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Original Article

Dose-dependent effects of sevoflurane exposure during early lifetime on apoptosis in hippocampus and neurocognitive outcomes in Sprague-Dawley rats

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Abstract: Sevoflurane has become a main method for induction of anesthesia in pediatric populations. Preclinical evidence suggest the neurotoxic effect of volatile anesthetics on the developing brain including sevoflurane. This study investigates the effect of different doses of sevoflurane on the developing brain. In this study, Sprague-Dawley rats of postnatal (P) day 7 were exposed to 0.3%, 1.3% and 2.3% sevoflurane for 6 hours. 6 hours after exposure, Nissl staining was performed to observe the morphological changes of the hippocampus and western-blot was done to evaluate the expression changes in cytochrome c, cleaved caspase-3, Bcl-2 and Bax. At P28, we used the step-through test and novel object recognition test to evaluate the influence of sevoflurane exposure on learning and memory of juvenile rats. We found that neonatal exposure to 2.3% but neither 0.3% nor 1.3% sevoflurane on P7 induced histopathological damage in the CA1 and CA3 subfields of the hippocampus. Only 2.3% sevoflurane induced hippocampal neural apoptosis via the mitochondrial-dependent pathway. Moreover, 2.3% sevoflurane deteriorated the learning and memory in juvenile rats, but 1.3% sevoflurane showed its positive effect. In conclusions, higher dose of sevoflurane lead to histopathological changes and apoptosis in neonatal rat hippocampus, as well as temporal neurocognition deficits.

Keywords: Sevoflurane, apoptosis, hippocampus, neurocognitive outcomes

Introduction

Every year, millions of children are treated with complex surgeries and procedures owing to the development of general anesthetics. For children, volatile anesthetics are an attractive option for anesthesia. However, recent preclinical experiments suggest that early exposure to commonly used general anesthetics could cause widespread neuroapoptosis and long-term neurocognitive deficits [1-5]. Retrospective epidemiologic studies showed that young children who had anesthetic exposures early in life could have potential learning problems [6-9].

Sevoflurane has become a mainstay of the inhalational anesthesia, especially in pediatric populations. Our previous study showed that exposure of neonatal rats to 2.3% sevoflurane for 6 hours caused neuronal apoptosis with pathological changes in brain hippocampus [10, 11]. Other preclinical findings also reported that sevoflurane leads to apoptosis in the developing brain and causes long-term cognitive deficits [12-15]. These results have raised concern about the possible detrimental effects of anesthesia in the young population. But not all studies report behavior deficits after exposure to sevoflurane. Chen et al recently found out that a lower dose of sevoflurane not only promotes hippocampal neurogenesis in neonatal rats, but also facilitates their performance in dentate gyrus-dependent learning tasks [16]. This means that there may not be a safe anesthetic for young children, but rather only a safe anesthetic exposure duration and concentration. Although dose-dependent neuronal apoptosis in response to anaesthetics has been documented, few studies have focused on the effect of different concentrations of sevoflurane on

developing brain neurocognitive outcomes. In this study, we aim to find out the injurious doses of sevoflurane and its long-term neurological effect.

Apoptosis is triggered by extrinsic and/or intrinsic cellular pathways [17]. The central role of mitochondria in regulating the intrinsic apoptotic death pathway has been well established. Mitochondria influences apoptosis of various types of cells, including neurons, by releasing proapoptotic substance into cytoplasm. The most prominent among these substances is cytochrome c, which causes activation of caspase proteases and cell death [18]. In the intrinsic pathway, apoptosis is regulated by a well balance of antiapoptotic bcl-2 family proteins and proapoptotic bcl-2 family proteins [19]. A proapoptotic member of the Bcl-2 family proteins, such as Bax, can be provoked by the apoptotic stimulus and permeabilized the outer mitochondrial membrane, which leads to the redistribution of cytochrome c from the mitochondrial intermembrane space into the cytoplasm [20]. Antiapoptotic bcl-2 family proteins, including Bcl-2, can protect the mitochondrial pathway of apoptosis by inhibiting Bax [19]. It has been reported that isoflurane may induce apoptosis through Bcl-2 family proteins and ROS-associated mitochondrial pathway of apoptosis [21]. However, studies on pathways involved in the sevoflurane induced developing brain apoptosis are limited. Therefore, we performed this study to determine the effects of different concentrations of sevofluran on apoptotic signaling in neonatal brain hippocampus. Our results demonstrated that higher dose of sevoflurane induced hippocampal neural apoptosis via the mitochondrial-dependent pathway, and this effect could be seen to be dose-dependent.

