Isoflurane Causes Greater Neurodegeneration Than an Equivalent Exposure of Sevoflurane in the Developing Brain of Neonatal Mice

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ABSTRACT

Background: We hypothesized that isoflurane has a greater potency to induce neurodegeneration than sevoflurane in the developing brains of neonatal mice based on our previous studies in cell culture.

Methods: We treated 7-day-old mice with either 0.75% isoflurane or 1.1% sevoflurane ($\sim$0.5 minimum alveolar concentration) for 6 h and then obtained blood and brain samples at 2 h after the anesthesia treatment for determination of neuroapoptosis in different brain regions and the neurodegenerative biomarker S100β in the blood. The mechanisms of neurodegeneration induced by isoflurane or sevoflurane were also compared by determining protein expressions of the cell cycle and apoptosis-related proteins. In separate groups, memory and learning ability were evaluated through the use of Morris Water Maze testing in mice at postnatal day 42 after anesthesia treatment at postnatal day 7.

Results: Isoflurane but not sevoflurane significantly increased the neurodegenerative biomarker S100β in the blood. Isoflurane treatments significantly increased apopto-sis indicated by the activation of caspase-3 and elevation of poly-(ADP-ribose) polymerase in different brain regions. An equipotent exposure of sevoflurane tended to increase apoptosis in hippocampal and cortex areas but was significantly less potent than isoflurane. Neither isoflurane nor sevoflurane significantly changed protein levels of glyceraldehyde-3-phosphate dehydrogenase, β-site amyloid β-precursor protein-cleaving enzyme, and cell cycle regulatory proteins (CDK4, cyclin D1). Isoflurane and sevoflurane at the selected exposures did not significantly alter memory and learning ability.

Conclusion: At equipotent exposures, isoflurane has a greater potency than sevoflurane to cause neurodegeneration in the developing brains of neonatal mice.

What We Already Know about This Topic

❖ Anesthetic exposure increases programmed cell death in the brains of neonatal animals, and cell culture experiments suggest that isoflurane is more potent than sevoflurane in this regard

What This Article Tells Us That Is New

❖ In neonatal mice, 6-h exposure of approximately 0.5 MAC isoflurane led to greater brain cell death than sevoflurane, although neither drug affected subsequent memory or learning ability

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NUMEROUS studies during the past few years have demonstrated the potential deleterious effects of anesthetic exposure to neonatal animals with regard to neurohistopathologic changes and long-term abnormal social behavior and cognitive dysfunction. Studies on a variety of animals ranging from rodents to rhesus monkeys have shown increased neuroapoptosis in the postnatal developing brains of these newborn animals when exposed to both intravenous and inhaled anesthetic agents.[1–4] Our recent study also demonstrated isoflurane-induced neurodegeneration represented by the elevation of a neurodegenerative biomarker in blood, S100β, and apoptosis in various brain regions in the prenatal developing rat brain.[5] Rodent studies have also demonstrated persistent learning deficits and social behavior

dysfunction after anesthetic exposure as neonates.[1,6,7] Recently, a retrospective study examining anesthetics given to children younger than 4 years detected a possible association between multiple anesthetics and the development of reading, written language, and math learning disabilities.[8,9]

It seems that the period of synaptogenesis in the developing brain is especially vulnerable to anesthesia neurotoxicity.[1] The mechanisms of anesthetic-mediated neurodegeneration in the developing brain are still not clear. It has been proposed that inhalational anesthetics induced neurodegeneration in the developing brain through activation of γ-aminobutyric acid and inhibition of N-methyl-D-aspartate receptors, which may be similar to the neurotoxicity induced by ethanol.[1,10,11] Activation of cell cycle events has been associated with ketamine-induced neurodegeneration in neonatal rat brains.[12] Our recent studies both in tissue culture and animals suggested that inhalational anesthetics, especially isoflurane, induce cell apoptosis and neurodegeneration in the developing brain *via* disruption of intracellular calcium homeostasis, particularly by causing excessive calcium release from the endoplasmic reticulum *via* activation of inositol-1,4,5-trisphosphate receptors.[13–15] It is interesting to note that isoflurane had significantly greater potency compared with sevoflurane to cause cell damage. This phenomenon resulted from the greater ability of isoflurane to induce calcium release from the endoplasmic reticulum *via* inositol-1,4,5-trisphosphate receptors in cell cultures.[14,16]

Together, sevoflurane and isoflurane constitute the majority of inhaled anesthetic agents given to children around the world. Therefore, it is important to know whether isoflurane also has a significantly greater potency to induce neurodegeneration than sevoflurane in the developing brain and whether this is correlated with their effects on cognitive function. Here, we studied the potential differences of sevoflurane and isoflurane to cause neuroapoptosis and cognitive function when exposed to neonatal mice and investigated possible mechanisms through activation of the cell cycle and changes in apoptosis-related proteins. In addition, we investigated whether blood S100β levels could be used as a neurodegenerative biomarker during an anesthesia neurotoxicity study in neonatal mice, similar to that in the rat fetus of our previous study.[5]

Methods and Materials

Animals

The Institutional Animal Care and Use Committee at the University of Pennsylvania (Philadelphia, Pennsylvania) approved all experimental procedures and protocols used in this study. All efforts were made to minimize the number of animals used and their suffering. C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were housed in polypropylene cages, and the room temperature was maintained at 22°C, with a 12-h light–dark cycle. At 4 weeks of age, the mice were weaned and housed in groups of four animals per cage. Mice had continuous access to water and

food. Both male and female mice were used in the experimental and control aspects of this study. A total of 68 mice were used in this study.

