Original Article

Cognitive Impairments Induced by Repeated Sevoflurane Exposure During Pre-adolescence in Adult Male and Female Rats: Involvement of Biochemical, Histological and Neuroplasticity Approaches

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Abstract

Background: In some therapeutic interventions, repeated exposure to pre-adolescence anesthesia is necessary. According to research, exposure to general anesthetics during pre-adolescence can lead to cell death, cognitive and behavioral problems, and neurobehavioral difficulties as an adult. The current study aimed to provide detailed morphological and functional evaluations of the long-term impacts of repeated sevoflurane exposure in male and female rats.

Materials and Methods: Seventy-two pre-adolescent rats were randomly divided into male and female control and inhaled sevoflurane groups (concentration of 2%) daily for 15 days. Animals received care for 20-30 days. The influence of repeated exposure to sevoflurane on cognitive functions was tested using the Morris Water Maze, novel object, and social interaction tests. As a measure of oxidative stress, superoxide dismutase (SOD) and glutathione levels were measured. Toluidine blue stain was utilized to evaluate the number of dark neurons in the hippocampus. Effects of sevoflurane on synaptic plasticity were compared in the performant pathway of the CA1 of the hippocampus.

Results: Repeated sevoflurane exposure in pre-adolescence led to behavioral disorders in male and female adult rats; there was no significant difference in levels of superoxide dismutase and glutathione. We found a significant quantifiable increase in dark neurons. Electrophysiological recordings indicated impaired long-term potentiation and pair-pulse in adult animals that received repeat sevoflurane exposure.

Conclusion: According to our findings, repeated exposure to sevoflurane during pre-adolescence can cause changes in the hippocampus and neuroplasticity in the adult brain. Results from this study may provide a new perspective on how repeated exposure to anesthesia can lead to toxic effects in pre-adolescent rats.

Keywords: Sevoflurane, Anesthesia, Pre-adolescence, Cognitive problems, Neurobehavioral problems, Cell damage

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Introduction

In patients with severe burns, for example, repeated changes to the burn dressing may require multiple sedation or anesthesia. Hence, anesthetic agents may have to be administered during or after a critical period of brain development. Despite the requirement, the safety of anesthesia agents for children is a concern for their parents. It has been shown in previous studies that exposure to general anesthesia as an infant or in childhood is associated with later neurobehavioral problems in adulthood. Clinical studies have demonstrated a correlation between anesthesia exposure and various clinical disorders, including impaired memory and executive function, neuroapoptosis, and long-term neurocognitive deficits (1, 2). Moreover, laboratory observations in rodents and nonhuman primates indicate that exposure to general anesthetics during the neonatal and early postnatal period can induce cell damage, impaired synapse growth, neurogenesis, subsequent cognitive and behavioral problems and may cause neurobehavioral issues later in childhood and even adulthood (3, 4).

In addition to behavioral changes and impaired learning and memory, anesthesia can also affect neuroplasticity. Extensive research has been conducted in the hippocampus on the mechanisms underlying long-term potentiation (LTP), which is thought to involve the mechanisms involved in forming memories (5). Hippocampal synaptic plasticity has been implicated in many learning and memory processes. Therefore, the mechanism that underlies learning disabilities found after exposure to anesthesia is assumed to be an impairment in hippocampal synaptic plasticity (5, 6).

Sevoflurane, a volatile, highly fluorinated methyl isopropyl ether anesthetic, is the most common agent for pediatric patients due to its sweet-smelling property, fast onset, and rapid recovery (7). Prior studies found that early-life sevoflurane exposure impairs social behavior and emotional cognition later in life. Some preclinical and clinical studies have already investigated the possible side effects of sevoflurane on cognition (8, 9). Several animal studies have consistently reported that sevoflurane causes brain injury and behavioral changes during critical periods of brain development in animals (10, 11). However, due to the complexity of learning and memory processes and the targets those general anesthetics possess, more studies are warranted to investigate the effects of sevoflurane on memory.

Several recent experimental studies on rodents suggest that repeated exposure to anesthetics during the neonatal and after can induce lasting impairment of CNS function (12, 13). Despite several studies on the long-term effects of anesthetics during the neonatal period, there are few findings regarding the long-term effects of repeated short-term exposure to anesthetic drugs during adolescence. Since there have been few studies on this issue, we aimed to provide an evaluation of the long-term impact of repeated short-term exposure to sevoflurane from a morphological and functional perspective. For this purpose, we evaluated repeated exposure of a pre-adolescence rat (after weaning) model on induced sevoflurane-related neurotoxicity in the hippocampus and the effects of its long-term use on behavior, biochemical, and histological methods. In addition, we aimed to characterize the electrophysiological alterations caused by pre-adolescence sevoflurane exposure by investigating in vivo long-term potentiation induction in the hippocampal perforant pathway in adult rats.

Methods

Animals and anesthetic procedures

This study used pre-adolescence rats weighing between 35-40g. This age was selected based on previous literature outlining the time for adolescence in rodents (14, 15). More specifically, in male and female rats, early adolescence begins at postnatal day (PND) 25–30, and early adulthood begins at PND 60-70. Seventy-two pre-adolescence male and female Wistar rats from 8 different mothers were randomly assigned to either the experimental (anesthesia) or control group. Mother rats were obtained from the Tehran University of Medical Sciences experimental study center. The rats were kept in four per cage at 22 ± 2 °C under 12-h light/dark cycle conditions. Animals were provided with free access to standard food and water throughout the study. All experiments were carried out between 9 AM and 4 PM. All the experimental protocols and procedures were performed according to guidelines for the care and use

of laboratory animals stipulated by the National Institutes of Health (NIH No: 8023, revised 1978). In addition, this experiment was approved by the Ethics and Research Committee of the Tehran University of Medical Sciences (IR TUMS.NI.REC.1400,05).

After weaning, the pre-adolescent animals were randomly divided into four equal groups: (n=18) male and female control groups and male and female sevoflurane inhalation groups (inhalation 2% daily for 15 days). Pre-adolescent rats (PND25) in the sevoflurane treatment groups were placed in a plastic chamber, then using an anesthesia apparatus (Bonther®, SP, Brazil), sevoflurane (Bremer Pharma GMBH, Germany) applied at 8% for induction (for the first 1 min of the exposure session). Then, it was reduced to 2% for maintenance of deep anesthesia (for the last 5 min of the exposure session) with air/ oxygen as a carrier at a gas flow of 2 L/minute. Deep anesthesia was confirmed by loss of eyelid and righting reflexes. During sevoflurane exposure, the chamber was heated to 38°C. When the rats moved freely, they were replaced into their cage. An investigator monitored the rat's respiratory frequency and skin color during anesthesia. After 15 days of anesthesia, the animals of all groups received care for 20-30 days and were then prepared for the designed experiments. The animals of each experimental group were weighed on days 0, 5, 10, and 15 of anesthesia, and the weight gain of the animals was monitored. Figure 1 shows a schematic diagram of the experimental design of the study.