Materials and methods

Animals

The Sprague-Dawley (SD) rats (all male) used in this study were obtained from the Experimental Animal Center at Sun Yat-sen University. The use of these animals was approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University (Guangzhou, Guangdong, China). We made every effort to minimize the number of rats used as well as to minimize their suffering. The room they were housed in was illuminated with a 12-h light-dark cycle (light from 07:00 to 19:00), and the room temperature was maintained at 21±2°C.

Sevoflurane exposure

SD rats at postnatal day 7 (P7, weight 16-17 g) were randomly divided into an air-treated control group (Control group), a 0.3% sevoflurane-treated group (0.3% Sevo group), a 1.3% sevoflurane-treated group (1.3% Sevo group) and a 2.3% sevoflurane-treated group (2.3% Sevo group). Rats of three sevoflurane-exposed groups were placed in a plastic container and exposed to 0.3%, 1,3% and 2.3% sevoflurane, respectively, for 6 h using air as a carrier, with a total gas flow of 2 L/min. During sevoflurane exposure, the containers were heated to 38°C using a heating device (NPS-A3 heated device, Midea, Co., Guangdong, China). Sevoflurane, oxygen and carbon dioxide levels within the chamber were monitored using a gas monitor (Detex-Ohmeda, Louisville, Co.). After 6 h, the different doses of sevoflurane were stopped, and the pups were exposed to only air until they could move freely. Then, we placed them back into the maternal cage. During sevoflurane exposure, the respiratory frequency and skin color of the rats were monitored by an investigator. If signs of apnea or hypoxia were detected, the rat was exposed to air immediately and excluded from the experiment. Pups in the control group were placed into the same container as those in sevoflurane-exposedgroups; however, they were exposed only to air for 6 h.

Arterial blood gas analysis

The arterial blood analysis was done in both control group and sevoflurane groups. Blood samples were immediately obtained from the left cardiac ventricle after removal from the maternal cage (0 h, n = 3 in each subgroup) and at the end of 6 hours exposure (6 h, n = 3 in each subgroup). The samples were stored in heparinized glass capillary tubes. The samples were analyzed immediately after blood collection by blood gas analyzer (Gem premier 3000). The pH, arterial carbon dioxide tension, arterial oxygen tension, and blood glucose levels of arterial blood were analyzed. The analysis of each sample was repeated independently at least three times.

Histopathological examination

Brains of rats from the sevoflurane-exposed groups and the control group (n = 3/each group) underwent Nissl staining using the same procedure as in our previous studies [10]. Pups were anaesthetized with a lethal dose of 10% chloral hydrate 6 hours after exposure and perfused transcardially with saline until the liver and lungs became white, followed by an aldehyde fixative with a concentration of 4% formaldehyde in 0.1 M PB (pH 7.4). The duration of the perfusion was 15-25 min, after which the brains were removed and kept in a similar fixative as described above overnight. Tissue blocks (0.5 mm thick) from the hippocampus were embedded in paraffin, sliced in 5 μm thick sections and stained with Nissl. The results were examined in detail under a light microscope (Nikon ECLIPSE, 50i, Japan) to study morphological changes in the CA1 and CA3 regions of the hippocampus. We counted cells imaged from three sections per animal (n = 3 for each group). Nissl-positive cells were counted only if the structures were of the appropriate size and shape, possessed a Nissl-positive nucleus and cytoplasmic Nissl-positive particles. The number of Nissl-positive neurones in the pyramidal cell layers of the bilateral CA1 regions was counted at × 400 magnification by two individuals in a blinded manner [10]. Questionable structures were examined under × 1000 magnification and were not counted if identification remained uncertain.