Anesthesia Exposure

Postnatal day 7 mice were placed in plastic containers resting in water baths to maintain a constant environmental temperature of 38°C. In these boxes, the mice were either exposed to 0.75% isoflurane or 1.1% sevoflurane in a humidified 30% oxygen carrier gas or simply humidified 30% oxygen without any inhalational anesthetic for 6 h. Six liters of total gas flow were used to ensure a steady state of anesthetic gas and prevent accumulation of expired carbon dioxide within the chamber. The inhalational agents, oxygen and carbon dioxide levels in the chamber, were monitored and maintained using infrared absorbance (Ohmeda 5330, Detex-Ohmeda, Louisville, CO) as described in our previous study.[17]

The duration and level of anesthetic exposure were determined after a pilot study, which demonstrated that 6 h of 0.75% isoflurane did not cause any changes in arterial blood gas, glucose, or temperature values (data not shown). Arterial blood gases were measured at the end of the 6-h exposure with an arterial blood gas analyzer (Nova Biomedical, Waltham, MA). Blood glucose level was simultaneously measured with a glucometer (ACCU-CHECK Advantage; Roche Diagnostics Corporations, Indianapolis, IN). The rectal temperature was periodically checked to ensure the maintenance of normothermia (Dual Thermometer; Fisher Scientific, Pittsburgh, PA) at 37° ± 0.5°C. The level of exposure for sevoflurane was determined after calculating the minimum alveolar concentration (MAC) equivalent to isoflurane.[18–20] The 0.75% isoflurane or 1.1% sevoflurane is about equipotent to ~0.5 MAC in mice. We used the adult human equivalent because others have already shown that (1) the MAC for neonatal mice for isoflurane is higher than that for both adult and neonatal humans and (2) 6-day-old C57BL/6 mice can be exposed to up to 3% sevoflurane for 6 h without any detrimental effect on cerebral perfusion, arterial blood gas, or glucose values.[21] It has also been shown that 7-day-old C57BL/6 mice exposed to 0.75% isoflurane for as little as 4 h will display as much as a 477% increase in neuroapoptosis when compared with the control.[22]

Determination of Plasma S100β

S100 proteins are a family of dimeric cytosolic calcium-binding proteins made up of an α- and a β-isomer and are found in abundance in astroglial and Schwann cells in the nervous system. S100β, the β-isomer of S100, seems to be released into the extracellular space near the injured tissue and can enter into the serum from the brain through a disrupted blood brain barrier after even mild brain injury secondary to trauma, hypoxia, ischemia, and neurotoxin.[23] We measured S100β levels in the blood of newborn mice using Sangtec 100 ELISA kits (DiaSorin, Inc., Stillwater, MN) following the manufacturer's protocol as described previously.[5] Briefly, blood from each mouse was obtained from the left ventricle

and was centrifuged to get the plasma. Plasma (50 μl) was placed in each well of 96-well plates and mixed with 150-μl tracer from kit, incubated for 2 h, followed by 3,3',5,5'-tetramethylbenzidine substrate and stop solution. The optical density was recorded at 450 nm. The sensitivity was determined by plotting the standard curve and then measuring concentrations of the samples from the standard curve.

Neurodegeneration Studies in the Developing Mouse Brain

Previous studies from us and others have demonstrated anesthesia-induced cell death by apoptosis both in cell cultures[16,24] and in the developing brains.[1,25] Activation of caspase-3 has been commonly used in these studies as a biomarker for anesthesia-mediated cell death by apoptosis,[1,25] although caspase-3 activation may also be essential for neuroprotection in preconditioning.[26] In this study, we have determined neurodegeneration in the developing brains by a combination of various methods to detect neuronal damage with activation of caspase-3 and poly-(ADP-ribose) polymerase (PARP, a DNA repair enzyme) and examination of general neurodegenerative biomarker S100β in the blood, understanding that any one of above methods alone is not sufficient to confirm anesthesia-mediated neurodegeneration. The technical aspects of collecting these samples involved the following measures. Two hours after the completion of the anesthetic treatment, animals from the control, isoflurane, and sevoflurane groups were anesthetized with 2–3% isoflurane for less than 1 min, and the blood and brain tissues were collected immediately after anesthesia induction. Isoflurane has widely been used for killing animals and should not significantly influence the extent of neurodegeneration in this study because the brains were removed immediately after this brief use of isoflurane. Blood (0.1 ml) was collected from the fetal left ventricle for determination of S100β levels, a recommended useful biomarker for fetal brain damage during pregnancy[27] and has been previously shown to significantly increased in fetal blood exposed to an isoflurane treatment *in utero*.[5] Thereafter, the fetal brains were perfused transcardially with ice-cold phosphate-buffered saline (pH 7.4, NaCl 137 mM, phosphates 11.9 mM, KCl 2.7 mM). The brains were then removed and postfixed with 4% paraformaldehyde in phosphate-buffered saline overnight in the same fixative at 4°C and cryoprotected in 30% (wt/vol) sucrose at 4°C for 24 h. Thereafter, the brains were frozen in isopentane at −20°C and stored at −80°C until use. Serial coronal sections (10 μm) were cut in a cryostat (Dolbey–Jamison Optical Company, Inc., Pottstown, PA), mounted on gelatin-coated slides, and then stored at −80°C until use.

