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Original Article

Arachidonic acid attenuates learning and memory dysfunction induced by repeated isoflurane anesthesia in rats

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Abstract: This study aims to explore the effects of arachidonic acid (ARA) on learning and memory dysfunction in rats exposed to repeated isoflurane anesthesia and the underlying mechanisms. Fifty rats were randomly divided into five groups: sham control group, isoflurane group, low dose ARA + isoflurane group, moderate dose ARA + isoflurane group, high dose ARA + isoflurane group. The Morris water maze test was performed to assess learning and memory function and the hippocampus tissues were obtained for biochemical analysis. The results showed that administration of ARA improved learning and memory deficit induced by repeated isofluane anesthesia in Morris water maze test and in a dose-dependent manner. Additionally, ARA increased the activities of choline acetyl transferase (ChAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and the levels of acetycholine (Ach) and γ-amino-butyric acid (GABA), whereas decreased the activity of acetylcholine esterase (AchE), the content of glutamate (Glu) and malondialdehyde (MDA), and the radio of Glu/GABA. Meanwhile, ARA elevated the ratio of Bcl-2/Bax and inhibited the activity of caspase-3. In conclusion, ARA has potential therapeutic value in alleviating isoflurane-induced learning and memory impairment. The mechanism might be involved in regulating the cholinergic and Glu/GABA regulatory system, decreasing oxidative damage and inhibiting cell apoptosis.

Keywords: ARA, isoflurane anesthesia, learning and memory dysfunction, cholinergic system, Glu/GABA regulatory system

Introduction

Postoperative cognitive dysfunction refers to the neurocognitive impairment, which is a common postoperative neurological complication. The clinical symptoms are the personality changes including decreased memory, attention, social adaptability, cognitive ability and comprehension ability [1]. The symptoms of cognitive decline may persist for a long time and have negative influence on the rehabilitation and the quality of life in patients. Although the pathogenesis of postoperative cognitive dysfunction is not yet clear, numerous studies have shown that it related to preoperative anesthesia.

Inhaled anesthetics are widely used in modern surgical procedures. However, there has been extensive concern about their involvement in postoperative cognitive dysfunction [2]. Isoflurane is a commonly used inhalation anesthetic. Substantial evidences illustrated that isoflurane exposure could lead to learning and memory impairment and promote AD (one of the most serious neurodegenerative diseases characterized by progressive dementia and cognitive dysfunction) development by aggravating cognitive dysfunction [2-6]. Hence, it is increasingly urgent to explore neuroprotective candidates for the postoperative cognitive dysfunction.

New attempts are focusing on agents that target the progression of cognitive dysfunction from different pathways and on developing multi-functional compounds to combat the

causes and symptoms. Arachidonic acid (ARA), an n-6 polyunsaturated fatty acid, is a main constituent of biomembranes and plays crucial role in maintaining cell function [7]. At the same time, ARA is one of the major components of hippocampus and important in brain function such as synaptic plasticity, membrane fluidity, signal transduction synaptogenesis, and neurogenesis [8]. Brain ARA level is a well-characterized form of synaptic plasticity and a strong candidate for a cellular mechanism of learning and memory, decrease with increasing age [9]. Previous study has shown that supplementation with ARA could ameliorate age-related decrease in cognitive function in healthy elderly men [10]. Similarly, animal studies also indicate that ARA plays critical role in maintaining brain function in aged rats and that cognitive function can be improved by ARA supplementation [11].

Because ARA has strong positive effects on brain tissue, it was hypothesized in present study that treatment with ARA might ameliorate isoflurane induced learning and memory dysfunction. To the best of our knowledge, no study to date has focused on the protective effect of ARA on isoflurane induced learning and memory deficit. Therefore, this study aims to investigate ARA treatment on learning and memory dysfunction induced by isoflurane based on Morris water mater test, and the relationship to cholinergic system, amino acid neurotransmitters, oxidative stress, Bcl-2/Bax ratio and caspase-3 activation.

