

# Apoptosis inhibition is involved in improvement of sevoflurane-induced cognitive impairment following normobaric hyperoxia preconditioning in aged rats

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**Abstract.** Sevoflurane, a commonly used anesthetic agent has been confirmed to induce cognitive impairment in aged rats. Normobaric hyperoxia preconditioning has been demonstrated to induce neuroprotection in rats. The present study aimed to determine whether normobaric hyperoxia preconditioning could ameliorate cognitive deficit induced by sevoflurane and the possible mechanism by which it may exert its effect. A total of 66, 20-month-old male Sprague-Dawley rats were randomly divided into 3 groups (n=22 each): Rats in the control (C) and sevoflurane anesthesia (S) groups received no normobaric hyperoxia preconditioning before sevoflurane exposure, rats in the normobaric hyperoxia pretreatment (HO) group received normobaric hyperoxia preconditioning before sevoflurane exposure (95% oxygen for 4 continuous h daily for 6 consecutive days). The anesthesia rats (S and HO groups), were exposed to 2.5% sevoflurane for 5 h, while the sham anesthesia rats (C group) were exposed to no sevoflurane. The neurobehavioral assessment was performed using a Morris water maze test, the expressions of the apoptosis proteins were determined using western blot analysis, and the apoptosis rate and cytosolic calcium concentration were measured by flow cytometry. Normobaric hyperoxia preconditioning improved prolonged escape latency and raised the number of platform

crossings induced by sevoflurane in the Morris water maze test, increased the level of bcl-2 protein, and decreased the level of bax and active caspase-3 protein, the apoptosis rate and cytosolic calcium concentration in the hippocampus 24 h after sevoflurane exposure. The findings of the present study may imply that normobaric hyperoxia preconditioning attenuates sevoflurane-induced spatial learning and memory impairment, and this effect may be partly related to apoptosis inhibition in the hippocampus. In conclusion, normobaric hyperoxia preconditioning may be a promising strategy against sevoflurane-induced cognitive impairment by inhibiting the hippocampal neuron apoptosis.

## Introduction

As the global population ages, and with improvements in medical and health technologies, there are more elderly patients and more are requiring surgical interventions and procedures (1). Globally, 50% of all elderly individuals are estimated to undergo at least one surgical procedure (2). Postoperative cognitive dysfunction (POCD) is a complication of anesthesia and surgery associated with significant morbidity and even mortality that is widely considered an important clinical problem, particularly in elderly patients. In a prospective multicenter trial performed by ISPOCD (international study of postoperative cognitive dysfunction) in 1998, the incidence of POCD in patients undergoing non-cardiac surgery was present in 26% at 1 week, and approximately 10% of the patients still had cognitive dysfunction after 3 months (3). Currently, there is no effective clinical treatment for POCD (4) and an urgent need to develop a novel treatment strategy to improve the prognosis of this condition (5).

The risk and precipitating factors of POCD are multifarious including increasing age, low education level, burden of illness, pain, anesthesia, repeated surgeries, postoperative infections and respiratory complications (6). Especially volatile anesthetics, one class of the most widely used drugs since the 19th century for general anesthesia is often mentioned as a possible cause of POCD (7). For instance, isoflurane (8), sevoflurane (9) and desflurane (10), which are commonly used

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*Abbreviations:* ANOVA, one-way analysis of variance;  $[Ca^{2+}]_c$ , cytosolic calcium concentration; DMEM, Dulbecco's modification Eagle's medium; LORR, loss of the righting reflex; MWM, Morris water maze; NBO, normobaric hyperoxia; NBO-PC, normobaric hyperoxia preconditioning; PI, propidium iodide; POCD, postoperative cognitive dysfunction; PS, phosphatidylserine

*Key words:* normobaric hyperoxia, apoptosis, aged, sevoflurane, POCD

in clinic, have been found to cause cognitive impairment. Previous studies have provided evidence of neurotoxicity caused by volatile anesthetics (11-13), and the safety of these anesthetic agents has come under scrutiny (14). Impairment of memory and spatial learning were observed in aged rodents receiving volatile anesthetics including sevoflurane (12). Although the mechanisms underlying the volatile anesthetic neurotoxicity have not been clarified, neuroapoptosis (15), neuroinflammation (16) and neurodegeneration (17) may be involved.

Normobaric hyperoxia (NBO) preconditioning (PC) has been demonstrated to protect against heart and cerebral ischemia as well as renal ischemia (18-20). Similarly, NBO-PC also attenuates cognitive impairment in an Alzheimer's disease mouse model (21). The protective effects of NBO-PC are attributed to antiapoptotic and anti-inflammatory effects (22). Since apoptosis and inflammation are involved in the neurotoxicity induced by volatile anesthetics (15,16), NBO-PC may be an effective and feasible method to alleviate cognitive impairment related to volatile anesthetics including sevoflurane.