Caspase-3-positive cells were detected using the immunohistochemical methods we have described previously.[5,17] Briefly, brain sections were first incubated in 3% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity. Sections were then incubated with blocking solution containing 10% normal goat serum in phosphate-buffered saline with 0.1% Tween-20 for 1 h at room temperature. The antiactivated caspase-3 primary antibody (1:200, Cell Signaling Technology, Inc., Danvers, MA) was then applied in blocking solution and incubated at 4°C overnight. Tissue sections were biotinylated with goat anti-rabbit antibody (1/200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 0.1% Tween 20 for 40 min, followed by incubation with the avidin-biotinylated peroxidase complex (Vectostain ABC-Kit, Vector Lab, Burlingame, CA) for 40 min. Tissue sections were colorized with diaminobenzidine (DAB, Vector Lab) for 8 min and counterstained with modified hematoxylin. Negative control sections were incubated in blocking solution that did not contain primary antibodies. Images were acquired and assessed at 200× using IPLab 7.0 software linked to Olympus IX70 microscope (Olympus Corporation, Tokyo, Japan) equipped with a Cooke Sensi-Cam camera (Cooke Corporation, Romulus, MI). Three brain tissue sections at 10 μm corresponding to the Atlas of the Developing Mouse Brain at P6 figure 131–133[28] were chosen from each animal and analyzed for caspase-3 positive cells in the hippocampal CA1 region. Two persons blinded to the treatments counted the total number of caspase-3 positive cells in the hippocampal CA1 region. The areas of hippocampal CA1 region were defined according to the Atlas of the Developing Mouse Brain, and the area measured using IPLab Suite v3.7 imaging processing and analysis software (Biovision Technologies, Exton, PA). The density of caspase-3 positive cells in a particular brain region was calculated by dividing the number of caspase-3 positive cells by the area of that brain region.

Western Blot Analysis

Western blot analyses were performed as described previously.[17,29] Proteins from the P7 mouse cerebral cortex were separated by 12% gel electrophoresis, and proteins were transferred to a nitrocellulose membrane. The blots were incubated with a monoclonal antibody against cleaved caspase-3 and PARP and then probed with horseradish peroxidase-conjugated secondary antibody to determine cell apoptosis in various brain regions after anesthesia treatments. To study the possible mechanisms of anesthesia-mediated neurodegeneration, we also examined the changes of protein levels of the cell cycle regulatory proteins (CDK4, Cyclin D1; 1:1000; Cell Signaling Technology, Inc.), β-site amyloid β-precursor protein-cleaving enzyme (BACE), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000; Abcam, Inc., Cambridge, MA) using Western Blotting. Detection was performed by using the ECL-PLUS system and photographed. β-Actin protein was used as a loading control. All three Western blots were from three different animals, and the density was measured by Quantity One software (version 4.5.0; BIO-RAD, Hercules, CA) and GS-800 Densitometer (BIO-RAD).

Memory and Learning Studies

We determined spatial reference memory and learning with the Morris Water Maze as described previously.[17] A round,

fiberglass pool, 150 cm in diameter and 60 cm in height, was filled with water to a height of 1.5 cm above the top of the movable clear 15-cm-diameter platform. The pool was located in a room with numerous visual cues (including computers, posters, and desks) that remained constant during the studies. Water was kept at 20°C and opacified with titanium dioxide throughout all training and testing. A video tracking system recorded the swimming motions of animals, and the data were analyzed using motion-detection software for the MWM (Actimetrics software, Evanston, IL). After every trial, each mouse was placed in a holding cage under an infrared heat lamp, before returning to its regular cage.

Cued Trials. The cued trials were performed at postnatal day 31 to determine whether any noncognitive performance impairments (*e.g.*, visual impairments or swimming difficulties) were present, which might affect performance on the place or probe trials. A white curtain surrounded the pool to prevent confounding visual cues. All mice received four trials per day. For each trial, mice were placed in a fixed position of the swimming pool facing the wall and were allowed to swim to a platform with a rod (cue) 20 cm above water level randomly placed in any of the four quadrants of the swimming pool. They were allotted 60 s to find the platform on which they sat for 30 s before being removed from the pool. If a mouse did not find the platform within 60 s, the mouse was gently guided to the platform and allowed to remain there for 30 s. The time for each mouse to reach the cued platform and the swim speed were recorded and analyzed.

Place Trials. After completion of the cued trials, we used the same mice to perform the place trials to determine the mouse's ability to learn the spatial relationship between distant cues and the escape platform (submerged, no cue rod), which remained in the same location for all place trials. The starting points were random for each mouse. The time to reach the platform was recorded for each trial. The less time it took a mouse to reach the platform, the better the learning ability. The juvenile mice at postnatal day 40 received two blocks of trials (two trials per block with 30 s apart, 60 s maximum for each trial, and 2-h rest between blocks) each day for 5 days.

Probe Trials. Probe trials were conducted after the last place trial for the juveniles at postnatal day 45 to evaluate memory retention capabilities. After all mice completed the last place trial on the fifth day, the platform was removed from the water maze and the mouse was placed in the opposite quadrant. The mice were allowed to swim for 60 s during each probe trial, and the time a mouse spent in each quadrant was recorded. The percentage of the swimming time spent in the target (probe) quadrant in which the platform had been placed before being removed was calculated. The time spent in the target quadrant compared with other quadrants was used as an indication of memory retention.

