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Effects of Vitamin B_{12} on postoperative cognitive dysfunction induced by isoflurane anesthesia in rats

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Abstract. – OBJECTIVE: Postoperative cognitive dysfunction is a clinical syndrome associated with cognitive decline in patients after anesthesia. This study aimed to investigate the effect of VB_{12} (Vitamin B_{12}), a kind of necessary micronutrients promoting the growth and development of the nervous system, on cognitive dysfunction induced by isoflurane anesthesia.

MATERIALS AND METHODS: Eighteen-month-old rats were exposed to or were not exposed to 1.4% isoflurane for 2 h. Two hours before isoflurane exposure, rats in groups with VB_{12} were injected intramuscularly with VB_{12} at 10 or 20 μg. Two weeks later, rats were subjected to Barnes maze and Morris water maze.

RESULTS: Rats exposed to isoflurane had significant impairments in long-term spatial memory assessed by Barnes maze. There was no statistical significance in the percentage of swimming time and path length in the Morris water maze tests among five groups, suggesting that isoflurane may not impair the recall of learned information in rats. Isoflurane increased the expression of interleukin 1β (IL-1β) and activated caspase 3 in the hippocampus, but not cortex of the rats. The increase of IL-1β and activated caspase 3 was attenuated by VB_{12}. However, isoflurane did not change the amount of tumor necrosis factor-α (TNF-α) and β-amyloid peptide in the hippocampus and cerebral cortex.

CONCLUSIONS: VB_{12} can attenuate cognitive dysfunction induced by isoflurane anesthesia. At the same time, IL-1β may play an important role in this isoflurane effect.

Key Words:
Cognitive function, Isoflurane anesthesia, Cell injury, VB12, Cytokines.

Introduction

Postoperative cognitive dysfunction[1] (POCD) is a kind of clinical syndrome mostly in elderly patients, which happens after the operation with characteristics of disturbance of consciousness and memory loss. It can last several weeks, several years or a lifetime, which influences the patients' final recovery and increase their mortality rate[2]. Volatile anesthetics have been shown to affect the learning and memory functions of rodents many days after the exposure[3,4], indicating that volatile anesthetics may play a role in the development of postoperative cognitive decline (POCD), a recognized clinical entity that has drawn significant attention from the scientific community and the public[5]. Isoflurane, a common inhalation anesthetic, can cause the cognitive dysfunction of rodents[6,7], which are widely used in clinical surgery. Several clinical kinds of research have reported that isoflurane anesthesia can increase the incidence of POCD. So, it has significance to find out the intervention to POCD caused by anesthetics. Molecular and zoology research found that isoflurane affecting the cognitive function depends on different doses: it protects the cells at low concentration and causes cognitive dysfunction at high concentration. Vitamin B_{12} (VB_{12}) has a complex structure composed of porphyrin compounds containing cobalt. It's a kind of necessary micronutrients promoting the growth and development of the nervous system, maintaining the normal function of nerve cells[8]. In the study of Monk et al[9], the rats with

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shortage of Vitamin B (VB) showed a lower spatial resolution, along with the behavior changed from mild cognitive impairment to badly damage in learning and memory ability. Healton et al[10] summarized that lower levels of VB_{12} can lead to chronic brain dysfunction, memory change, depression, acute psychosis, etc. Ito et al[11] explored that it's effective to supply VB_{12} in the early stage of cognitive impairment. In animal models of cognitive dysfunction, taking VB_{12} in advance can improve the cognitive function of animals. Up to now, there is no literature reporting whether VB_{12} can intervene cognitive dysfunction caused by isoflurane anesthesia. Thus, we designed this study to determine whether VB_{12}, a kind of B vitamins composed of porphyrin compounds containing cobalt, play an important role in cognitive dysfunction induced by isoflurane anesthesia. We exposed 18-month old rats to isoflurane. Then, we evaluated their cognitive functions, the levels of β-amyloid peptide and caspase 3 and cell death in the brain, as well as the levels of the pro-inflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α).

