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

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Directed by Professor Jeong-Rim Lee

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ABSTRACT

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

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(Directed by Professor Jeong-Rim Lee)

General anesthesia is inevitable for pediatric patients undergoing surgery, though volatile anesthetic agents may cause neuroinflammation and be related to neurodevelopmental impairment; however, the underlying pathophysiology remains unclear. We hypothesized that sevoflurane induces neuroinflammation in the developing brain which is related to specific damage-associated molecular patterns (DAMPs) Therefore, we aimed to investigate the mechanism of neuroinflammation in developing rat brains and their association with sevoflurane exposure time, by identification of the specific DAMP pathway, and evaluate the effects of non-steroidal anti-inflammatory drugs (NSAIDs) in alleviating neuroinflammation.

We conducted a three-step experiment to investigate neuroinflammation induced by sevoflurane. The first step involved determining the exposure time required for sevoflurane to cause neuroinflammation. In the second step, we identified the specific pathway of DAMPs that were involved in neuroinflammation by sevoflurane. Finally, in the third step, we investigated the effects of NSAIDs on sevoflurane-induced neuroinflammation. 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 (ELISA).

We utilized a total of 112 age of seven days rats for our study, out of which six rats expired during the experiment, resulting in a mortality rate of 5.3%. We observed a significant increase in the expression of CD68; HMGB-1 and galectin-3; and TLR4, TLR9, and phosphorylated NF-κB upon 6 hr sevoflurane exposure. Conversely, the transcriptional levels of TNF-α and IL-6 significantly increased, and IFN-γ significantly decreased after 6 hr of sevoflurane exposure. Co-administration of NSAIDs while sevoflurane anesthesia significantly attenuated TNF-α and IL-6 levels and restored IFN-γ levels. The protective effect of NSAIDs was repeatedly valicated using western blot analysis and serum ELISA.

Collectively, the findings suggest that 6 hr sevoflurane exposure induces neuroinflammation through the DAMPs pathway neuropeptides, HMGB-1 and galectin-3, in neonatal rat brains. The co-administration of NSAIDs reduced this sevoflurane-induced neuroinflammation.

Key words : sevoflurane, neuroinflammation, damage-associated molecular patterns, high mobility group box-1, galectin-3, non-steroidal anti-inflammatory drugs

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I. INTRODUCTION

Every year, tens of thousands of children worldwide require surgery to save their lives 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 the concerns that almost all anesthetic drugs currently used have been linked to unfavorable effects, such as apoptosis of neuronal cells, inappropriate synaptic formation, changes in dendritic architecture, inhibition of myelination, and faulty axonal targeting, on the developing brain or neuronal system. [1-6] Moreover, early life-time 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 disease 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), which are regulated by microglial cells. [16,17] Microglial cells are resident macrophages present in the central nervous system (CNS) 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 commonly used inhalational anesthetic in pediatric surgery, several studies also suggested neuroinflammation as a mechanism of anesthetic-induced brain damage in preclinical neonatal research.[19] 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.

II. MATERIALS AND METHODS

1. Animal preparation

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University College of Medicine (IACUC 2017-0137). All studies reported herein were conducted in accordance with ARRIVE guidelines (https://arriveguidelines.org). A total of 23 gravid Wistar rats were obtained from the central laboratory of SLC Inc. (Hamamatsu, Shizuoka 431-1103, Japan). A total of one hundred and twelve 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-hr 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), offspring were kept with their parents until day seven of life (P7), and litter sizes ranged from 8–14 animals. At least one animal from each litter was assigned to each treatment group, and their body weights ranged from 9.5–15.5 g. Animals of both sexes were used in the study.

2. 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 four groups: a control group without sevoflurane exposure (Con), a 2 hr sevoflurane group (Sevo-2hr), a 4 hr sevoflurane group (Sevo-4hr), and a 6 hr sevoflurane group (Sevo-6hr). The sevoflurane groups were exposed to 2.5% sevoflurane with 30%

oxygen for the assigned time period, 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-6hr group in each set. As in the first experiment, the Sevo-6hr group was exposed to 2.5% sevoflurane with 30% oxygen for 6 hr. 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-6hr group, and seven in the ibuprofen group (IBU) in each set. The Sevo-6hr group was exposed to 2.5% sevoflurane for 6hr, and the IBU group was treated with a combination of 2.5% sevoflurane for 6 hr and intra-peritoneal injections of ibuprofen at 30 mg/kg. 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. Sevoflurane-anesthetized animals were briefly removed from the anesthetizing chamber for injections. The allocation of the number of used rats was summarized in table 1.

Table 1. The allocation of number of used P7 rats

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

Con, control group; IBU, administration of ibuprofen; P7, age of seven days; Sevo-2hr, exposure to sevoflurane for 2 hr; Sevo-4hr, exposure for 4 hr; Sevo-6hr, exposure for 6 hr

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-minute 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). After anesthesia, the sternum was incised, and blood serum was obtained from the heart. The rat was then perfused using a phosphate-buffered saline (PBS) solution, including heparin, and sacrificed. Brain tissue was obtained for analysis and stored at −80 ºC.

3. Immunohistochemistry

The level of 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 immunohistochemistry (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, Cambridge, UK), anti-HMGB-1 (1:100; Cell Signaling Technologies, Danvers, MA, USA), and anti-galectin-3 (1:500; LSBio, Seoul, South Korea). The staining was followed by using 3,3′-diaminobenzidine as a chromogen (Abcam, Cambridge, UK). The slides were then viewed using an Olympus IX73P2F microscope (Olympus America, NY, USA) equipped with an Olympus DP71 digital camera (20X magnification).

4. Immunofluorescence

Tissue samples for immunofluorescence (IF) 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., Dallas, Texas, USA), anti-TLR4 (1:100; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), anti-TLR9 (1:100; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), and anti-phospho-nuclear factor kappa B (p-NF-κB, 1:100; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) 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 hr at room temperature protected from light. Cells were counterstained with 4′,6-diamidino-2-phenyldole (DAPI, 1:2,500; Molecular Probe Inc., Eugene, Oregon, USA). Images were obtained using a Carl Zeiss LSM700 (Carl Zeiss, Oberkohen, Germany) confocal microscope fitted with the appropriate filters.

