; Santa Cruz Biotechnology, Inc.), and anti-phospho-nuclear factor kappa B (p-NF-κB, 1:100; Santa Cruz Biotechnology, Inc.) primary antibodies in 2.5% NGS overnight at 4°C. The next day, the cells were washed three times with PBS-Tween 20 (PBS-T, 0.01%) and then incubated with fluorescein-5-isothiocyanate (FITC)-conjugated secondary antibodies in 2.5% NGS for 1 h at room temperature protected from light. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:2,500; Molecular Probe Inc.). Images were obtained using a Carl Zeiss™ LSM700 (Carl Zeiss) confocal microscope fitted with the appropriate filters.

Enzyme-linked immunosorbent assay (ELISA)

The levels of HMGB-1, heparan sulphate, and galectin-3 (MyBioSource), as well as interferon-gamma (IFN-γ; Biolegend) in the serum, were measured using commercial ELISA kits following the manufacturer's instructions.

Western blot analysis

The obtained brain tissues were washed with cold PBS, minced, and lysed in a cell lysis buffer (Cell Signaling Technologies) containing protease inhibitors (10 μg/ml each of aprotinin,

Table 1. Allocation of Number of Used P7 Rats

	Control	Sevo-2h	Sevo-4h	Sevo-6h	IBU	Set	Total
1st experiment	6	6	6	6		1	24
2nd experiment	5			7		4	48
3rd experiment	6			7	7	2	40
Total	38	6	6	48	14		112

Con: control group, IBU: administration of ibuprofen, P7: aged 7 days, Sevo-2h: exposure to sevoflurane for 2 h, Sevo-4h: exposure for 4 h, Sevo-6h: exposure for 6 h.

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bestatin, L-leucine, and pepstatin A) dissolved in a solution of 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM benzene sulfonyl fluoride, and 1% NP-40. The total protein concentration was determined using Quick Start Bradford reagent (Bio-Rad; Hercules). Whole-cell extracts (50 μg) were separated on 10%–15% sodium dodecyl sulfate–polyacrylamide electrophoresis gels and transferred to Immobilon®-P transfer membranes (Millipore). The membranes were then incubated with primary antibodies that were detected using horseradish peroxidase-conjugated IgG antibody (Cell Signaling Technologies). Bands were determined using West Glow™ FEMTO Chemiluminescent Substrate (BIOMAX). The primary antibodies used targeted CD68 (1:1000; Abcam), HMGB-1 (1:1000; Abcam), galectin-3 (1:1000; LSBio), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000; Cell Signaling Technologies) as loading controls.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen). Complementary DNA was synthesized from 2 μg of total RNA using AccuPower® real time premix kits (BIONEER). Real-time PCR analysis was performed using TB Green™ Premix Ex Taq II kit (Takara Bio Inc.) and QuantStudio3™ (Applied Biosystems) according to the manufacturer's instructions. Each sample was analyzed in quadruplicate, and target genes were normalized to the reference housekeeping gene, GAPDH. Fold differences were then calculated for each group using the normalized cycle of normalization values for the control groups. The primer sequences for real-time PCR are given in Table 2.

Sample size and statistical analysis

We utilized the minimum number of animals required for this study in accordance with the 3R (replacement, reduction, and refinement) principles of animal experiment ethics [22,23]. Each in vivo assay was performed in triplicate and repeated at least thrice. All data from the in vivo experiments are presented as the mean ± standard deviation and were analyzed using one-way analysis of variance with Bonferroni's correction applied to post hoc multiple comparisons tests. P values < 0.05 were considered statistically significant. Statistical analyses were performed using SAS® version 9.4 (SAS Institute Inc.).

Results

We utilized a total of 112 P7 rats for our study, out of which six rats expired during the experiment, resulting in a mortality rate of 5.3%. All mortality cases occurred during the maintenance of sevoflurane anesthesia.

Assessment of sevoflurane-induced neuroinflammation according to the exposure time

We performed IHC to measure the expression of CD68 as a marker of microglial cell activation induced by sevoflurane exposure time (Fig. 1). The expression of CD68 had significantly increased after exposure to sevoflurane for 6 h compared with that in the control group. However, no significant differences were observed between the controls and the 2 h or 4 h exposure groups. To further assess the activation of microglial cells via the DAMPs pathway, we conducted brain IHC and serum ELISA using anti-