Materials and Methods

Animal groups

Eighteen-month-old rats were divided into five groups: control, isoflurane only, VB_{12} only, isoflurane + VB_{12} (10 μg), isoflurane + VB_{12} (20 μg). Rats in groups with isoflurane were exposed to 1.4% isoflurane in oxygen for 2 h. Rats in the control and VB_{12} only groups were kept in a chamber gassed with 100% O_2 for 2 h.

VB_{12} Application to Rats

2 h before isoflurane exposure, rats were injected intramuscularly with VB_{12} at 10 or 20 μg according to preliminary experiment. Rats were given intramuscular injection twice a week. The rats in the isoflurane only group and control group were given the same volume of saline.

Isoflurane Exposure

The rats were exposed to isoflurane by being placed in a chamber, and the chamber was gassed with 1.4% isoflurane in oxygen for 2 h, whose part was submerged in a water-bath at 37°C. The inhaled and exhaled gas concentrations were monitored continuously with a Datex™ infrared analyzer (Capnomac, Helsinki, Finland). After a 2 h isoflurane exposure, isoflurane application was stopped. Since isoflurane was carried by 100% O_2, rats in the control and VB_{12} only group were kept in a chamber that was gassed with 100% O_2 for 2 h.

Physiological Index Monitoring

During anesthesia the lip color of rats was observed every 15 min and respiratory rate was recorded. Three rats of each group were punctured and drawn blood for blood gas analysis immediately after anesthesia.

Barnes Maze

Two weeks after isoflurane exposure, rats were subjected to Barnes maze. Barnes maze is designed to test spatial learning and memory, which is a circular platform with 20 equally spaced holes. There was one hole connected to a dark chamber (also called target box) under the platform. At the beginning of each experiment time, rats were all placed in the middle of the platform, and then were encouraged to find the target box by using aversive noise (85 dB) and bright light (200 W) shed on the platform. The spatial acquisition phase for the rats consisted of training on 4 days with 2 trials per day, 3 min per trial and interval 15 min between each trial. One trial was performed on each of these two days. The rats' reference memories were tested on the 5th day (short-term retention) and 12th day (long-term retention), but rats were subjected to no tests during the period from day 5 to day 12. The latency and count of errors of finding the target box were recorded with the help of ANY-Maze video tracking system (SD Instruments, San Diego, CA, USA) during each trial.

Morris Water Maze

The rat was placed on the platform for 20 s to allow orientation to extra-maze cues. After orientation, rats were gently put into the pool facing the wall. The rat in each different quadrant not housing the platform was at the center of the wall. The maximum time of swim was 60 s. If the rat could not swim to the platform in 60 s, it was gently guided to the platform and allowed to re-orient to the distal visual cues for an extra 20 s before being removed from the pool. Removed from the pool, rats were dried with a towel and placed in a warming cage for at least 5 min to ensure complete dryness before returning to the home cage. Rats were tested in two trials per day. The interval time of two trials was about 30 min. A probe test was conducted 24 h after the last training session to examine spatial reference memory, during which the platform was removed. 30 min after the probe test, a visual cue test

was administered to evaluate sensorimotor ability and motivation[12]. To make sure the rats could reach the platform relying on a local visual stimulus rather than spatial orientation to extra-maze cues, we set the platform 1 cm above the water level and marked with black tape. The tracks from all tests were analyzed for a series of behavioral parameters using SMART software.

Brain Tissue Harvest

6 h after isoflurane exposure, rats were deeply anesthetized with isoflurane, and were followed to be perfused transcardially with saline. Immediately, the right hippocampi and cortex were cut-off to be analyzed for the subsequent Western blotting of caspase 3 and amyloid β, while the left hippocampi and cortex were taken out for enzyme-linked immunosorbent assay (ELISA) to analyze the levels of IL-1β and TNF-α.