Western-blot

The harvested brain tissues were subjected to Western blot analyses as described by our previous study [10]. The blots were incubated with, anti-Bcl-2 (1:1,000, mouse monoclonal; Cell Signaling Technology, Inc.), anti-BAX (1:1,000, rabbit polyclonal; Cell Signaling Technology), anti-cleaved caspase-3 (1:1,000, rabbit polyclonal, Asp175; Cell Signaling Technology), anti-cytochrome c (1:1000, rabbit polyclonal; Cell Signaling Technology) and antibody β-actin (1:1,000, Sigma, St. Louis, MO). The β-actin protein was used as a loading marker. The changes of protein levels were also examined using the ECL-PLUS system and photographed. The optical density was measured by analyzing scanned images using the Image J software. The changes of the protein levels ratio corresponding to β-actin were determined by the optical density measurements on Western immunoblot. All studies were performed a minimum of three times for each hippocampus sample of each rat.

Step-through test

The step-through test was performed 3 weeks after sevoflurane exposure in order to assess the learning and memory deficit caused by sevoflurane. The test was based on a protocol performed in a previous study [22]. The apparatus used consisted of six separated rooms. Every room (15 × 10 × 11 cm) could be divided into an illuminated compartment and a dark compartment with an interconnecting semicircular door (3 cm in diameter). The copper grid floor under the dark compartment was connected to a shock-maker that could deliver an electric current (36 V). For training trials, each animal was gently placed in the illuminated compartment with its back facing the door and allowed to enter the dark compartment freely for 3 min. When entering the dark compartment, the rat immediately received an electric shock to the paws and went back to the illuminated space through the door. After adaptation, formal training started as mentioned above and lasted for 5 min. The tests were repeated 24 h and 48 h later. During retention trials, the number of mistakes and the latency to initially enter the dark compartment were recorded within5 min. If the rat did not receive a shock within 5 min, it was assigned 300 s for the retention latency value. No rats excluded from the test. n = 10/each group.

Novel object recognition test

We used an apparatus that consisted of a round arena (diameter, 80 cm). Each rat in four groups received two 10-min sessions in the empty box to become accustomed to the apparatus and test room. Twenty-four hours later, each rat was placed in the box; the rat was first exposed to two identical objects for 10 min (sample phase); subsequently, one object was replaced with a new object; the rat was returned to the box for an additional 10 min (acquisition phase). The box and the objects were cleaned between trials to stop the olfactory cues. The short-term memory was tested 10 min after the sample phase, and the long-term memory was tested 24 h after the sample phase. Time spent in exploring each object was recorded. The rec-

Table 1. Arterial Blood Analysis

	Time, h	n	Arterial Blood Analysis			
			PH	$PaCO_2$, Kpa	PaO_2, Kpa	Glucose, mmol L^{-1}
Control group	0	3	7.38±0.05	3.54±0.53	13.32±0.44	5.6±0.4
	6	3	7.38±0.07	3.55±0.41	13.31±0.50	5.5±0.7
2.3% sevoflurane	0	3	7.37±0.05	3.57±0.49	13.34±0.64	5.4±0.6
	6	3	7.38±0.04	3.59±0.41	13.32±0.51	5.5±0.4

Neonatal exposure to sevoflurane does not induce significant cardiorespiratory dysfunction. Analysis of arterial blood gas revealed no significant differences in any of the measured parameters between mice exposed for 6 h to sevoflurane and control group exposed to air for 6 h (t test, all *P* values > 0.05). $PaCO_2$ = arterial carbon dioxide tension; PaO_2 = arterial oxygen tension; Glucose = Blood glucose levels.

