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Original Article

Sevoflurane impairs acquisition learning and memory function in transgenic mice model of Alzheimer's disease by induction of hippocampal neuron apoptosis

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Abstract: Objectives: To investigate the mechanism and effect of sevoflurane on learning and memory function in transgenic mice model of Alzheimer's disease (AD). Methods: A total of 45 Tg2576 mice were used and randomly assigned to control, sham, and sevoflurane group. Spatial learning and memory ability were measured before and after sevoflurane exposure using morris water maze (MWM) and Y-maze behavioral tests. Moreover, TUNEL assay was carried out to determine the cell death in hippocampal cornuammonis (CA) 1, CA3, and dentate gyrus (DG) region. Apoptosis-related protein (caspase-3 and Bcl-xL) expression in the hippocampus was analyzed by Western blotting. Results: The MWM results showed that there were no significant differences in the swimming speed after sevoflurane exposure among the three groups. However, the escape latency, time spent in original quadrant, and the number of correct trials (Y-maze) were significantly lower after sevoflurane anesthesia exposure in the sevoflurane group than the sham group and control group ($P < 0.05$). Besides, the apoptotic cell numbers of the CA1 and CA3 region in the sevoflurane group were significantly higher than those in the sham group and control group ($P < 0.05$). Western blotting results showed that the protein expression levels of Bcl-xL were significantly higher, but the caspase-3 levels were significantly lower in the sevoflurane group than those in the control group and sham group (both $P < 0.05$). Conclusion: Our results indicate that sevoflurane might impair acquisition learning and memory function in AD by induction of hippocampal neuron apoptosis.

Keywords: Alzheimer's disease, sevoflurane, acquisition learning and memory function, apoptosis

Introduction

Alzheimer's disease (AD) is the most common type of dementia, accounting for an estimated 60% to 80% of cases [1]. It is a progressive neurodegenerative disorder characterized by decline of cognitive function and the presence of extracellular β-amyloid plaques. Clinically, AD represents spatial memory loss in early stage of the onset and impaired judgment, disorientation, confusion, behavior changes in later symptoms [2, 3]. It has been reported that approximately 13% of all adults over age 65 are affected by AD and related forms of dementia [4]. AD not only impacts on an individual's health, but also imposes a substantial financial burden to society [5, 6]. It has been well demonstrated that age, family history, female gender, education, and specific genetic mutations are the strongest risk factors of AD [7]. The mechanism underling AD, however, is not fully illumined, and a sufficient treatment for the disease has not been found yet [8]. Therefore, it is imperative to characterize the potential risk factor to prevent AD.

Recently, the association between anesthesia and AD has been gained great attention [9]. Exposure to general anesthesia (GA) has been proposed as a potential risk factor for the subsequent development of postoperative cognitive dysfunction (POCD) and AD [10]. Some animal researches have demonstrated that inhaled anesthetics could increase the formation of bamyloid plaques and neurofibrillary tangles [11-14]. However, controversy concerning the association exists. A recent meta-analysis and randomized controlled trials have

suggested that GA is not associated with an increased risk of AD [4, 15]. Sevoflurane is the most widely-used inhalation anesthetic. Previous studies have shown that sevoflur aneanesthesia not only induces neurotoxicity in the normal brain tissues of adult and pregnant mice [16, 17], but also promote AD neuropathogenesis by inducing apoptosis and increasing β-amyloid protein levels [12]. However, Callaway *et al.* suggested that sevoflurane anesthesia does not impair acquisition learning or memory in young adult and aged rats [18]. Hence, further studies should be performed to confirm the association.

In our study, we therefore set out to investigate the effects of sevoflurane on learning and memory function in transgenic mice model of AD, as well as the underling associated mechanism.