Thus far, to the best of our knowledge whether NBO-PC can ameliorate cognitive deficit induced by sevoflurane and the possible mechanism by which it may exert its effect has not yet been clarified. We previously found that sevoflurane induces apoptosis in hippocampal neurons and causes cognitive deficit in aged rats (15). Based on these findings, it was hypothesized that NBO-PC may ameliorate cognitive deficit induced by sevoflurane through inhibiting hippocampal apoptosis. In the present study, this hypothesis was tested in an aged rat model of cognitive dysfunction induced by sevoflurane and the underlying molecular mechanism of this phenomenon was explored. In brief, if the neuroprotection of NBO-PC could be proved, it may indicate a potential novel target for the treatment of POCD.

## Materials and methods

**Study design.** The present study was a prospective, randomized, controlled animal study, which started in September 2018 and ended in October 2019, with the duration of the animal experimentation for 5 months. It was approved by the Ethics Committee for Animal Experimentation (Ethical approval no. Guo A2017-026-1), and the animals were studied at Hebei Medical University (Shijiazhuang, China).

**Animals.** A total of 66 male Sprague-Dawley 20-month-old rats (450-550 g) were divided randomly into 3 groups (n=22 each): i) Control group (C group); ii) sevoflurane group (S group); and iii) sevoflurane + NBO-PC group (HO group). To adapt to the animal care facility for 5 days before the experiment, these rats were housed in plastic cages under standard conditions, with free access to food and water, and maintained on a 12/12 h light/dark cycle. The rationale for choosing rats of this age was previous evidence that sevoflurane inhalation resulted in learning and memory deficits (23). There was no death in each group prior to decapitation.

**Preconditioning protocol and experimental procedure.** NBO-PC was performed as previously described (24). Briefly, animals assigned to the HO group received 4 h of NBO

treatment at 1 atm absolute (ATA) in 95% oxygen each day for 6 consecutive days in environmental chambers comprised air-tight boxes (65x25x45 cm) with gas inlet and outlet ports at room temperature (22±1°C). The C and S groups were exposed to normobaric normoxia (21% oxygen) according to the same protocol. After the last preconditioning (the 6th preconditioning cycle) 24 h later, rats assigned to the S and HO groups were placed in sealed transparent anesthesia induction chambers with soda lime at the bottom near the side opening of where the anesthetic machines were connected. The aforementioned rats received 2.5% sevoflurane (Maruishi Pharmaceutical Co., Ltd.) via humidified 50% O<sub>2</sub> carrier gas from a calibrated vaporizer for 5 h. Rats in the C group were also placed in the same chambers for 5 h with no sevoflurane given. Following 24 h sevoflurane-exposure, rats were subdivided for the Morris water maze (MWM) test, apoptosis detection in the hippocampus and cytosolic calcium concentration measurement of hippocampal cells. The experimental procedure was shown in Fig. 1.

**Morris water maze test.** Cognitive function of 10 rats randomly selected from each group was tested using the MWM test 24 h after sevoflurane exposure. The MWM (Shanghai Jiliang Software Technology Co., Ltd.) was a circular pool (180 cm diameter, 50 cm depth) with black walls and a water (23±1°C) depth of 30 cm, which was divided into 4 equal quadrants. A hidden round platform (10 cm diameter) was submerged 2 cm below the surface of water, located in one quadrant. The MWM test consisted of 5 days of place navigation test (a test of ability to learn the location of the hidden platform) and a probe trial (a test of ability to remember the previously-learned location of the escape platform having been removed) on the 6th day. In the test, rats were given 4 trials/day for 5 consecutive days. A trial was terminated and recording was stopped when the animal reached the platform, where it was allowed to remain for 15 sec. If the rat failed to find the target before 90 sec, it was usually guided to the goal and allowed to stay on the platform for 15 sec with the escape latency being recorded as 90 sec. On the sixth day, the hidden platform was removed and the rat was placed in the opposite quadrant. Each rat was allowed to swim freely in the pool for 90 sec and the platform crossing times were recorded.

**Measurements of apoptosis rate and cytosolic calcium concentration ([Ca<sup>2+</sup>]<sub>c</sub>) in the hippocampus.** Apoptosis rate of the hippocampus was measured by flow cytometry with an Annexin-V-FITC/propidium iodide (PI) apoptosis detection kit (Vazyme Biotech Co. Ltd.). Phosphatidylserine (PS) normally located on the inner side of the cell membrane, flips to the outer side of the cell membrane at the early stage of apoptosis (24). Annexin-V, a Ca<sup>2+</sup> dependent phospholipid-binding protein, with affinity for PS is routinely used to label externalization of PS (25). PI (propidium iodide, a nucleic acid dye), which does not permeate cells with an intact plasma membrane, can penetrate the cell membrane to make the nucleus red in the late stage of apoptosis and necrosis (26). Annexin-V and PI dual staining allows discrimination of apoptotic cells (Annexin-V<sup>+</sup>, PI<sup>-</sup>), late apoptotic/necrotic cells (both Annexin-V<sup>+</sup> and PI<sup>+</sup>) and live cells (both Annexin-V<sup>-</sup> and PI<sup>-</sup>) (27). A total of 6 rats randomly selected from each group, 24 h after sevoflurane-exposure