Table 2. Primers Used in Real-time PCR

Gene	Sequence	Annealing temp (°C)
HMGB-1	F: 5′-CAAGAAGAAGCACCCGGATG-3′	58
	R: 3′-CATAACGAGCCTTGTCAGCC-5′	
CD68	F: 5′-GCATCTTGTACCTGACCCAG-3′	58
	R: 3′-GGGAATGAGAGAGCCAAGTG-5′	
LGAL3	F: 5′-CAAAGGGGAAGCTGACTGT-3′	58
	R: 3′-CGACATCGCCTTCCACTTTA-5′	
IFNγ	F: 5′-TCGAATCGCACCTGATCACT-3′	58
	R: 3′-CTTTGTGCTGGATCTGTGGG-5′	
TNFα	F: 5′-ATGAGCACGGAAAGCATGA-3′	58
	R: 3′-GAGCCAGGAATGAGAAGAGGC-5′	
TLR2	F: 5′-TGGAACTGATGGAGGTGGAG-3′	58
	R: 3′-GATGTGCAGGCTCCGTATTG-5′	
TLR4	F: 5′-CCAGAGCCGTTGGTGTATCT-3′	58
	R: 3′-TACAATTCGACCTGCTGCCT-5′	
TLR9	F: 5′-TCCCTTCGAGTGCTTGATGT-3′	58
	R: 3′-ATGGAAAGTCTGAGGGTGCA-5′	
IL-6	F: 5′-GCAAGAGACTTCCAGCCAGT-3′	58
	R: 3′-TACTGGTCTCTTGTGGGTGG-5′	
IL-18	F: 5′-TGGCTGTGACCCTATCTGTG-3′	58
	R: 3′-CCTGGCACACGTTTCTGAAA-5′	
TGFβ	F: 5′-GTGGAGCAACACGTAGAACT-3′	58
	R: 3′-ACGTCAAAAGACAGCCACTC-5′	
IL1β	F: 5′-ATCTCACAGCAGCATCTCGA-3′	58
	R: 3′-AAAGAAGGTGCTTGGGTCCT-5′	
GAPDH	F: 5′-AACGACCCCTTCATTGACCT-3′	58
	R: 3′-TGACCAGCTTCCCATTCTCA-5′	

F: forward (5′-3′), R: reverse (3′-5′), HMGB: high mobility group box, CD: cluster of differentiation, IFN: interferon, LGAL: galectin 3, TNF: tumor necrosis factor, TLR: toll-like receptor, IL: interleukin, TGF: transforming growth factor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, PCR: polymerase chain reaction.

bodies against HMGB-1 and galectin-3 (Fig. 2A). IHC staining showed that the expression of HMGB-1 (P = 0.044) and galectin-3 (P = 0.002) had significantly increased in the Sevo-6h group compared with that in the control group (Fig. 2B). Similarly, serum ELISA results confirmed that HMGB-1 and galectin-3 had significantly increased in the Sevo-6h group compared with levels in the control group, whereas heparan sulfate levels were not significantly different (Fig. 2C). Based on these results, we confirmed that microglial cells were activated by 6 h of sevoflurane exposure, possibly mediated by HMGB-1 and galectin-3.

Identification of DAMPs pathway activated by sevoflurane exposure via TLRs

After confirming that exposure to sevoflurane for 6 h significantly increased the expression of HMGB-1 and galectin-3, we conducted a second experiment to identify the specific DAMPs pathway. We performed an IF assay on the TLRs known to be receptors for HMGB-1 and galectin-3. We evaluated the TLR expression by IF analysis using anti-TLR2, TLR4, and TLR9 antibodies. The nuclei were stained with DAPI in blue. The expression of TLR2 was hardly visible, but there was a visible increase in TLR4 and TLR9; specifically, TLR9 showed more robust expression than TLR4 (Fig. 3). We also performed IF analysis of phosphorylated NF-κB, a known downstream signal transcription factor of the TLRs. The expression of phosphorylated NF-κB also increased in the sevoflurane group compared with that in the control group (Fig. 4).

Effect of NSAIDs on sevoflurane-induced neuroinflammation

Lastly, we assessed the effect of NSAIDs on sevoflurane-induced neuroinflammation by measuring the relative expression of CD68, galectin-3, and TLR9 in P7 brain tissue exposed to sevoflurane for 6 h with ibuprofen injections using quantitative real-time PCR (qPCR; Figs. 5A–C). Consistent with our first and second experiments, 6 h of sevoflurane exposure increased the transcription level of CD68, galectin-3, and TLR9. However, the expressions of CD68, galectin-3, and TLR9 were significantly lower than in the IBU group compared with that in the sevoflurane-only (Sevo-6h) group. We also conducted qPCR for pro-and anti-inflammatory cytokines related to the DAMPs pathway (Figs. 5D–F). The level of proinflammatory TNF-α and IL-6 cytokines was significantly higher in the Sevo-6h group compared with that in the control group, whereas it was attenuated in the IBU group. The expression level of IFN-γ, a cytokine associated with innate immunity, was significantly lower in the Sevo-6h group and showed recovery in the IBU group. There was no difference in transcription levels of TLR2, TLR4, IL-18, TGF-β, and IL-1β between the Sevo-6h group and the IBU group (Supplementary Fig. 1).