Materials and methods

Animals

In our study, a total of 45 young Tg2576 mice (23 male, 22 female) purchased from the Chinese Academy of Medical Sciences were used in the experiment. After weaning, the mice were single housed in individually ventilated cages with free access to standard food and water. They were maintained on 12:12 h reversed light-dark cycle, with controlled temperature (20-22°C) and humidity (50-60%). The animal experiments were in line with the governmental guidelines for care and use of laboratory animals and approved by the Committee for Animal Research of our hospital.

Anesthesia and treatment

The 45 young Tg2576 mice were randomly allocated to three groups (n = 15): control group, sham group, and sevoflurane group. The mice in the control group received no special intervention. The mice in the sevoflurane group were placed in an anesthetic induction chamber containing sevoflurane (2.5%) in 100% O_2 for about 2 h, whereas the sham group mice only received 100% O_2 at a matched flow rate for about 2 h in an identical chamber. The animals breathed spontaneously, and anesthetic concentration and O_2 concentration were determined continuously. The temperature (37 ± 1°C) was controlled using warming mats and rectal temperatures of the mice were assessed per 30 min interval. The mean arterial blood pressure of the mice was monitored noninvasively using a tail cuff (BP-2000; Visitech Systems, Inc., Apex, NC, USA).

Morris water maze (MWM) test

MWM behavioral tests were performed to examine the spatial learning and memory ability of the animals. The animals were placed in a closed, quiet room with dimmed lights and were acclimatized to facility environment for approximately half an hour before this test. The facility included a circular tank (160 cm in diameter and 30 cm deep, maintained at 23 ± 1°C) with black walls. The tests were carried out 4 consecutive days (six trials per day) before and after anesthetic exposure. The last time for the trial was about 10 min, with an interval of 30 min between two trials. At the beginning of trials, mice in each group were immersed in the water maze individually in the same quadrant. Each animal was allowed 60 s to look for the platform, but, if the mouse failed within 60 s, it was remained for another 10 s. The time was recorded and compared with evaluate vision and swimming ability. Swimming speed (cm/s), escape latency(s), time in original quadrant (%), were recorded and compared. At the end of trials, the mice were removed from the tank, towel-dried and returned to the home cage. After 30 min, the mice were back to the maze tank for subsequent trials. Performances were videotaped and analyzed using Ethovision Video-Tracking Software (v3.1.16 Noldus, The Netherlands).

Y-maze test

The Y-maze apparatus consisted of three equally spaced arms arranged 120° apart (41 cm long × 12 cm high × 3 cm wide) and positioned 60 cm above the floor. A signal light was positioned at end of each arm. Each mouse was placed in a random arm of the Y-maze center and then allowed to explore freely for 5 min. The correct response was recorded if the mouse succeeded to escape to a safe region within 10 s, whereas an error was recorded if the mouse failed to escape to a safe region within 10 s. Number of correct trials in Y-maze was recorded to assess memory.