Materials and methods

Animals

Sprague-Dawley rats (half male and half female, 220 ± 20 g) were purchased from the Experimental Animal Center of Suzhou Aiermaite Technology Co. Ltd. (SPF grade, Certificate No. SCXK20140007). All rats were housed in groups of five with free access to food and water and kept in a regulated environment with a 12 h light/dark cycle at 23 ± 3°C and 40-70% humidity. Each rat was examined for clinical signs of ill health on receipt and observed within 7 days of arrival. All procedures were in accordance with the Guidelines of the Animal Care and Use of Laboratory Animals from the Association of Laboratory Animal Science and the Center for Laboratory Animal Science of Nangang Branch of Heilongjiang Provincial Hospital.

Experimental procedure

Fifty rats were randomly divided into 5 groups (n = 10 for each): sham control group (group A), isoflurane group (group B), 0.3 g/kg ARA + isoflurane group (group C), 1.0 g/kg ARA + isoflurane group (group D), 3.0 g/kg ARA + isoflurane group (group E). Rats in groups B, C, D and E were exposed to 1.2% isoflurane for 2 h once daily for consecutive 5 days. Since isoflurane was carried by 100% O_2, rats in group A were kept in a chamber gassed with 100% O_2 for 2 h and were not exposed to isoflurane at any time. Rats in groups C, D and E received ARA at a single oral dose of 0.3, 1 and 3 g/kg for consecutive 10 days prior to isoflurane exposure, respectively. Morris water maze test was carried out 1 h after the last administration and was assessed blind to the treatments by the observer.

Morris water maze

Hippocampus-dependent spatial learning and memory was evaluated *via* Morris water maze test as described previously with slight modifications [12]. A circular galvanized steel water tank (150 cm in diameter × 60 cm in depth) with four quadrants was used. The water temperature was adjusted to 23 ± 2°C. A hidden platform (10 cm in diameter) that served as the escapes platform was submerged 2 cm below the water surface and placed at the midpoint of a fixed quadrant. Training was performed four times daily for 6 days and escape latencies were recorded. After this test, the platform was removed and the rats were put into water from the same quadrants, the times of crossing the former platform in 120 s were recorded.

SOD, GSH-Px and MDA level and cholinergic system analyses

After training, the animals were sacrificed and the hippocampuses were dissected out and blotted dry, and then weighed and prepared as a 5% tissue homogenate in ice-cold 0.9% saline solution. The homogenate was centrifuged at 1000 × g for 10 min at 4°C and the supernatant was taken and used for the analyses. Superoxide dismutase (SOD), glutathione per-

Table 1. Effect of ARA on the escape latency in hidden-platform acquisition of Water maze test

Group	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
group A	43.2 ± 7.5	33.7 ± 6.7	22.8 ± 4.1	16.5 ± 3.9	11.4 ± 2.8	10.9 ± 3.3
group B	79.8 ± 11.1**	66.4 ± 8.9**	52.9 ± 8.6**	38.8 ± 6.6**	29.1 ± 6.6**	24.6 ± 4.8**
group C	65.8 ± 11.5	54.3 ± 9.3	40.8 ± 7.6	31.9 ± 7.4	25.3 ± 6.5	20.1 ± 4.3
group D	57.2 ± 7.8$^{\#\#}$	42.8 ± 8.1$^{\#\#}$	31.3 ± 6.7$^{\#\#}$	23.4 ± 7.2$^{\#}$	16.7 ± 4.3$^{\#}$	13.3 ± 3.2$^{\#}$
group E	46.6 ± 8.1$^{\#\#}$	34.8 ± 6.3$^{\#\#}$	24.1 ± 5.5$^{\#\#}$	18.1 ± 6.1$^{\#\#}$	14.3 ± 4.1$^{\#\#}$	12.1 ± 2.6$^{\#\#}$

Note: Date are expressed as mean ± S.D. for N =10. $^{**}P < 0.01$ versus sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus isoflurane group.

