

Original Article

Folic acid pretreatment attenuates isoflurane-induced cognitive dysfunction in aged rats

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Abstract: POCD is an important complication characterized with cognitive attenuated. Although the underlying mechanism of isoflurane-induced cognitive impairments is still unclear, neuronal inflammation, oxidative stress and neuronal apoptosis are increasingly being pointed as the major contributors. Folic acid is a kind of necessary dietary, related neurogenesis and programmed cell death. This study aims to investigate the effects of folic acid pretreatment on cognitive dysfunction induced by isoflurane anesthesia in aged rats and explore the underlying mechanisms. Aged rats were randomly divided into five groups: Group A (control); Group B (isoflurane); Group C (6 mg/kg.bw/d folic acid + isoflurane); Group D (10 mg/kg.bw/d folic acid + isoflurane); Group E (14 mg/kg.bw/d folic acid + isoflurane). Learning and memory behavioral changes were evaluated by Morris water maze. The activities of antioxidant enzymes, such as MDA, GSH-Px, SOD, and CAT were detected. The level of bcl-2, bax and caspase-3 were also examined. We found that folic acid reversed the decline of SOD, GSH-Px and CAT, as well as the rise of MDA in hippocampal tissue of isoflurane anesthesia aged rats. The rats in group C, D, E went along with Bax raise and Bcl-2 reduction, the Bcl-2/Bax ratio showed an obvious increase. In conclusion, folic acid pretreatment attenuates the hippocampus-dependent cognitive impairment induced by isoflurane anesthesia via anti-apoptosis and anti-oxidative stress.

Keywords: Folic acid, cognitive functions, isoflurane, aged rats

Introduction

Post-operative cognitive dysfunction (POCD) is a common complication of central nervous system following major surgery, which is characterized by cognitive functions decreased, including working memory, long term memory, information processing, attention and cognitive flexibility [1, 2]. In recent years, technological development in surgery and anesthesia has achieved great improvement; however, the incidence of POCD remains relatively high, especially in the elderly. POCD is likely to be several weeks or months in most patients, while it can develop into long-term or even permanent in some patients [3], which prolongs the patient's hospital stays, increases the cost of hospitalization, decreases the patient's life quality, and leads to higher incidence of dementia, even mortality [4]. Therefore, POCD has been a major clinical issue in geriatric surgical patients. Previous studies illustrated that inhalational anesthetic such as isoflurane, played an impor-

tant role in cognitive impairments and relevant neurotoxicity. It might exert neurotoxic effects by inducing structural and functional changes in the central nervous system and ultimately lead to cognitive dysfunction. Accumulating evidence has demonstrated that isoflurane anesthesia can induce serious oxidative damage, activate the caspase-3, and reduce the Bcl-2/Bax ratio in the hippocampus [5, 6]. The oxidative damage and caspase-3 activity could impair the synaptic plasticity which leads to the neurodegeneration and consequent deficits in the cognitive function.

Dietary supplementation of antioxidants, such as copherol (vitamin E), ascorbic acid (vitamin C), and carotenes, has been studied to improve the cognitive deficit, but the results and corresponding mechanisms remain controversial. Dietary folic acid, as the members of the B9 family, is required for normal development of the nervous system, playing important roles in neurogenesis and programmed cell death [7].

Protective effects of folic acid on cognitive dysfunction

An epidemiological study showed that decreased levels of folic acid in the blood are associated with a two-fold increase in the risk of AD [8]. A study showed folic acid attenuated cognitive dysfunction in streptozotocin-induced diabetic rats [9]. In clinical practice, people with a low level of folic acid are more at a risk of diminished cognitive function, while a higher dose of folic acid can contribute to slow the cognitive decline of ageing [10]. Folic acid deficiency may compromise the antioxidative reserve at multiple levels, and increase oxidative stress, ultimately increase neuronal excitotoxicity and cell death [11]. Folic acid deficiency and elevation of homocysteine have been shown to potentiate the effects of oxidative stress increase and apoptosis in vitro [12]. Because folic acid has strong positive effects on brain tissue, it was hypothesized that treatment with folic acid might ameliorate the cognitive dysfunction.