5. Enzyme-linked immunosorbent assay

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

6. Western blot analysis

Obtained brain tissues were washed with cold phosphate-buffered saline, minced, and lysed in a cell lysis buffer (Cell Signaling Technologies, Danvers, MA, USA) containing protease inhibitors (10 μg/mL each of aprotinin, 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, CA, USA). Whole-cell extracts (50 μg) were separated on 10–15% sodium dodecyl sulfate–polyacrylamide electrophoresis gels and transferred to Immobilon P-transfer membranes (Millipore, Burlington, MA, USA). The membranes were then incubated with primary antibodies, which were detected using a horseradish peroxidase HRP-conjugated IgG antibody (Cell Signaling Technologies, Danvers, MA, USA). Bands were determined using West Glow FEMTO Chemiluminescent Substrate (BIOMAX; Seoul, South Korea). The primary antibodies

used targeted CD68 (1:1000; Abcam, Cambridge, UK), HMGB-1 (1:1000; Abcam, Cambridge, UK), galectin-3 (1:1000; LSBio, Seoul, South Korea), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000; Cell Signaling Technologies, Danvers, MA, USA) as loading controls.

7. Quantitative real-time polymerase chain reaction

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, Maryland, USA). Complementary DNA was synthesized from 2 μg of total RNA using AccuPower RT premix kits (BIONEER, Daejeon, South Korea). Real-time polymerase chain reaction (PCR) analysis was performed using TB GreenTM Premix Ex Taq II kit (Takara Bio Inc., Kusatsu, Japan) and QuantStudio3 (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' 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 normalized cycle of normalization values for the control groups. The primer sequences for real-time PCR are given in Table 2.

Table 2. Primers used in real-time PCR.

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

F, forward (5′-3′); R, reverse (3′-5′); HMGB, high mobility group box; CD, cluster of

differentiation; IFN, interferon; TNF, tumor necrosis factor; TLR, toll-like receptor; IL, interleukin; TGF, transforming growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

8. Statistical analysis

Each in vivo assay was performed in triplicate and repeated at least thrice. All data from the in vivo experiments are presented as the median ± interquartile range deviation and were analyzed using one-way analysis of variance with Bonferroni's correction applied to post hoc multiple comparisons tests. *P*-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

III. 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 maintenance of sevoflurane anesthesia.

1. 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 (Figure 1). The expression of CD68 had significantly increased after exposure to sevoflurane for 6 hr compared with that in the control group. However, no significant differences were observed between the controls and the 2 hr or 4 hr exposure groups. To further assess the activation of microglial cells via the DAMPs pathway, we conducted brain IHC and serum ELISA using antibodies against HMGB-1 and galectin-3 (Figure 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-6hr group compared with that in the control group (Figure 2B). Similarly, serum ELISA results confirmed that HMGB-1 and galectin-3 had significantly increased in the Sevo-6hr group compared with that in the control group, whereas heparan sulfate levels were not significantly different (Figure 2C). Based on these results, we confirmed that microglial cells were activated by 6 hr of sevoflurane exposure, possibly mediated by HMGB-1 and galectin-3.

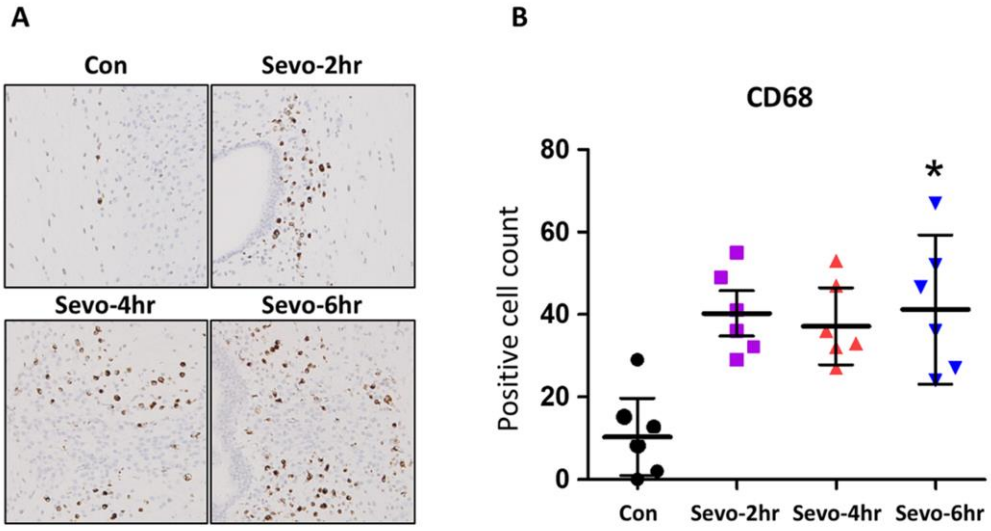


Figure 1. Comparison of CD68 expression according to sevoflurane exposure time in P7 brain tissue. (A) Representative immunohistochemistry image stained with anti-CD68. (B) Quantification of CD68-positive cells between the four exposure groups. Values are presented as median and IQR. *P<0.05 vs. Con group. CD68, cluster of differentiation 68; Con, control group; IQR, interquartile range; P7, age of seven days; Sevo-2hr, exposure to sevoflurane for 2 hr; Sevo-4hr, exposure for 4 hr; Sevo-6hr, exposure for 6 hr.