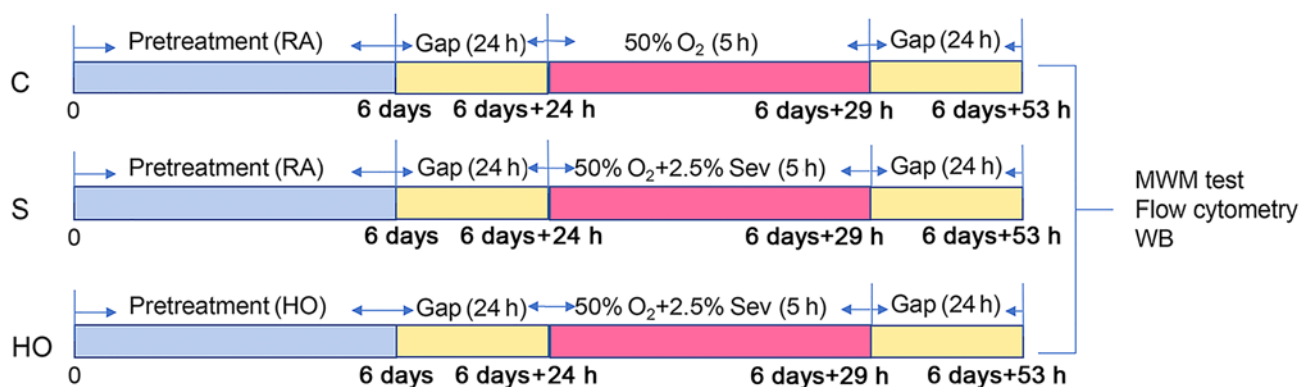


Figure 1. Experimental protocol for the study of normobaric hyperoxia preconditioning. RA, normoxia; HO, normobaric hyperoxia; Gap, ordinary air room without any procedure; d, day; h, hour; Sev, sevoflurane; WB, western blotting; MWM, Morris water maze; C group, control group; S group, sevoflurane group; HO group, sevoflurane + NBO-PC group; NBO-PC, normobaric hyperoxia preconditioning.

were anesthetized with an intraperitoneal injection of 10% chloral hydrate (250 mg/kg) without any signs of peritonitis observed. According to previous literature (28), the loss of the righting reflex (LORR, an indicator of hypnosis), ambulation and voluntary movement were considered to be indicators of the success of chloral hydrate anesthesia. LORR was assessed by attempting to place the rat in left lateral recumbency, followed by dorsal recumbency. If the rat remained on its back for 10 sec, LORR was considered to be achieved (29). After successful induction of chloral hydrate, A total of 18 rats from three groups were sacrificed by decapitation. Brain tissue was immediately removed and the left hippocampi were separated and cut into blocks. Hippocampal tissue (~100 mg) was placed on a 100-mesh copper net and cut up with tweezers and then gently rubbed and filtered. The filtrate was centrifuged at  $167.7 \times g$  for 5 min at room temperature. Cells collected after centrifugation were resuspended in 500  $\mu$ l Annexin-V binding buffer to prepare a single cell suspension ( $1-5 \times 10^5/l$ ). Subsequently, the cells were incubated with 5  $\mu$ l Annexin-V-FITC at 5°C for 10 min in dark and then 5  $\mu$ l PI was added and the cells were incubated at 5°C for 10 min in dark. A flow cytometer (FC500; Beckman Coulter Inc.) was used to detect the apoptosis rate of early apoptotic cells with an analyzing software (EXPO32 ADC v1.2; Beckman Coulter Inc.).

Hippocampus cells from the right hippocampi of the 18 aforementioned rats were collected in the way described above and suspended in 3 ml Dulbecco's modification Eagle's medium (DMEM) and made into a single cell suspension loaded ( $1-5 \times 10^5/l$ ) to measure the  $[Ca^{2+}]_c$ . Next, 3  $\mu$ l Fluo-3/AM (calcium ion fluorescence probe; Hangzhou MultiSciences (Lianke) Biotech, Co., Ltd.) was added and cells were incubated at 37°C for 30 min, washed twice with DMEM, and then resuspended in DMEM at 37°C for 15 min. Flow cytometry instrument (FC500; Beckman Coulter Inc.) was used to measure fluorescence intensity, with the excitation wavelength of 488 nm and emission wavelength of 525 nm with an analyzing software (EXPO32 ADC v1.2; Beckman Coulter Inc.).

