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Original article

Neuronal apoptosis may not contribute to the long-term cognitive dysfunction induced by a brief exposure to 2% sevoflurane in developing rats

Yi Lu, Yan Huang, Jue Jiang, Rong Hu, Yaqiong Yang, Hong Jiang*, Jia Yan*

Department of Anesthesiology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiao Tong University, School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, China

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ABSTRACT

Background: Sevoflurane is an inhaled anesthetic commonly used in the pediatric. Recent animal studies suggest that early exposure to high concentration of sevoflurane for a long duration can induce neuroapoptosis and later cognitive dysfunction. However, the neurodevelopmental impact induced by lower concentration and shorter exposure duration of sevoflurane is unclear. To investigate whether early exposure to 2% concentration of sevoflurane for a short duration (clinically relevant usage of sevoflurane) can also induce neuroapoptosis and later cognitive dysfunction.

Methods: Rat pups were subjected to control group, 2% sevoflurane for 3 h and 3% sevoflurane for 6 h. TUNEL assay and apoptotic enzyme cleaved caspase-3 measured by western blot were used for detection of neuronal apoptosis in frontal cortex and CA1 region of hippocampus 24 after sevoflurane treatment. Long-term cognitive function was evaluated by Morris water maze and passive avoidance test as the rats grew up.

Results: The apoptotic levels in frontal cortex and CA1 region were significantly increased after rats exposed to 3% sevoflurane for 6 h ($P < 0.05$), but not 2% sevoflurane for 3 h ($P > 0.05$). Exposure to both 2% sevoflurane for 3 h and 3% sevoflurane for 6 h could cause long-term cognitive dysfunction and animals exposed to 3% sevoflurane for 6 h exhibited worse neurodevelopmental outcomes ($P < 0.05$).

Conclusion: It was suggested that neuronal apoptosis might not contribute to long-term cognitive dysfunction induced by 2% concentration and short exposure time of sevoflurane. Our findings also suggested that the mechanisms of sevoflurane-induced neurodevelopmental impact might be various, depending on the concentration and exposure duration.

1. Introduction

Over the past two decades, a series of animal experiments have indicated that various general anesthetics may be neurotoxic to the developing brain [1–5]. Thus, the potential for anesthetics-induced developmental neurotoxicity is of concern to the anesthesiologists, surgeons and parents of children undergoing surgery. The risks of childhood anesthesia have recently emerged as a public health concern [6,7]. In 2009, the U.S. Food and Drug Administration (FDA) established a partnership with the International Anesthesia Research Society (IARS) SmartTot to offer funds for preclinical and clinical studies concerning anesthetics-related neurodevelopmental issues [8]. Moreover, the Pediatric Anesthesia and NeuroDevelopment Assessment (PANDA) study team holds a biennial scientific symposia to review recent preclinical and clinical data related to anesthetic neurotoxicity [9,10].

Every year, millions of children are exposed to inhaled anesthetics [3]. Sevoflurane is a commonly used inhaled anaesthetic for the induction and maintenance of general anesthesia during surgery. Because it has the advantage of a low blood–gas partition coefficient and pungency, sevoflurane is widely used as a pediatric anesthetic. Recently, several animal studies reported that early-life long (6–9 h) exposure to high concentrations (3–4%) of sevoflurane could cause neuronal apoptosis and subsequent long-term cognitive impairment [11–15]. However, 3 h exposure to the lower concentrations (1–2%) of sevoflurane more closely approximates typical general pediatric anesthetic episodes for anesthesia maintenance [16]. Whether lower concentrations and a shorter duration of exposure to sevoflurane can induce neuronal apoptosis and later cognitive impairment is unclear.

* Corresponding authors.
E-mail addresses: mzkyanj@163.com (J. Yan), dr_hongjiang@163.com (H. Jiang).

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The immature human brain is most vulnerable to neurotoxic agents during the "brain growth spurt" (BGS), which begins at mid-gestation and continues for 2–3 years after birth [17–19]. In rodents, the window of vulnerability to neurotoxic agents occurs primarily during the first 2–3 weeks after birth [20,21]. In this study, we aimed to investigate whether 3 h of exposure to 2% sevoflurane, as used in clinical practice, has pro-apoptotic effects on the developing brain and impairs the long-term cognitive function in rats in the same manner as prolonged exposure to high concentrations.

2. Materials and methods

2.1. Animals

Because peak anesthesia-induced neurodegeneration in rodents occurs on postnatal day (PND) 7 [22], Sprague-Dawley (SD) PND7 rats weighing 14–18 g, provided by the Animal Center of Shanghai Jiao Tong University School of Medicine (Shanghai, China) were used in this study. The housing and treatment of the animals were in accordance with the National Institutes of Health guidelines for animal experimentation and approved by the institutional animal care and use committee. The animals were kept on a 12-h light/dark cycle (light from 7 am to 7 pm) with room temperature (23 ± 1 °C).