The aim of our present work was to investigate whether folic acid pretreatment to aged rats could attenuate the learning and memory dysfunction induced by isoflurane anesthesia in aged rats, and the underlying mechanism was also examined.

Materials and methods

Animal groups

Sprague Dawley rats, aged twenty months, weighing 260 ± 20 g, were obtained from Experimental Animal Center of Suzhou Aiermaite technology Co. Ltd. (SPF grade, Certificate No. SCXK20150004). All procedures including the use of the laboratory animals were approved by the Institutional Animal Care Committee of Binzhou Medical College. All animals were treated in accordance with the Care and Use of Laboratory Animals. The rats were randomly divided into five groups ($n=15$ rats/group): control group (Group A), isoflurane group (Group B), 4 mg/kg.bw/d folic acid + isoflurane group (Group C); 8 mg/kg.bw/d folic acid + isoflurane group (Group D); 12 mg/kg.bw/d folic acid + isoflurane (Group E). Folic acid (Sigma Chemical Company, St. Louis, USA) was dissolved in physiological saline and was dosed by intragastric administration once daily for consecutive 30 days before isoflurane exposed. Respectively, the rats in group C, D, E received a dose of 4, 8 or 12 mg/kg.bw/day folic acid. An equal

volume of physiological saline was given to the rats in group B.

Isoflurane exposure

Rats in group B, C, D, E were put into an translucent Plexiglas chamber ($25 \times 30 \times 20$) within a thermostatic bath ($37 \pm 2^\circ\text{C}$). 1.2% isoflurane was delivered by a calibrated vaporizer, and gas consisting of oxygen and nitrogen (3:7) served as the carrier. Rats were continuously exposed to isoflurane of 4 ml/min for 2 h. Rats in group A were placed in the same chamber and environment, but the chamber was only flushed with carrier gas at a rate of 4 L/min for 2 h. The concentration of isoflurane, oxygen and carbon dioxide in the chamber were monitored continuously with a DatexTM infrared analyzer (Capnomac, Helsinki, Finland).

Morris water maze

24 h after isoflurane exposure, 5 rats from each group were chosen to evaluate the spatial learning and memory ability by the Morris water maze experiments as previously described [13] with minor modifications. A round pool (150 cm in diameter; 60 cm in depth) was filled with warm ($23 \pm 2^\circ\text{C}$) opaque water and divided into four imaginary quadrants. A movable clear platform located 2 cm below the water surface was fixed in the third quadrant. The rats received 4 trials daily for consecutive 6 days. In the trials, and the escape latency was recorded. After the last trials, the platform was removed immediately for a probe trial, the rats were put into the pool and allowed to swim freely and the numbers of original platform crossings were recorded.

Brain tissue harvest

Four hours after exposure, 5 rats from each group were sacrificed by overdose of Nembutal. The hippocampal tissues were quickly removed. The right was dissected out immediately for western blot analysis and ELISA examination, while the left was used for hematoxylin and eosin (H&E) staining.

H&E staining

Hippocampus tissues containing CA1 and dentate gyrus area were fixed in 10% neutral buffered formalin solution overnight, and then embedded in paraffin. Coronal 8- μm -thick sec-