**Western blotting.** Western blotting was performed to determine expression of bcl-2, bax and active caspase-3

in the hippocampus. The proteins were extracted from the hippocampal tissue samples of the 6 remainder rats of each group. Briefly, the hippocampus was homogenized in RIPA lysis buffer (Wuhan Servicebio Technology Co., Ltd.), and the total protein concentration of the supernatant was determined using a BCA protein quantification kit (Wuhan Servicebio Technology Co., Ltd.). Proteins were separated by 12% SDS/PAGE gel (SDS-PAGE kit; Beijing Zoman Biotechnology Co., Ltd.) with 50  $\mu$ g protein loaded per lane and then transferred on to PVDF membranes (EMD Millipore). After blocking with 5% skimmed milk at 37°C for 2 h, the membranes were incubated at 4°C overnight with primary antibodies: bcl-2 (1:1,000; Proteintech Group Inc.; cat. no. 12789-1-AP), bax (1:1,000; Arigo Biolaboratories Corp.; cat. no. ARG66247), active caspase-3 (1:2,000; ImmunoWay Biotechnology Company; cat. no. YM3431). Anti- $\beta$ -actin (1:1,000; ProteinTech Group Inc.; cat. no. 66009-1-ig) was also used as a protein loading control for each sample. Membranes were further incubated with secondary antibodies (1:10,000; Rockland Immunochemicals Inc.; cat. no. 36595) for 2 h at room temperature. The signals were detected using an Odyssey CLx infrared imaging system (LI-COR Biosciences). ImageJ software (v1.8.0; National Institutes of Health) was used for quantification of the signals.

**Statistical analysis.** Statistical analyses were performed with the SPSS software v.23.0 (IBM Corp), and the data were expressed as mean  $\pm$  SD. Three biological repetitions were conducted for each experiment. Escape latency were analyzed by two-way repeated-measures ANOVA followed by the post hoc Bonferroni test. The statistical differences of all the other data were analyzed by one-way ANOVA without repeated measures followed by the post hoc Bonferroni test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of NBO-PC on cognitive deficit induced by sevoflurane.** To evaluate the cognitive function of the rats the MWM test was conducted. All rats had a tendency of reduced escape latency as training progressed indicating that the rats were learning from the day by day practice (Fig. 2A and B).

