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Pathologic changes and effect on the learning and memory ability in rats exposed to fluoride and aluminum

Mang Li, Jing Cui, Yanhui Gao, Wei Zhang, Liyan Sun, Xiaona Liu, Yang Liu, and Dianjun Sun

Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University, Harbin 150081, China

Key Lab of Etiology and Epidemiology, Education Bureau of Heilongjiang Province & Ministry of Health (23618504), Harbin 150081, China

China and Russia Medical Research Center, Harbin Medical University, Harbin 150081, China

Corresponding author : Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University; Key Lab of Etiology and Epidemiology, Education Bureau of Hei Long Jiang Province & Ministry of Health (23618504), 157 Baojian Road, Harbin 150081, China.

Tel.:+86 451 86612695;

Fax: +86 451 86657674.

E-mail address: hrbmusdj@163.com (D. Sun).

Abstract

Background:The aim of this study is to establish a single and combined intoxication model of fluoride and aluminum so as to observe the impact of these chemicals on the learning and memory ability and the pathologic changes in brain of rats.

Methods: Forty male Wistar rats were randomly assigned into control (distilled water), fluoride (50 mg/ L F^-), aluminum (100 mg / L Al^{3+}) and combined groups (50 mg / L F^- and 100 mg / L Al^{3+}), the experiment lasted for 3 months. The short-term memory ability and learning and memory ability of rats were then assessed using Y maze and Morris water maze, respectively. At the same time, the concentrations of fluoride and aluminum in urine and brain were measured. The pathologic and microstructural changes in the hippocampus were observed via light microscope and transmission electron microscopy, and the expression of the $A\beta_{1\text{-}42}$ protein was detected by use of immunohistochemistry.

Results: The results showed that the learning and memory ability of each toxicant-exposed group was decreased, the most severe was in the aluminum group, followed by combined group, and the lightest was in the fluoride group. Although there was no significant difference between all groups, both fluoride and aluminum could lower the short-term memory ability of rats. In addition, different pathologic and microstructural changes were seen in fluoride, aluminum and combined groups. Compared with the control group, the expression of $A\beta_{1\text{-}42}$ protein in aluminum group was highest, followed by combined group, and that in fluoride group was lowest.

Conclusions: In conclusion, combined intake of fluoride and aluminum may alleviate the deficits to learning and memory ability caused by aluminum intoxication.

Key words: learning and memory, fluoride, aluminum

1. Introduction

Drinking-tea fluorosis is a unique type of fluorosis that mainly distributed in western China. Individuals who live in these areas where drinking-tea fluorosis is prevalent often drink tea daily. Brick tea, which these residents have a long history of drinking, abounds in fluoride and aluminum[1].

Fluoride participates in the formation of osseous tissue and enamel. A low fluoride

level in the body can result in various abnormity, such as caries, decreased bone density and growth retardation. However, excessive intake of fluoride leads to chronic fluorosis, which is primarily characterized by phrenological damages, such as dental fluorosis and skeletal fluorosis[2,3], and non-phrenological damages to nervous, cardiovascular and endocrine systems[2, 4-7].Fluoride-induced neurologic damages included the morphological and functional changes of the brain[2,8].

Currently, aluminum is acknowledged as a chronic cumulative toxicant to the nervous system. Excess intake of aluminum causes cognitive impairment and short-term memory dysfunction[9], and its characteristic pathological changes are neurofibrillary tangles and senile plaques[10]. Senile plaques mainly composed of amyloid β(Aβ) proteins and deposited in neurons or synapses. It has neurotoxic effects, leads to neuronal degeneration and apoptosis, ultimately impairs recognition, learning and memory ability[11-12].

Previous animal studies indicated that both fluoride and aluminum exert adverse impacts on learning and memory ability [2,8-9], the targeted region of brain is the hippocampus[8,13-14]. Distinct reactions would occur when different proportions of fluoride and aluminum, which might be synergistic or independent effects. However, the damages to the nervous system when individuals drink brick tea (the proportion of fluoride to aluminum is approximately 1:2[15]), is remain unclear.

In this study, simulating the proportion of fluoride and aluminum in brick tea, a rat toxicant model was established using 50 mg/L F^- and 100 mg/L Al^{3+}, and impact of these chemicals on experimental animals was observed.