Protective effects of folic acid on cognitive dysfunction

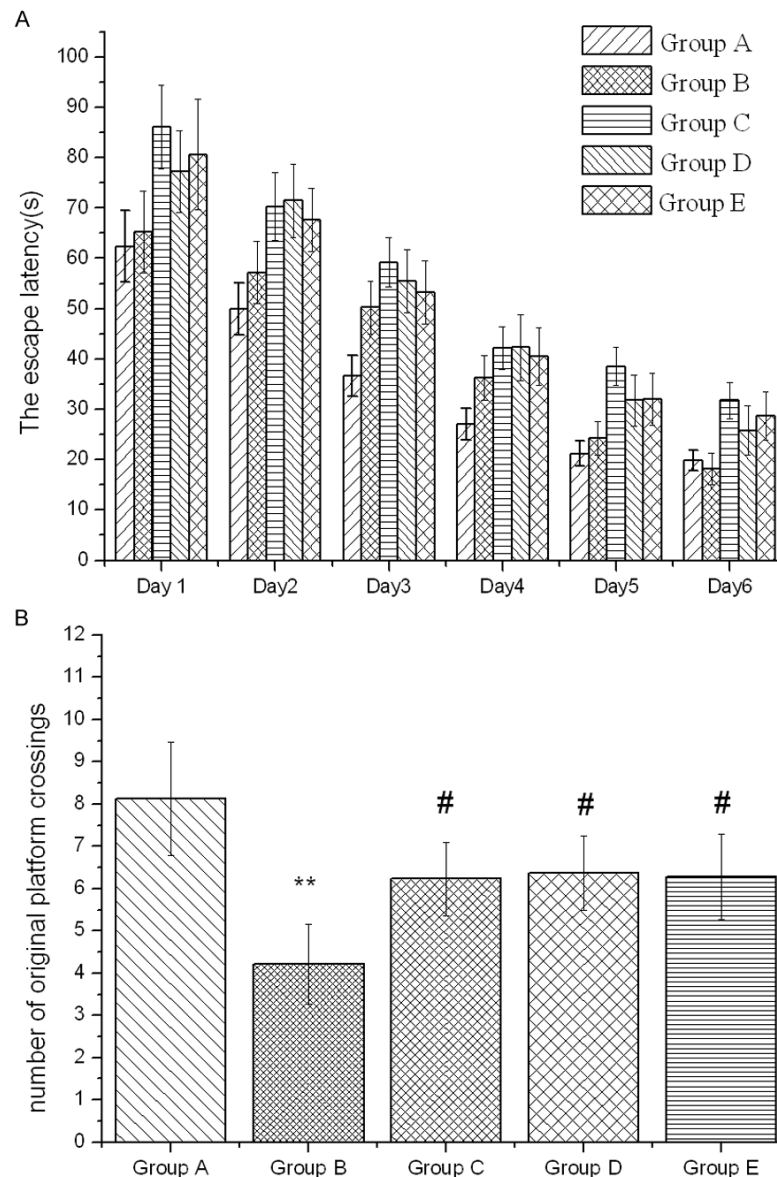


Figure 1. Effects of folic acid on isoflurane induced cognitive impairments. A. The escape latency; B. Numbers of crossing the former platform. Dates were shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, VS Group A; # $P < 0.05$.

tions were cut and subjected to H&E staining for light microscopic examination.

Quantification of SOD, GSH-Px, CAT, and MDA

The hippocampus were isolated and blotted dry. After weighted, the samples were homogenized in ice-cold isotonic phosphate buffer. After centrifuged, the supernatant was collected and quantitatively assayed for the levels of Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and malondialdehyde (MDA) by corresponding ELISA kits (Nanjing Jiancheng Bioengineering Institute,

Nanjing, China) according to the manufacturer instructions.

Western blotting analysis

The level of Bax, Bcl-2, capase-3 in hippocampus tissues were assessed using Western blotting method. Total proteins of hippocampal tissues were homogenized with RIPA lysis buffer, containing protease inhibitor cocktail EDTA-free, and then were centrifuged at 15000 r for 15 min. The proteins concentration was detected the bicinchoninic acid (BCA) protein assay kit (Chengdu Must Biotechnology Co., Ltd, Chengdu, China). After separated by 12% SDS-PAGE gels, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Wuhan Boster Biological Technology, Ltd.). The membranes were blocked by 5% non-fat dry milk at room temperature for 2 h and then were incubated overnight at 4°C by corresponding primary antibody anti-caspase-3 (1:500, Cell Signaling Technology, MA, USA), anti-Bax (1:1000, Cell Signaling Technology, MA, USA), anti-Bcl-2 (1:1000, Cell Signaling Technology,

MA, USA), and anti- β -Actin (1:1000, Cell Signaling Technology, MA, USA). Subsequently, the membranes were incubated with secondary antibody and visualized with enhanced chemiluminescence (ELC) detection reagent (Amersham). The protein bands were scanned and analyzed by Lab Image version 2.7.1 (Kapelan GmbH, Halle, Germany).