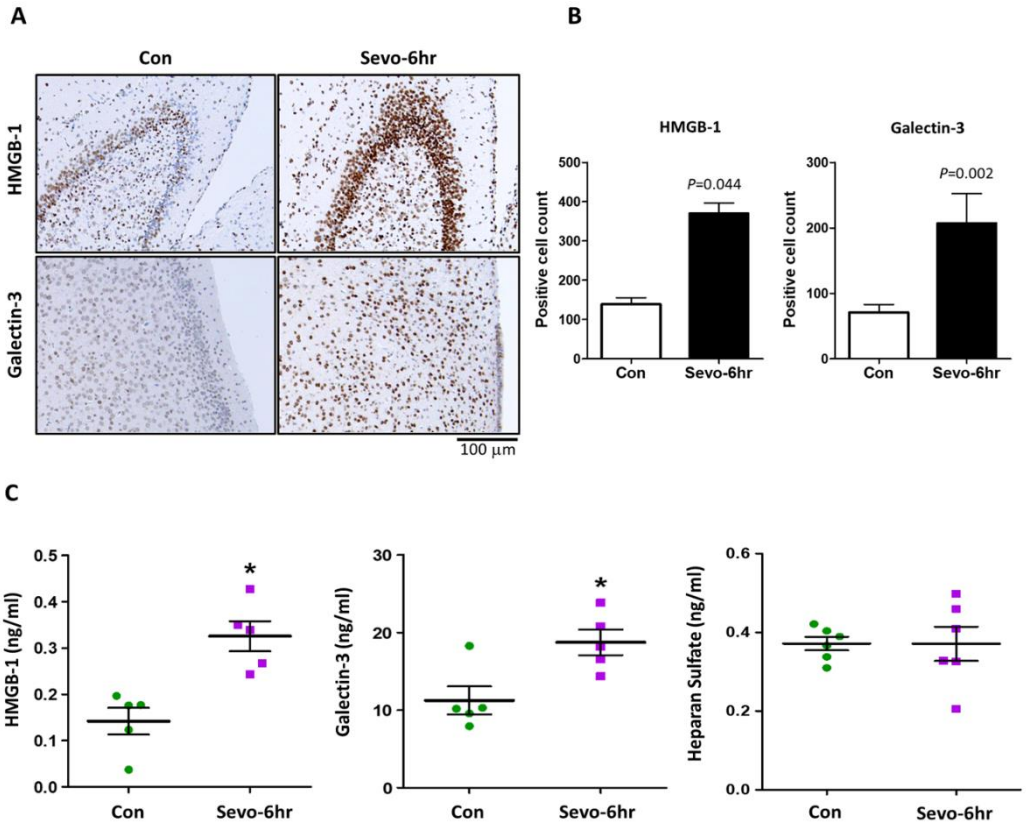
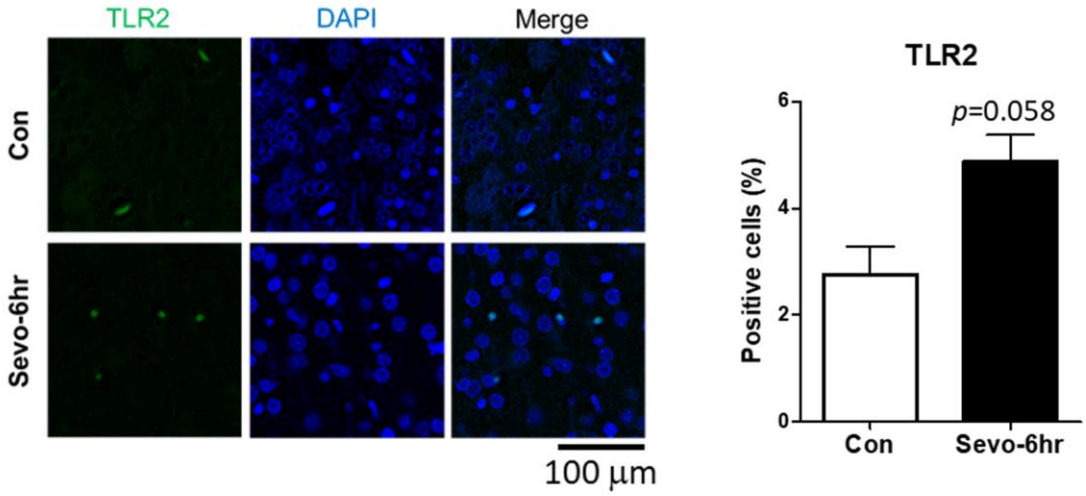


Figure 2. Expression of DAMPs molecules in P7 brain tissue and blood serum. (A) Representative immunohistochemistry image stained with anti-HMGB-1 and anti-galectin-3 in brain tissue. (B) Quantification of positive cell count of HMGB-1 and galectin-3 in brain tissue. (C) Quantification of serum ELISA concentration with anti-HMGB-1, galectin-3, and heparan sulfate in P7 rat blood serum. Values are presented as median and IQR. *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; IQR, interquartile range; P7, age of seven days; Sevo-6hr, exposure to sevoflurane for 6 hr.

2. Identification of DAMPs pathway activated by sevoflurane exposure via TLRs

After confirming that exposure to sevoflurane for 6 hr 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 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 it had visibly increased in TLR4 and TLR9; specifically, TLR9 showed more robust expression than TLR4 (Figure 3). We also performed IF analysis of phosphorylated NF-κB, which is known as a downstream signal transcription factor of TLRs. The expression of phosphorylated NF-κB also increased in the sevoflurane group compared with that in the control group (Figure 4).