We performed western blot analysis on brain tissue and serum ELISA to confirm our qPCR results (Fig. 6). Consistent with the qPCR findings, we observed a significant increase in the protein levels of CD68 and galectin-3 in the Sevo-6h group compared with that in the control group. Notably, the galectin-3 levels were significantly reduced in the IBU group compared with that in the

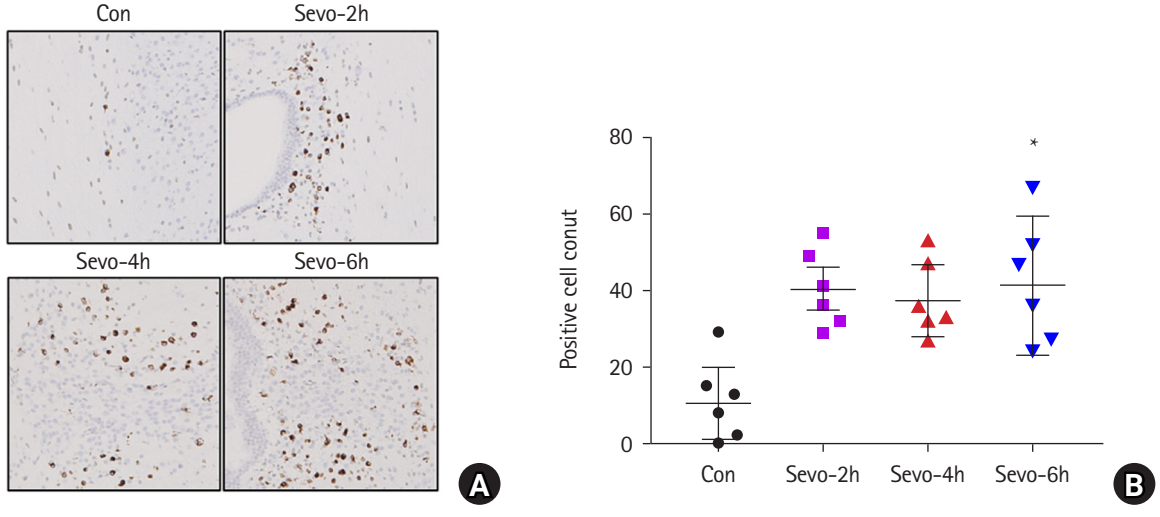

Fig. 1. Comparison of CD68 expression according to sevoflurane exposure time in P7 brain tissue. (A) Representative IHC image stained with anti-CD68. (B) Quantification of CD68-positive cells between the four exposure groups. Values are presented as median (Q1, Q3). *P < 0.05 vs. Con group. CD68: cluster of differentiation 68, Con: control group, IHC: immunohistochemistry, P7: aged 7 days, Sevo-2h: exposure to sevoflurane for 2 h, Sevo-4h: exposure for 4 h, Sevo-6h: exposure for 6 h.

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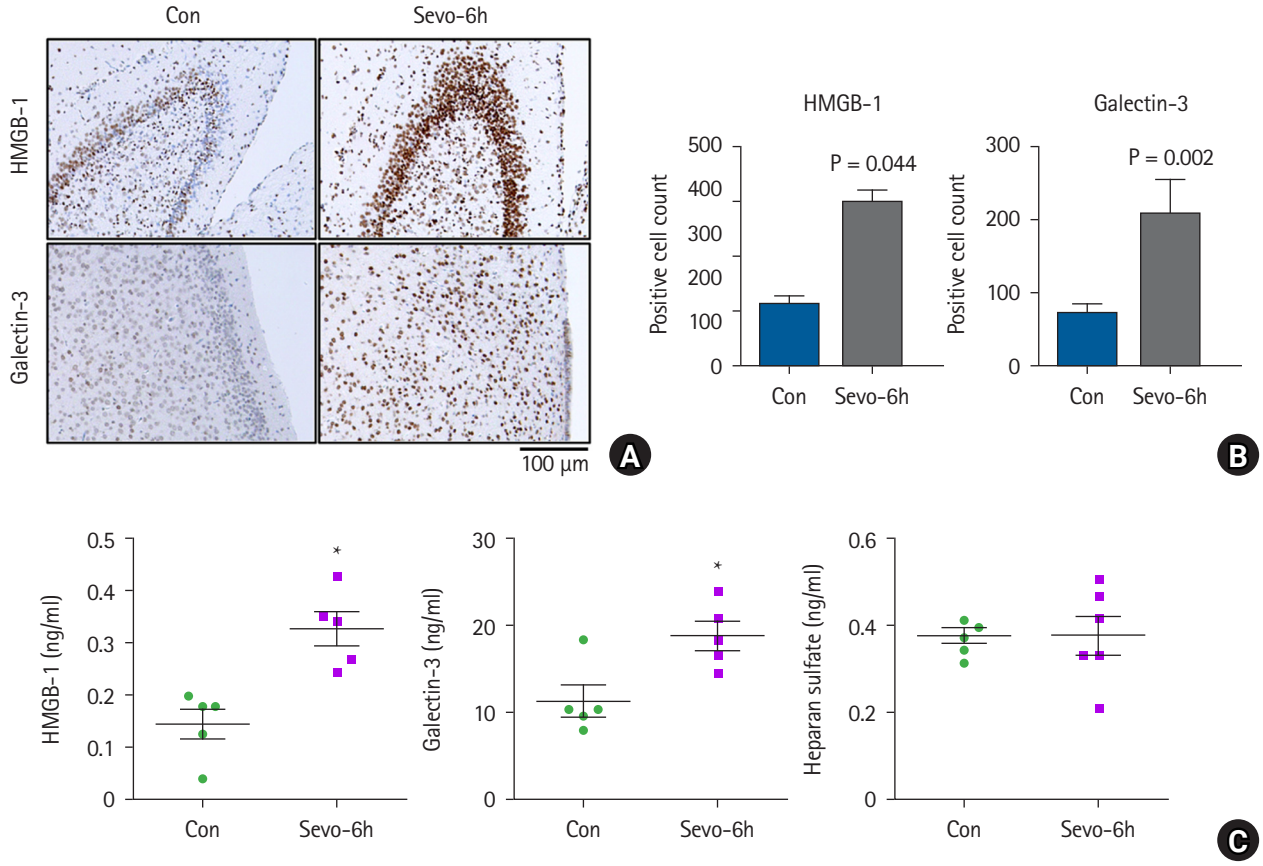