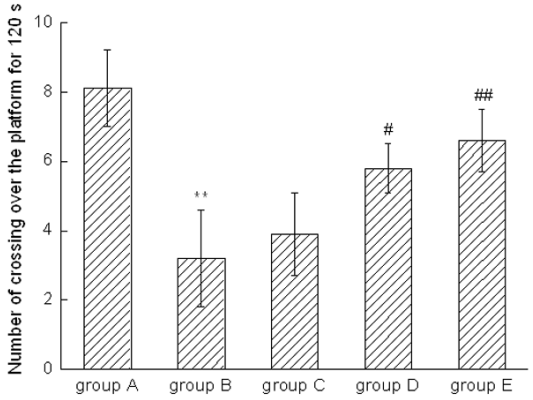

Figure 1. Effects of ARA on the number of crossing over a position where the former platform had been for 120 s in Morris water maze test. Data are expressed as mean ± S.D. for N = 10. $^{**}P < 0.01$ *vs.* sham control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* isoflurane group.

oxidase (GSH-Px) and malondialdehyde (MDA) activities as well as acetylcholine (Ach) content, acetylcholine esterase (AchE) and choline acetyl-transferase (ChAT) levels were determined with the corresponding ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions, respectively.

High-performance liquid chromatography (HPLC) analysis

The levels of the amino acid neurotransmitters glutamate (Glu) and γ-aminobutyric acid (GABA) in hippocampus tissue were determined by HPLC with fluorescent detection. Analyses were performed on Agilent 1200 series HPLC system (Palo Alto, CA, USA) and Agilent Zorbax Extend-C_{18} analytical column (250 mm × 4.6 mm i.d., 5 μm, Agilent Corp, USA). The excitation and emission wavelengths were set to 355 and 450 nm, respectively. The column oven temperature was maintained at 30°C. The mobile phase consisted of 0.1 M Na_2HPO_4 in H_2O (A) and methanol: acetonitrile = 3:1 (B). Compounds were eluted isocratically (A:B = 64:36) over a 35 min runtime at a flow rate of 1 mL/min after a 10 μL injection. The contents of Glu and GABA were quantified by comparison with the standard curves for each amino acid. The formula for calculating Glu and GABA content in the hippocampus is as follows:

Glu or GABA (μg/g) = [concentration of sample (μg/μl) × volume of sample (μl)]/weight of hippocampus (g).

Western blot analysis

The levels of Bcl-2 and Bax were detected by western blot analysis. The hippocampus tissue samples were homogenized in lysis buffer containing complete protease inhibitor cocktail (1 M Tris-HCl (pH 8.0), 5 M NaCl, 10% Nonidet P-40 and 1 M 1,4-dithio-dl-threitol (DTT)). After quantitated with the bicinchoninic acid (BCA) protein assay kit (Beyotime Biotech, Shanghai, China), the total proteins were electrophoresed in 12% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes. After the blots were blocked with 5% fat-free dried milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with corresponding primary antibodies. Subsequently, the membranes were incubated with the corresponding secondary antibodies at room temperature for 2 h. The blots were visualized with enhanced chemiluminescence (ECL) detection system (Amersham), and the results were analyzed by LabImage version 2.7.1 (Kapelan GmbH, Halle, Germany). Bax, Bcl-2 and *β*-actin anti-bodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Measurement of caspase-3 activity

The caspase-3 activity of the hippocampus tissue samples were detected using Caspase-Glo