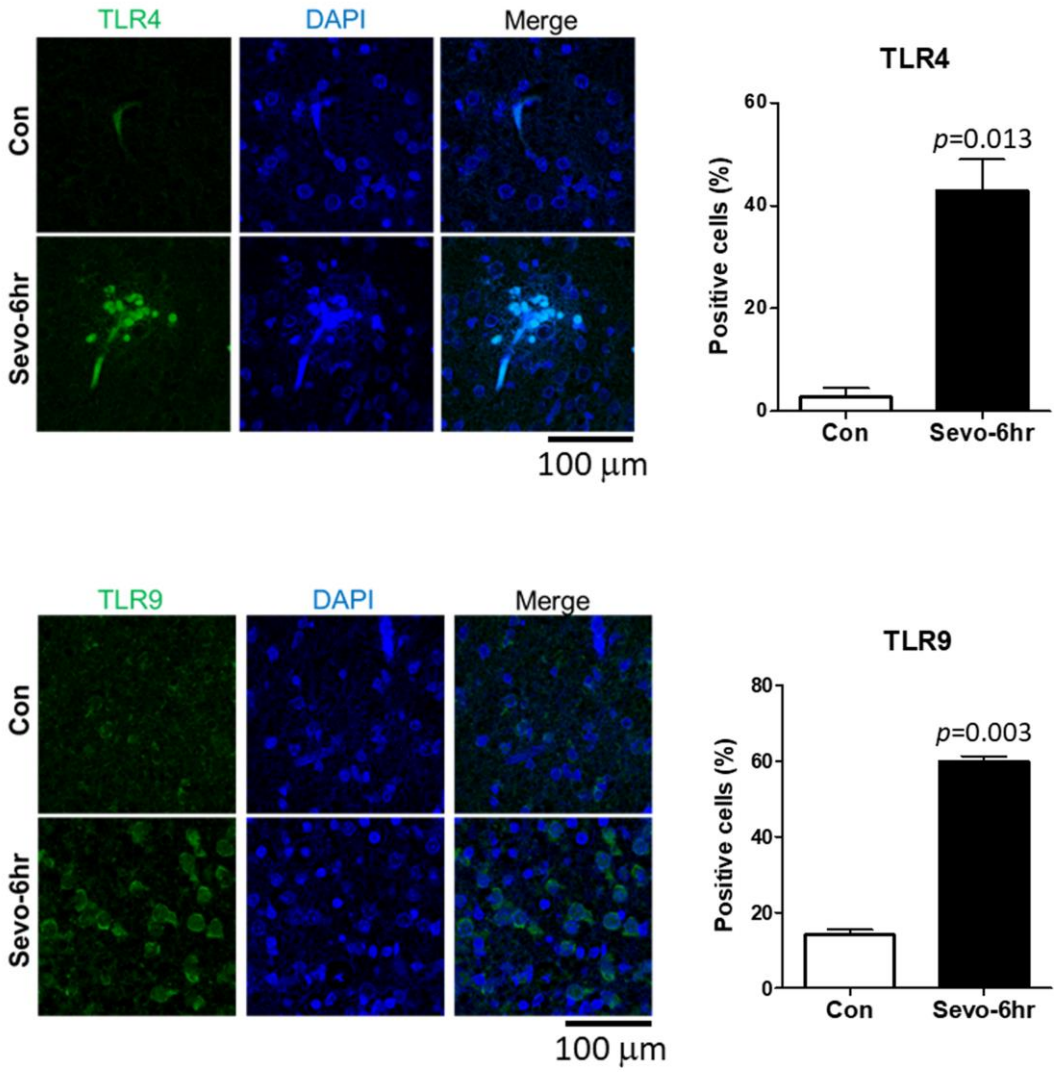


Figure 3. Expression levels and quantification of TLRs 2, 4, and 9 in the P7 rat brain were assessed using immunofluorescence. Values are presented as median and IQR. Con, control group; DAPI, 4',6-diamidino-2-phenylindole; IQR, interquartile range; P7, age of seven days; Sevo-6hr, exposure to sevoflurane for 6 hr; TLR, toll-like receptor.

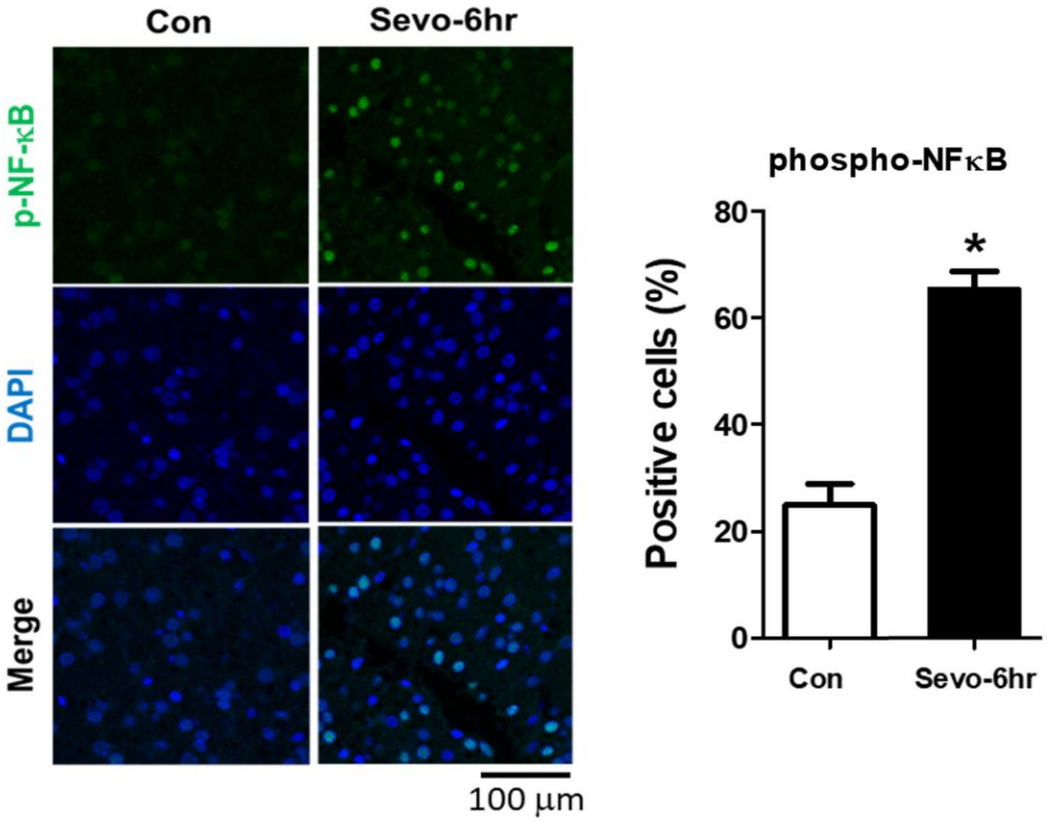


Figure 4. Expression levels and quantification of phosphorylated NF-κB assessed using immunofluorescence according to sevoflurane exposure time in the P7 brain tissue. Values are presented as median and IQR. *P<0.05 vs. Con group. Con, control group; DAPI, 4',6-diamidino-2-phenylindole; P7, age of seven days; IQR, interquartile range; phospho-NF-κB, phosphorylated nuclear factor κB; Sevo-6hr, exposure for 6 hr.

3. 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 hr with ibuprofen injections using quantitative real-time PCR (qPCR; Figure 5A-C). Consistent with our first and second experiments, 6 hr of sevoflurane exposure increased the transcription level of CD68, galectin-3, and TLR9. However, the expression of CD68, galectin-3, and TLR9 had significantly lower than in the IBU group compared with that in the sevoflurane-only (Sevo-6hr) group. We also conducted qPCR for pro- and anti-inflammatory cytokines related to the DAMPs pathway (Figure 5D-F). The level of pro-inflammatory TNF-α and IL-6 cytokines was significantly higher in the Sevo-6hr 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-6hr 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-6hr group and the IBU group (Figure 6).