Western Blotting

The above obtained hippocampal tissues were homogenate in RIPA buffer (catalog number: 89900; Thermo Scientific, Worcester, MA, USA) containing protease inhibitor cocktail (catalog number: P2714; Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor mixture (catalog number: P2714; Sigma-Aldrich, St. Louis, MO, USA). Then, the homogenates were centrifuged at 13,000 g for 30 min at 4°C. After centrifugation, the supernatant was preserved to determine the protein concentration by Bradford assay. 50 μg proteins per lane were electrophoresed on a polyacrylamide gel, then blotted onto a polyvinylidene difluoride membrane, finally blocked with ProteinFree T20 Blocking Buffer (catalog number: 37573; Thermo Scientific, Lot LB141635). Next, the following primary antibodies-rabbit monoclonal anti-cleaved caspase-3 antibody (1:1000; catalog number: 9664; Cell Signaling Technology, Inc., Danvers, MA, USA) and rat monoclonal anti-β-actin polyclonal antibody (1:2000; catalog number: A2228; Sigma-Aldrich, St. Louis, MO, USA) were used to incubate these membranes, and appropriate secondary antibodies were used. Protein bands were visualized by using a Genomic and Proteomic Gel Documentation (Gel Doc) Systems from Syngene (Frederick, MD, USA). β-actin from the same samples was chosen as reference protein and normalized the protein band intensities of cleaved-caspase 3, amyloid β and IL-1β to reduce loading errors. The results from animals then were normalized by mean values of the corresponding control animals under various experimental conditions.

Statistical Analysis

In this study, all results were analyzed by Rank Sum test or by one-way repeated measures analysis of variance, followed by the Tukey test (for the data generated during the training sessions of Barnes maze). All statistical analyses were performed with the SigmaStat (Systat Software, Inc., Point Richmond, CA, USA). All data were finally presented as means ± standard deviation (SD) ($n \geq 4$). A $p<0.05$ was considered to be significant.

Results

Isoflurane Impaired the Cognitive Functions of Rats and that VB_{12} Attenuated this Effect

The rats had no episode of hypoxia (defined as $SpO_2<90\%$) during the isoflurane exposure. During isoflurane exposure, the blood pressures and heart rates of rats were similar to those in control group. The time for 18-month old rats in control and other groups to find the target hole was decreased with increased training in the Barnes maze (Figure 1A). Isoflurane exposed rats took more time to identify the target hole than that in control group at one day after the training sessions. The use of VB_{12} before isoflurane anesthesia reduced the latency for rats to identify the target hole than those exposed in isoflurane only. Rats injected with VB_{12} at 20 μg took less time to identify the target hole than those injected with VB_{12} at 10 μg (Figure 1B).

In the Morris water maze testing, the rats were demanded to find the target platform to escape from swimming in the pool with water. To achieve this goal, the rats used visual stimuli from extra-maze cues in the testing room to form a "spatial orientation map" in brains. During the test, learning abilities was evaluated by the time the rats elapsed before they arrived at the platform to escape from swimming in the pool (escape latency) and by the percentage of time or path length spent in the quadrant housing the platform (target quadrant). In the test, escape latency decreased during 7 d training period in all groups. The escape latency of isoflurane-exposed rats was obvious longer than that of control group, respectively on the 2^{nd}, 3^{rd}, 4^{th} day. However, isoflurane exposed rats which injected with VB_{12} at 20 μg in advance took less time to find the platform than rats exposed in isoflurane without VB_{12} (Figure 2A). The percentage of swimming time and path length rats spent in the tests were significantly increased