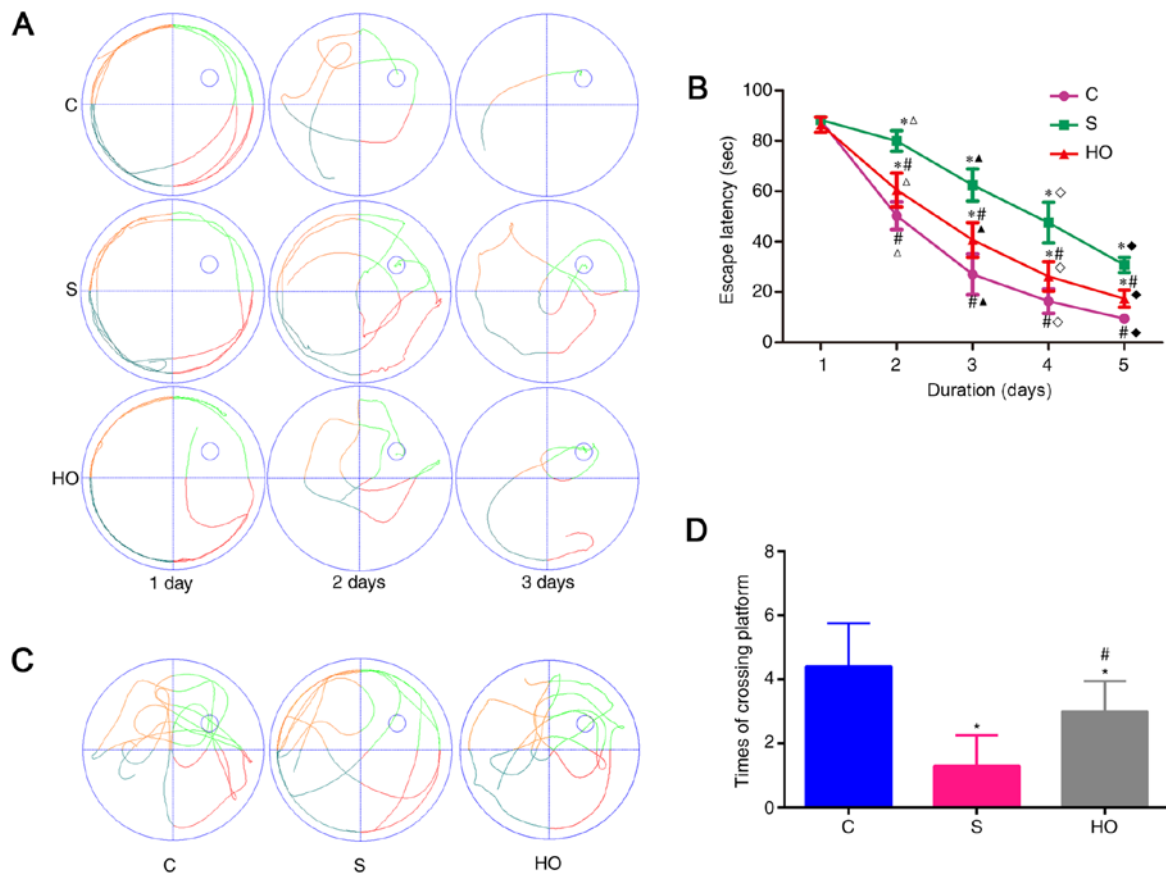


Figure 2. Effects of normobaric hyperoxia preconditioning on sevoflurane-induced learning and memory impairment by the MWM test. (A) Typical paths during place navigation test for each group on days, 1, 3 and 5 after anesthesia. (B) Escape latency (time to find the hidden platform) plotted against training day. 1, 2, 3, 4 and 5 d means the 1-5 day after rats were anesthetized. Escape latency in each group decreased significantly ( $P < 0.05$ ) compared with 1d. (C) A typical path during the probe trial for each group. (D) Platform crossing times during the probe trial of the MWM test. Sevoflurane exposure increased the times of platform crossing compared with the control condition ( $^{\#}P < 0.05$ ). Normobaric hyperoxia preconditioning attenuated the sevoflurane-induced decrease in the number of platform crossings ( $^{\#}P < 0.05$  vs. S group). ( $n = 10/\text{group}$ ).  $^{\ast}P < 0.05$  vs. control group,  $^{\Delta}P < 0.05$  vs. 1d,  $^{\blacktriangle}P < 0.05$  vs. 2d,  $^{\circ}P < 0.05$  vs. 3d,  $^{\ast}P < 0.05$  vs. 4d. MWM, Morris water maze; C, control group; S group, sevoflurane group; HO group, sevoflurane + NBO-PC group; NBO-PC, normobaric hyperoxia preconditioning.

The escape latency was significantly longer and the platform crossings were lower in S group rats compared with C group rats, indicating significant cognitive impairment induced by sevoflurane (Fig. 2B-D). Notably, the learning and memory impairment induced by sevoflurane was ameliorated by NBO-PC, as indicated by shorter escape latency (Fig. 2B) and increased platform crossings (Fig. 2C and D). Taken together, these results imply that NBO-PC can ameliorate the dysfunction of learning and memory induced by sevoflurane in aged rats.

**Effects of NBO-PC on apoptosis in the hippocampus.** Next, to determine whether the neuroprotection of NBO pretreatment is associated with the level of apoptosis in the hippocampus, the apoptosis rate in the hippocampus was measured by flow cytometry 24 h after sevoflurane-exposure. Compared with C group, the apoptosis rate was significantly increased in the S and HO groups (Fig. 3A and B). However, the increase of apoptosis rate in the HO group was lower compared with that in the S group (Fig. 3A and B).

In addition, the protein expression level of bcl-2, bax and active caspase-3 in the hippocampus were also tested to estimate the level of apoptosis. Downregulation of antiapoptosis

protein (bcl-2) (30) and upregulation of pro-apoptotic protein (bax) (31) and apoptosis protein (caspases-3) (32) are an indication of apoptosis. Caspase-3 has classically been defined as the main executioner of apoptosis, and the activated form of caspase-3 is involved in the execution phase of apoptosis (33). In the present study, the 'caspase-3' in Fig. 3C and D represented active caspase-3.

As shown in Fig. 3C and D, compared with the C group, the anesthesia with 2.5% sevoflurane (S group) induced significant increase of bax and active caspase-3 and a decrease of bcl-2 in the hippocampus. Although similar variations were observed in the HO group, the change in the HO group was lower compared with the S group (Fig. 3C and D). All the aforementioned data suggested that NBO-PC inhibited apoptosis in the hippocampus caused by sevoflurane.

**Effects of NBO-PC on  $[Ca^{2+}]_c$  in the hippocampus.** It has been confirmed that sevoflurane increases  $[Ca^{2+}]_c$  to induce neuroapoptosis (34). To evaluate the role of  $Ca^{2+}$  in the NBO-PC mediated neuroprotective effect, the  $[Ca^{2+}]_c$  in the hippocampus was measured after sevoflurane anesthesia in the aged rats. Sevoflurane induced an elevation of  $[Ca^{2+}]_c$  compared with the C group (Fig. 4A and B), while NBO-PC (HO group)