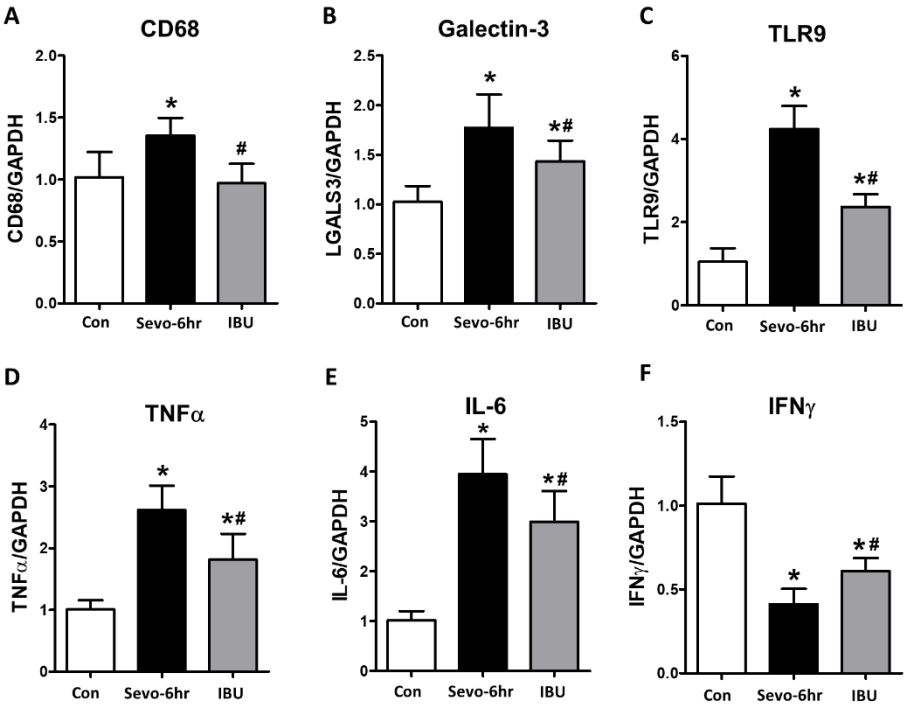


Figure 5. Effect of ibuprofen on the expression of DAMPs pathway and the level of cytokines. (A-F) Quantification of mRNA levels of CD68, galectin-3, TLR9, TNF-α, IL-6, and IFN-γ, normalized to GAPDH and assessed using quantitative PCR. Values are presented as median and IQR. **P*<0.05 vs. Con group, #*P*<0.05 vs. Sevo-6hr 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; IQR, interquartile range; PCR, polymerase chain reaction; Sevo-6hr, exposure to sevoflurane for 6 hr; TLR9, toll-like receptor 9; TNF-α, tumor necrosis factor-alpha.

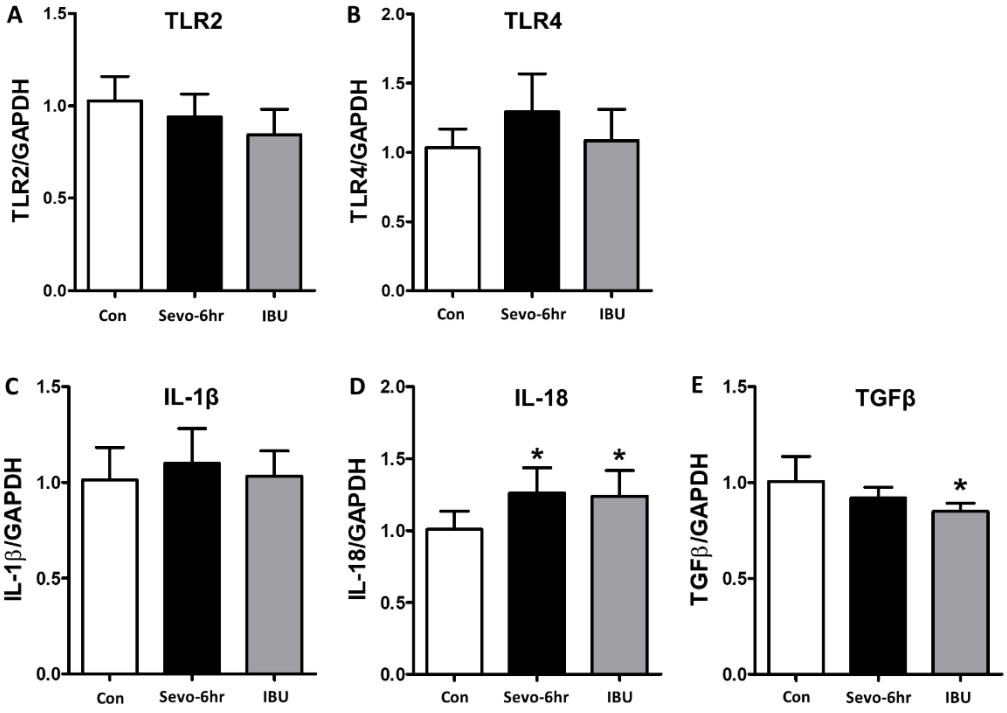


Figure 6. Effect of ibuprofen on the expression of DAMPs pathway and the level of cytokines. (A-E) Quantification of mRNA levels of TLR2, TLR4, IL-1β, IL-18, and TGF-β, normalized to GAPDH and assessed using quantitative PCR. Values are presented as median and IQR. **P*<0.05 vs. Con group. Con, control group; DAMPs, damage-associated molecular patterns; IBU, administration of ibuprofen; IL-1β, interleukin-1-beta; IL-18, interleukin-18; IQR, interquartile range; Sevo-6hr, exposure to sevoflurane for 6 hr; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; TGF-β, transforming growth factor-beta.

We performed Western blot analysis on brain tissue and serum ELISA to confirm our qPCR results (Figure 7). Consistent with the qPCR findings, we observed a significant increase in the protein levels of CD68 and galectin-3 in the Sevo-6hr group compared with that in the control group. Notably, galectin-3 levels were significantly reduced in the IBU group compared with that in the Sevo-6hr group, whereas CD68 levels showed no significant difference between the IBU group and the Sevo-6hr group (Figure 7A). Furthermore, the serum ELISA results reaffirmed that HMGB-1 and galectin-3 levels were significantly higher in the Sevo-6hr group but significantly lower in the IBU group, whereas IFN-γ levels were significantly higher in the IBU group (Figure 7B).