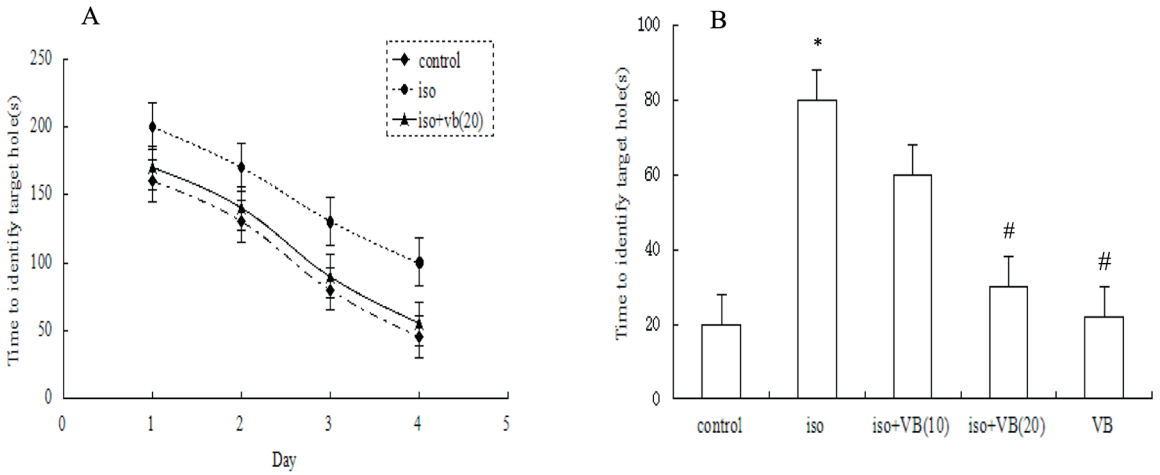

Figure 1. Isoflurane induced cognitive impairment measured by Barnes Maze. Eighteen-month-old rats were exposed to or were not exposed to 1.4% isoflurane for 2 h. They were subjected to Barnes maze 2 weeks later. ***(A)*** Performance in the training sessions of Barnes maze test. ***(B)*** Latency to identify the target hole at 1 day after the training sessions. Results are means ± SD (n=4). **p*<0.05 compared to control, #*p*<0.05 compared to isoflurane alone.

in the target quadrant during the training course in all groups (Figure 2B). During the training courses on the same day, there was no statistical significance in the percentage of swimming time and path length rats spent in the tests among the control, isoflurane-exposed and isoflurane plus VB_{12} exposed rats. These results suggest that isoflurane may not impair the recall of learned information in rats. The above results suggest that isoflurane impairs the cognitive functions of rats and that VB_{12} can attenuate this effect.

Isoflurane Increased the Expression of IL-1β and Activated Caspase 3 in the Hippocampus of Rats, and that VB12 Weakened this Effect

6 h after isoflurane exposure, the level of IL-1β in the hippocampus of rats was significantly higher than that in control rats (p<0.05); anyway this increase was attenuated by VB_{12}. The inhibition effect of VB_{12} was more obvious in high dosage (Figure 3A). To compare the level of IL-1β and activated caspase 3 in control and VB_{12} only group, the difference