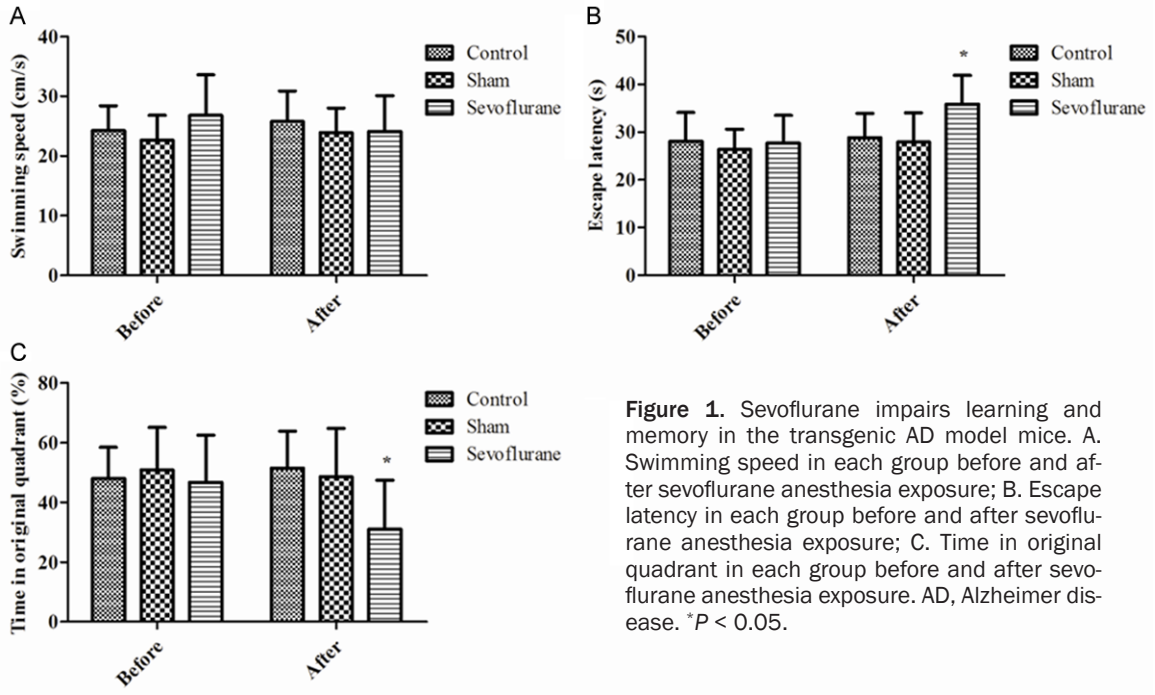

Figure 1. Sevoflurane impairs learning and memory in the transgenic AD model mice. A. Swimming speed in each group before and after sevoflurane anesthesia exposure; B. Escape latency in each group before and after sevoflurane anesthesia exposure; C. Time in original quadrant in each group before and after sevoflurane anesthesia exposure. AD, Alzheimer disease. $^{*}P < 0.05$.

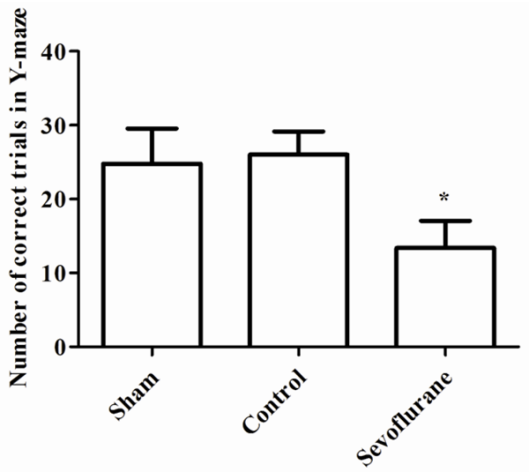

Figure 2. Number of correct trials in Y-maze. $^{*}P < 0.05$.

Apoptosis assay

TUNEL assay was performed to analyze cell death according to the manufacturer's instructions using TACS 2 TdT®-DAB In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA). The mice were killed under anesthesia with pentobarbital sodium (80 mg/kg). The brain tissues including hippocampalcornu-ammonis (CA) 1, CA3, and dentate gyrus (DG) were harvested, immersed in 3% H_2O_2, and washed with phosphate buffer saline (PBS), incubated with proteinase K solution (Life Technologies, Ambion) at 37°C for 20 min. Then the sections were maintained in TUNEL reaction mixture for 1 h at 37°C, followed by incubation in 50 μl converter-POD for another 30 min at 37°C. After washing with PBS, the sections were incubated with diaminobenzidine (DAB) substrate solution for 10 min. Finally, the sections were counterstained by methyl green and analyzed under × 400 magnifications by fluorescent microscope (Fluoview FV1000, Olympus, Tokyo, Japan) in 5 vision fields.