Statistical Analysis

We used GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA) for all statistical analysis and graph
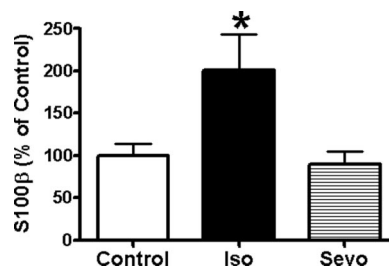

Fig. 1. Isoflurane but not sevoflurane significantly increased S100β in the blood of neonatal mice. S100β levels in the blood obtained from left ventricle puncture were determined using a Sangtec 100 ELISA kit (DiaSorin, Inc., Stillwater, MN). Data represent mean ± SE. N = 8, 9, and 10 for control, isoflurane (Iso), and sevoflurane (Sevo) groups, respectively. * $P < 0.05$ compared with both control and sevoflurane groups.

generation. Data for caspase-3 densities in the different neonatal brain regions, as well as western blot analysis for cleaved caspase-3, PARP, glyceraldehyde-3-phosphate dehydrogenase, and β-site amyloid β-precursor protein-cleaving enzyme were analyzed using one-way ANOVA followed by Tukey multiple comparison testing. Behavioral studies were also analyzed using two-way ANOVA (anesthesia treatment as between-groups and time as repeated measures factors) followed by Bonferroni multiple comparison testing to compare the three groups. In all experiments, differences were considered statistically significant at $P < 0.05$.

Results

Neonatal Exposure to Isoflurane Induced a Significantly Greater Amount of Apoptotic Neurodegeneration Than an Equipotent Exposure of Sevoflurane

Our previous study demonstrated that S100β, a useful neurodegenerative biomarker, significantly increased in rat fetal blood after their pregnant mothers were treated with isoflurane. In this study, we further tested whether S100β could also be used as a useful neurodegenerative biomarker in anesthesia-mediated neurodegeneration in the developing brains of newborn mice. A 6-h exposure of 0.75% isoflurane to 7-day-old mice significantly increased S100β in the blood by twofold than that in the control (fig. 1). However, an equipotent exposure of sevoflurane did not significantly increase S100β as compared with control.

We further examined the effects of equipotent exposures of isoflurane and sevoflurane on apoptosis in the different brain regions of 7-day-old mice. Cleaved caspase-3 detects endogenous levels of activated caspase-3, a known and useful marker of neuronal cell death from apoptosis. Consistent with its effect on blood S100β levels, isoflurane exposure significantly increased the amount of caspase-3-stained apoptosis observed in CA1 hippocampal brain slices prepared 2 h after anesthesia exposure (fig. 2). Sevoflurane tended to increase apoptosis in the hippocampus but not statistically significant compared with control (fig. 2B, sevoflurane *vs.* control, $P > 0.05$). A 6-h exposure of 0.75% isoflurane
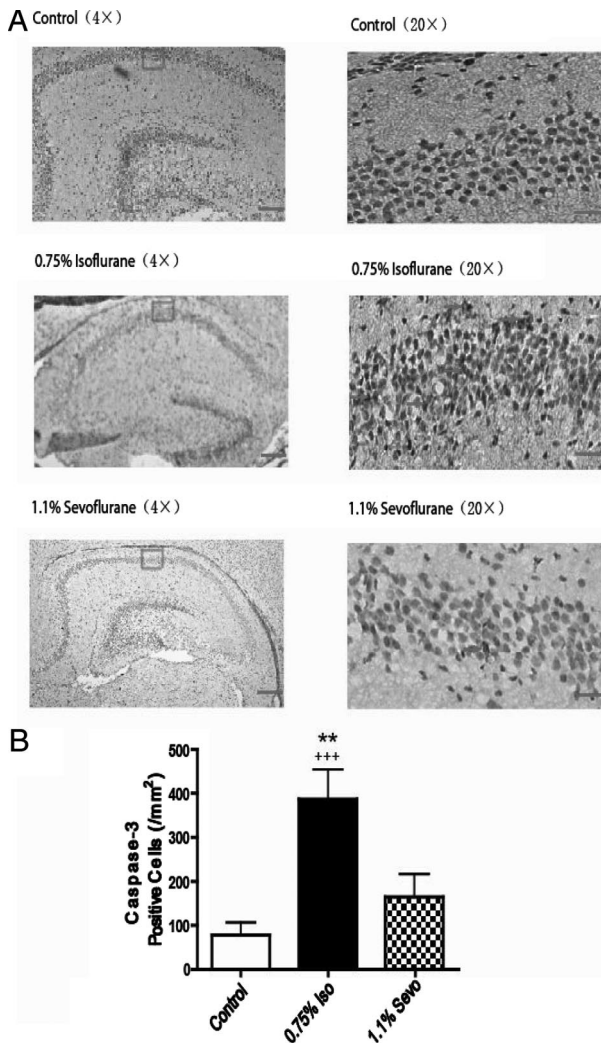
Fig. 2. Isoflurane induced greater apoptosis than equipotent exposure of sevoflurane in the hippocampus CA1 region of neonatal developing brains. (*A*) Brain sections containing hippocampus from various experimental groups were immunostained to determine caspase-3 positive cells, and the sample sections were illustrated at different magnifications. *Arrows* indicate caspase-3 positive cells in the hippocampus CA1 region located within the *rectangles* illustrated on the *left* panel demonstrating whole hippocampus of neonatal mice developing brains at low magnification. Scale bar is in the right low corner of each brain section and is 50 μm for the low magnification on the *left panel* and 10 μm for the high magnification on the *right panel* of brain sections. (*B*) Statistical analysis of caspase-3 positive cell from various experimental groups. Data represent mean ± SE. N = 12, 12, and 9 for control, isoflurane (Iso), and sevoflurane (Sevo) groups, respectively. +++ P < 0.001 compared with control. ** P < 0.01 compared with sevoflurane.