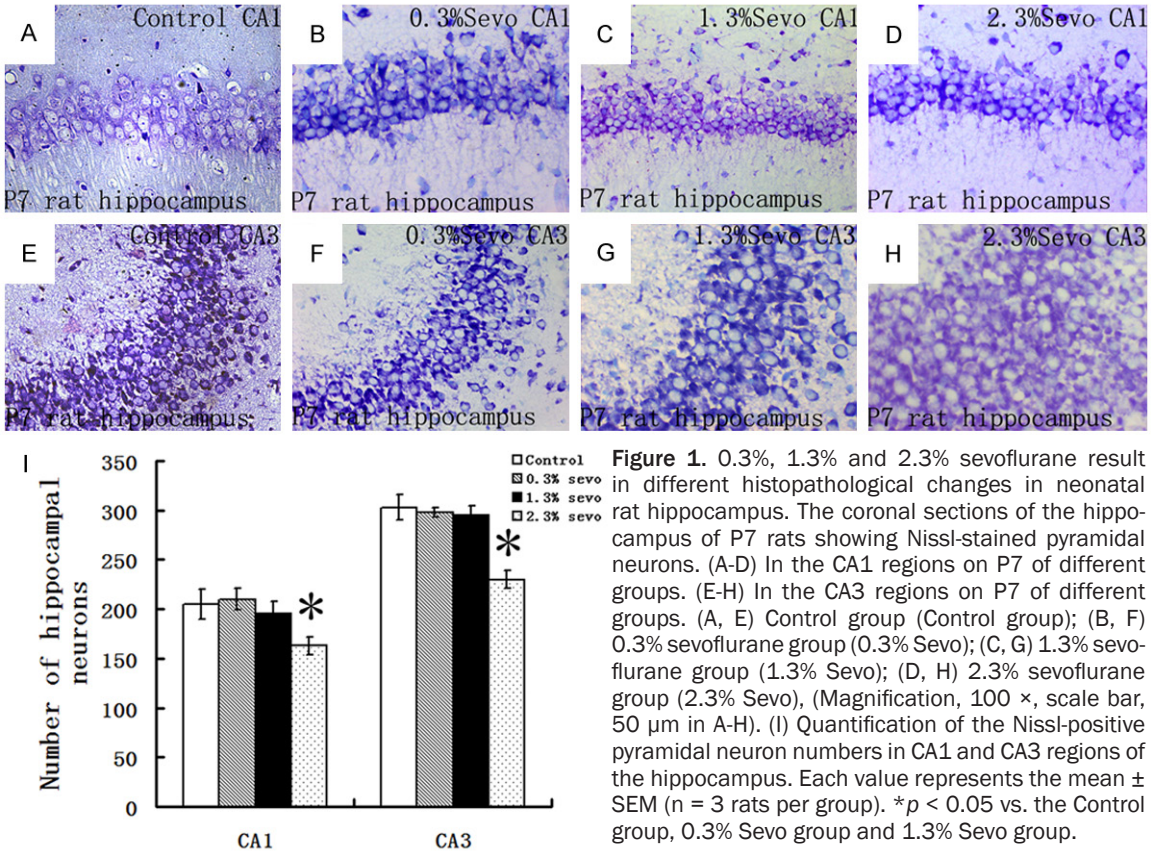

Figure 1. 0.3%, 1.3% and 2.3% sevoflurane result in different histopathological changes in neonatal rat hippocampus. The coronal sections of the hippocampus of P7 rats showing Nissl-stained pyramidal neurons. (A-D) In the CA1 regions on P7 of different groups. (E-H) In the CA3 regions on P7 of different groups. (A, E) Control group (Control group); (B, F) 0.3% sevoflurane group (0.3% Sevo); (C, G) 1.3% sevoflurane group (1.3% Sevo); (D, H) 2.3% sevoflurane group (2.3% Sevo), (Magnification, 100 ×, scale bar, 50 μm in A-H). (I) Quantification of the Nissl-positive pyramidal neuron numbers in CA1 and CA3 regions of the hippocampus. Each value represents the mean ± SEM (n = 3 rats per group). *$p < 0.05$ vs. the Control group, 0.3% Sevo group and 1.3% Sevo group.

ognition index was defined as the ratio of the time spent in exploring the novel object over the total exploration time during the acquisition phase (n = 10/each group).

Statistical analysis

All of the data are expressed as the mean ± SEM, and we performed the statistical tests using SPSS 15.0 software. The data from the histopathological examination was analyzed by one-way ANOVA and individual post-hoc comparisons (Bonferroni post-hoc test). We compared the data between the two groups using independent-samples t-tests. We analyzed the data from the step-through test between groups using a repeated measures two-way ANOVA; we analyzed the results of novel object recognition test using one-way ANOVA followed by individual post hoc comparisons (Bonferroni post hoc test). For the detailed statistical analysis, we used a post-hoc test. $p < 0.05$ was considered to be statistically significant.