Western blotting

For Western blotting analysis, protein density was determined using abicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Pierce Protein Research Products, Rockford, USA). Protein samples were resolved by 10-12% sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis, transferred to polyvinylidenedifluoride (PVDF) membranes (Sigma), blocked with 5% skim milk powder in Tris-buffered saline Tween (TBST) 20 for 2 h. Then the membranes were incubated overnight at 4°C with the following the primary antibody: anti-caspase-3 antibody (Cell Signaling), anti-Bcl-xL antibody (Cell Signaling). After washing, membranes were then incubated with horseradish peroxidase-conjugated labelled secondary antibody (Cell Signaling). Mouse anti-β-actin monoclonal antibody (Cell Signaling) was used as an internal control. The blots were finally developed an enhanced chemiluminescence

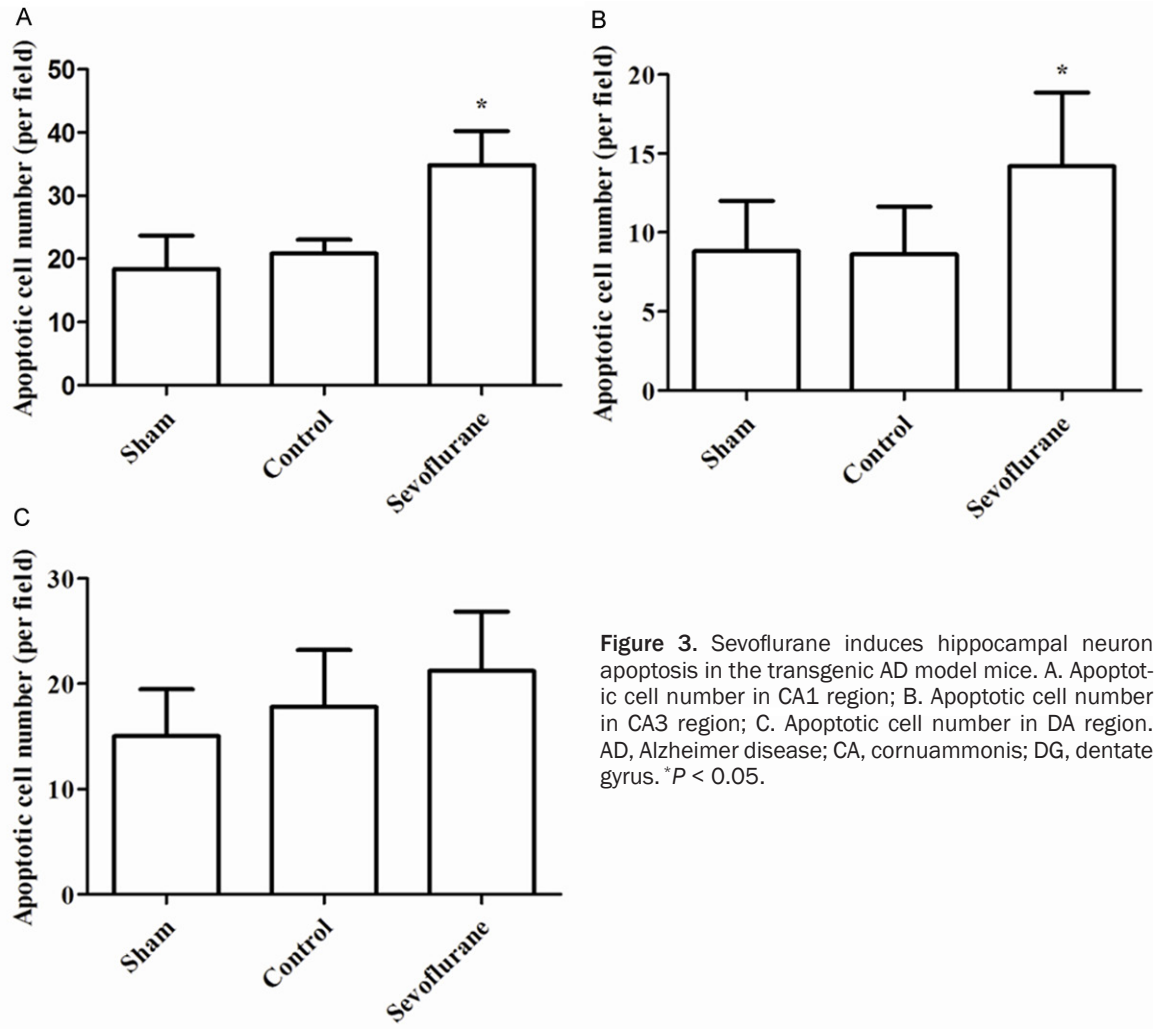

Figure 3. Sevoflurane induces hippocampal neuron apoptosis in the transgenic AD model mice. A. Apoptotic cell number in CA1 region; B. Apoptotic cell number in CA3 region; C. Apoptotic cell number in DA region. AD, Alzheimer disease; CA, cornuammonis; DG, dentate gyrus. $^{*}P < 0.05$.

(ECL Plus Western Blotting Detection System; GE Healthcare).

Statistical analysis

The collected data, expressed as means ± standard deviation (SD), were analyzed using the GraphPadPrism 5.0 software (GraphPad Inc., San Diego, CA). One-way analysis of variance (ANOVA) and Student's t test were performed to compare means within groups, followed by the post-hoc Tukey test if necessary. Differences were considered significant if $P < 0.05$.

Results

Sevoflurane impaired learning and memory in the transgenic AD model mice

To explore the effect of sevoflurane anesthesia on learning and memory in the transgenic mice of AD model, MWM test and Y-maze were performed. The MWM results showed that there were no significant differences in the swimming speed, escape latency and time spent in original quadrant before sevoflurane exposure among the three groups. Besides, no significant differences were found in the swimming speed after sevoflurane anesthesia exposure among the three groups. However, the escape latency and time spent in original quadrant were significantly decreased after sevoflurane anesthesia exposure in the sevoflurane group compared with the sham group and control group ($P < 0.05$) (**Figure 1A-C**). Moreover, the Y-maze test results demonstrated that the number of correct trials was significantly reduced in the sevoflurane group compared with the sham group and control group ($P < 0.05$) (**Figure 2**). These results indicated that sevoflurane impaired learning and memory in the AD model mice.