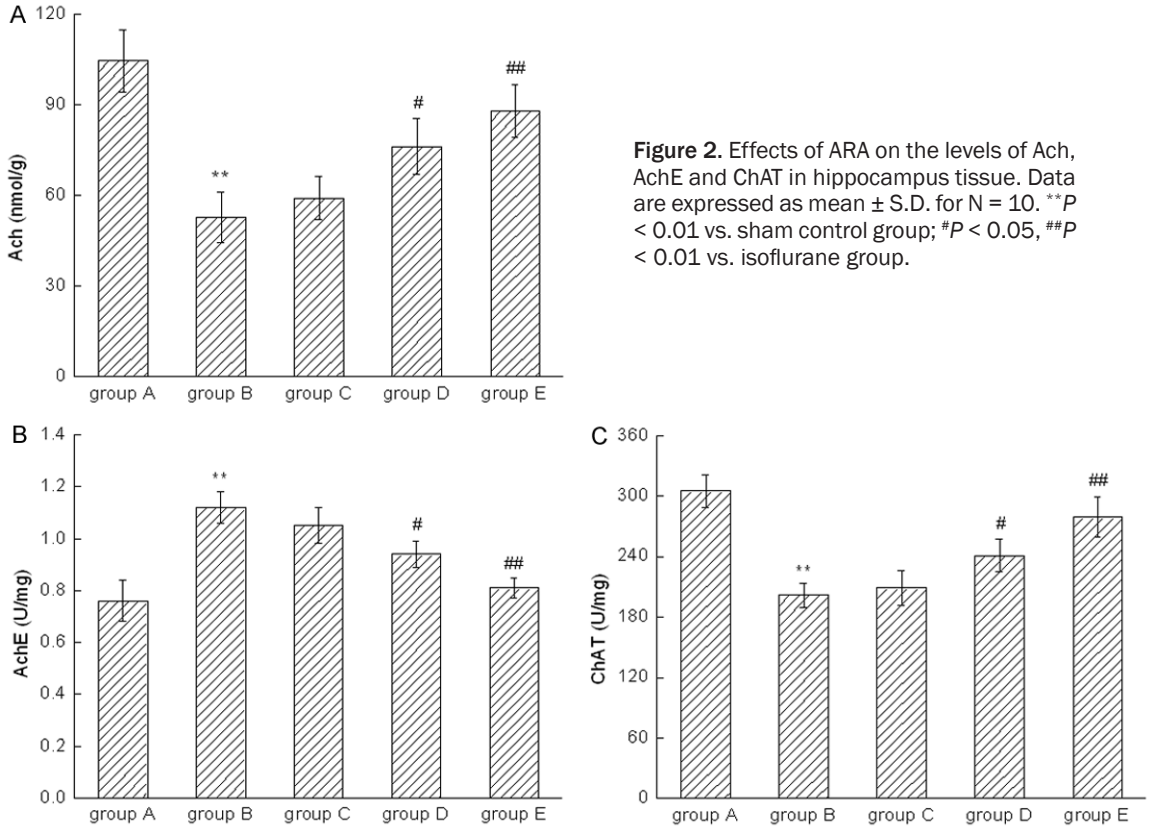

Figure 2. Effects of ARA on the levels of Ach, AchE and ChAT in hippocampus tissue. Data are expressed as mean ± S.D. for N = 10. $^{**}P < 0.01$ vs. sham control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. isoflurane group.

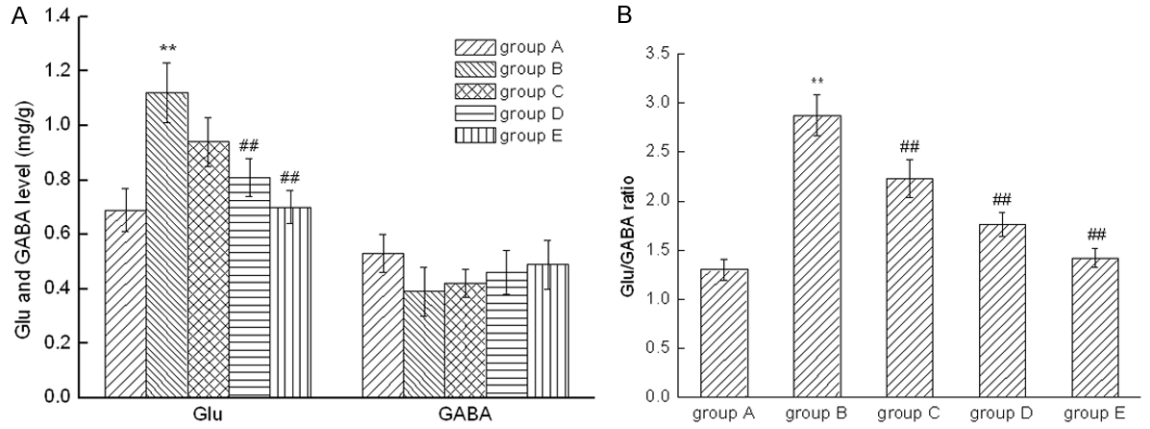

Figure 3. Effects of ARA on the levels of Glu and GABA and the ratio of Glu/GABA in hippocampus tissue. Data are expressed as mean ± S.D. for N = 10. $^{**}P < 0.01$ *vs.* sham control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* isoflurane group.