Behavior tests

Morris Water Maze (MWM)

Behavioral tests were performed at P60-70 (200-230 gr in male or female rats). MWM was conducted over four days using adult rats. The MWM was constructed in a circular black tank (120 cm in diameter and 70 cm in height). It was placed in a dim room with visual cues. The tank was filled to a depth of 40 cm with water (22±1 °C). The maze was divided into four equal quadrants. A circular platform (10 cm in diameter) was located at the center of one of the quadrants, submerged 1.5 cm beneath the water's surface. The platform's location did not change until the end of the test, and fixed, extra-maze visual cues were present at various locations around the maze.

The task consisted of two stages: the acquisition phase for spatial learning assessment and the probe test for retention retrieval of memory. The acquisition phase comprised one session with four trials lasting 90 seconds over three consecutive days. A 60-s probe test with the platform removed was also given 24 hours after completing the last session. In each session, there was a 30-second interval between each trial. During each trial session, the path the animal swam was automatically recorded using a video camera-based System (EthoVision, Noldus, Version 14). The parameters analyzed in the acquisition phase were the latency time (latency to first) and the movement to find the hidden platform (distance to move). The latency of the first entry to the target zone (escape latency to platform time) and time spent in the target quadrant (time in the target zone) were the parameters analyzed for the probe test. One rat was deemed a 'poor performer' in all groups and was excluded from the study.

Novel object recognition (NOR) task

This test was done over three days to evaluate the discrimination aspect of memory, as reported before (16). For habituation, the day before starting the test, the animals were allowed to explore the empty arena (60×60×50 cm) for 5 minutes. Twenty-four hours later, the animals were individually placed in the arena containing two similar objects fixed to the floor at an equal distance from the walls and each other. The rats explored two similar objects during the first (familiarization) session for 10 minutes. On the third day, the rat was again placed into the arena for 10 minutes, but the novel object was replaced by one of the familiar objects. Exploration of objects was defined as the animal's nose being in the zone at a distance of ≤ 2 cm from the object. The total exploring time for each object *was automatically recorded using a video camera-based System (EthoVision, Noldus, Version 14). The recognition Index* (RI) was calculated based on the following formula:

[RI = $T_N / (T_N + T_F)$ multiplied by 100]

TN is the novel object that spends time, and TF is the familiar object that spends time (16).

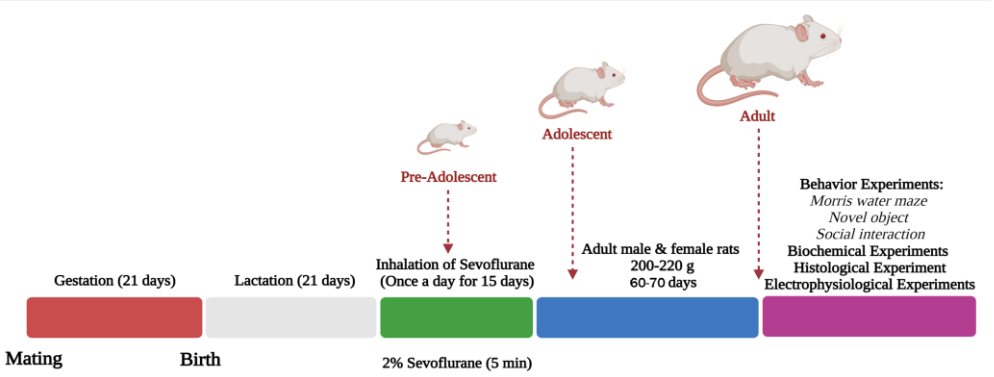

Figure 1. Schematic diagram of the experimental design of the study showing the four rat groups, control male, control female sevoflurane male, and sevoflurane female.

Social interaction test

In this test, rats were studied in a social interaction apparatus of three communicating chambers (90 cm × 45 cm × 45 cm and elevated 50 cm from the floor). The Social interaction test comprised three 10-minute sessions of habituation, sociability, and preference for social novelty (experimental sessions). After the habituation period, a stimulus rat is placed under a wire cage in the left chamber of the test apparatus. A similar wire cage is located without the stimulus rat in the right chamber of the test apparatus. An unfamiliar conspecific (Stranger 1) was introduced into one enclosure, and the test rat was allowed to sniff Stranger 1 or an empty cage. The stranger rat was from an identical background, the same gender, and the same age as the test rat, but they had no previous contact. After 24 h, another unfamiliar conspecific (Stranger 2) was introduced into the other enclosure, and the test rat was allowed to sniff Stranger 1 and Stranger 2. The total exploring time for each object *was automatically recorded using a video camera-based System (EthoVision, Noldus, Version 14). The* placement of Stranger 1 on the left or right side was altered between trials, and the social apparatus was cleaned with 70% alcohol after each trial to minimize olfactory disturbance. Measures were taken of the time in exploration in the target chamber in sessions for sociability and preference.

Biochemical study

After the behavioral tests, the adult rats were decapitated under ketamine (150 mg/ kg) anesthesia. Their brains were removed from the cranium, and the hippocampal tissue (n = 5) was removed and homogenized in cold Tris–HCl buffer solution (150 mM, pH 7.4). After centrifuging, the supernatant was used for the following assays (1,000 g, 4°C, 10 min). The Bradford method was applied to determine the total protein (17). Reduced glutathione (GSH) was determined using a specific assay kit (Kiazist, Lifescience, Iran). For this purpose, the supernatant was first mixed with 5% trichloroacetic acid (TCA) and centrifuge, then 0.1 ml of obtained supernatant, 0.5 ml of 5′5 dithiobis (2-nitrobenzoic acid) (DTNB), 2 ml of phosphate buffer (pH 8.4), and 0.4 ml of distilled water were added. After 30 min, the absorbance of 412 nm. was obtained. Superoxide dismutase (SOD) activity was determined using a specific assay kit (Kiazist, LiveScience, Iran). In brief, the supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer for 30 min (n=5/group). Then nitroblue tetrazolium was added. Blue formazan production was monitored at 550 nm. One unit of superoxide dismutase was defined as the amount of the enzyme required for 50% dismutation of the superoxide radicals.

Electrophysiological study

Long term potential

Field Excitatory Postsynaptic Potential (fEPSP) was recorded with an extracellular bipolar stainless-steel stimulating electrode with a diameter of 0.125 mm that was placed in the medial perforant pathway (4.2 mm lateral to the lambda, -3.2 mm ventrally). A stainless-steel recording electrode was positioned in the DG with the maximum response (-3.8 mm posterior

and ±2.2 mm lateral to the bregma). Extracellular field potentials were amplified 1000x, digitized at 10 kHz, and filtered at a band of 0.1 Hz-10 kHz with a differential amplifier. The applied stimuli were biphasic square waves (a width of 200 ms). Signals were passed through the A/D interface (Science Beam Co., Iran) to a computer, and data were analyzed using eprobe software. Stimulation intensity was adjusted at a level to evoke 40% of the maximal response (Population Spike (PS) and field Excitatory Post-Synaptic Potential (fEPSP)). Before LTP induction, we executed stimulus-response curves using a range of stimulus intensities (100-1200 μA). For LTP experiments, after stable baseline recording for at least 30 min, LTP was induced through high-frequency stimulation (HFS) (10 trains of 10 pulses at 200 Hz separated by 10 s). After the tetanic stimuli, the baseline stimulation was resumed, and the recording continued for at least 60 min. Five consecutive evoked responses were averaged at stimulus intervals of 10 seconds.