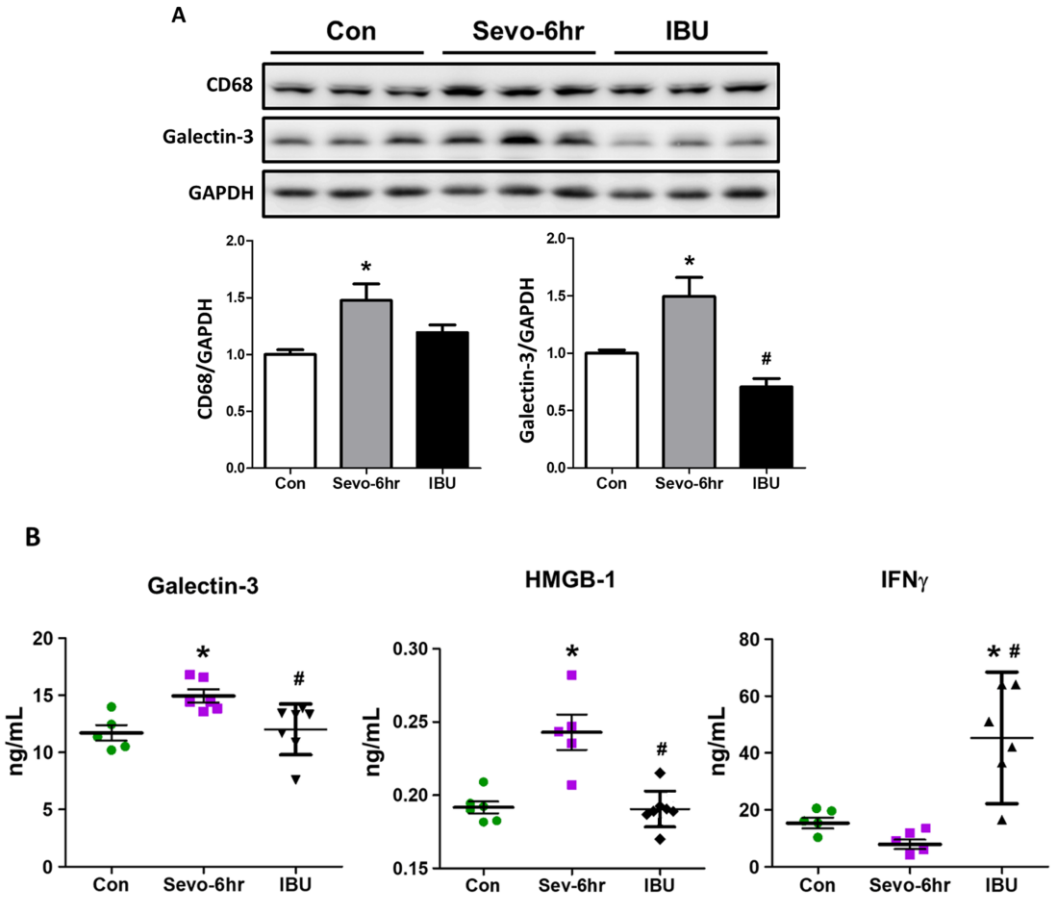


Figure 7. Effects of ibuprofen on the expression of CD68 and galectin-3 in the P7 rat brain tissue and serum. (A) Representative western blots and quantification of protein levels of CD68 and galectin-3 normalized to that of GAPDH. (B) Blood serum levels of galectin-3, HMGB-1, and IFN-γ. Values are presented as median and IQR. **P*<0.05 vs. Con. group, #*P*<0.05 vs. Sevo-6hr group. CD68, cluster of differentiation 68; Con, control group; HMGB-1, high mobility group box 1; IBU, administration of ibuprofen with Sevo-6hr; IFN-γ, interferon-gamma; IQR, interquartile range; P7, age of seven days; Sevo-6hr, exposure to sevoflurane for 6 hr.

IV. DISCUSSION

Our study confirmed that sevoflurane-induced neuroinflammation and activation of the microglial cells by sevoflurane exposure in 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 DAMPs-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 that 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 hr or 4 hr but significantly increased in the group exposed for 6 hr. This is consistent with the results of previous studies showing that short-duration, single-dose anesthetic drug exposure did not significantly affect neuro-impairment in the developing brain. [21]

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 subarachnoid hemorrhage. [22] Furthermore, HMGB-1 is reportedly a common biomarker for neurologic dysfunctions such as traumatic brain injury, epilepsy, cognitive dysfunction, and neuroinflammation. [13] Galectin-3 is upregulated in mice with Alzheimer's disease and related to Huntington's disease in humans, [23,24] and galectin-3 levels have been considered as a new prognostic factor for patients with Parkinson's disease.[25] 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 as a prognostic factor for cognitive dysfunction.

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, TLR9, and receptors for advanced glycation end-products (RAGE). Like HMGB-1, galectin-3 activates the pro-inflammation pathway via TLR2 and TLR4. [26] In this study, TLR4 and TLR9 were identified as PRRs of HMGB-1 and galectin-3 (Figure 3). TLR4 was suggested as a major pro-inflammatory pathway for HMGB-1 and galectin-3 in previous studies; [13,27] however, these studies induced neuroinflammation with lipopolysaccharide (LPS), whereas our study induced neuroinflammation with sevoflurane.

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

Ibuprofen is an anti-inflammatory drug that inhibits the cyclooxygenase enzyme. In the cell, ibuprofen exhibits anti-inflammatory effects through cell cycle modulation and apoptosis in activated microglial cells.[28] In the Parkinson's model mice, ibuprofen administration reduced dopaminergic neuron reduction, decreased microglial cells, decreased pro-inflammatory cytokines (TNF-α, IL-6), and improved behavioral changes.[29] In this study, ibuprofen reduced the pro-inflammatory mediators (TNF-α, IL-6) that were increased by sevoflurane and showed a restorative effect on the expression of decreased IFN-γ (Figure 5). Furthermore, co-administering ibuprofen with sevoflurane reduced HMGB-1 and galectin-3 expression in the rats (Figure 7). The treatment with ibuprofen resulted in a decrease in galectin-3 and HMGB-1 expression and an increase in IFN-γ expression; however, no difference was observed in CD68 expression compared with that in the Sevo-6hr 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, in the current clinical situation where in vivo studies are not possible, serum HMGB-1 and galectin-3 were presented as prognostic factors for neuroinflammation induced by anesthetic agents.