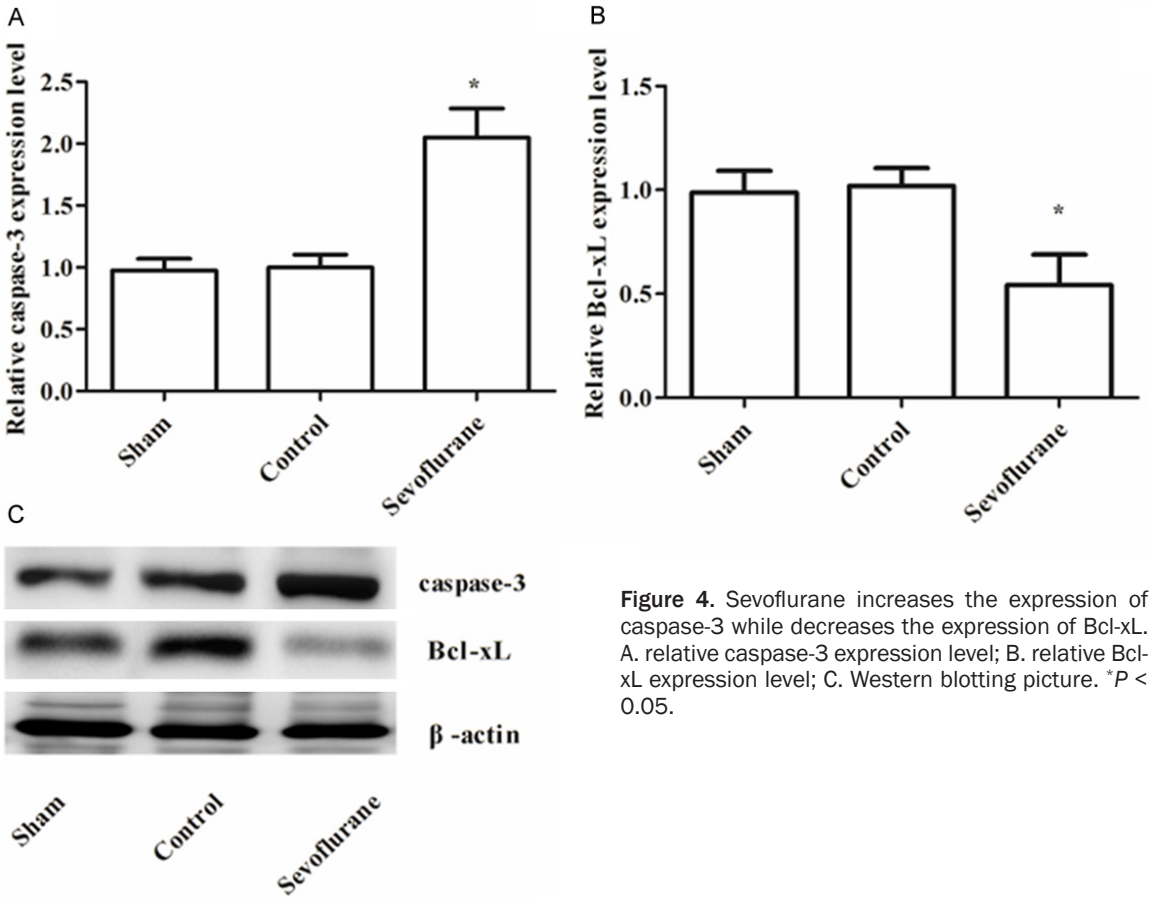

Figure 4. Sevoflurane increases the expression of caspase-3 while decreases the expression of Bcl-xL. A. relative caspase-3 expression level; B. relative Bcl-xL expression level; C. Western blotting picture. $^{*}P < 0.05$.

Sevoflurane induced hippocampal neuron apoptosis in the transgenic AD model mice

In order to observe the effect of sevoflurane on hippocampal neurons, we carried out TUNEL assay. Apoptotic cell number was counted in the CA1, CA3 and DG region. We found that the apoptotic cell number of the CA1 and CA3 region in the sevoflurane group were significantly higher than that in the sham group and control group ($P < 0.05$) (**Figure 3A** and **3B**). But, there were no significant differences in the DG region among the three groups (**Figure 3C**). Our results suggested that sevoflurane further aggravated the learning and memory ability by inducing hippocampus neuron apoptosis.

Sevoflurane increased the expression of caspase-3 while decreased the expression of Bcl-xL

To further explore the related mechanisms respect to apoptosis, we verified the protein expression levels of Bcl-xL and caspase-3 in the mice hippocampus in each group by Western blotting. The results showed that the protein expression levels of Bcl-xL were significantly higher, but the caspase-3 levels were significantly lower in the sevoflurane group than those in the control group and sham group (both $P < 0.05$) (**Figure 4A-C**).

Discussion

To date, though, there have been no definite conclusions strongly suggesting the relationship between anesthesia and AD; there have been a good deal of studies suggesting that inhalation anesthetics, such as isoflurane [12, 19, 20], desflurane [21], and sevoflurane [12], may be responsible for the increase of β-amyloid protein in human tissue culture studies. In the present study, we explored the effects of sevoflurane on the learning and memory function in transgenic Tg2576 mice model of AD. Our results suggest that sevoflurane might impair acquisition learning and memory function in AD by inducing hippocampal neuron apoptosis.