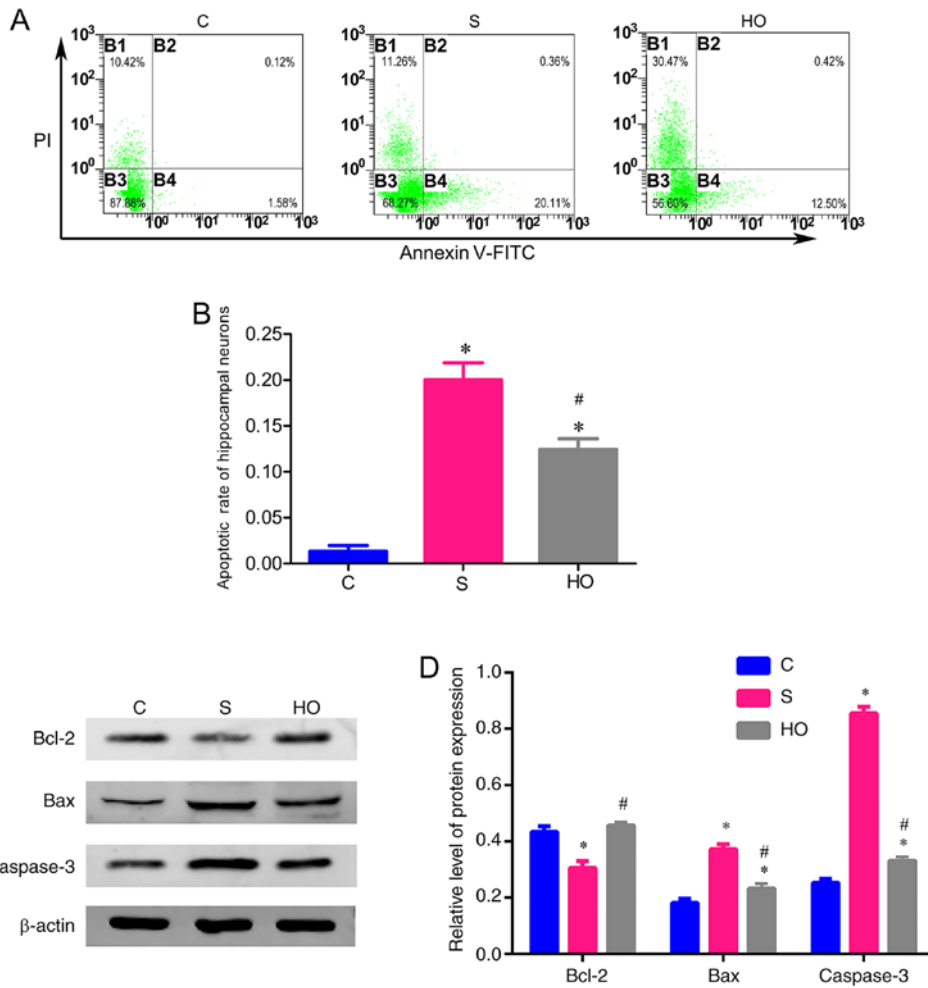


Figure 3. Effects of normobaric hyperoxia preconditioning on the apoptosis in the hippocampus. (A) Representative plots of apoptosis detection by flow cytometry in the hippocampus of aged rats in each group, early apoptotic cells (B4, Annexin-V<sup>+</sup>/PI<sup>-</sup>), late apoptotic or necrotic cells (B2, Annexin-V<sup>+</sup>/PI<sup>+</sup>), live cells (B3, Annexin-V<sup>-</sup>/PI<sup>-</sup>), mechanical damaged cells (B1, Annexin-V<sup>-</sup>/PI<sup>+</sup>). (B) Apoptosis rate in the hippocampus 24 h after sevoflurane exposure in each group. (C) Representative western blot image from each group. (D) Western blot analysis of Bcl-2, Bax and active caspase-3 expression in the hippocampus of each group. \*P<0.05 vs. control group, #P<0.05 vs. S group. C, control group; S group, sevoflurane group; HO group, sevoflurane + NBO-PC group; NBO-PC, normobaric hyperoxia preconditioning.