Statistical analysis

Statistical analysis was implemented using SPSS18.0 for windows. All dates were reported as the mean $\pm$ SD, differences between groups

Protective effects of folic acid on cognitive dysfunction

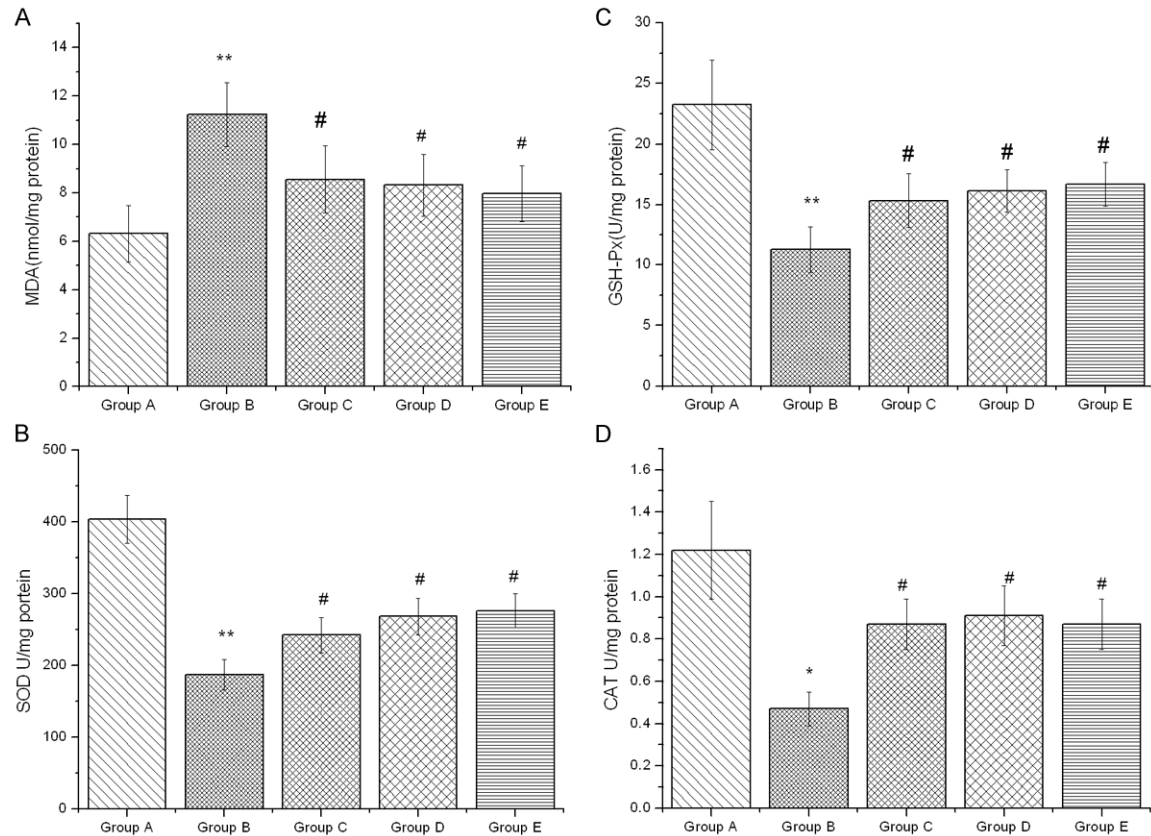


Figure 2. Effects of folic acid on the level of MDA, SOD, GSH-Px and CAT in hippocampi homogenate. Dates were shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, VS Group A; # $P < 0.05$.

were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. The level of statistical signification was set at $P < 0.05$.