induced more than a twofold increase in the amount of apoptosis observed as compared with a 6-h exposure of MAC equivalent sevoflurane (fig. 2B, isoflurane *vs.* sevoflurane, P < 0.01) and almost a fourfold increase as compared with the control group (fig. 2B, isoflurane *vs.* control, P < 0.001 for both). Similarly, in the brain cortex, isoflurane induced

significantly more apoptosis than an equipotent exposure of sevoflurane, represented by increased cleaved caspase-3 determined with Western blotting (fig. 3). Compared with the control group, sevoflurane also increased apoptosis in the cortex, although significantly less than that by isoflurane (fig. 3B). Cerebral cortex immunostaining showed an almost twofold increase in the amount of cleaved caspase-3 caused by exposure to isoflurane as compared with the sevoflurane group and an almost sixfold increase when compared with the control group (figs. 3A and B). To further verify the cell death by apoptosis observed in the three different groups of mice, we also determined the changes of cleaved nuclear enzyme PARP, which is another useful biomarker for confirming cell death by apoptosis.[30] The changes of cleaved PARP after isoflurane or sevoflurane are similar to those of cleaved caspase-3 (figs. 3C and D), further validating the degree of cell death by apoptosis in the cerebral cortex.

Effects of Isoflurane and Sevoflurane on Cell Cycle Biomarkers, BACE and GAPDH

We further investigated the possible mechanisms by which inhalational anesthetics induce apoptosis in the developing mouse brain. Effects of anesthetics (ketamine or isoflurane) on cell cycle and proliferation have been proposed as one of the mechanisms for anesthesia-mediated cell death.[12,31] We determined the changes of cell cycle activation biomarkers (CDK4, Cyclin D1) using Western blotting after the treatments of isoflurane or sevoflurane. Neither isoflurane nor sevoflurane significantly changed the level of cell cycle activation biomarkers (figs. 4A–D). Activation of the β-site amyloid precursor protein-cleaving enzyme (BACE) contributes to the isoflurane-induced vicious cycle of cell apoptosis and production of β-amyloid, a neuropathologic feature in Alzheimer's disease. Therefore, we compared the effects of isoflurane and sevoflurane on the level of BACE protein in cortex. Although isoflurane seems to cause an increase in the amount of BACE detected with Western blot analysis as compared with the control and sevoflurane, it was not statistically significant (figs. 4E and F). GAPDH has been shown to initiate apoptotic cell death induced by multiple stress factors in various models.[32] We further determined whether inhaled anesthetics induced apoptosis by changing the levels of GAPDH. Neither isoflurane nor sevoflurane significantly changed the levels of GAPDH (figs. 4G and H).

Neonatal Exposure to neither Sevoflurane nor Isoflurane Impaired Learning or Memory Significantly

To evaluate the effect of neonatal exposure with sevoflurane or isoflurane on potential learning and memory deficits, we subjected mice to Morris Water Maze testing. The cued trials began at postnatal day 31 to evaluate swimming and visual abilities to establish that any differences obtained in the place or probe trials were not due to physical impairments and did not demonstrate a significant difference among the three groups (data not shown). Place trials conducted beginning at postnatal day 40 examined the mice's ability to learn and
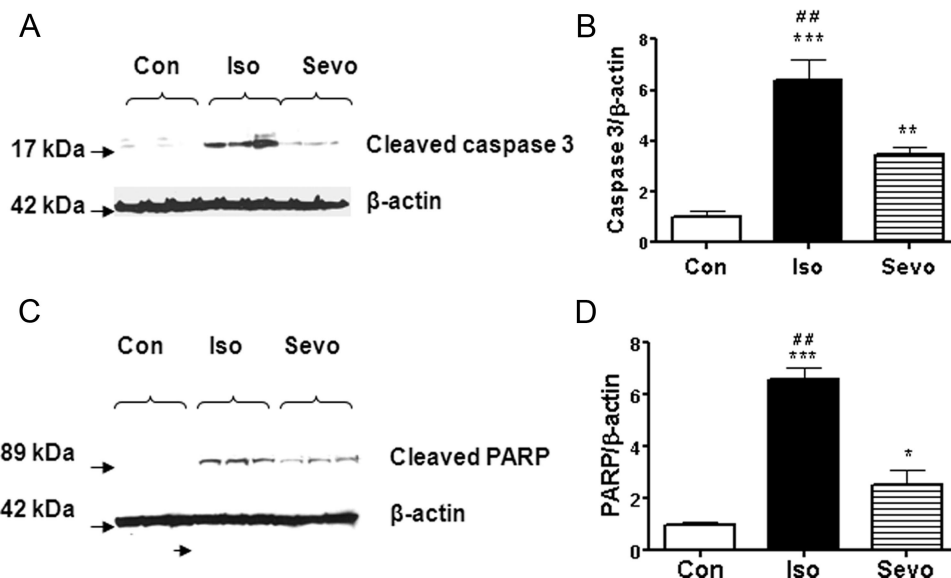
Fig. 3. Isoflurane induced greater apoptosis than equipotent exposure of sevoflurane in the cerebral cortex of neonatal developing brains. (*A*, *C*) Representative changes of cleaved caspase-3 (*A*) and poly-(ADP-ribose) polymerase (PARP) (*C*) in cerebral cortex by Western Blot using specific antibody targeted to cleaved caspase-3 and PARP, respectively. (*B*, *D*) The quantified cleaved caspase-3 (*B*) and PARP (*D*) bands were normalized to the loading control β-actin. N = 6 for control, isoflurane, and sevoflurane groups. * $P < 0.05$ compared with control. ** $P < 0.01$ or *** $P < 0.001$ compared with control. ## $P < 0.01$ compared with sevoflurane treatment. con = control, iso = isoflurane, sevo = sevoflurane.

remember the location of a new platform. As shown in figure 5A, although the results indicated that the performance during the place trials improved as the training progressed, the average time for each group to reach the submerged platform did not overall differ significantly. The only statistical significance that could be detected was the comparison between sevoflurane and control group at day 4. The probe trial performed after the last series of place trials on P45 further tested the mice's retention memory. As demonstrated in figure 5B, although mice receiving an anesthesia treatment with either isoflurane or sevoflurane tended to spend less percentage of time in the target quadrant than mice in the control group, there was no significant statistical difference between the groups ($P > 0.05$).