Fig. 2. Expression of DAMP molecules in P7 brain tissue and blood serum. (A) Representative IHC image stained with anti-HMGB-1 and anti-galectin-3 in brain tissue. (B) Quantification of positive cell count of HMGB-1 and galectin-3 in brain tissue. (C) Quantification of serum ELISA concentration with anti-HMGB-1, galectin-3, and heparan sulphate in P7 rat blood serum. Values are presented as median (Q1, Q3). *P < 0.05 vs. Con group. Con: control group, DAMPs: damage-associated molecular patterns, ELISA: enzyme-linked immunosorbent assay, HMGB-1: high mobility group box 1, IHC: immunohistochemistry, P7: aged 7 days, Sevo-6h: exposure to sevoflurane for 6 h.

Sevo-6h group, whereas the CD68 levels showed no significant difference between the IBU group and the Sevo-6h group (Fig. 6A). Furthermore, the serum ELISA results reaffirmed that the HMGB-1 and galectin-3 levels were significantly higher in the Sevo-6h group but significantly lower in the IBU group, whereas the IFN-γ levels were significantly higher in the IBU group (Fig. 6B).

Discussion

Our study confirmed sevoflurane-induced neuroinflammation and activation of the microglial cells in the neonatal rat brain. The degree of neuroinflammation was related to the anesthesia exposure time. We also showed that the activation of microglial cells was associated with an increase in DAMP-related molecules, HMGB-1, and galectin-3, through TLRs and the NF-κB pathway. The combined use of ibuprofen with sevoflurane reduced neuroinflammation, revealing a potentially protective effect.

Through this study, we confirmed the occurrence of neuroinflammation induced by sevoflurane by using CD68 expression. The expression of CD68 did not increase significantly in the group exposed to sevoflurane for 2 h or 4 h but significantly increased in the group exposed for 6 h. This is a consistent with the result of previous study showing that short-duration, single-dose anesthetic drug exposure did not significantly affect neuro-impairment in the developing brain [24].

In the present study, we confirmed that sevoflurane-induced neuroinflammation was mediated by HMGB-1 and galectin-3. In clinical studies, the HMGB-1 levels were increased in the cerebrospinal fluid of patients with subarachnoid hemorrhage, and that of TNF-α, IL-6, and IL-8 were correlated with this increase. The HMGB-1 level is also associated with poor outcomes in patients with subarachnoid hemorrhage [25]. Furthermore, HMGB-1 is reportedly a common biomarker for neurologic dysfunctions such as traumatic brain injury, epilepsy, cognitive dysfunction,