At the present time, the issue regarding the effects of inhaled anesthetics on brainin cludes apoptosis, neuronal damage and durable cognitive dysfunction [22-25]. Though the underling mechanisms are complex and may involve in some shared physiological pathways, recent studies indicate that protein misfolding and aggregation, one of the related mechanisms for cell death in neurodegenerative diseases, can be enhanced by anesthetics [26, 27]. Inhaled anesthetics, small haloalkanes or haloethers, bind to hydrophobic cavities of proteins [28]. They could increase the levels of small oligomers [29] such as amyloid b (Ab) peptides aggregates that are the major component of senile plaques. Then these small toxic oligomers may initiate a second sequence involving the abnormal hyperphosphorylation of tau [30, 31]. The hyperphosphorylation of tau in combination with accumulation and aggregation of amyloid-β peptides are hallmarks of AD neuropathogenesis in humans. Sevoflurane, currently the most used anaesthetic, is widelyused in pediatric anesthesia. It has been reported that sevoflurane causes less cytotoxicity, a smaller increase in intracellular calcium, no long-term neurocognitive sequelae, and no impairment on working memory compared toisoflurane, propofol, and desflurane. But, there are also contradictive conclusions. For example, Satomoto *et al.* [32] suggested that sevoflurane induced neurodegeneration and abnormal social behaviors in mice. Istaphanous *et al.* reported an equal toxicity between sevoflurane and isofluarne [33]. Moreover, a recent neuro imaging study indicated that human emotional memory could be blocked by sevoflurane though suppressing cerebral metabolism [34]. Hence, whether or not sevoflurane causes neurocognitive dysfunction is still unclear. In our study, we chose sevoflurane to investigate the possible effect of sevoflurane on learning and memory function in transgenic mice model of AD, as well as the related mechanism.

Spatial learning and memory ability was assessed before and after sevoflurane exposure using MWM behavioral tests that has been well established to be sensitive to hippocampal impairments. We found that there were no significant differences in the swimming speed, escape latency and time spent in original quadrant before sevoflurane exposure among the three groups. However, after sevoflurane anesthesia exposure, the escape latency and time spent in original quadrant were significantly decreased. To further confirm the results, we performed the Y-maze test. The Y-maze-behavioral test is regarded as an indicator of short-term memory. This behavioral test showed similar results as the MWM results, indicating the learning and memory function were impaired by sevoflurane anesthesia exposure. The possible mechanism respect to our MWM and Y-maze test results were analyzed by TUNEL assay. The results demonstrated that the apoptotic cell number of the CA1 and CA3 region in the sevoflurane group were significantly higher than that in the sham group and control group, suggesting that sevoflurane aggravated the learning and memory ability by inducing hippocampus neuron apoptosis. To further reveal the apoptosis mechanisms, the protein expression levels of Bcl-xL and caspase-3 in the mice hippocampus were determined. Bcl-xL, an important member of anti-apoptotic Bcl-2 family, suppresses cell death by binding the α-helical BH3 domain of a family of proapoptotic proteins such as Bak, Bad, Bid, and Bax [35]. Caspases family plays an important role in mediating programmed cell death, namely apoptosis. Among the caspases family, caspase-3 is a frequently activated death protease that directly cleaves apoptotic substrates [36]. Our results showed that the protein expression levels of Bcl-xL were significantly higher, but the caspase-3 levels were significantly lower, indicting the activation of caspase and apoptosis. These results were in line with Dong *et al.* [37] and Lu et al. [17]. In their studies they both showed the caspase and apoptosis could be activated by exposure to sevoflurane.

Taken together, we would like to indicate that sevoflurane might impair acquisition learning and memory function in AD by induction of hippocampal neuron apoptosis. Our current findings provided experimental evidence that inhalational anesthetic sevoflurane might be associated with AD neuropathogenesis. Therefore, it should be noteworthy that the use of sevoflurane as an anesthetic, especially in those patients with AD and those are highly suspicious of AD.

Disclosure of conflict of interest

None.

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