3/7 assay kit (Promega, Madison, WI, USA) in the present study according to the kit instruction.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software for windows. All data were presented as mean ± S.D., One-way analysis of variance (ANOVA) followed by Dunnett's test. The level of statistical significance was set at $P < 0.05$.

Results

Effects of different treatment on Morris water maze test

Isoflurane administration (group B) led to learning and memory impairment in rat in Morris

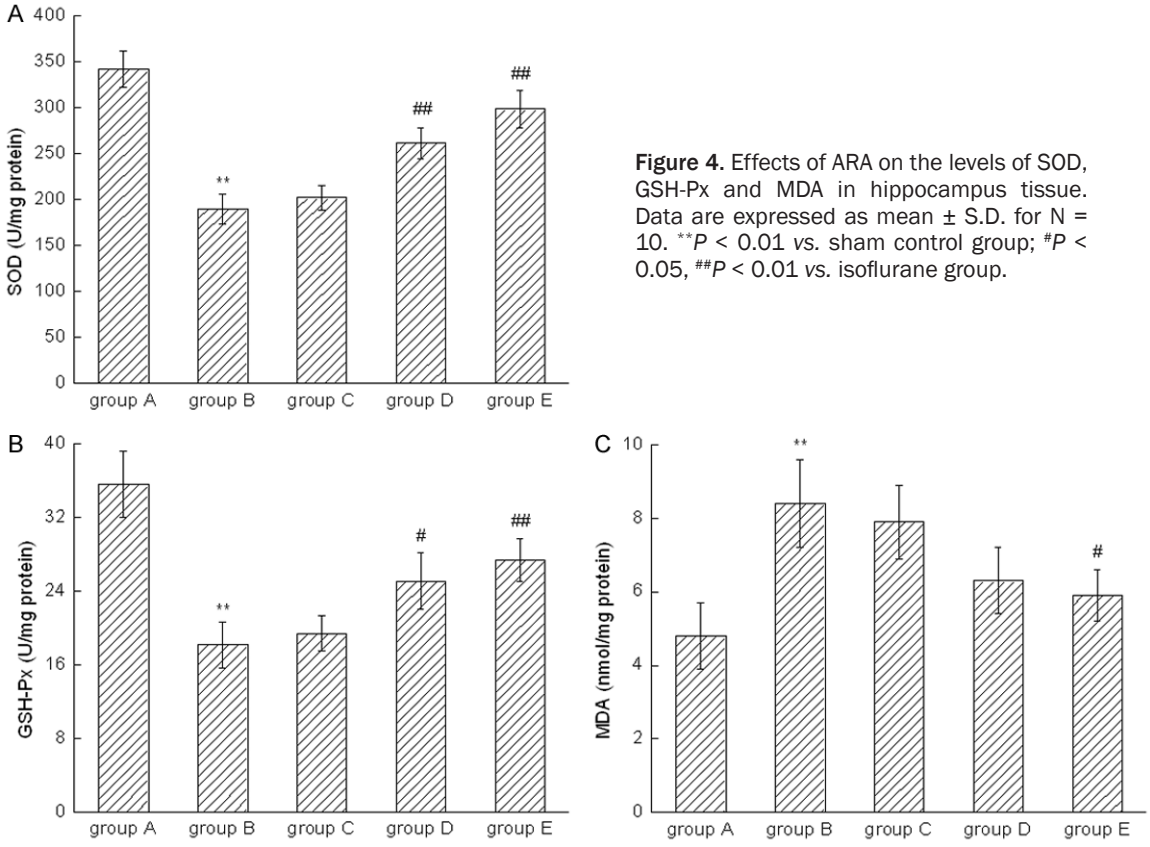

Figure 4. Effects of ARA on the levels of SOD, GSH-Px and MDA in hippocampus tissue. Data are expressed as mean ± S.D. for N = 10. $^{**}P < 0.01$ *vs.* sham control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* isoflurane group.