A limitation of this study is that 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. Second, we did not confirm the long-term effect of sevoflurane-induced neuroinflammation on behavior or cognitive dysfunction. Lastly, factors like surgical stress were not considered in this study. More research is needed for clinical application.

V. CONCLUSION

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 anti-inflammatory drugs may result in a reduction of neuroinflammation, and further investigations into the appropriate dosage and duration of treatment are needed.

REFERENCES

1. Briner A, De Roo M, Dayer A, Muller D, Habre W, Vutskits L. Volatile anesthetics rapidly increase dendritic spine density in the rat medial prefrontal cortex during synaptogenesis. Anesthesiology. 2010 Mar;112(3):546-56.
2. Creeley C, Dikranian K, Dissen G, Martin L, Olney J, Brambrink A. Propofol-induced apoptosis of neurones and oligodendrocytes in fetal and neonatal rhesus macaque brain. Br J Anaesth. 2013 Jun;110 Suppl 1(Suppl 1):i29-38.
3. Milanovic D, Pesic V, Loncarevic-Vasiljkovic N, Pavkovic Z, Popic J, Kanazir S, et al. The Fas Ligand/Fas Death Receptor Pathways Contribute to Propofol-Induced Apoptosis and Neuroinflammation in the Brain of Neonatal Rats. Neurotox Res. 2016 Oct;30(3):434-52.
4. Deng X, Chen B, Wang B, Zhang J, Liu H. TNF-α Mediates the Intrinsic and Extrinsic Pathway in Propofol-Induced Neuronal Apoptosis Via PI3K/Akt Signaling Pathway in Rat Prefrontal Cortical Neurons. Neurotox Res. 2017 Oct;32(3):409-419.
5. Mottahedin A, Ardalan M, Chumak T, Riebe I, Ek J, Mallard C. Effect of Neuroinflammation on Synaptic Organization and Function in the Developing Brain: Implications for Neurodevelopmental and Neurodegenerative Disorders. Front Cell Neurosci. 2017 Jul; 11;11:190.
6. Liu B, Ou G, Chen Y, Zhang J. Inhibition of protein tyrosine phosphatase 1B protects against sevoflurane-induced neurotoxicity mediated by ER stress in developing brain. Brain Res Bull. 2019 Mar;146:28-39.
7. Coleman K, Robertson ND, Dissen GA, Neuringer MD, Martin LD, Cuzon Carlson VC, et al. Isoflurane Anesthesia Has Long-term Consequences on Motor and Behavioral Development in Infant Rhesus Macaques. Anesthesiology. 2017 Jan;126(1):74-84.
8. Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, et al. Early exposure to anesthesia and learning disabilities in a population-based birth cohort.

Anesthesiology. 2009 Apr;110(4):796-804.

9. Hu D, Flick RP, Zaccariello MJ, Colligan RC, Katusic SK, Schroeder DR, et al. Association between Exposure of Young Children to Procedures Requiring General Anesthesia and Learning and Behavioral Outcomes in a Population-based Birth Cohort. Anesthesiology. 2017 Aug;127(2):227-240.
10. Ing C, Hegarty MK, Perkins JW, Whitehouse AJO, DiMaggio CJ, Sun M, et al. Duration of general anaesthetic exposure in early childhood and long-term language and cognitive ability. Br J Anaesth. 2017 Sep 1;119(3):532-540.
11. Ing C, Sun M, Olfson M, DiMaggio CJ, Sun LS, Wall MM, et al. Age at Exposure to Surgery and Anesthesia in Children and Association With Mental Disorder Diagnosis. Anesth Analg. 2017 Dec;125(6):1988-1998.
12. Davidson AJ, Sun LS. Clinical Evidence for Any Effect of Anesthesia on the Developing Brain. Anesthesiology. 2018 Apr;128(4):840-853.
13. Paudel YN, Shaikh MF, Chakraborti A, Kumari Y, Aledo-Serrano Á, Aleksovska K, et al. HMGB1: A Common Biomarker and Potential Target for TBI, Neuroinflammation, Epilepsy, and Cognitive Dysfunction. Front Neurosci. 2018 Sep; 11;12:628.
14. Huang X, Ying J, Yang D, Fang P, Wang X, Zhou B, et al. The Mechanisms of Sevoflurane-Induced Neuroinflammation. Front Aging Neurosci. 2021 Aug 5;13:717745.
15. Li Z, Zhu Y, Kang Y, Qin S, Chai J. Neuroinflammation as the Underlying Mechanism of Postoperative Cognitive Dysfunction and Therapeutic Strategies. Front Cell Neurosci. 2022 Mar 28;16:843069.
16. Wu X, Lu Y, Dong Y, Zhang G, Zhang Y, Xu Z, et al. The inhalation anesthetic isoflurane increases levels of proinflammatory TNF-α, IL-6, and IL-1β. Neurobiol Aging. 2012 Jul;33(7):1364-78.
17. Shen X, Dong Y, Xu Z, Wang H, Miao C, Soriano SG, et al. Selective anesthesia-induced neuroinflammation in developing mouse brain and cognitive impairment. Anesthesiology. 2013 Mar;118(3):502-15.

18. Carniglia L, Ramírez D, Durand D, Saba J, Turati J, Caruso C, et al. Neuropeptides and Microglial Activation in Inflammation, Pain, and Neurodegenerative Diseases. Mediators Inflamm. 2017;2017:5048616.

19. Dai J, Li X, Wang C, Gu S, Dai L, Zhang J, et al. Repeated neonatal sevoflurane induced neurocognitive impairment through NF-κB-mediated pyroptosis. J Neuroinflammation. 2021 Aug 21;18(1):180.

20. Baud O, Saint-Faust M. Neuroinflammation in the Developing Brain: Risk Factors, Involvement of Microglial Cells, and Implication for Early Anesthesia. Anesth Analg. 2019 Apr;128(4):718-725.