Discussion

Given our work with tissue cultures in which differences in the disruption of intracellular calcium and subsequent cell apoptosis were demonstrated between isoflurane and sevoflurane exposures,[14,16] we hypothesized that a difference in the amount of neuroapoptosis and potential learning and memory deficits would occur after single exposures of MAC equivalents of these two inhalational agents in the animal study. Consistent with our hypothesis and the finding in the tissue culture studies,[14,16] this study showed that a single exposure of isoflurane, but not an equipotent exposure of sevoflurane, caused a significantly increased blood S100β level compared with control, a novel finding that needs to be further investigated. Furthermore, isoflurane induced a significantly greater amount of apoptosis in different brain regions than the equivalent exposure of sevoflurane, which is a

novel finding in an animal model of anesthesia-mediated neurotoxicity. The combination of an elevation of S100β, activation of caspase-3 and PARP in different brain regions, particularly in the hippocampus CA1 area, suggests that anesthetic-induced damage most likely originates from the nervous tissues. Neither isoflurane nor sevoflurane significantly changed the levels of cell cycle regulatory proteins, BACE, and GAPDH. Isoflurane and sevoflurane at the selected exposure ($\sim$0.5 MAC) did not impair memory or learning in this animal model.

One of the limitations for studying anesthesia-mediated neurodegeneration in the brain in various animal models, especially in clinical trials, is the lack of a detectable neurodegenerative biomarker in the blood with high sensitivity and selectivity. S100β is a calcium-binding protein in the astrocytes of the central nervous system, which may be increased even after mild injury to the brain secondary to trauma, hypoxia, ischemia, or neurotoxin.[23] Consistent with our previous study showing that isoflurane inhaled by pregnant rats increased S100β in their fetus' blood in a dose-dependent manner,[5] we demonstrated that 0.75% isoflurane for 6 h but not its equipotent exposure of sevoflurane significantly increased plasma S100β levels in newborn mice. It is unclear at this time whether plasma S100β levels could be used as a valuable biomarker for anesthesia-mediated neurodegeneration mainly due to the large percentage of natural apoptosis already occurring at this age in developing brains.[33] In addition, sevoflurane did not increase the plasma level of S100β but clearly activated caspase-3 in the cortex, implicating a potential dissociation between the plasma S100β level and degree of
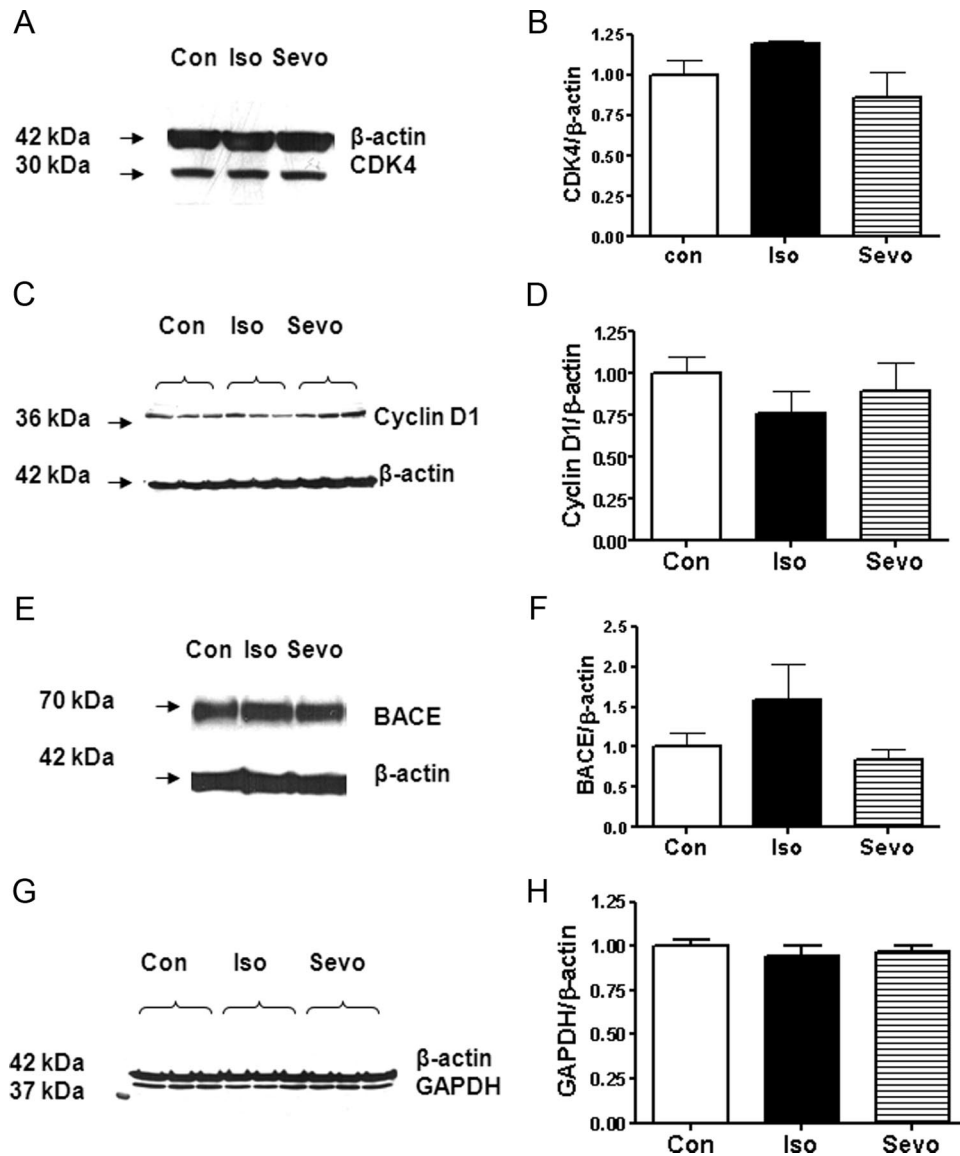
Fig. 4. Effects of isoflurane or sevoflurane on cell cycle regulatory proteins, β-site amyloid β-precursor protein-cleaving enzyme (BACE), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Proteins from cerebral cortex of neonatal mice were used for Western blotting to compare their changes among different experimental groups. (*A*, *C*, *E*, *G*) Representative changes of cell cycle regulatory proteins (CDK4, Cyclin D1), BACE, and GAPDH in the Western blot listed on the *left panel*. (*B*, *D*, *F*, *H*) Statistical analysis and comparison of changes of four proteins listed on the *right panel*. Each protein was quantified and normalized to the loading control β-actin and compared among control, isoflurane, and sevoflurane groups. Data represents mean ± SE. N = 3 for control, isoflurane, and sevoflurane. con = control, iso = isoflurane, sevo = sevoflurane.