Results

Learning and memory behaviors

Rats in all groups showed a rapid decrease in escape latencies (**Figure 1A**). While the rats in isoflurane group exhibited significant longer escape latencies (**Figure 1A**, $P < 0.01$) and a significant decrease in numbers of crossing the former platform (**Figure 1B**, $P < 0.05$) compared to other groups. Folic acid intervention (group C, D, E) led to significant improvement in escape latencies and numbers of crossing the former platform than group B ($P < 0.05$), while there was no significant difference among group C, D, E ($P > 0.05$).

Tissue MDA, SOD, GSH-Px and CAT analysis

The changes of MDA, SOD, GSH-Px and CAT in hippocampi homogenate after treatment were

shown in **Figure 2**. Group B showed significantly higher level of MDA, and significantly lower level of SOD, GSH-Px and CAT than other groups ($P < 0.05$; $P < 0.01$). After treated with folic acid, the MDA level for the rats in groups D, E and F have a significantly decrease ($P < 0.05$), with the level of SOD, GSH-Px and CAT increase ($P < 0.05$). Treatment with folic acid attenuated the rise in the level of MDA and decline in the level of SOD, GSH-Px and CAT ($P < 0.05$).

Histopathological evaluation

As **Figure 3** showed, in Group A, neuron arrangement is regular, and the nuclei is large (**Figure 3A**). The number of positively stained in a field of hippocampal tissue were counted for groups, the neuronal density of rats in Group B were decreased than Group A ($4267.32 \pm 234.12 / \text{mm}^3$ VS $2024.27 \pm 103.12 / \text{mm}^3$, $P < 0.05$). In Group B, the nucleus is more deeply stained, and nuclear fusion phenomenon is more obvious than control group. The condition improved obviously with folic acid pretreatment (**Figure 3C-E**).