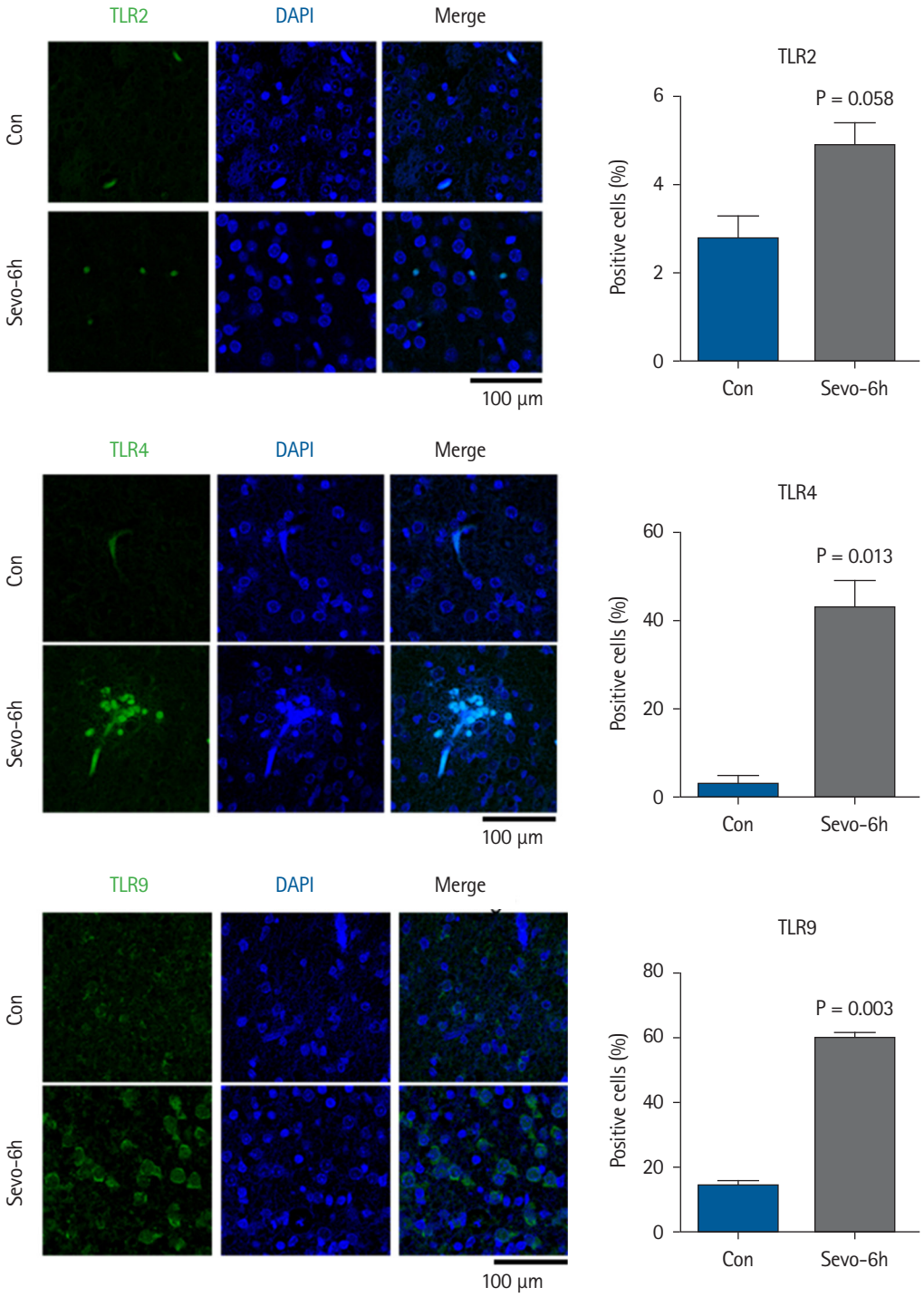

Fig. 3. Expression levels and quantification of TLRs 2, 4, and 9 in the P7 rat brain assessed using IF. Values are presented as median (Q1, Q3). Con: control group, DAPI: 4′,6-diamidino-2-phenylindole, IF: immunofluorescence, P7: aged 7 days, Sevo-6h: exposure to sevoflurane for 6 h, TLR: toll-like receptor.

and neuroinflammation [13]. Galectin-3 is upregulated in mice with Alzheimer's disease and is related to Huntington's disease in humans [26,27]. Galectin-3 levels have been considered as a new prognostic factor for patients with Parkinson's disease [28]. Considering these points, the degree of sevoflurane-induced neuroinflammation might be predicted through the level measurement of HMGB-1 or galectin-3 through serum ELISA, and it could be considered a prognostic factor for cognitive dysfunction.

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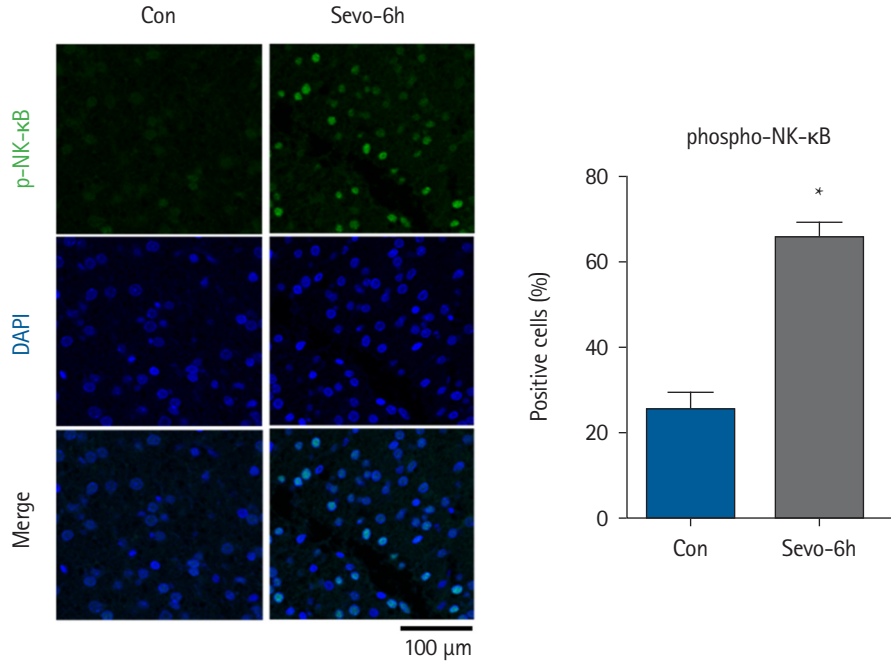