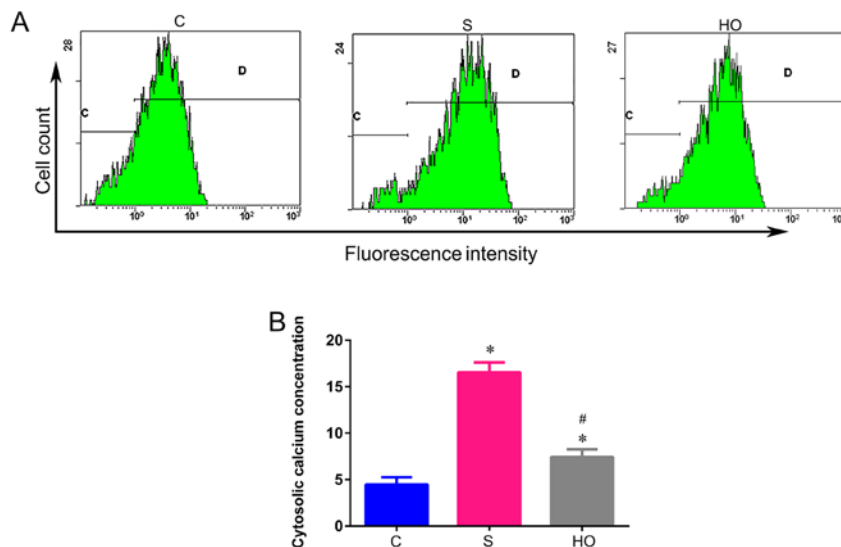


Figure 4. Effects of normobaric hyperoxia preconditioning on the  $[Ca^{2+}]_c$  in the hippocampus. (A) Intracellular calcium homeostasis in hippocampus cells 24 h after sevoflurane exposure in each group. (B) Representative histograms of  $[Ca^{2+}]_c$  by flow cytometry in the hippocampus of aged rats in each group. The 'C' and 'D' gates in the figure represent negative and positive cells respectively. \*P<0.05 vs. control group, #P<0.05 vs. S group.  $[Ca^{2+}]_c$  cytosolic calcium concentration; C, control group; S group, sevoflurane group; HO group, sevoflurane + NBO-PC group; NBO-PC, normobaric hyperoxia preconditioning; PI, propidium iodide.

significantly decreased the elevation of  $[Ca^{2+}]_c$  caused by sevoflurane (Fig. 4A and B). Hence, it was proposed that the inhibition of apoptosis induced by NBO-PC was mediated by reducing  $[Ca^{2+}]_c$ .

## Discussion

The present study investigated whether NBO-PC ameliorates cognitive deficit after sevoflurane anesthesia, and whether NBO-PC influences the hippocampal apoptosis in aged rats. The major findings of the present study are as follows: i) NBO-PC reduced cognitive deficit induced by sevoflurane-exposure; ii) antiapoptosis protein (bcl-2) expression increased, pro-apoptotic protein (bax) and apoptosis protein (caspase-3) decreased in the hippocampus after NBO-PC; and iii) the apoptosis rate and  $[Ca^{2+}]_c$  decreased in the hippocampus after NBO-PC. These results demonstrated that the protective role of NBO-PC in sevoflurane-induced spatial and learning impairment was partly related to inhibition of apoptosis in the hippocampus via reducing  $[Ca^{2+}]_c$ .

The potential detrimental effects of anesthetics on aged brains has become a hot topic in recent years due to concerns about the safety of general anesthesia/anesthetics (35). Although clinical evidence regarding the association between anesthetic exposures of aged patients and subsequent cognitive impairments remains unclear, repeated or consistent exposures to general anesthetics may be a potential harmful risk in aged brains (36,37). Sevoflurane with properties of fast onset and rapid recovery (38) is commonly used as a general anesthesia drug in various types of surgery (for example, orthopedic surgery, gynecological surgery and gastrointestinal surgery).

Notably, numerous *in vitro* and *in vivo* studies have demonstrated that sevoflurane induces neurotoxicity (12,15,39). Associated with sevoflurane-induced cell injury and death in neurocyte (40), sevoflurane also has been demonstrated to impair the cognitive functions of the aged animals (12,41). Mechanisms underlying sevoflurane-induced neurotoxicity have not been clarified (40), nevertheless, neuroapoptosis may be a key mechanism (15,42). An *in vitro* study demonstrated that sevoflurane induces apoptosis of neural stem cells by activating  $\gamma$ -aminobutyric acid (43). Chen *et al* (44) found that sevoflurane initiates endoplasmic reticulum stress mediated apoptosis in hippocampal neurons of aging rats. The present study revealed that 2.5% sevoflurane exposure for 5 h decreased the expression of bcl-2, increased the expression of bax and caspase-3, and increased the apoptosis rate in the hippocampus, which were consistent with the changes in cognitive function. Additionally, in the present study the increased  $[Ca^{2+}]_c$  in the hippocampus after sevoflurane-exposure was also found indicating a calcium-mediated neuroapoptosis. Apart from neuroapoptosis, there are other mechanisms underlying-induced neurotoxicity, such as neuroinflammation (12), neurodegeneration (45).