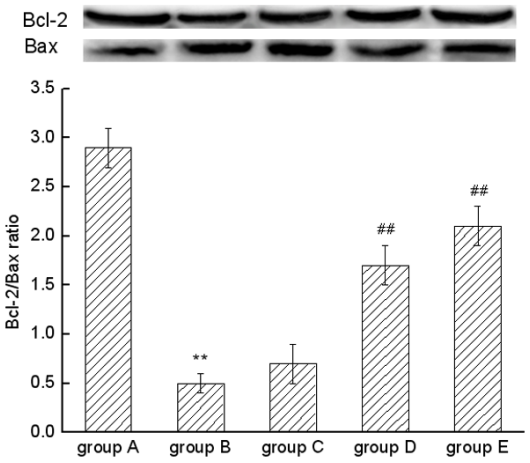

Figure 5. Effects of ARA on the ratio of Bcl-2/Bax in hippocampus tissue. Data are expressed as mean ± S.D. for N = 10. $^{**}P < 0.01$ *vs.* sham control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* isoflurane group.

water maze test, as demonstrated by a significant longer escape latencies (**Table 1**, $P < 0.01$) and a significant decrease in the times of crossing the former platform (**Figure 1**, $P < 0.01$) compared to group A. Intervention with ARA could induce significant improvement in the prolonged escape latencies and lowered the times of crossing the former platform (**Table 1** and **Figure 1**). Meanwhile, the effect of ARA was dose dependent. These results indicated that isoflurane led to learning and memory dysfunction, while administration of ARA ameliorated these learning and memory impairments.

Effect of different treatment on the levels of Ach, AchE and ChAT

Figure 2A-C presented the effects of different treatment on Ach, AchE and ChAT levels in hippocampus homogenate. As shown in **Figure 2A**, the content of the Ach in groups B significantly declined compared with group A ($P < 0.01$), while administration ARA (1.0 and 3.0 g/kg) reversed the decreased tendency ($P < 0.05$, $P < 0.01$). As demonstrated in **Figure 2B** and **2C**, isoflurane anesthesia significantly elevated AchE level ($P < 0.01$) and decreased ChAT level ($P < 0.01$) compared with group A. However, treatment with ARA (1.0 and 3.0 g/kg) could significantly upregulate the level of AchE ($P <$

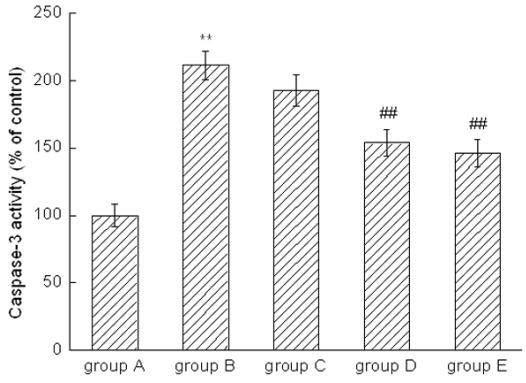

Figure 6. Effects of ARA on the activity of caspase-3 in hippocampus tissue. Data are expressed as mean ± S.D. for N = 10. $^{**}P < 0.01$ *vs.* sham control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* isoflurane group.

0.05, $P < 0.01$) and downregulate the level of ChAT ($P < 0.05$, $P < 0.01$). In addition, ARA at the dose of 0.3 g/kg had no significant protective effect ($P > 0.05$).

Effect of different treatment on levels of Glu and GABA

Figure 3A, **3B** demonstrated the results obtained for Glu and GABA levels in hippocampus tissue. The level of GABA significantly decreased in group B ($P < 0.01$), while Glu content ($P < 0.01$) and Glu/GABA ratio ($P < 0.01$) increased compared with group A. After intervention with ARA, the levels of Glu and the ratio of Glu/GABA in both groups D ($P < 0.05$) and group E declined ($P < 0.01$), while there was no significant difference in GABA level ($P > 0.05$) in groups C, D and E compared with group B.