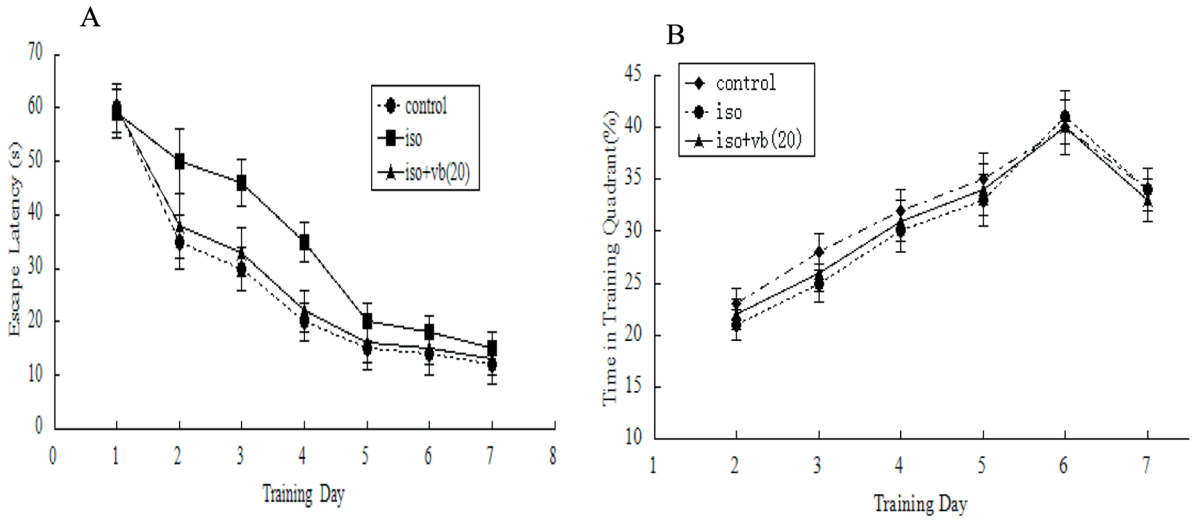

Figure 2. Isoflurane affected spatial learning ability mearsured by Morris water maze. Spatial learning was assessed as a function of training day with respect to the following parameters: ***(A)*** escape latency, ***(B)*** percentage of time spent in the target quadrant, and ***(C)*** percentage of total path length spent in the target quadrant. Data are means ± SD (n = 4).

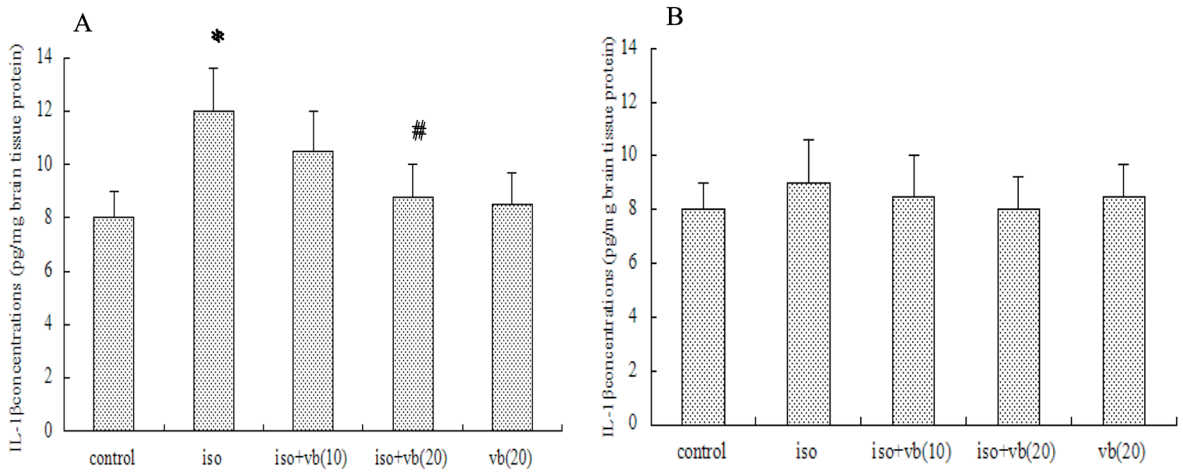

Figure 3. Isoflurane effects on interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) contents in rat brain tissues. Eighteen-month-old rats were exposed to or were not exposed to 1.4% isoflurane for 2 h. Hippocampus and cerebral cortex was harvested at 6 h after anesthetic exposure for ELISA of IL-1β or TNF-α content. Results are means ± SD (n=4). *p<0.05 compared to control, #p<0.05 compared to isoflurane alone.

didn't reach statistical significance. However, there was no statistical significance in IL-1β of cerebral cortex (p>0.05) (Figure 3B). At the same time, there was no significant difference in tumor necrosis factor α (TNF-α) content in the hippocampus and cerebral cortex among five groups of rats (p>0.05).

Similar to the change patterns with IL-1β, the level of activated caspase 3 expression in the hippocampus of rats exposed to isoflurane was obviously higher than that in control rats (p<0.05) and this increase was attenuated by VB_{12} (Figure 4A and 4B). The difference between the levels of activated caspase 3 expression in isoflurane only and isoflurane plus VB_{12} groups reached statistical significance (p<0.05). However, there was no statistical difference between the five groups in the content of amyloid β, suggesting that amyloid β may not play an important role in the cognitive dysfunction.