NBO, in addition to serving as a tool for enhancement of oxygen delivery, has been demonstrated to provided neuroprotection in various models, including ischemia-reperfusion brain injury (18), newborn hypoxia-ischemia brain injury (46), cerebral hemorrhage (47) and brain trauma (48). Notably, Gao *et al* (21) found that NBO treatment improves spatial learning and memory deficits in APP/PS1 transgenic

mice, suggesting that the NBO treatment may have a similar effect on cognitive impairment caused by exposure to anesthetics. Hence, the present study, investigated the effect of NBO pretreatment on the neurotoxicity of sevoflurane in aged rats. In one previous dose-response study (18), protective effect against cerebral ischemia-reperfusion injury was induced by exposing rats to a normobaric hyperoxic environment (95%  $O_2$  exposure for 16 and 24 h consecutively), with no protective effect of NBO exposure less than 16 h. Simultaneously, attention should be paid to the toxicity of long-term oxygen inhalation. It has previously been reported that exposure to 95%  $O_2$  for 24 h resulted in severe pulmonary congestion with extravasations of red blood cell, edema and alteration in the alveolar structure (49). Mohammadi and Bigdeli (24) confirmed that normobaric hyperoxia preconditioning (exposure to 95% inspired NBO for 4 h/day for 6 consecutive days) had neuroprotective effect on rats in the middle cerebral artery occlusion model. Hence, the regimen for NBO-PC in the present study was based on the current study. The findings of the present study demonstrated that NBO-PC was neuroprotective in aged rats exposed to 2.5% sevoflurane for 5 h. The MWM test clearly demonstrated that NBO-PC greatly improved cognition impairment caused by sevoflurane in aged rats. As expected, the apoptosis in the hippocampus was in concert with the changes in cognitive function in the present study. In addition, the present study demonstrated that antiapoptosis protein (bcl-2) expression increased, pro-apoptotic protein (bax) and apoptosis protein (caspases-3) decreased in the hippocampus after NBO-PC, and the apoptosis rate decreased in the hippocampus after NBO-PC.

Concurrently, it was observed in the present study that NBO-PC reduced the increase of  $[Ca^{2+}]_c$  to sevoflurane in the hippocampus. Intracellular  $Ca^{2+}$ , one of the most widely used intracellular messengers is involved in controlling almost all cell processes including muscle contraction, exocytosis, proliferation, differentiation, protein synthesis and gene expression (50). Disruption of the intracellular calcium homeostasis, particularly due to a persistent and excessive increase in the intracellular  $Ca^{2+}$  can induce cell death by apoptosis (51). In addition, apoptosis cell death mediated by calcium dysregulation serves an important role in anesthetic neurotoxicity (52). The results of the present study implied that NBO-PC may play a neuroprotective role by reducing the intracellular calcium increase caused by sevoflurane anesthesia through some molecular mechanisms, which need further study.

In the present study, a lower chloral hydrate dose (250 mg/kg) was used to anaesthetize the rats in accordance with some previous studies (53,54). Some studies (55,56) use 300-400 mg/kg chloral hydrate to perform surgery or establish animal models, the process of which requires deep anesthesia and long duration. However, in the present study just a mild anesthesia was needed to ensure that the rats were hypnotized without struggling. In addition, the potential effects of chloral hydrate on experimental variables should also be taken into consideration (57). Therefore, in view of the low requirements for the depth and the duration of anesthesia and the possible influence of high dose of chloral hydrate on detection indicators, the lower dose of chloral hydrate was used to anaesthetize the rats in the present study.

The present study had some limitations. In the present study, 20-month-old rats were selected as aged rats. Among the published articles on cognitive dysfunction, the ages of old rats were very different ranging from 18-24 months (58-65). According to the aforementioned studies, the rats selected by the present study are equivalent to the aged. It was reported that 1 rat month is comparable to 3 human years in adulthood (66), which indicates differences in anatomy, physiology and developmental processes of different months old rats. In addition, these differences may lead to differences in research results. Although the precise correlation between age of laboratory rats and human is still a subject of debate, it has been accepted that 24 month old rats correspond to 60 year old humans (66). Hence, the age of the old rats in this experiment may be relatively young. In addition, some assays, such as histopathological staining of the hippocampal sections were not performed to visually reflect the brain damage caused by sevoflurane due to funding. So future studies must select much older rats as subjects and optimize the assessment of brain damage through histopathological staining if funding is available.

In conclusion, the present study demonstrated that NBO-PC decreased the hippocampal apoptosis as well as alleviated the memory deficits in aged rats who were exposed to sevoflurane. The results of the present study, suggested that neuron apoptosis inhibited by NBO-PC is in association with a decrease of cytosolic  $Ca^{2+}$  in the hippocampus, which may at least partially be the molecular mechanism by which NBO-PC induces neuroprotection.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YW was responsible for designing the study, performing the experiment, collecting the data and writing the manuscript. CPY was responsible for designing the study, performing the experiment, and collecting the data. YLT and ZJZ were responsible for collection of experimental specimens and the extraction of proteins and reviewing the manuscript. ZYH was responsible for analyzing and interpreting the data.

QJW was responsible for providing experimental ideas and reviewing the manuscript. YW and QJW were responsible for the confirming the authenticity of all the raw data. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

This study was approved by the Ethics Committee for Animal Experimentation (Ethical approval no. Guo A2017-026-1), and the animals were studied at Hebei Medical University (Shijiazhuang, China). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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