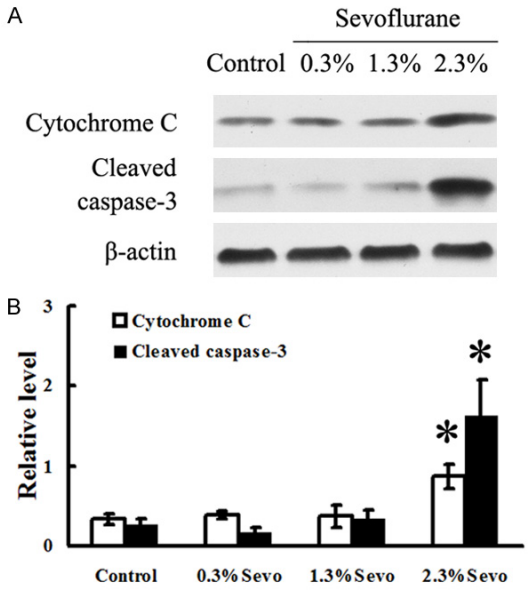

Figure 2. The effect of 0.3%, 1.3% and 2.3% Sevoflurane on the expression of cytochrome C and cleaved caspase-3. Samples were obtained from the hippocampus of rats subjected to air (Control, lane 1 in A) or different concentrations of sevoflurane treatment (lanes 2-4, 0.3% Sevo, 1.3% Sevo and 2.3% Sevo respectively in A) at 6 h after treatme. (B) 2.3% sevoflurane significantly increased the expression level of cytochrome c and cleaved caspase-3. *$p < 0.05$ vs. the Control group, 0.3% Sevo group and 1.3% Sevo group.

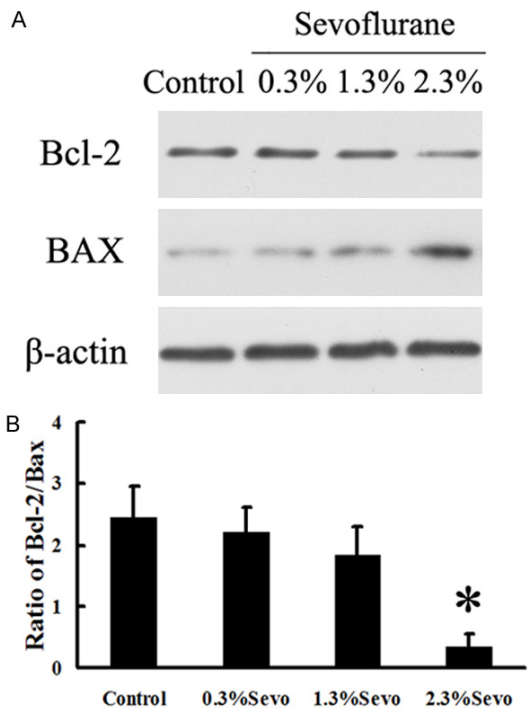

Figure 3. The effect of 0.3%, 1.3% and 2.3% Sevoflurane on the expression of Bcl-2 and Bax. Samples were obtained from the hippocampus of rats subjected to air (Control, lane 1 in A) or different concentrations of sevoflurane treatment (lanes 2-4, 0.3% Sevo, 1.3% Sevo and 2.3% Sevo respectively in A) at 6 h after treatme. (B) The ratio of Bcl-2 and Bax were calculated and 2.3% sevoflurane significantly decreased the ratio .*$p < 0.05$ vs. the Control group, 0.3% Sevo group and 1.3% Sevo group.

Results

Arterial blood analysis

According to the arterial blood analysis, there were no significant differences between the Control group and Sevo group in PH, oxygen tension, carbon dioxide tension and glucose levels (see supplementary data, **Table 1**).

2.3% but not 0.3% or 1.3% sevoflurane resulted in histopathological changes in neonatal rat hippocampus

In the Control group, the 0.3% Sevo group and the 1.3% Sevo group, no morphological changes were observed. However, in the 2.3% Sevo group, the cells were disorganized and not closely ranked with regard to some typical neuropathological changes including nucleus shrinkage and neuron loss (**Figure 1A-H**). Statistical analysis showed that there was a significant decrease in the density of healthy pyramidal neurons in the CA1 and CA3 regions of the hippocampus in the 2.3% Sevo group compared with the other three groups (*$p < 0.05$ compared to Control group, 0.3% Sevo group and 1.3% Sevo group, **Figure 1I**).