Discussion

Learning and memory function are important aspects of cognitive function. In our studies, the Barnes Maze and Morris Water Maze tests were used to testing the cognitive function of rats. They are behavior tests which commonly used in cognitive dysfunction induced by anesthesia[13,14]. This task has the advantage of being acquired quickly without pre-training or restriction of food and water[15,16]. Also, learning, memory and factors that influence these behaviors, such as visual acuity, motor function and motivation, can be dissociated by manipulating the testing protocol[17]. The results showed that exposure of rats to isoflurane for 2 h didn't affect the performance of rats in the training sessions of Braze maze, suggesting that the learning abilities of spatial reference tasks weren't affected by isoflurane. In the Morris Water Maze test, the escape latency of isoflurane exposed rats was obvious longer than that of control group respectively on the 2nd, 3rd, 4th day[18,19]. However, isoflurane exposed rats which injected with VB_{12} at 20 μg in advance took less time to find the platform than rats exposed in isoflurane without VB_{12}. The percentage of swimming time and path length rats spent in the tests were significantly increased in the target quadrant during the training course in all groups. During the training courses on the same day, there was no statistical significance in the percentage of swimming time and path length rats spent in the tests among the control, isoflurane-exposed and isoflurane plus VB_{12} exposed rats. These findings suggest that isoflurane may not impair the recall of learned information in rats. Our results suggest that isoflurane used in a clinically relevant manner impairs the cognitive functions of rats. This conclusion is consistent with the researches carried on by others[20-22]. However, this cognitive dysfunction caused by isoflurane was blocked by VB_{12}. The inhibition