2.2. Sevoflurane exposure

Rat pups were separated from their mothers for acclimatization prior to sevoflurane exposure. Pups from the same litter were randomly allocated to three different groups. Totally, ninety PND7 rats were included in this study ($n = 30$ for each group). Rats in the control group received 100% oxygen for 6 h in a chamber at 37 °C. Rats in the other two groups were exposed to either 2% sevoflurane (SEVOFRANE®, Osaka, Japan) for 3 h (Sevo1 group) or 3% sevoflurane for 6 h (Sevo2 group) under 100% oxygen in the same chamber at 37 °C as described previously [13]. The concentration of sevoflurane in the chamber was monitored and maintained by a vaporizer as we described previously [23]. The gas flow to the chamber was 2 l/min. We chose these treatments because 3 h exposure to 2% seveflurane more closely approximates typical general pediatric anesthetic episodes for anesthesia maintenance [16] and 6 h exposure to 3% sevoflurane can cause neuronal apoptosis in developing animals [11,12,14,15].

2.3. Arterial blood gas analysis

To determine adequacy of ventilation and oxygenation, arterial blood samples ($n = 6$) were obtained from the left cardiac ventricle in each group at the end of anesthesia, and the samples were immediately analyzed by a blood gas analyzer (Radiometer, ABL800, Denmark). We compared the pH, pO_2, pCO_2, oxygen saturation (sO_2), and the concentrations of blood glucose (Glu), lactic acid (Lac) and bicarbonate (HCO_3^-) among the groups. Animals were killed by lethal injection of pentobarbital at the time of blood sampling.

2.4. Analysis of apoptotic levels

2.4.1. TUNEL assay of brain

Twenty-four hours after sevoflurane exposure, six rats from each group ($n = 6$) were anesthetized with sodium pentobarbital and the brains were perfused, fixed, dehydrated and made into paraffin sections (5 μm), as described previously [24]. Apoptotic cells in the brain sections were detected by TUNEL Assay using the FragEL™ DNA Fragmentation Detection Kit (Merck, Darmstadt, Germany), according to the manufacturer's protocol. Briefly, brain sections were permeabilized with proteinase K (20 μg/ml) at room temperature for 20 min. Endogenous peroxidase was inactivated by 3% H_2O_2. Specimens were incubated for 1.5 h with terminal deoxynucleotidyl transferase (TdT) labelling reaction mixture, and apoptotic cells were visualized with 3,3′-diaminobenzidine (DAB), and normal nuclei were counterstained with methyl green. Because the cerebral cortex reaches peak vulnerability to anesthetics at PND7 and the hippocampus is closely related to learning and memory [25], the number of apoptotic neurons in the frontal cortex and the CA1 region of the hippocampus was quantified. We selected two random viewing fields (400×) per region (frontal cortex and CA1) from one brain section per animal for analysis in a double blinded manner.

2.4.2. Western blot

Apoptosis was also assessed using western blot to quantify cleaved caspase-3 (Cl-Csp3) in all groups ($n = 6$). Briefly, tissue samples of the frontal cortex and CA1 region were collected from three groups twenty-four hours after sevoflurane exposure. Tissues were lysed in a buffer containing a protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) and homogenated. The homogenate was centrifuged and the supernatant was collected for further analysis. Protein concentrations were measured by BCA Protein Assay Kit (Novagen, San Diego, CA, USA). Equal amounts of protein were boiled in loading buffer (Beyotime, Beijing, China) and separated by 10% polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose, and the blots were probed overnight with anti-cleaved caspase-3 (1:200, Millipore, Darmstadt, Germany) and β-actin antibodies (1:500, internal standard, Santa Cruz, San Diego, CA, USA) at 4 °C. Primary antibodies were visualized using secondary antibodies conjugated to horseradish peroxidase (Santa Cruz, San Diego, CA, USA) and ECL reagent (Pierce, Rockford, IL, USA). Quantitative analysis of Cl-Csp3 was normalized to β-actin using the Quantity One software.

2.5. Neurologic assessment

2.5.1. Morris water maze

To assess neurodevelopmental outcomes, particularly the learning and memory functions of juveniles, rats from all groups

Table 1
Arterial blood gas analysis for the three groups.

	pH	pCO_2 (mmHg)	pO_2 (mmHg)	sO_2 (%)	Lac (mmol/l)	HCO_3^- (mmol/l)	Glu (mmol/l)
Control	7.463 ± 0.030	29.4 ± 1.2	117.5 ± 4.8	99.6 ± 0.1	1.7 ± 0.2	20.2 ± 0.5	5.5 ± 0.3
2%Sevo 3h	7.417 ± 0.025	30.6 ± 1.1	113.9 ± 6.9	99.5 ± 0.1	1.8 ± 0.2	21.2 ± 0.7	5.5 ± 0.4
3%Sevo 6h	7.404 ± 0.045	32.3 ± 1.5	108.0 ± 5.4	99.3 ± 0.2	1.9 ± 0.2	21.0 ± 0.8	5.9 ± 0.4

[a] $P < 0.05$ compared to the control group.