Paired-pulse response

After recording a baseline for 30 minutes, the paired-pulse depression/facilitation was determined. The response to the paired-pulse stimulation was subsequently recorded and delivered at 40% of maximal stimulus intensity with Inter-Pulse Intervals (IPI) of 20, 30, 50, 70, 100, 150, and 300 ms. For each IPI, ten consecutive evoked responses were averaged. The fEPSP slope ratio [percentage of second fEPSP slope/first fEPSP slope; fEPSP2/fEPSP1%] and the population spike amplitude ratio [PS2/PS1%] were determined at various inter-stimulus intervals.

Histological evaluation

Euthanasia and tissue preparation

Histological studies were done on the hippocampal samples of randomly chosen rats in each group (n = 6/experimental group). Rats were deeply anesthetized with a high dose of ketamine (150 mg/kg) and transcardially perfused with 50 ml of normal heparinized saline and 50–75 ml of a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4). Then, the brains were removed from the cranium and kept in closed plastic containers filled with 10% formaldehyde solution at ambient temperature for 24 hours. The samples were dehydrated with an ascending ethanol series, cleared with xylene, and embedded in paraffin. A hippocampal block was cut into 7μm coronal sections and prepared for tolonidine blue staining. Neuronal counting was done in the CA1 and CA2 areas and the hippocampus's dentate gyrus (DG). Dark neurons (DNs) and cells with visible cytoplasmic boundaries and clear nucleolus were included in the count. The counting process was repeated twice for each section.

Statistical analysis

Statistical analysis was conducted using Graph-Pad Prism Version 8.0 for Windows (Graph-Pad Software, USA). Statistical differences were determined using two-way ANOVA and one-way ANOVA analysis with a Bonferny post hoc test. P values less than 0.05 indicated statistical significance; all values were expressed as the mean±SEM.

Ethics Approval All experiments were conducted according to the Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80–23, revised 1996). Additionally, all procedures were reviewed and confirmed by the Research and Ethics Committee of the Tehran University of Medical Sciences (IR TUMS.NI.REC.1400.05).

Results

Effect of repeated sevoflurane exposure on weight gain

Figure 2 shows the effects of repeated exposure to sevoflurane on weight gain. As can be seen, two-way ANOVA revealed an immediate increase in body weight gain pattern in male and female sevoflurane groups during days 1 to 15 after the first anesthesia treatment compared to the control groups ($F_{(9,108)}$ = 23.62; P<0.0001). This increase in body weight was statistically significant on days 10 and 15. In contrast, no significant change in weight gain was observed in the first five days after administering anesthetic drugs. In addition, there was no significant gender difference within the groups (Figure 2).

Morris Water Maze Data

To determine the behavioral effects of sevoflurane, adult animals' spatial memory was assayed at three to four weeks (P60-70, 220-230 gr weight) after sevoflurane exposure. Figure 3 (A and B) indicates the learning results in the acquisition phase of

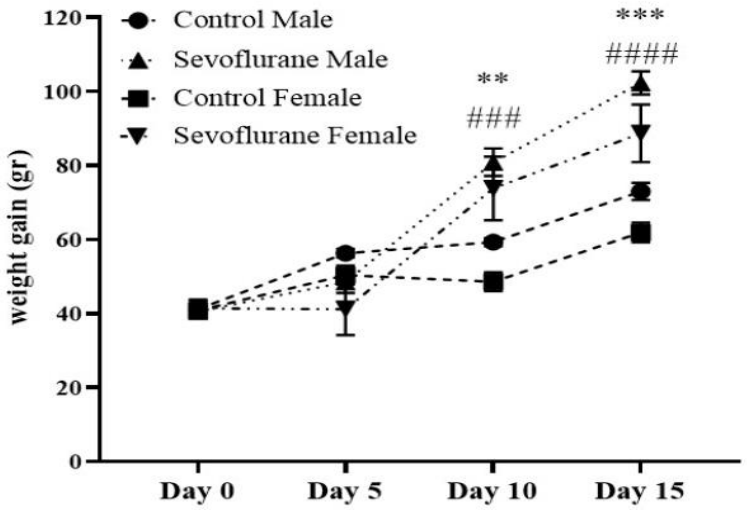

Figure 2. Effects of repeated sevoflurane exposure on weight gain. Compared to the control groups, bodyweight gain patterns in the sevoflurane groups (male and female) during day 1 to 15 after the first anesthesia treatment. Two-way ANOVA with Bonferroni's post Hoc (**P < 0.05, **** P<0.0001 male sevoflurane compared to male control group, (###P < 0.001, #### P<0.0001 female sevoflurane compared to female control group).

the MWM. Distance traveled and latency to reach the platform during the learning phase were analyzed by Two-way ANOVA and Bonferroni's post hoc (mean ± SEM).

Results of latency to reach the platform during the learning phase revealed that there was a significant influence on the factor groups [$F_{(2.3, 21)}$: 3.64; P=0.036] and day factor [$F_{(1.5, 14)}$=33.04, p=0.0001], but the interaction of between the day and group had no significant influence [$F_{(2, 24)}$=1.8, p=0.707]. When this interaction was analyzed with One-way ANOVA, it became evident that the animals in the sevoflurane female group had significantly longer latencies to reach the platform compared with the control female on day 2 (P<0.05). There was also no significant difference between the sevoflurane male and male control groups in the latency to reach the platform over three days (Figure 3A). Regarding the total distance traveled, the two-way ANOVA analysis revealed anesthesia exposure had a significant effect on the factor of days [$F_{(1.5,10)}$: 29.8, P<0.0001] but not group factor [$F_{(2, 14)}$: 1.063, P=0.37]. The interaction between days and groups failed to reach a statistical significance [$F_{(2, 18)}$: 0.57, P=0.619]. The one-way ANOVA further revealed an increase in the total distance traveled in sevoflurane female groups compared with the control group on three days (n=10). However, there was no significant difference between groups in total distance traveled (Figure 3B).

A probe trial test assessed memory retrieval 24 hours after the last training session. Two parameters of latency to the first entry to the target (platform) and the total time spent in the target region revealed that sevoflurane had a significant effect on latency in the first entry to the target [$F_{(3, 28)}$: 8.58, P<0.001]. Bonferroni's post hoc test confirmed that the sevoflurane groups had a higher latency upon first entry to the target zone than the control groups (P=0.004 in the male groups and P=0.01 in the female groups) (Figure 3D). The one-way ANOVA also showed that there was a significant difference between the sevoflurane group and the male control group for the total time spent in the target quadrant [$F_{(3, 32)}$: 3.601, P<0.05] (Figure 2E). Post hoc analyses indicated that the male sevoflurane groups spent less time than the male control group (P<0.05). Therefore, we conclude that reference memory impairment is induced by repeat exposure to sevoflurane. Comparing the heat map between the groups also confirms these results (Figure 3C).