2.3%, but not 0.3% or 1.3% sevoflurane affected the expression of cytochrome c, cleaved caspase-3, Bcl-2 and Bax in neonatal rat hippocampus

Statistical analysis showed that 2.3% sevoflurane significantly increased the expression level of cytochrome c, cleaved caspase-3, Bax and significantly decreased the expression level of Bcl-2 compared to the other three groups (*$p < 0.05$ compared to Control group, 0.3% Sevo group and 1.3% Sevo group, **Figures 2** and **3**). However, lower concentrations of sevoflurane, including 0.3% and 1.3%, did not led to any difference in the expression of cytochrome c, Bcl-2, Bax and cleaved caspase-3 compared to the control group ($p > 0.05$, **Figures 2** and **3**).

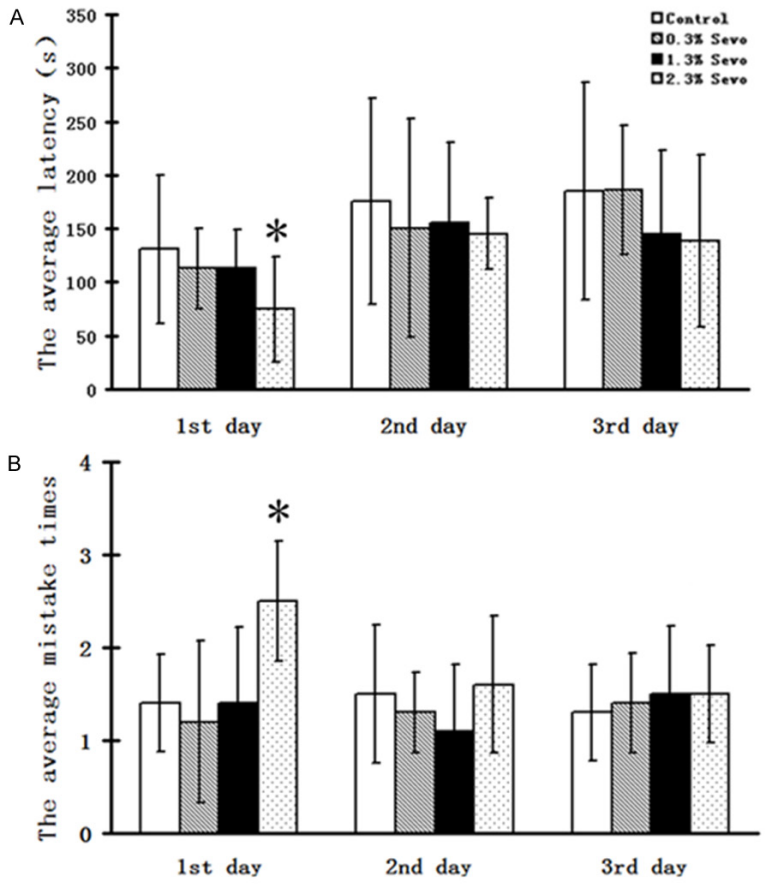

Figure 4. Effects of different doses of sevoflurane on step-through test. A: The average latency of all the four groups to the initial entry into the dark compartment. Exposure to 2.3% sevoflurane at a very young age significantly decreased the average latencies during the training and retention trials, and the difference exists on the first day. B: The average number of mistake times in the passive avoidance response after sevoflurane exposures. A significant difference was found forthe average number of mistakes only in the 2.3% sevoflurane group on the first day. *$p < 0.05$ vs. the Control group, 0.3% Sevo group and 1.3% Sevo group.