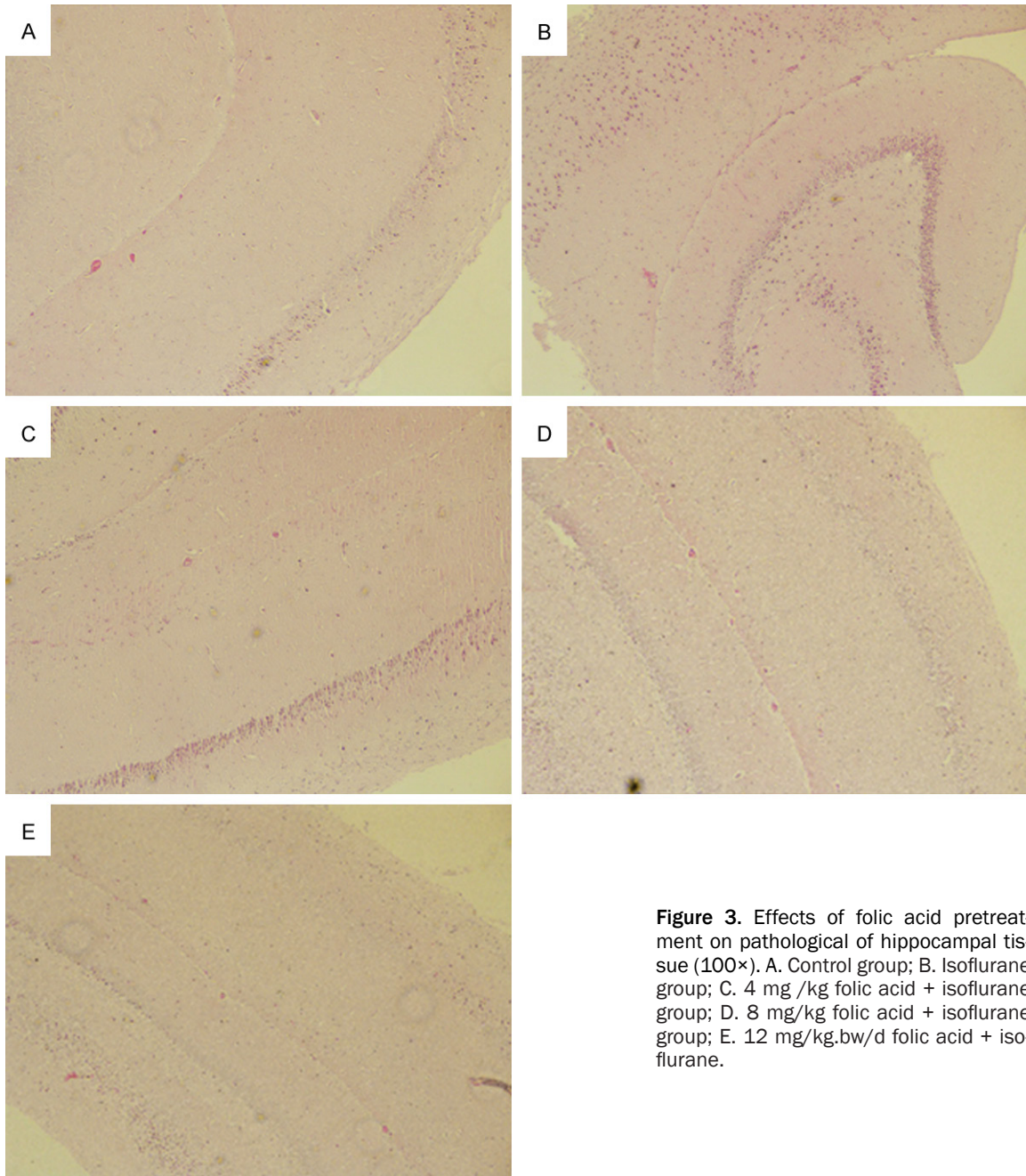


Figure 3. Effects of folic acid pretreatment on pathological of hippocampal tissue (100×). A. Control group; B. Isoflurane group; C. 4 mg /kg folic acid + isoflurane group; D. 8 mg/kg folic acid + isoflurane group; E. 12 mg/kg.bw/d folic acid + isoflurane.

The expression of Bax, Bcl-2 and caspase-3

We examined the protein levels of Bax, Bcl-2 and caspase-3 which are known as the indicators of apoptosis. The rats with isoflurane exposed (Group B) showed a significant increase in Bax and a significant decrease in Bcl-2 than control group; The Bcl-2/Bax ratio decreased 5.31 fold compared with control group. With folic acid treatment at doses of 6,

10 and 14 mg/kg.bw/day, the rats went along with Bax raise and Bcl-2 reduce, the Bcl-2/Bax ratio increased by about 3.06, 3.59 and 3.74 fold than group B. As **Figure 4** displayed, the caspase-3 protein expression in group B was significantly higher than group A. After folic acid treatment at doses of 6, 10 and 14 mg/kg.bw/day, level of caspase-3 significant decreased by about 1.76, 1.79 and 1.91 fold compared with Group B.