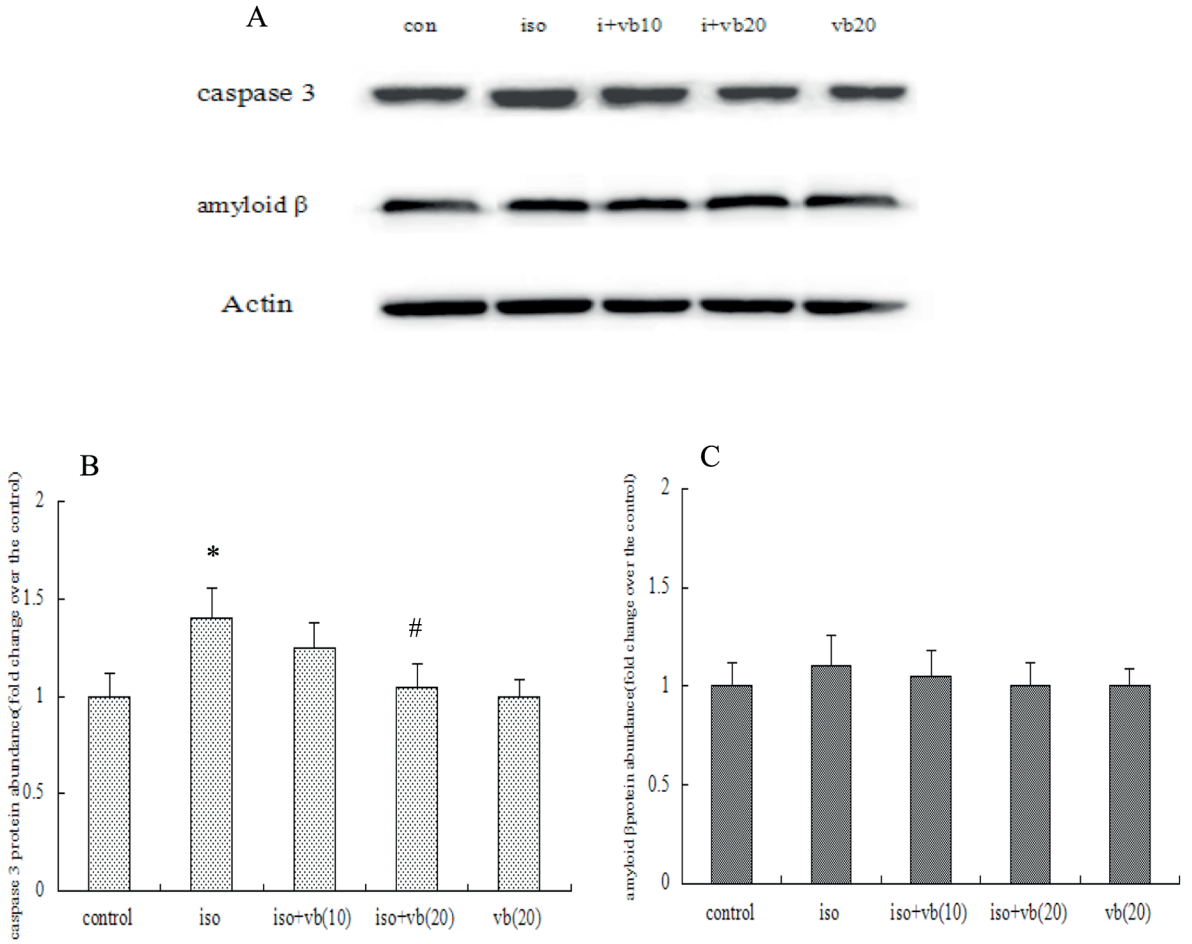

Figure 4. Isoflurane effects on the expression of caspase 3 and amyloid β in rats brain tissues. Eighteen-month-old rats were exposed to or were not exposed to 1.4% isoflurane for 2 h. Hippocampus and cerebral cortex were harvested at 6 h after isoflurane exposure for Western blotting. ***(A)*** Representative Western blot images. ***(B-C)*** Graphic presentation of the caspase 3 and amyloid β protein abundance quantified by integrating the volume of autoradiograms from 4 rats for each experimental condition. Results are means ± SD (n=4). *p<0.05 compared to control, #p<0.05 compared to isoflurane alone. Con: control, iso: isoflurane, iso + vb10: isoflurane plus VB_{12} at 10 μg, iso + vb20: isoflurane plus VB_{12} at 20 μg.

effect of VB_{12} was more obvious in high dosage. VB_{12} has a complex structure composed of porphyrin compounds containing cobalt. It's a kind of necessary micronutrients promoting the growth and development of the nervous system, maintaining the normal function of nerve cells[8]. Different dosage (10 μg and 20 μg) of VB_{12} was studied on the effect of cognitive dysfunction caused by isoflurane anesthesia. The results showed that VB_{12} attenuated isoflurane-induced cognitive impairment. The inhibition of VB_{12} was more obvious with 20 μg than 10 μg. Up to now, the mechanisms for isoflurane-induced cognitive impairment are poorly understood. Researchers have proved that neuroinflammation such as IL-1β[23] and TNF-α, β can induce the cognitive dysfunction[24]. We showed that isoflurane increased the level of IL-1β in hippocampi and that VB_{12} reduce this increase, suggesting that IL-1β plays an important role in cognitive impairment. However, we observed no change of TNF-α in hippocampi and cortex. At the same time, we didn't observe changes of IL-1β in cortex, which similar to previous studies.

Increased amyloid β-production and caspase 3 activation in the brain have been observed in acute phase after volatile anesthetic exposure[25]. However, it is not known whether these brain changes result in the cognitive dysfunction caused by isoflurane exposure. In our work, a significant increase of caspase 3 was observed in the hippocampi of rats after isoflurane exposure and that VB_{12} weakened this increase. What's more, the inhibition effect of VB_{12} was more obvious in

high dosage. The results suggested that caspase 3 may play an important role in cognitive dysfunction. However, the content of amyloid β in the hippocampi of rats didn't reach the statistical significance in all groups, suggesting that amyloid β may not play an important role in cognitive dysfunction.

Conclusions

We showed that isoflurane damaged the long-term spatial reference memory and hippocampus-dependent memory and learning ability in old rats. Increased level of IL-1β and caspase 3 activations were observed in the hippocampi of rats after isoflurane exposure, suggesting that isoflurane may activate the IL-1β-caspase 3-neuronal injury/death pathway in the hippocampus. VB_{12} can attenuate isoflurane-induced cognitive impairment and the inhibition was more obvious with high dosage at 20 μg. The mechanisms for isoflurane-induced cognitive impairment warrant further study.

Authors' Contribution

Mingxuan Sha conceived, designed and did statistical analysis & editing of manuscript. Hongyu Sha and Jianbo Zhao did data collection and manuscript writing. All the authors did review of manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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