Fig. 4. Expression levels and quantification of phosphorylated NF-κB assessed using IF according to sevoflurane exposure time in the P7 brain tissue. Values are presented as median (Q1, Q3). *P < 0.05 vs. Con group. Con: control group, DAPI: 4′,6-diamidino-2-phenylindole, IF: immunofluorescence, P7: aged 7 days, p-NF-κB: phosphorylated nuclear factor κB, Sevo-6h: exposure for 6 h.

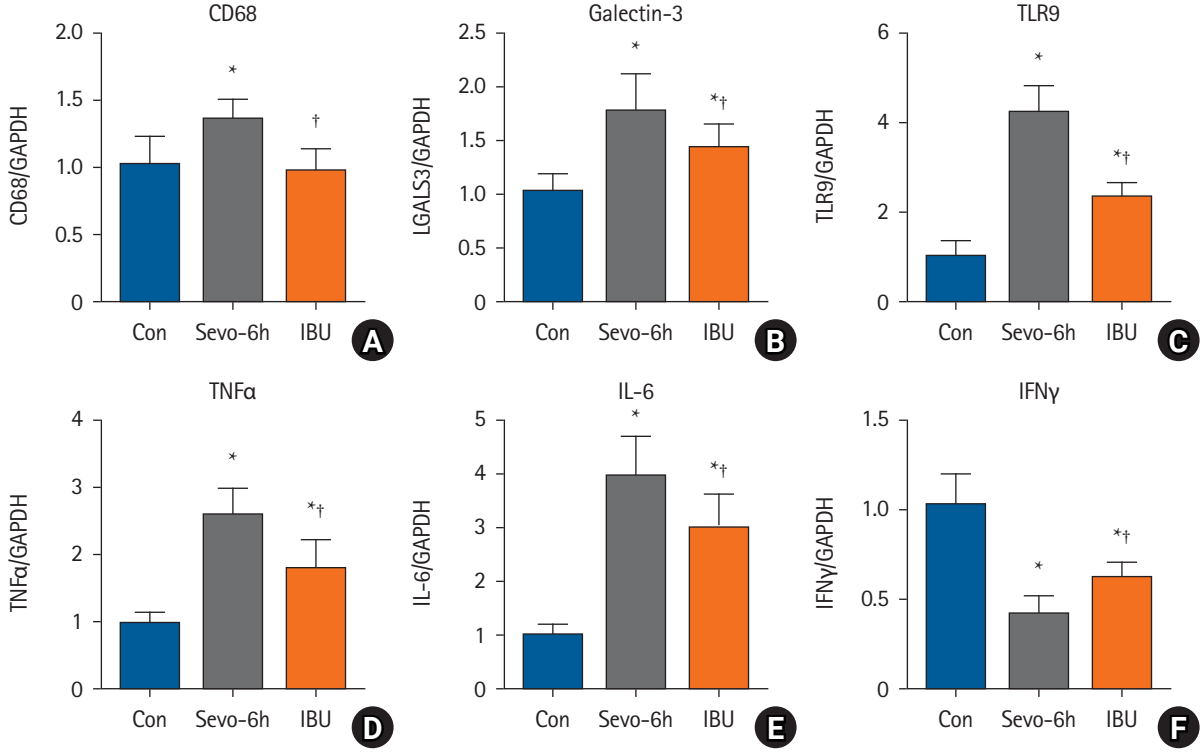

Fig. 5. Effect of ibuprofen on the expression of DAMPs pathway and the level of cytokines. (A-F) Quantification of mRNA levels of CD68, galectin-3, TLR9, TNF-α, IL-6, and IFN-γ, normalized to GAPDH, and assessed using quantitative PCR. Values are presented as median (Q1, Q3). *P < 0.05 vs. Con group. †P < 0.05 vs. Sevo-6h group. CD68: cluster of differentiation 68, Con: control group, DAMPs: damage-associated molecular patterns, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, IBU: administration of ibuprofen, IFN-γ: interferon-gamma, IL-6: interleukin-6, PCR: polymerase chain reaction, Sevo-6h: exposure to sevoflurane for 6 h, TLR9: toll-like receptor 9, TNF-α: tumor necrosis factor-alpha.