21. Sun LS, Li G, Miller TL, Salorio C, Byrne MW, Bellinger DC, et al. Association Between a Single General Anesthesia Exposure Before Age 36 Months and Neurocognitive Outcomes in Later Childhood. JAMA. 2016 Jun 7;315(21):2312-20.

22. Nakahara T, Tsuruta R, Kaneko T, Yamashita S, Fujita M, Kasaoka S, et al. High-mobility group box 1 protein in CSF of patients with subarachnoid hemorrhage. Neurocrit Care. 2009 Dec;11(3):362-8.

23. Boza-Serrano A, Ruiz R, Sanchez-Varo R, García-Revilla J, Yang Y, Jimenez-Ferrer I, et al. Galectin-3, a novel endogenous TREM2 ligand, detrimentally regulates inflammatory response in Alzheimer's disease. Acta Neuropathol. 2019 Aug;138(2):251-273.

24. Siew JJ, Chen HM, Chen HY, Chen HL, Chen CM, Soong BW, et al. Galectin-3 is required for the microglia-mediated brain inflammation in a model of Huntington's disease. Nat Commun. 2019 Aug 2;10(1):3473.

25. Yazar HO, Yazar T, Cihan M. A preliminary data: Evaluation of serum Galectin-3 levels in patients with Idiopathic Parkinson's Disease. J Clin Neurosci. 2019 Dec;70:164-168.

26. Chaudhry SR, Hafez A, Rezai Jahromi B, Kinfe TM, Lamprecht A, Niemelä M, et al. Role of Damage Associated Molecular Pattern Molecules (DAMPs) in Aneurysmal Subarachnoid Hemorrhage (aSAH). Int J Mol Sci. 2018 Jul 13;19(7):2035.

27. Burguillos MA, Svensson M, Schulte T, Boza-Serrano A, Garcia-Quintanilla A, Kavanagh E, et al. Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation. Cell Rep. 2015 Mar 10;10(9):1626-1638.

28. Elsisi NS, Darling-Reed S, Lee EY, Oriaku ET, Soliman KF. Ibuprofen and apigenin induce apoptosis and cell cycle arrest in activated microglia. Neurosci Lett. 2005 Feb 28;375(2):91-6.

29. Costa T, Fernandez-Villalba E, Izura V, Lucas-Ochoa AM, Menezes-Filho NJ, Santana RC, et al. Combined 1-Deoxynojirimycin and Ibuprofen Treatment Decreases Microglial Activation, Phagocytosis and Dopaminergic Degeneration in MPTP-Treated Mice. J Neuroimmune Pharmacol. 2021 Jun;16(2):390-402.

ABSTRACT (IN KOREAN)

발달중인 소아 쥐의 뇌에서 흡입 마취제인 세보플루란 의한 손상 기전으로서 신경 염증의 매커니즘 규명

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조 영 은

소아의 수술에 있어서 전신마취는 필수불가결하다. 흡입마취제의 사용이 신경염증반응과 발달 지연과 연관이 있다는 전임상 연구들이 알려져 있으나 아직까지 그 정확한 기전에 대해 밝혀진 바는 없다. 세보플루란은 소아환자의 마취에 있어서 가장 많이 쓰이는 마취 약제이다. 본 연구에서는 손상 관련 분자 패턴 중 HMGB-1과 galectin-3가 흡입마취제인 세보플루란에 의해 유발되는 신경염증반응을 매개한다고 가정하였다.

본 연구에서는 세가지 단계로 실험을 구성하였다. 첫번째로, 발달 중인 소아 쥐에서 세보플루란 노출 시간에 따른 신경 염증 발생의 정도를 확인하였다. 두번째로는 세보플루란에 의해 유발된 신경 염증과 연관 있는 손상 관련 분자 패턴 인자를 규명하고자 하였다. 마지막으로 세보플루란과 비스테로이스 소염제인 이부브로펜을 함께 투여하여 유발된 신경 염증에 미치는 그 효과를 확인하고자 하였다. 실험 중 소아 쥐의 뇌에서 발현하는 여러가지 인자는 면역조직염색, 면역형광염색, 실시간 중합효소연쇄반응, 웨스턴 블랏, 효소결합 면역 흡착 검사 등을 이용하여 확인하였다.

총 112마리의 7일된 소아 쥐가 실험에 사용되었고, 그 중 6마리가 실험 중 사망하여 사망률은 5.3%였다. 세보플루란에 의해 신경 염증이 유발되는 노출 시간을 확인하기 위하여 7일된 소아 쥐를 세보플루란에 2시간, 4시간, 6시간에 각각 노출하였을 때, 대조군에 비하여 6시간 노출된 그룹에서 미세아교세포의

활성을 나타내는 분화 클러스터 68의 발현이 증가함을 확인하였다. 또한 HMGB-1, galectin-3의 발현이 증가하였으며, 그 하위 단계의 수용체로 알려진 톨유사수용체 4와 9, 그리고 인산화 핵인자 카파비의 발현이 증가함을 확인하였다. 또한 증성 사이토카인인 종양괴사인자 알파와 인터루킨 6의 발현은 증가한 반면, 인터페론 감마의 발현은 감소하였다. 세보플루란의 노출과 함께 비스테로이드 소염제인 이부브로펜을 투여하였을 때, 종양괴사인자 알파와 인터루킨 6의 발현은 감소하였고 인터페론 감마의 발현은 회복되었다. 똑같은 결과를 뇌조직의 웨스턴 블랏을 통해 확인하였고, 혈청 효소 결합 면역흡착검사를 통해 다시 확인할 수 있었다.
본 연구를 통해 6시간의 세보플루란에 노출되었을 때, 발달중인 쥐의 뇌에서는 신경 염증이 유발되었고, 이것은 손상 관련 인자 패턴 중 HMGB-1과 galectin-3에 의해 발생하였음을 확인하였다. 또한 이부브로펜의 투여는 세보블루란에 의해 발생한 신경 염증을 감소시켰다. 추후 다른 마취 약제에 의한 신경 염증 반응의 발생 및 이부브로펜의 용량 등에 대한 추가적인 연구가 더 필요할 것으로 보인다.

핵심되는 말: 세보플루란, 신경 염증, 손상 관련 분자 패턴, HMGB-1, galectin-3, 비스테로이드성 소염제