Novel object

According to our results, In the novel object recognition test, the animals in the control groups indicated a tendency to spend more time exploring a

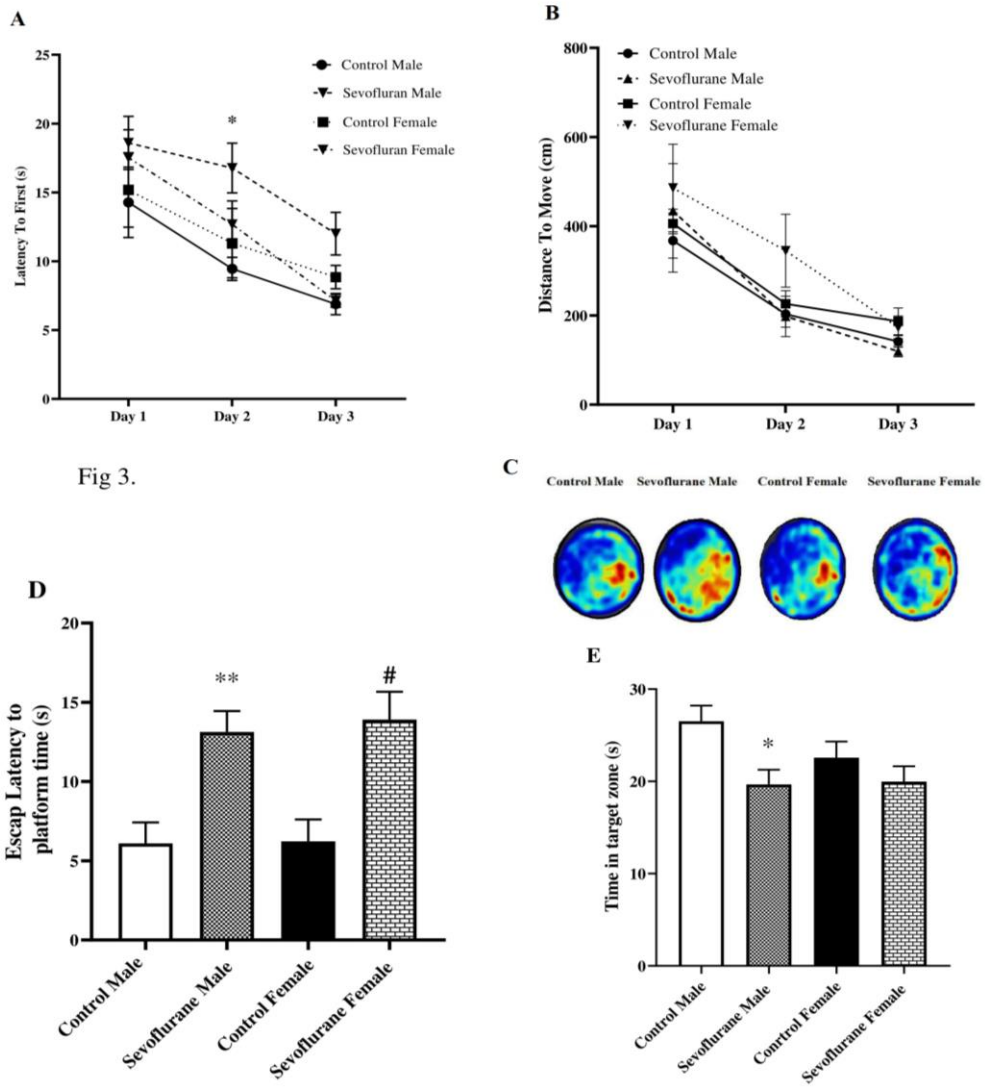

Fig 3.

Figure 3. Effect of sevoflurane exposure on spatial learning. (A) Rats were evaluated at P60-70 for their ability to determine the location of a hidden platform. A Two-way ANOVA of escape latency (the time to find the hidden platform) and (B) distance traveled indicated that the performance of sevoflurane-exposed rats was inferior to that of control rats during place training. (C) A heat map showing time in the target zone by groups (# $P < 0.05$ female sevoflurane compared to the female control group). (D) During the probe trial, sevoflurane-exposed rats spent less time in escape latency at the former location of the platform than did the control (**$P < 0.01$ male sevoflurane compared to a male control group; # $p < 0.05$ female sevoflurane compared to the female control group). (E) During the probe trial, sevoflurane-exposed male rats spent less time searching for the missing platform in the target quadrant than the controls (*$P<0.05$).

novel object than a familiar object than the animals of the anesthesia exposure groups (both male and female) (Figure 4A). There is a significant difference in total exploration time for each object between the control groups and the sevoflurane groups [$F_{(7,60)}$= 3.99, P= 0.0012) (Figure 4A). Our results indicated the discrimination index is significantly lower in the repeated exposure anesthesia groups (45.3 ± 3.1%; P < 0.0001 for male vs. 46.9% ± 2.9% for female) than in the control groups (64.9% ± 2.9% for male and 61.8% ± 3.1% for female animals). Post hoc analyses indicated significantly lower in the male (no female) sevoflurane group as compared to the male control group (P<0.05; n=9, Figure 4B). Our results indicate