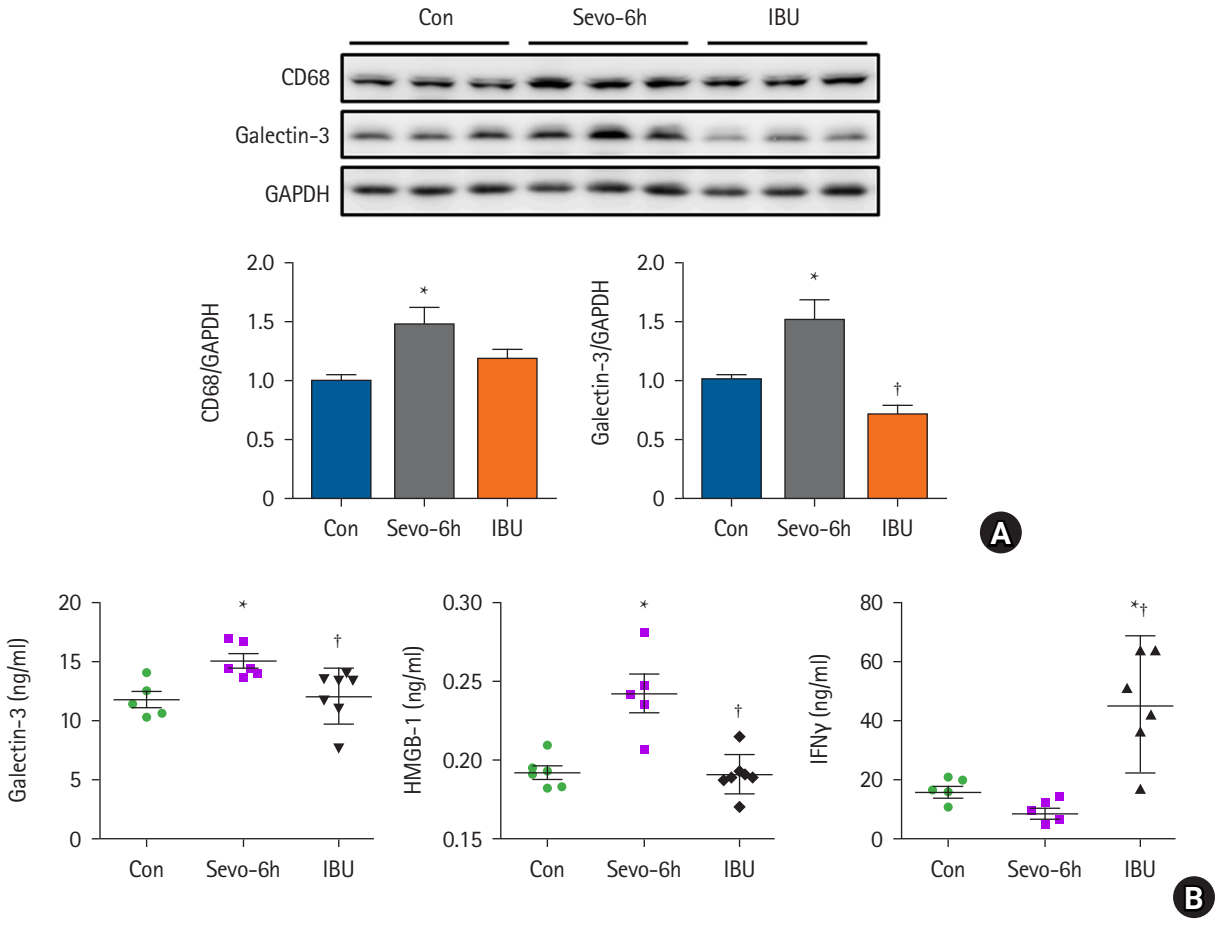

Fig. 6. Effects of ibuprofen on the expression of CD68 and galectin-3 in the P7 rat brain tissue and serum. (A) Representative western blots and quantification of protein levels of CD68 and galectin-3 normalized to GAPDH. (B) Blood serum levels of galectin-3, HMGB-1, and IFN-γ. Values are presented as median (Q1, Q3). *P < 0.05 vs. Con. group, †P < 0.05 vs. Sevo-6h group. CD68: cluster of differentiation 68, Con: control group, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, HMGB-1: high mobility group box 1, IBU: administration of ibuprofen with Sevo-6h, IFN-γ: interferon-gamma, P7: aged 7 days, Sevo-6h: exposure to sevoflurane for 6 h.

HMGB-1 acts as a non-histone DNA binding transcription factor present in all nucleated cells, and it can act as a danger signal when released from damaged cells into the extracellular space. The released HMGB-1 is recognized by pattern recognition receptors (PRRs) such as TLR2, TLR4, and TLR9, and receptors for advanced glycation end-products (RAGE). Like HMGB-1, galectin-3 activates the pro-inflammation pathway via TLR2 and TLR4 [29]. In this study, TLR4 and TLR9 were identified as PRRs of HMGB-1 and galectin-3 (Fig. 3). TLR4 was suggested as a major proinflammatory pathway for HMGB-1 and galectin-3 in previous studies [13,30]; however, these studies induced neuroinflammation with lipopolysaccharide, whereas our study induced neuroinflammation with sevoflurane.