caspase-3 activation at least during neurotoxicity mediated by ∼0.5 MAC sevoflurane. Although the elevation of plasma S100β alone may not provide enough information on the exact damage of various brain regions induced by anesthesia, the consistent elevation of plasma S100β levels after isoflurane exposure compared with controls with their effects on apoptosis in brain hippocampus and cortex in this study strengthens the finding of anesthesia-mediated neurodegeneration. Because of the extreme difficulty or impossibility of obtaining brain samples for histologic study in almost all human clinical trials, it is worthwhile to investigate whether S100β and other neurodegenerative biomarkers may possibly be used as a valuable neurodegenerative biomarker for clinical studies investigating anesthesia neurotoxicity in developing brains in the future. The practicality of S100β as a neurodegenerative biomarker in the adult brain after anesthesia exposure still needs further investigation as there is usually more of an intact blood brain barrier in adult brains blocking the release of S100β into the blood.

Given the recent concerns over the possible implications of the various anesthetic drugs on neonatal learning and memory and the overwhelming use of sevoflurane as the induction and most often maintenance agent of choice in pediatric anesthesia, we deemed it prudent to compare whether there existed differences between isoflurane and sevoflurane on neurodegeneration
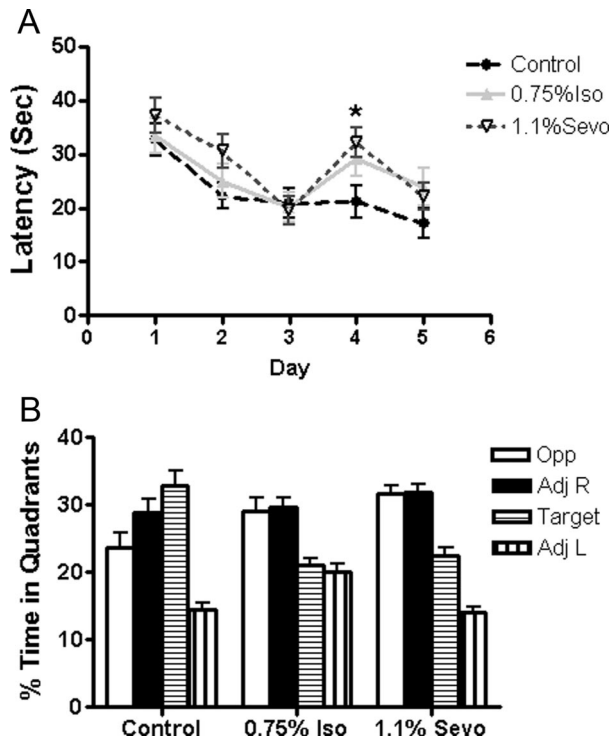
Fig. 5. Effects of anesthesia treatments at neonatal age on memory and learning ability at juvenile age. Learning and memory were determined using place and probe trials with the Morris Water Maze beginning at postnatal day 42 mice after at postnatal day 7 were exposed to isoflurane or sevoflurane or carrying gas (control). (*A*) Place trial demonstrating latency for mice to reach platform measuring spatial reference memory and learning. * $P < 0.05$ compared with control. (*B*) Probe trial demonstrating the percentage of time mice spent in the target quadrant measuring the short retention memory. N = 12, 10, and 12 for control, isoflurane, and sevoflurane, respectively, in both place and probe trials. Adj L = adjacent quadrant to the left of the target quadrant; Adj R = adjacent quadrant to the right of the target quadrant; Iso = isoflurane; Opp = opposite quadrant of the target quadrant; Sevo = sevoflurane.

and subsequent memory and learning deficits. Although more research is needed to determine the degree of interspecies variation in terms of sensitivity to anesthetic drugs during the brain growth spurt period, differences in the degree of pathologic changes induced by these different agents may allow anesthesiologists to better limit the amount of damage potentially caused by these drugs. The different potencies between isoflurane and sevoflurane for causing apoptosis in the developing brains of newborn mice are likely due to their different potencies to cause calcium release from the endoplasmic reticulum as indicated in the tissue cultures in our previous studies.[14] Unfortunately, because of technical difficulties, we were unable to measure the changes in neuronal calcium levels in the developing mice brains in this study.