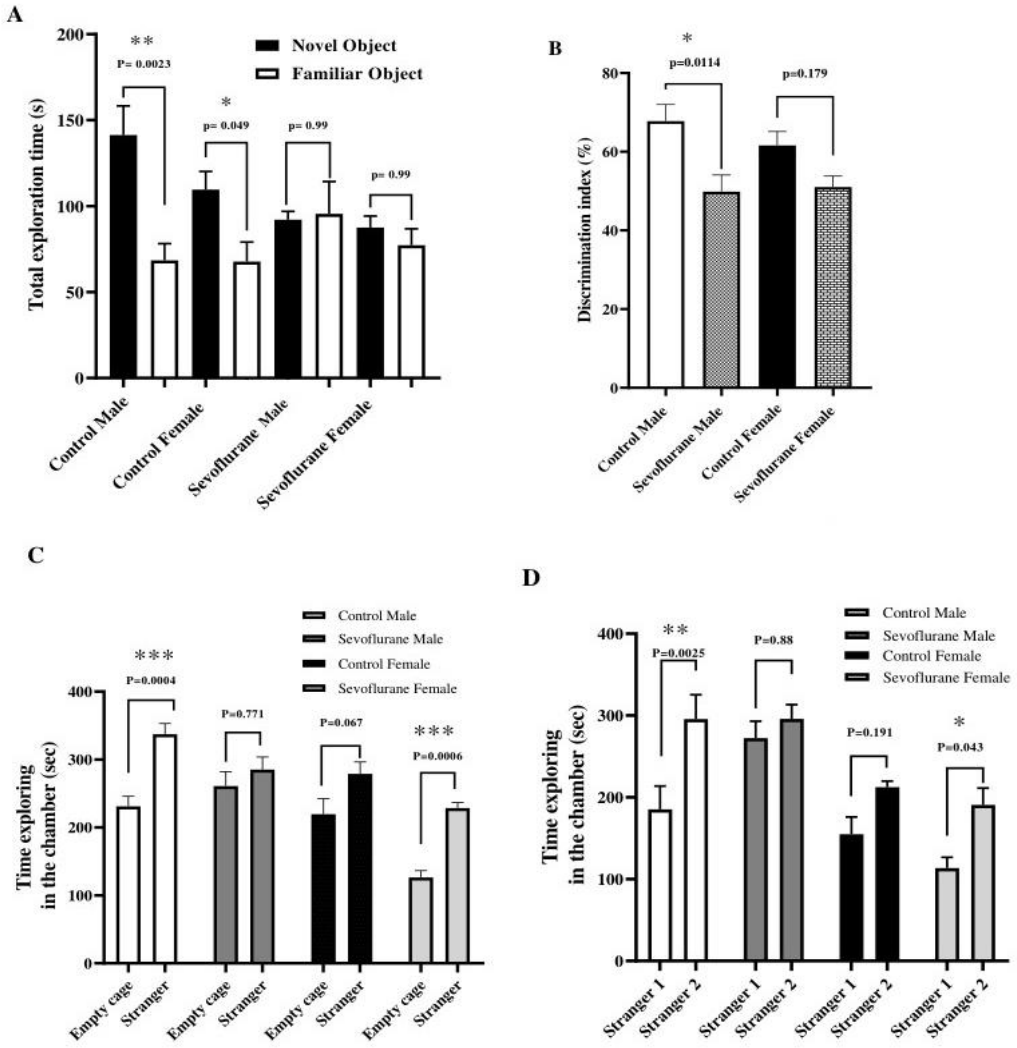

Figure 4. Performance on the NOR task for total exploration time, the recognition index, and the three-chamber test measuring social recognition memory. (A) One-way ANOVA indicated a significant difference between the sevoflurane groups compared to the control group for the total exploration time (**P< 0.01 in males and # P<0.05 in females) and on the discrimination index for the NOR task only in male Animals (*P<0.05). (B) Bonferroni's post hoc analyses indicated the anesthesia groups spent less time on familiar objects than novel objects (n= 7). All graphs were plotted as mean± SEM. (C). Exploration time for the empty cage (E) and the cage with stranger rat (S1) in the control and sevoflurane groups during the socialization in the three-chamber sociability test (n =7; S1 vs. E ***P < 0.001in the male control group and ###P < 0.001in female sevoflurane group). (D) Exploration time for the cage with the familiar mouse (S1) and the cage with the stranger mouse (S2) for the control and sevoflurane groups during the sociability in the three-chamber test (n =7; S1 vs. S2 **P < 0.01 in male control group and #P < 0.05 in female sevoflurane group). All graphs were plotted as mean± SEM.

that repeated exposure to sevoflurane anesthesia in pre-adolescence leads to persistent impairment in recognition memory in adulthood.

Social interaction

As seen in Figure 3C, animals with normal sociability preferred the wire cage with a strange rat over an empty cage. Animals of the male sevoflurane group showed comparable social interaction as their control animals' littermates. However, during the social recognition memory task, which requires normal hippocampal function, the male sevoflurane animals failed to distinguish the novel stranger rat from the

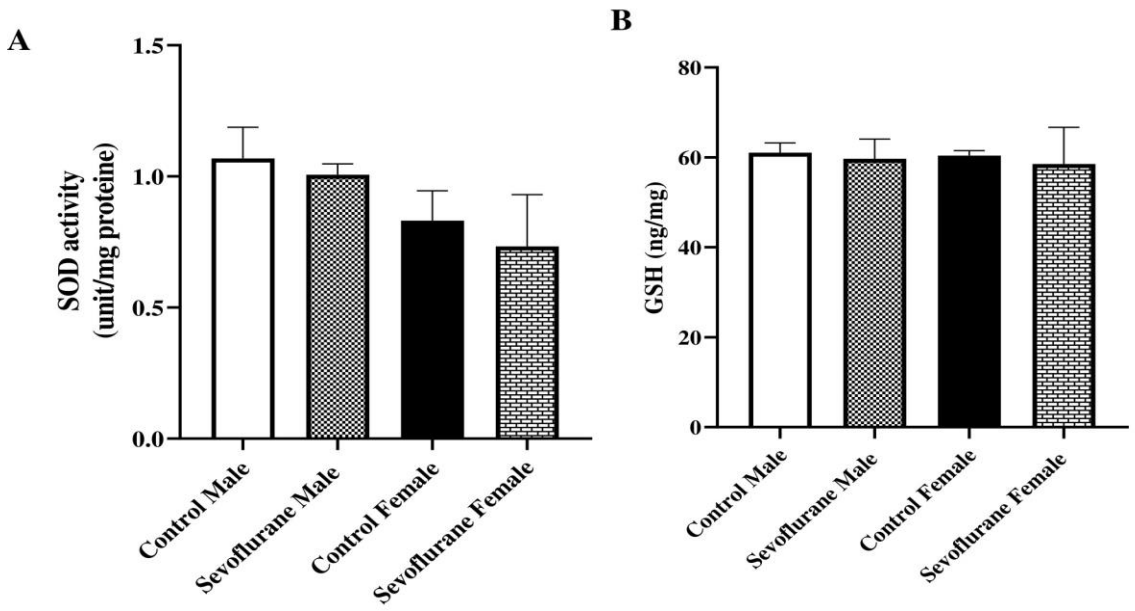

Figure 5. Effect of sevoflurane exposure on stress oxidative in the hippocampus. (A) The superoxide dismutase activity (SOD) and (B) the GSH content in the hippocampus homogenate in control and repeat exposure anesthesia groups (n = 5). All graphs were plotted as mean± SEM.

familiar mouse. In contrast, the control rat spent significantly more time exploring the novel stranger rat (Figures 4C and D), suggesting a social memory deficit in sevoflurane animals, primarily male. Our results with two-way ANOVA Bonferroni's post hoc test showed there are significant differences between the male control groups and sevoflurane groups in sociability [$F_{(3,28)}$= 15.26, P < 0.0001) and social memory [$F_{(3,24)}$=13.54, P<0.0001]. However, according to our results, in female animals, there was no significant difference between the sevoflurane group and the control group for both sociability and social memory (n=8).