Effect of different treatment on the levels of SOD, GSH-Px and MDA

Figure 4A-C demonstrated the effects of different treatment on SOD, GSH-Px and MDA levels in hippocampus homogenate. The activities of SOD and GSH-Px in group B were decreased significantly (189.9 ± 16.5 U/mg protein, P < 0.01; 18.2 ± 2.5 U/mg protein, $P < 0.01$), while the MDA level was increased significantly (8.4 ± 1.2 nmol/mg protein, $P < 0.01$) compared with group A (SOD: 341.7 ± 20.1 U/mg protein; GSH-Px: 35.6 ± 3.6 U/mg protein; MDA: 4.8 ± 0.9 nmol/mg protein; P < 0.01). Treatment with ARA at dosage of 1.0 and 3.0 g/kg prior to isoflurne anesthesia ameliorated the oxidant damage by increasing the homogenate level of SOD (to 261.3 ± 17.2 and 298.2 ± 19.8 U/mg protein; $P < 0.01$), and that of GSH-Px (to 25.1 ± 3.1 U/mg protein and 27.4 ± 2.3 U/mg protein; $P < 0.05$, $P < 0.01$), but decreasing the homogenate MDA level (to 6.3 ± 0.9 and 5.9 ± 0.7 nmol/mg protein; $P > 0.05$, $P < 0.05$).

Effect of different treatment on Bcl-2/Bax ratio and caspase-3 activation

The expression of Bax and Bcl-2 proteins in the hippocampus tissue is demonstrated in **Figure 5**. The increase of apoptosis in group B went along with a significant upregulated in Bax expression ($P < 0.01$), downregulated in Bcl-2 expression (P < 0.01) and the Bcl-2/Bax ratio ($P < 0.01$) compared with group A. However, in the treatment groups, ARA (at dosage of 1.0 and 3.0 g/kg) reduced Bax expression, elevated the Bcl-2 expression and Bcl-2/Bax ratio ($P < 0.01$) compared with the group B. As displayed in **Figure 6**, the activity of caspase-3, considered as an indicator of cell apoptosis, was significantly higher in group B (211.2 ± 10.4%, $P < 0.01$) than these in group A. Administration of ARA (1.0 and 3.0 g/kg) induced significant decrease caspase-3 expressions in groups D (153.8 ± 8.1%, $P < 0.01$) and E (146.2 ± 10.2%, $P < 0.01$) compared with group B.

Discussion

Isoflurane, a commonly used inhalation anesthetic, can induce postoperative cognitive dysfunction and have negative influence on the rehabilitation and the quality of life in patients. This study aims to investigate the protective effect of ARA on learning and memory impairment induced by isoflurane and the underlying mechanisms involved. The results showed that treatment with ARA can significantly ameliorate isoflurane-induced learning and memory deficit in Morris water maze test.

Although the mechanism of isoflurane on central nervous system is largely unknown, one possible pathway may be correlated with the central cholinergic system, such as cholinergic receptor insensitivity, affinity of Ach and receptor descending, Ach release of peripheral presynaptic membrane decreasing [3]. It is reported that cholinergic changes in the hippocampus are in relation to spatial learning and memory impairment [3, 12, 13]. Ach, a major neu-

rotransmitter in brain, plays crucial role in learning and memory processes. AchE can promptly terminate the action of Ach released into the synapse, so it is essential to normal function of nervous system [14]. ChAT, a cholinergic marker, is also involved in the synthesis of Ach. The cognitive dysfunction is correlated with decline in ChAT level and loss of cholinergic neurons. Therefore, AchE and ChAT are key enzymes regulating the availability of Ach. Isoflurane-induced amnesic animals have been used as a model for assessment of anti-amnesic effect of new drugs, following administration of isoflurane an elevation in AChE activity and a decrease in ChAT level and Ach content in hippocampus tissue have been observed [3]. In our study, injection of isoflurane can significantly upregulate the AchE level and downregulate ChAT level and Ach content in hippocampus, which agree with previous reports. However, treatment with ARA can significantly alleviate these changes. Therefore, the role of ARA on the Ach, ChAT and AchE levels might be involved in the improvement of the learning and memory impairment treated with isoflurane.