2.3% but not 0.3% or 1.3% sevoflurane significantly affected the passive avoidance response in the step-through test on the first testing day

A significant effect of 2.3% sevoflurane on the latency to the initial entry into the dark compartment was observed on the first testing day (*$p < 0.05$ compared to Control group, 0.3% Sevo group and 1.3% Sevo group, **Figure 4A**). Exposure to 2.3% sevoflurane in postnatal day 7 significantly decreased the latencies during the training and retention trials, but these differences occurred only briefly on the first day. During the next retention trials, no significant difference between the four groups with regard to the latency to initially enter into the dark compartment was observed ($p > 0.05$, **Figure 4A**). A significant effect of 2.3% sevoflurane on the number of mistakes was observed on the first testing day ($p < 0.05$, **Figure 4A**). No significant difference was found for the number of mistakes among the four groups on the second and third days ($p > 0.05$, **Figure 4B**).

1.3% sevoflurane significantly improved short term memory in juvenile rat

In this trial, we recorded the recognition index for short-term memory (STM) and long-term memory (LTM), and found that the recognition index of rats in 0.3% Sevo group was significant higher than the Control group, 0.3% Sevo group and 1.3% Sevo group (*$p < 0.05$ compared to Control group, 0.3% Sevo group and 1.3% Sevo group, **Figure 5A**). For the LTM, we observed no significant differences between the four groups ($p > 0.05$, **Figure 5B**).

Discussion

The widespread use of anesthesia in children currently makes its safety a major health issue of interest. Sevoflurane is a general inhalational anesthetic commonly used in pediatric surgery because it is fast acting and has a very short recovery time [23]. In the past few years, growing preclinical evidence has continually showed that commonly used anesthetic agents can cause widespread neuronal cell death in developing brains [24]. And some clinical foundings suggest that exposure to anesthesia lead to deficiency in cognition in children [6-9]. However, it is still difficult to determine the exact neurotoxicity effect associated with exposure to specific anesthetic like sevoflurane. So, in this study, we exposed postnatal 7-day rat pups to different doses of sevoflurane (0.3%, 1.3%, 2.3%) for 6 hours and evaluated their effects

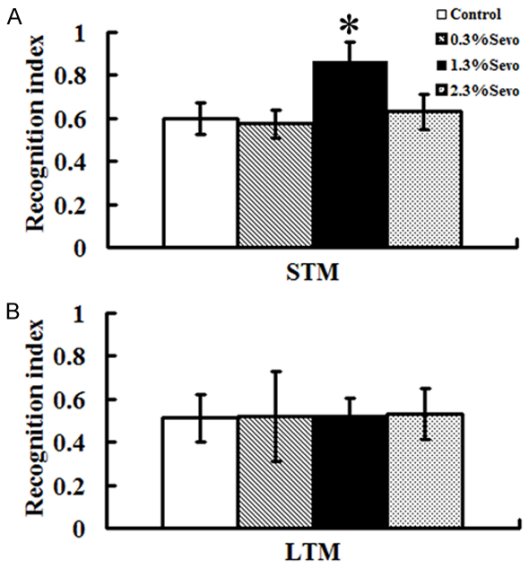

Figure 5. Different doses of sevoflurane on the the recognition index for short-term memory and long-term memory. A: For STM, the recognition index of rats in 0.3% Sevo group was significant higher than the other groups (*$p < 0.05$ compared to Control group, 0.3% Sevo group and 1.3% Sevo group). B: For the LTM, no significant differences were found between the four groups ($p > 0.05$).

on developing brain. We chose postnatal 7-day rats because peak synaptogenesis occurs during this period and brains are quite vulnerable to outside enviroment [25]. The commonly used clinical dose of inhalation anesthetics is between 0.65 minimum alveolar concentration (MAC) to 1.3 MAC. And the MAC of sevoflurane in neonatal rats is 3.28% [10]. Thus, 2.3% sevoflurane is comparable with that used in the clinical setting. We also observed the possible detrimental effects of below clinical doses of sevoflurane (0.3% and 1.3%) on developing brain.