Biochemical results

Regarding hippocampus stress oxidative markers (Figures 5A and B), sevoflurane reduction of superoxide dismutase enzyme (SOD) activity compared to the control group in brain structures of adult rats is confirmed, but this reduction was not significant statistically [$F_{(1.8,7.52)}$=1.39; p<0.30] (Figure 5A). One-way ANOVA also showed that there was no significant difference between groups in the increase of glutathione (GSH) content in the hippocampus of adult rats [$F_{(3, 16)}$=0.05, P=0.98] (Fig. 5B).

Electrophysiological result

To further investigate the long-term effects of sevoflurane anesthesia on neuronal activity, we assessed basal synaptic transmission and plasticity in the performant pathway of the hippocampal at one month after exposing rats to the anesthesia. Basal synaptic transmission was studied by analyzing stimulus-response (I/O) curves and short- and long-term potentiation by assessing paired-pulse facilitation (PPF) and long-term potentiation (LTP). Acquired input/output (I/O) curve from recorded 60 s periods revealed that there is no difference in the amplitude of PS between the control and the sevoflurane exposure groups [$F_{(1.7,6.9)}$ =0.958, P = 0.41] Fig.6A). In addition, the fEPSP slope of the exposure groups did not reduce significantly compared to the control group (data not shown). As shown in Figure 6B, in short intervals between two strictly paired stimuli, the amplitude of PS prompted by paired pulse did not increase significantly in the sevoflurane groups compared to the control group. Two-way ANOVA with Bonferroni's post hoc analyses indicated a significant difference in the paired-pulse ratio of PS amplitude [$F_{(2.0,8.1)}$ = 5.8 P< 0.01] in the male sevoflurane group compared to the male control group, especially in intervals of 30, 50

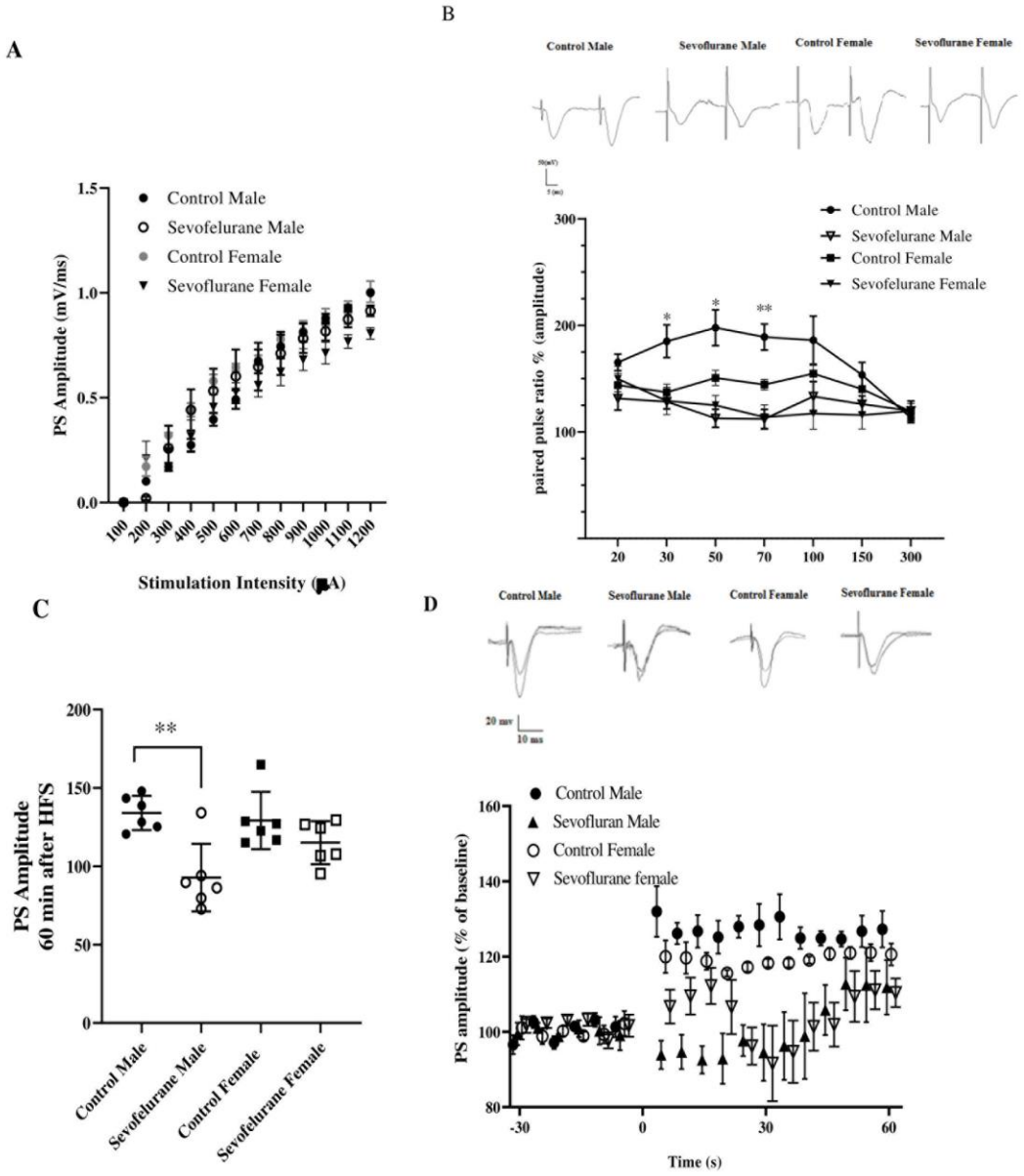

Figure 6. Effect of sevoflurane exposure on neuronal synaptic plasticity. (A) The acquired input/output curve from a 60 s recording and single traces of the DG neurons' reaction to numerous stimulus intensities (100–1200 mA). (B) short intervals between two strictly paired stimuli, the amplitude of PS prompted by a paired-pulse in all four groups, and the recorded single traces following paired stimuli with interruption of 30ms in the investigational groups (*P < 0.05; (**P < 0.01) (C) Mean of the PS amplitude recorded at 0 to 60 min after the application HFS (n=6 animals/group) (**P < 0.01 male sevoflurane compared to the male control group). (D) The effect of repeated exposure to sevoflurane on LTP induction and maintenance in the dentate gyrus up to 60 min after tetanization (HFS). Data were normalized against the baseline period (-30 - 0min), and the single traces were recorded before/after HFS.

and 70 msec. Analyses in female groups indicated no significant difference in the paired-pulse ratio of PS amplitude. Figures 6C and 6D illustrate the effect of repeated exposure to sevoflurane on LTP induction and maintenance in the dentate gyrus. A repeated measure two-way ANOVA followed by Bonferroni's post hoc test revealed that PS-LTP after tetanization (HFS) was significantly lower in the sevoflurane groups relative