The cholinergic system was noted to regulate learning and memory, but now the central Glu/GABA regulatory system is becoming more prominent [15]. Glu and GABA are two of the most abundant neurotransmitters in the central nervous system, play important role in the maintenance of normal brain functions, such as learning and memory [16]. Under pathological and physiological conditions, Glu and GABA have opposing regulatory effects. Glu is a key mediator of excitatory neurotransmission but GABA is inhibitory one in central nervous system [15]. A certain balance of neuronal transmission between Glu and GABA is required to maintain normal learning and memory function. The increase of Glu or decrease of GABA (i.e., elevation of the ratio of Glu/GABA) can lead to impairment of learning and memory [17]. In present study, isoflurane exposure led to upregulate Glu level and Glu/GABA ratio, downregulate GABA level. After the intervention of ARA, the level of Glu and the ratio of Glu/GABA were declined, while the level of GABA was elevated in hippocampus. These results suggest that ARA can inhibit the excess release of Glu, reduce the Glu/GABA ratio, suppress the excitotoxicity of Glu by reversing the imbalance of the ratio of Glu/GABA, increase GABA level, and thus, improve the learning and memory dysfunction.

Considerable evidences have shown that overproduction of reactive oxygen species (ROS) and/or its metabolites are potentially neurotoxic [18]. Oxidative stress plays a crucial role in the development and progression of AD [19]. Previous study suggested that ROS could induce leaning and memory impairment when activity of anti-ROS system is decreased [20, 21]. SOD and GSH-Px, respectively served as the first and second defense against the apoptosis caused by oxygen-derived free radicals, will be released in cells to attenuate the damage [22]. MDA is the production of the oxygen-derived free radicals and lipid peroxide caused by free radicals which could directly reflect the cell injury degree [23]. In order to determine the relationship between learning and memory function and oxidative stress, and the possible effect of ARA on this relationship, the present study determined marks of oxidative stress (MDA) and antioxidant defense (SOD and GSH-Px) in hippocampus. The results demonstrated that isoflurane exposure declined activities of SOD and GSH-Px, but enhanced MDA activity, which were correlated with learning and memory impairment. However, ARA could significantly ameliorate these abnormalities. This finding agrees with Wang et al [24] who found that ARA could defense against oxidative stress by boosting the internal antioxidant system of hippocampal slices. Above all, ARA might affect the level of endogenous antioxidants or/and oxidative stress, and this might be rational to understand the beneficial effects of it on neuroprotective against isoflurane via its antioxidant capacity.

Recent studies have shown that isoflurane can induce apoptosis and caspase activation, which might be consider as the major cause of the loss of cholinergic neurons in hippocampus [25-29]. The Bcl-2 family members are active mediators of apoptosis that either inhibit (e.g., Bcl-2) or facilitate (e.g., Bax) apoptotic cell death [30]. The balance of Bcl-2 and Bax plays an important role in determining the fate of the cells to either undergo proliferation or apoptosis [31]. Any agent that decreases the Bcl-2/Bax ratio may promote apoptosis. In this study, isoflurane-induced cytotoxicity upregulated the Bax protein expression and downregulated the

Bcl-2 protein expression in hippocampus, and this finally led to Bcl-2/Bax ratio significantly decrease. However, ARA supplementation could significantly reverse the decreased Bcl-2/Bax ratio. These results indicated that ARA might ameliorate apoptosis by regulating of the Bcl-2 family protein expression. Furthermore, the activiation of caspase-3 is a vitally important step in the execution phase of apoptosis and the inhibition hinders apoptosis [32]. Moreover, caspase-3 activity can be induced by Bax and inhibited by Bcl-2, and Bax can neutralize Bcl-2 actions by forming heterodimers with Bcl-2 [33]. In this study, the caspase-3 activity was detected to further support our findings. The results demonstrated that exposure to isoflurane significantly elevated the caspase-3 level. Since the Bcl-2/Bax ratio was low, a compensatory induction of Bcl-2 was not sufficient to overcome the proapoptotic actions of Bax on caspase-3 activation. However, treatment with ARA could attenuate these apoptosis induced by isoflurane. Therefore, the protective effects of ARA might be due to the inhibition of Bcl-2 family protein translocation and capase-3 activity.

Conclusion

In summary, isoflurane exposure markedly impairs spatial learning and memory function in rats. Intervention with ARA could ameliorate isoflurane induced learning and memory dysfunction. The underlying mechanisms appear to be attributed to regulating the cholinergic neurotransmission and Glu/GABA regulatory system, inhibiting oxidative stress, reducing Bcl-2 family protein translocation and preventing caspase-3 activation. Therefore, ARA demonstrates great potential as a candidate for the treatment of learning and memory dysfunction.

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Disclosure of conflict of interest

None.

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