Our results showed that exposure to 2.3% but not 0.3% or 1.3% sevoflurane for 6 hours leads to histopathological changes and apoptosis in the neonatal rat hippocampus. Moreover, only 2.3% sevoflurane affect the expression of cytochrome c, Bcl-2 and Bax in neonatal rat hippocampus. Anti-apoptotic factor Bcl-2 and the pro-apoptotic factor Bax belong to the Bcl-2 family proteins and they regulate the intrinsic, mitochondrial apoptotic pathway [26]. Mitochondria plays an important part in the apoptotic death of many types of cells, including neurons [18]. In intrinsic apoptotic pathway, the damaged mitochondria could release cytochrome *c* to cytosol, and then lead to caspase-3 activation and apoptosis [26]. Our findings suggest that sevoflurane can regulate Bax and Bcl-2 to cause the release of cytochrome *c*, which will initiate the mitochondrial pathway of apoptosis. Another research performed by Loop *et al* shown that sevoflurane caused disruption of mitochondrial membrane potential and release of cytochrome *c* from the mitochondria to the cytosol in human T lymphocytes [27]. But another *in vivo* study done by Wei et al told us that sevoflurane did not induce cytotoxicity in both PC12 cells and primary cortical neurons or change the Bcl-2/Bax ratio [28]. This might because different cells showed different sensitivity and in this situation sevoflurane at higher doses may also cause cytotoxicity.

Interestingly, we did not see significant changes in histopathological or proteins after expsoure to 0.3% or 1.3% sevoflurane. This indicates that lower concentrations of sevoflurane did not damage the developing brain but higher concentration dose. Dose-dependent neuronal apoptosis in response to anesthetics has been reported in both rodent and non-human primate studies, and these drugs included ketamine, propofol and isoflurane [3, 29, 30]. This was consistent with Shen's study [15]. In their research, they exposed the neonatal rats respectively to 1%, 2%, 3% or 4% sevoflurane. Behavior tests were performed and results showed that higher doses of sevoflurane in early life exposure can lead to spatial memory impairments in adulthood. But their results didn't reveal the reason why higher concentration lead to this long-term behavior changes. They used several behavior tests including Morris water maze test and in contextual-fear discrimination learning test. In our study, we used step-through test and recogonition test to learn behavior changes after sevoflurane expsoure.

The result of step-through test showed that 2.3% sevoflurane significantly affected the rats' passive avoidance response on the first testing day. This finding means that, in our experimental setting, sevoflurane might exert a significant but marginal effect on juvenile rats. Another study performed by Wang et al found that P7 rats exposed to 2.5% sevoflurane for 4 hours had significant spatial learning and memory impairments 7 weeks after anesthesia by using the Morris water maze test [14]. We assumed that, unlike Wang et al's study, the dose of sevoflurane (2.3%) used in our model was still

not high enough to cause long-term behavior deficits. And we kown that although hippocampus has been studied extensively as a part of the brain system responsible for spatial learning and memory, the step-through test used in this study involved other brain regions besides the hippocampus, such as the cerebellum, striatum, cerebral cortex and amygdala [31]. In further studies, we should perform more controls to show that the "learning and memory" phenotypes were not due to other simpler factors such as altered sensory/motor abilities. In novel object recognition test, we found that 1.3% sevoflurane improves object recognition memory in juvenile rats. This is quite interesting, because 0.3% or 2.3% sevoflurane had no influence on the object recognition memory, but 1.3% sevoflurane did. A recently published work done by Chen et al reported that a lower dose of sevoflurane (1.8%) promotes hippocampal neurogenesis in neonatal rats and improves their performance in dentate gyrus learning tasks [16]. But in our work, the effect of 1.3% sevoflurane on hippocampal neurogenesis and its relationship to object recognition need to be further studied. On the opposite, another work reported that high concentrations of sevoflurane (3-5%) decreased progenitor proliferation and increased cell death after anesthesia [32]. It indicates that different doses of sevoflurane affected death of the cells including progenitor cells in developing brain differently. Lower doses of sevoflurane did not lead to apoptosis in developing brain hippocampus, and even might have some protective effects on the developing brain. But futher studies are needed to find out the mechanism about the protective effect.

Conclusions

In conclusion, higher doses of sevoflurane lead to histopathological changes and apoptosis in the neonatal rat hippocampus as well as marginal neurocognitive damage. These results raised our concern about the possible detrimental effects of high concentrations of anesthetics on the young population. This means there might not be a safe anesthetic for young children, but rather a safe anesthetic exposure duration and concentration.

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Disclosure of conflict of interest

None.

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