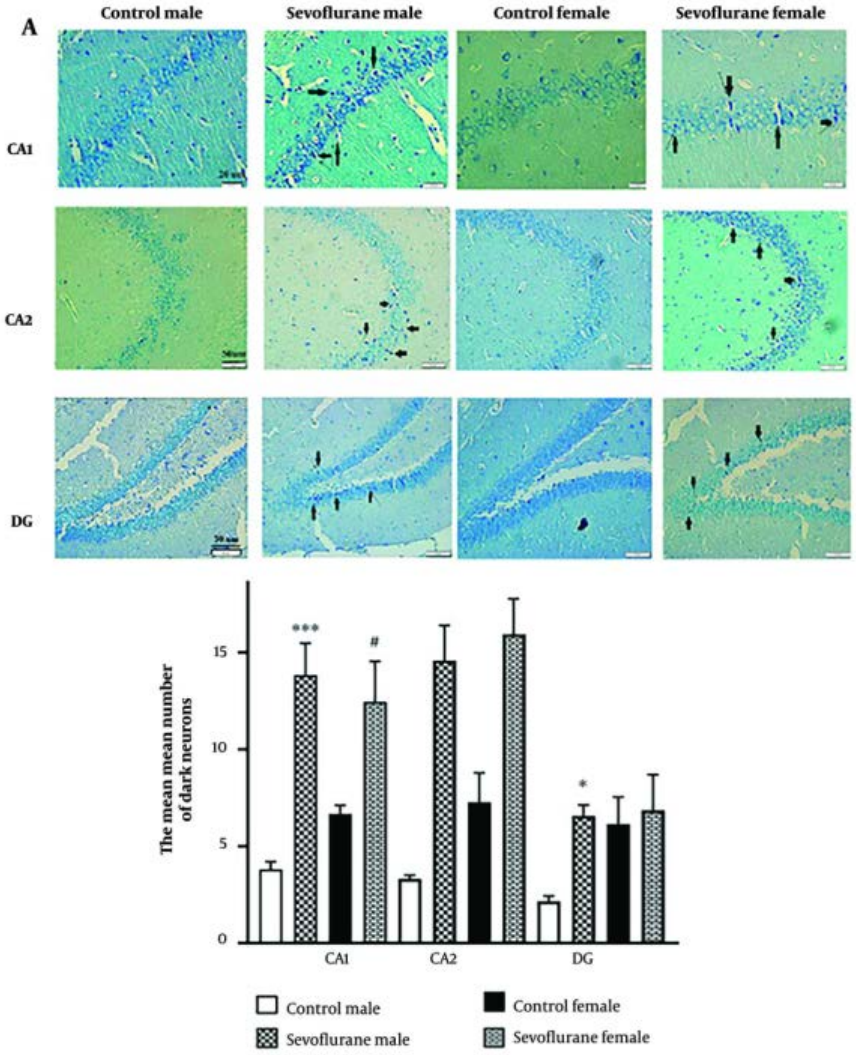

Figure 7. Production of dark neurons in the hippocampal CA1, CA2, and dentate gyrus in adult rats after repeated sevoflurane exposure in pre-adolescence. (A) The light-microscopic appearance of toluidine blue-stained dark neurons in 10-µm hippocampus sections CA1, CA2, and DG areas. (B) Dark neuron numbers in CA1 and CA2 regions of the sevoflurane groups were significantly more than the animals of control groups (***P < 0.001 male sevoflurane compared to the male control group in CA1 and CA2 regions; #P< 0.05 female sevoflurane compared to the female control group in CA1 and CA2 areas; *P<0.05 male sevoflurane compared to a male control group in DG region. All graphs were plotted as mean± SEM.

to the control group [$F_{(2.12)}$ =12.02, P<0.01]. High-frequency stimulation (400 Hz) of the medial perforant pathway produced a long-lasting synaptic potentiation in the control group compared to anesthesia groups (P<0.001) up to 60 min after HFS (Figure 6D). Our results indicated that lower PS-LTP after tetanization (HFS) in the sevoflurane group was significant compared to the control group (P<0.001). The LTP level, measured as the mean PS amplitude at 0 to 60 min after HFS stimulation, significantly differed in the male sevoflurane and male control groups (P<0.01; Figure 6C). Similar results were observed for the fEPSP slope by assessing pair pulse facilitation (PPF) and long-term potentiation (LTP) (data not shown).

Histological result

In the histological assessment, the appearance of neurons with condensed darkly stained nuclei and bright eosinophilic cytoplasm was used to identify the neuronal injury. The numbers of DNs per unit area (NA) in the hippocampus's CA1, CA2, and DG

subdivisions were counted. Then, the mean number of DNs in the experimental group's hippocampus was compared with the control groups. As shown in Figure 7A, only a few DNs were found in different hippocampus regions in the control group (male and female). Still, the DNs per unit area in the hippocampus's CA1, CA2, and DG increased in the sevoflurane groups. One-way ANOVA with Bonferroni's Post hoc analyses indicated that there was a significant increase in the mean number of dark neurons in the CA1, CA2, and dentate gyrus area in the sevoflurane groups compared to the control groups [$F_{(3,18)}$ =12, P<0.0001 for CA1, $F_{(3,17)}$ =11.96 P<0.001 for CA2, and $F_{(3,17)}$ =3.54, P<0.036 for DG]. Our results in adult rats indicate that repeated exposure to sevoflurane in pre-adolescence males and females causes cell death in the hippocampus (Figure 7B).

Discussion

Multiple sedation or anesthesia is required in some surgeries and clinical procedures on infants and adolescents. Due to the side effects of anesthetics, anesthesiologists should minimize these effects as much as possible. As part of the current study, we examined whether repeated sevoflurane exposure after childhood brain development might lead to cognitive deficits in adult rats. Repeated sevoflurane exposure on the pre-adolescent hippocampus was investigated biochemically, histologically, and neuroplasticity to address this issue.

Sevoflurane is the most commonly used anesthetic drug and has gained significant attention for its effects on the nervous system. Numerous studies have demonstrated its neurotoxicity. The results of these studies have been controversial due to differences in the duration and concentration of anesthesia, the age of the animals, and the methods used for behavioral testing (18, 19). In the past few years, several studies have reported on single or multiple sevoflurane treatments in low or high doses for the long- or short-term (12, 20). Since the duration of the pediatric surgery is often short but must be repeated, in this study, we set the minimum alveolar concentration (MAC) for sevoflurane in pre-adolescent rats at 2%. We administrated daily short-term doses for 15 days. According to recent literature, a single dose of sevoflurane is insufficient to induce behavioral changes; however, exposure to several doses of sevoflurane could be associated with learning deficits (7, 21).

According to previous studies, anesthesia-induced learning impairments can occur at different stages of development. There is a brain growth spurt in humans during the last three months of pregnancy, and in mice and rats, it occurs during the first two weeks following birth (22). As Irwin Feinberg explained in 1983, synaptic pruning is a natural process in the brain between early childhood and adulthood (23). During synaptic pruning, the brain eliminates extra synapses. Huttenlocher (1979) showed that synaptic density in the human cerebral cortex increases after birth and peaks at 1 to 2 years of age at about 50% above adult levels (24). It drops sharply during adolescence and then stabilizes in adulthood, with a slight possible decline late in life. During this process, the amount and timing of neural activity are central to determining and disruption causes neurodegenerative disorders. Various factors can disrupt this process, including stress, medications, genetics, and social aspects (25). There is a growing body of evidence suggesting that infants who receive anesthesia drugs (single or multiple doses, high or low doses) may develop memory impairment, neuroapoptosis, and long-term neurocognitive deficits as well as behavioral problems during the neonatal brain development period (2, 12, 26).