It is still not clear whether anesthesia-mediated neurodegeneration is a direct cause of anesthesia-induced memory and learning disability, although a previous study has suggested an association between isoflurane-induced neurode-

generation in the developing brain and subsequent memory and learning disability.[1] Later studies could not establish the association between the two phenomena.[34] The results from this study suggest that isoflurane at ~0.5 MAC induced significant neurodegeneration but not memory or learning impairment. We propose that the following factors may contribute to the dissociation between isoflurane-mediated neurodegeneration and memory and learning impairment. First, our exposure concentrations were in some instances about half the MAC equivalent exposure of inhalational agents in studies that showed behavioral differences.[1] We used much lower concentrations because 0.75% was the highest concentration of isoflurane in published studies that displayed neuroapoptosis in neonatal mice and did not require us to stimulate the mice during the exposure to maintain a minimum mortality for our animals. Therefore, the degree of neurodegeneration in this study is considerably less than previous studies demonstrating an association between anesthesia-mediated neurodegeneration and memory and learning impairment.[1] Second, the combination of several anesthetics may potentiate neurodegeneration or cognitive dysfunction as demonstrated in a previous study,[1] whereas our study used only a single anesthetic agent. Third, although there was no association between isoflurane- or sevoflurane-mediated neurodegeneration and memory and learning disability, it is still possible that the ability of isoflurane to decrease synapse number and function[35] may be correlated with its effects to impair memory and learning. A recent study demonstrated that isoflurane can damage the synapse,[35] supporting vulnerability of the period of synaptogenesis in the developing brain to anesthesia-mediated neurodegeneration. Unfortunately, we did not examine the effects of isoflurane or sevoflurane on synapse members or function in the current study. Fourth, although isoflurane has been shown to impair memory and learning in adult and aged rodents,[6,7] few studies demonstrated severe or persistent anesthesia neurodegeneration in adult or aged brains. Obviously, future studies would be helpful to determine whether isoflurane induces greater damage than sevoflurane to the synapse number and function of the developing brain and whether these possible effects originate from their different potencies to cause calcium dysregulation as demonstrated in the tissue culture of our previous study.[14] It should also be noted that inhalational anesthetics can have both neuroprotective and neurotoxic properties, depending on the dose and duration used as demonstrated in our previous study.[36]

The mechanisms of anesthesia-mediated neurodegeneration on cognitive dysfunction in the developing brain remain unclear. This study demonstrated no role in the activation of cell cycles or BACE, which has been a proposed mechanism for anesthesia-mediated neurodegeneration in other models.[12,24] This discrepancy with other studies could be explained by the different anesthetics, their concentrations and durations, and variations in the study models. Both ketamine and isoflurane are considered inhibitors of *N*-methyl-D-aspartate receptors, which may play a role in anesthe-

sia-mediated neurodegeneration in the developing brain.[11] However, isoflurane does not affect cell cycle regulatory proteins as ketamine, which was demonstrated in a different model in a previous study.[12] BACE has been shown to increase the production of β-amyloid and contribute to the isoflurane-induced vicious cycle in tissue culture studies.[24] Although isoflurane seemed to cause an increasing trend in BACE in the developing brains of mice with this study, it did not reach statistical significance. We speculate that a higher concentration of isoflurane may still be able to increase BACE in the developing brains of mice, thus needing further investigation. GAPDH activation is an important mediator to induce apoptosis by a variety of stress factors[32]; however, it does not seem to play an important role in isoflurane-mediated neurodegeneration in the developing brain, at least in this animal model.

Our study has several limitations. Although some anesthetics are given to children for imaging studies in which there is minimal stimulation, most anesthetics are performed to allow children to undergo painful surgical procedures. We did not simulate any type of surgical stimulus during our anesthetic exposures for mice. Studies have shown that painful stimuli in neonatal rats in the absence of anesthesia can also cause the development of neuroapoptosis.[37] At this time, it is unclear what effect the addition of a surgical stimulus would have on the degree of neuroapoptosis and potential learning, memory, and behavioral abnormalities incurred during an inhalational anesthetic as the addition of ketamine ameliorated the neuroapoptosis caused by unopposed painful stimuli in newborn rats. More importantly, a recent study examining human neonatal pain and its relation to cognitive and motor development later in life demonstrated an association between increased painful stimuli and diminished cognition and motor function later in life.[38] Second, we used low concentrations of isoflurane and sevoflurane to minimize their adverse effects on cardiopulmonary physiology and mortality. This may underestimate the effects of both inhaled anesthetics on neurodegeneration and cognitive dysfunction as both effects are dose dependent.[1] Third, we could not directly determine the anesthetic-induced calcium changes because of technical limitations, which may help explain the different potencies in which isoflurane and sevoflurane induced neurodegeneration in our animal model.

In summary, this *in vivo* study demonstrated that isoflurane at ~0.5 MAC induced significantly greater neurodegeneration than an equipotent MAC equivalent of sevoflurane, without affecting memory and learning abilities significantly. The activation of cell cycle proteins, GAPDH, and BACE do not seem to be involved in the neurodegeneration induced by either isoflurane or sevoflurane in this animal model. Given the results from our laboratory, S100β has the potential to be used as a biomarker for anesthesia neurotoxicity studies in the developing brains of rodents. Further investigation will be needed to determine the usefulness of S100β in human studies. More research related not only to the mechanisms of neuroapoptosis induced by anesthetic drugs but also to the differences between similar classes of anesthetic agents will provide information that would allow us to better provide safe anesthetics for our potentially vulnerable pediatric patients.

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