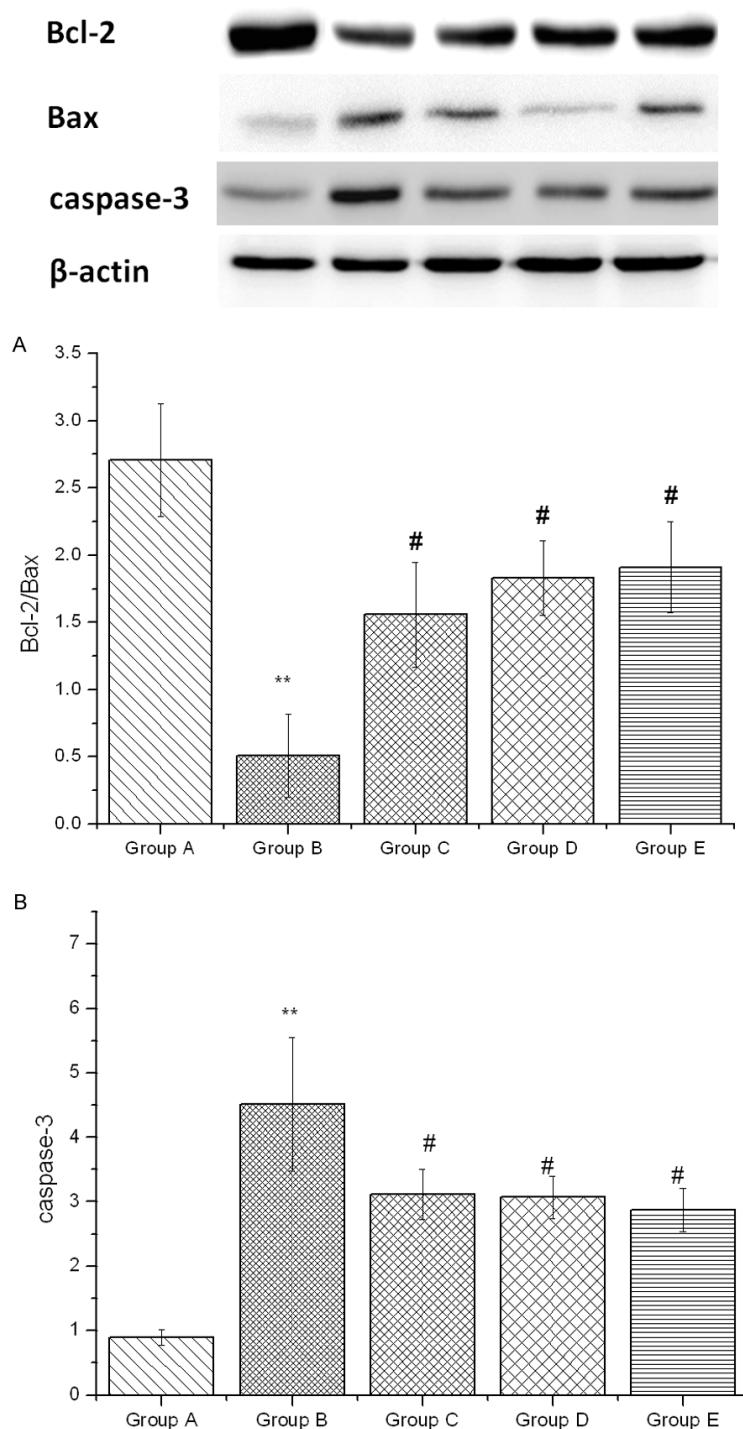


Figure 4. Effects of folic acid on the level of caspase-3, and Bcl-2/Bax ratio. A. The Bcl-2/Bax ratio; B. Caspase-3. Dates were shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, VS Group A; # $P < 0.05$.

Discussion

Cognitive dysfunction and hippocampal cells injury induced by isoflurane anesthesia have been verified by numerous studies [4, 14].

Effective therapeutic approaches have drawn significant attention from the public and scientific community. Recently research, many pharmacological agents, such as resveratrol [15], dexmedetomidine [1], have been used in experimental isoflurane anesthesia injury. The correlation with inflammatory cytokines and apoptosis were also demonstrated. In addition, mitochondrial oxidative damage has been linked to the early pathogenesis of POCD [23]. Therefore, protecting the spinal cord from oxidative damage, and decreasing the neuronal apoptosis might be an important topic of treatment for POCD. Several studies showed folic acid played an important role in neurogenesis and decline in the anti-ROS system [16, 17]. In the present study, we demonstrated the influence of folic acid on learning and memory dysfunction, oxidative stress and apoptosis induced by isoflurane anesthesia. The results demonstrate that isoflurane exposure markedly impairs spatial learning and memory function in aged rats.