Few studies have examined anesthesia drugs' long-term effects on pre-adolescents. However, many questions remain, including whether anesthesia in pre-adolescence has the same effects as that in neonates, what effects anesthesia has on the brain beyond development, and whether these adverse effects persist into adulthood. Research and current studies show that repeated exposure to sevoflurane appears to interfere with the synaptogenesis system and can lead to behavioral deficits in both cognitive and social spheres (27). Our behavioral tests found that repeated exposure to sevoflurane during pre-adolescence resulted in deficiencies in learning in male and female rats and deficits in social behavior in female rats. These findings are consistent with the results reported by other researchers in experimental studies where sevoflurane at doses from 0.5-2.6% has been

administered either during or after a learning task, which has shown inhibition of memory retention in rats (9, 28). Wu and colleagues (2018) reported that repeated exposure to sevoflurane in gestation causes decreased platform-crossing and time spent in the target quadrant in MWM in P28–33 male rats (29). In a study, Li and colleagues (2019) using the novel object recognition test and Morris Water Maze indicated that anesthesia with sevoflurane in 7-day-old neonatal both male and female mice for three h per day for three consecutive days led to mild behavioral abnormalities later in life (30). Shen and colleagues (2013) reported that exposure to 3% sevoflurane for two hours in neonatal mice (P6) did not induce cognition and memory impairment. However, they indicated that 3% sevoflurane for two hours daily for three days did cause cognitive impairment that was detected at one month of age (31). Consistent with our results in female rats, Yamasaki et al. (2015) reported that mice exposed to isoflurane (1.8% for two h) showed no changes in behavioral tests for social behavior or depression one week after its administration (32). It appears age and anesthetic exposure duration may effect on changes the behavior of animals. The behavioral test results were confirmed by histological assays.

Our results indicate that the activity of the stress oxidative enzymes (GSH and SOD) was not significantly reduced in animals of the sevoflurane groups (both male and female). Interestingly, our results regarding the antioxidants SOD and GSH and the neuro safety of sevoflurane also consist of other studies. Allaouchiche and colleagues reported that Sevoflurane does not induce a chemical reaction sequence leading to the generation of oxygen-free radicals (33). Zhou and colleagues (2015) indicated that stress reduction oxidative in low-dose sevoflurane exposure (once, 0.3% sevoflurane for six h) (34). Yılmaz Doğru et al. (2017) reported that sevoflurane (MAC 18 min/day for 7 and 14 days) does not affect the activity of antioxidant systems in a rat ovary (35). However, Unlike the behavioral results, our oxidative stress data do not corroborate the histological results. Although some studies have confirmed that repeated exposure to sevoflurane led to apoptosis and neuronal damage (13), the process of neuronal damage inducing sevoflurane has a different mechanism than oxidative stress. This field needs more studies at the molecular level and on cellular signaling pathways.

Our electrophysiological results revealed a significant decrease in LTP and PPF in the hippocampus of the sevoflurane male rats compared to control group animals exposed to sevoflurane at pre-adolescence. In correlating with histological, abnormal development results and synaptic connections' plasticity, inessential can have a role in anesthesia-induced behavioral deficits. Our results also indicate that exposure to sevoflurane anesthesia after P25 significantly affects cell damage. On the other hand, anesthetic agents that act as NMDA receptor antagonists and GABAA receptor agonists can induce extensive neuronal apoptosis in the developing brain (neonatal) age-dependently (26). It has been demonstrated that repeated exposure to an NMDA antagonist and a GABAA agonist was much more detrimental than either alone (11). This evidence suggested that not only was more severe neurodegeneration induced when both GABAA and NMDA receptors were simultaneously altered in the developing brain, but they also had similar effects on brain function. During synaptogenesis, signals via NMDA and/or GABA receptors mediate neuronal survival, migration, and synaptic formation (36). It has also been reported that abnormal or excess GABAA and NMDA receptor signals may negatively influence synaptic maturation (37). Sevoflurane anesthesia passively modulates those synaptic formations or synaptic developmental processes. It induces abnormal synaptic formation or ectopic neuronal cell distribution, impairing synaptic plasticity, such as facilitating paired pulls or the induction of hippocampal LTP in the CA1 region (38).

The present study's findings suggest that pre-adolescent anesthesia exposure induced lasting effects on neural transmission, at least at the presynaptic level. The changes we observed in LTP and PPF may be due to increased presynaptic calcium concentrations. Sevoflurane depresses neuronal activity and reduces Ca^{2+} influx into neurons primarily through the blockade of NMDA receptors (39). NMDA receptor-mediated Ca^{2+} influx is critical for neuronal differentiation, migration, and synaptogenesis (40). Therefore, sevoflurane-induced NMDA receptor inactivation might significantly reduce activity-

mediated calcium influx into neurons, affecting synapse formation and plasticity. In confirmation, Luhman and Kessels stated that synapse maturation accelerated after two weeks of postnatal development, including increased levels of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors and glutamatergic synaptic transmission as indicated by the increased amplitude of miniature and spontaneous excitatory postsynaptic currents (41).

In contrast, no significant changes in hippocampal LTP and PPF were found in the experimental group of sevoflurane females when evaluated 30 days after drug exposure. In female rats, estrogen can be protective in preventing the blockade of NMDA receptors. Some reports also have demonstrated neuroprotection effects from subclinical concentrations of sevoflurane in adults (34), which needs further study.

This study has some limitations despite providing evidence that repeated exposure to sevoflurane results in long-term cognition and memory impairments. Initially, this study investigated the electrophysiological effects, oxidative stress, and apoptosis of the hippocampus and memory formation dependent on the hippocampal formation. It has, however, been observed that sevoflurane-induced apoptosis also occurs in other brain areas, such as the thalamus and the somatosensory cortex (42). As a second component, we examined the mechanisms underlying the repeated exposure to sevoflurane-induced changes in hippocampal tissue; though more evidence regarding the changes that occur at the cellular and molecular levels after exposure to sevoflurane is undeniable, continuing research into the mechanisms of anesthesia-induced apoptosis pathways is crucial.

Conclusion

The present study demonstrated that repeated exposure to sevoflurane during pre-adolescence can cause rapid changes in the hippocampus. Acute changes to the brain persist into adulthood and lead to cell damage, impaired synapse growth, cognitive and behavioral difficulties, and neurobehavioral difficulties. Adult animals receiving anesthesia during adulthood have impaired PPF and LTP based on electrophysiological recordings after repeated exposure to anesthesia. This result may provide a new perspective in understanding the mechanism of repeated exposure to sevoflurane-induced toxicity.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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