We also confirmed that the signal was transmitted downstream by NF-κB (Fig. 4). The expression of NF-κB significantly differed from that of the control group at 2, 4, and 6 h after sevoflurane exposure, whereas the expression of CD68 only showed a difference in the Sevo-6h group (Fig. 1). Considering this difference, further research is required to establish the threshold duration for sevoflurane-induced neuroinflammation.

Ibuprofen is the most widely studied and used NSAID in children for the management of acute pain and is the only NSAID approved for use in children as young as six months [31,32]. In the cell, ibuprofen exhibits anti-inflammatory effects through cell cycle modulation and apoptosis in activated microglial cells [33]. In the Parkinson's mice model, ibuprofen administration reduced dopaminergic neuron reduction, decreased microglial cells, decreased proinflammatory cytokines (TNF-α, IL-6), and improved behavioral changes [34]. In this study, ibuprofen reduced the proinflammatory mediators (TNF-α, IL-6) that were increased by sevoflurane and showed a restorative effect on the expression of decreased IFN-γ (Fig. 5). Furthermore, co-administering ibupro-

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fen with sevoflurane reduced the HMGB-1 and galectin-3 expression in the rats (Fig. 6). The treatment with ibuprofen resulted in a decrease in the galectin-3 and HMGB-1 expression and an increase in the IFN-γ expression; however, no difference was observed in CD68 expression compared with that in the Sevo-6h group, indicating the need for additional research on the appropriate dosage.

A strength of this study is that it examined the effects of sevoflurane, the most widely used anesthetic agent in pediatric anesthesia and that it assessed mediators in the DAMPs pathway. In addition, by revealing the effect of ibuprofen on neuroinflammation, an in vitro rationale was provided for safe pediatric anesthesia in future clinical fields. Lastly, serum HMGB-1 and galectin-3 were presented as prognostic factors for neuroinflammation induced by anesthetic agents.

First, this study's limitation is that it is difficult to apply directly to the clinical field as it is an experimental animal study [35]. Nevertheless, it is crucial to weigh the potential implications, especially considering the millions of children worldwide who undergo surgical procedures requiring anesthesia [21]. While the six-hour sevoflurane exposure investigated in this study may seem prolonged in the context of clinical practice, it aligns with the common duration employed in toxicology animal studies [36]. In addition, in rare instances, anesthetic drugs may be administered in high concentrations for prolonged periods or repeatedly. Although the animal study conditions may not exactly match the clinical situations, the research can be considered to be still valuable in terms of its scientific implications.

Second, we were unable to perform additional experiments to verify the long-term impact of sevoflurane-induced neuroinflammation on cognitive dysfunction or behavior. Nevertheless, previous research has revealed that sevoflurane exposure causes neuroinflammation in the brain by activating microglial cells [14]. Our study proposes that it does so via the DAMPs pathway, using HMGB-1 and gelatin-3. As HMGB-1 and gelatin-3 are already known to be linked to poor prognoses in other brain diseases in animal and human studies [13,25–28], serum concentration of these two factors has the possibility to be used as a biomarker to predict long-term neurological issues in humans. Additional research is required to establish the specific concentration, exposure duration, and threshold at which sevoflurane induces neuroinflammation and long-term consequences in pediatric patients.

Third, loss-of-function experiments with HMGB-1 or galectin-3 were not conducted. Such additional experiments could clarify the roles of HMGB-1 and galectin-3 in neuroinflammation induced by anesthetics. Lastly, factors like surgical stress were not considered, and more research is needed for clinical application.

In conclusion, sevoflurane-induced neuroinflammation was mediated by the activation of microglial cells, particularly HMGB-1 and galectin-3 in the DAMPs pathway. Co-administration of ibuprofen may result in a reduction of neuroinflammation in this study. Further investigation is needed to ascertain whether the findings of this study can be generalized to other NSAIDs and, if applicable, to establish the appropriate dosage and duration.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Data Availability

The data that support the findings of this study are available upon request to the corresponding author. The data are not publicly available owing to privacy and ethical considerations.

Author Contributions

Young-Eun Joe (Investigation; Writing – original draft)
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Supplementary Material

Supplementary Fig. 1. Effect of ibuprofen on the expression of DAMPs pathway and the level of cytokines. (A-E) Quantification of mRNA levels of TLR2, TLR4, IL-1β, IL-18, and TGF-β, normalized to GAPDH and assessed using quantitative PCR. Values

are presented as median (Q1, Q3). *P < 0.05 vs. Con group. Con: control group, DAMPs: damage-associated molecular patterns, IBU: administration of ibuprofen, IL-1β: interleukin-1-beta, IL-18: interleukin-18, Sevo-6h: exposure to sevoflurane for 6 h, TLR2: toll-like receptor 2, TLR4: toll-like receptor 4, TGF-β: transforming growth factor-beta.

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