Learning and memory are two main aspects to evaluate the cognitive capability. In our study, to determine the role of folic acid on cognitive function, Morris Water Maze was conducted in aged rats. The results showed that the rats with isoflurane exposed had longer escape latencies and less numbers of crossing the original platform than control group, suggesting isoflurane impairs the hippocampus-dependent learning and spatial memory capability. Our result is consistent with many other related reports [17, 18]. It was reported that folic acid with a low-dose of 4 mg/kg and a high-dose of 12 mg/kg had potential to enhance cognitive function induced by

cerebral ischemia in rats. In our study, 4 mg/kg, 8 mg/kg, and 12 mg/kg were chosen. The study finds that folic acid pretreatment shortens the escape latencies and increases numbers of crossing the original platform. But among doses, there were no significant difference in cognitive function. These results indicated folic acid pretreatment might improve POCD induced by isoflurane anesthesia, and no dose-dependent.

Mechanisms for folic acid attenuating isoflurane-induced cognitive impairment are still poorly understood. However, related evidences have shown that overproduction of reactive oxygen species (ROS) and/or its metabolites are potentially neurotoxic [19]. Considerable evidences have demonstrated these oxidative stress parameters were associated with learning and memory deficits [19, 20]. SOD, GSH-Px, and CAT are three main enzymes to provide cellular protection against damage from oxygen-derived free radicals. MDA is a degradation product of the oxygen-derived free radicals and lipid oxidation, which reflects the damage caused by reactive oxygen species. In the present study, aged rats with isoflurane exposed showed a significantly decrease in the level of antioxidant defense enzymes including SOD, GSH-Px, and CAT, while the level of MDA significantly increased. During isoflurane exposed, large amounts of oxidative stress were produced. We found that folic acid treatment before isoflurane inhalation was able to ameliorate these abnormalities. In our study, folic acid pretreatment attenuated the rise in the level of MDA and decline in the level of SOD, GSH-Px and CAT in hippocampus. The histopathological evaluation showed the hippocampus neuronal density in rats with isoflurane exposure was decreased, and the nucleus was more deeply stained, with nuclear fusion phenomenon increased. However, folic acid improved the condition. Free radical activity and their oxidation have damage to biological macromolecules and a variety of cellular components, which induced the obvious toxic effects to nerve cells, and lead to the neuronal density decreased, nuclear fusion phenomenon. Folic acid has properties of free radical scavenging and antioxidant activity [21], which are consistent with our study.

It has been confirmed that neuron apoptosis is an important reason for cognitive impairments induced by isoflurane inhalation in rats model. The pro-apoptotic protein of Bax and anti-apop-

totic members of the Bcl family were related with the dysfunction of mitochondrial membrane on the mitochondrial apoptotic pathway [22]. In addition, the caspase-3 was a key enzyme associated with apoptosis, and isoflurane exposure might enhance its activity. In our study, exposure of aged mice to isoflurane increased the level of Bax, decreased the level of Bcl-2, and Bcl-2/Bax ratio decreased 5.31 fold than control group; Isoflurane exposed led to a high expression of caspase-3. However, with pretreated folic acid, we observed an up-regulation in the cleaved caspase-3 and Bax, a down-regulation in Bcl-2 in the hippocampus. Bcl-2/Bax ratio was obviously improved by about 3.06, 3.59 and 3.74 fold with folic acid treatment at doses of 4, 8 and 12 mg/kg.bw/day respectively. These results showed that pretreatment with folic acid could attenuate neuronal apoptosis by the regulation of Bcl-2 family protein. Moreover, our results showed that folic acid improved neuron apoptosis induced by isoflurane exposure, also suggested the important role of folic acid on apoptosis.

In conclusion, only pretreatment with folic acid showed obvious neuroprotective effects against isoflurane-induced cognitive impairment in aged rats. Furthermore, the mechanisms might attribute to decrease the oxidative stress and inhibit the protein level related to neuronal apoptosis in hippocampus. The present study provides a potential treatment strategy for POCD induced by isoflurane, but further mechanism and clinical trials are necessary.

Disclosure of conflict of interest

None.

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