2. Materials and Methods

2.1. Reagents

NaF (pure analytical grade) and $AlCl_3$ (pure analytical grade) were purchased from Tianjin Institute of Fine Chemicals. Fluoride standard preserved water and aluminum standard preserved water were both purchased from the National Center of Analysis and Testing for Nonferrous Metals and Electronic Materials. The Aβ1-42 antibody was purchased from Abcam Company, the SABC secondary antibody kit was purchased from Wuhan Boster Company, DAB chromogenic kit was purchased from

Beijing Zhongshan Golden Bridge Biotechnology Company.

2.2. Animals and treatment

Forty healthy, 5-week old, male Wistar rats (Beijing Weitong Lihua Experimental Animal Technology Co., Ltd.) weighing 90-120 g were housed in an environment of constant temperature with 12-h light/dark cycle, and provided with free access to food and water. All animal experiments were approved by the local Institutional Animal Care and Use Committee.

All of the rats were allowed to adapt for 7 days after arrival to the animal room and were then randomly assigned into four groups: control (distilled water, n=10), fluoride (50 mg/ L F^-, n=10), aluminum (100 mg / L Al^{3+}, n=10) and combined (50 mg / L F^- and 100 mg / L Al^{3+}, n=10). The water containing 50 mg / L F^- and 100 mg / L Al^{3+} was prepared using NaF and $AlCl_3$. After 3 months, the pigment and abrasion of the rat's teeth were observed by the naked eye and photographed with a digital camera. The short-term memory ability and learning and memory ability of the rats were examined by use of Y maze and Morris water maze, respectively. With the completion of Morris water maze, 24 h-urine was collected. Then, the rats were sacrificed under chloral hydrate anesthesia. The brain and hippocampus were collected, part of these tissues was used to determine the levels of fluoride and aluminum, part was fixed in 10% formaldehyde solution for HE staining and immunohistochemistry, and part was fixed in 0.25% glutaraldehyde solution at 4℃ for transmission electron microscopy.

2.3. Y maze

Each rat, which was naive to the maze, was placed at the end of any arm and allowed to move freely through the maze during 8 min. The total and serial entries of one arm were measured using digital counters containing infrared sensors [16-18]. The effect was calculated as the rate of alternation using the following equation:

The rate of alternation = (number of alternations /total number of arm entries−2) × 100

2.4. Morris water maze

The Morris water maze consisted of a black round tank (120 cm in diameter and 75 cm in height) filled with water to a depth of 40 cm, and the temperature of water was

26 ±1°C. The maze was divided into four quadrants. The experiments lasted for four days in total. On day 1, each rat was subjected to three pre-training trials to learn how to climb onto the platform to escape the water. The training phase lasted for 2 days (days 2 and 3). Eight consecutive trials were administered on each day of training. For each trial, the starting location was varied randomly between the four quadrants. The platform location remained the same for each individual rat for the duration of the training, but different animals were trained using different platform positions to avoid quadrant bias. The latency to reach the platform was measured for each trial. The fourth day was the probe test, in which the platform was removed from the pool. The animals were placed in a quadrant opposite from the location of the training platform and allowed to swim for 60 seconds. The number of entries of the rat into the target zone and the latency to the first entrance into the target zone were measured using a video tracking system to evaluate the learning and memory ability of the rats [19-20].

2.5. Fluoride and aluminum concentrations in urine and brain

The concentration of fluoride in urine was treated using theF^- ion-selective electrode according to a national standardized method in China (WS/T 89-1996), and the concentration of aluminum in urine was assayed by use of inductively coupled plasma (ICP) atomic emission spectrometry. The brain was cremated first, and then concentrations of fluoride and aluminum in brain were measured using the F^- ion-selective electrode and ICP atomic emission spectrometry, respectively.

2.6. Pathological and ultrastructural observations

One half of the hippocampus was fixed in 10% formaldehyde solution for four weeks. Then, the tissue was washed, dehydrated using an ethanol gradient, cleared, embedded in paraffin, cut into 4-μm sections, stained with hematoxylin-eosin and observed. Part of the other side of the hippocampus was fixed in 2.5% glutaraldehyde solution for four weeks and then fixed in 1% osmium tetroxide for 2 h, dehydrated using an acetone gradient, soaked with Epon 812, embedded and polymerized at 60℃ for 12 h. Ultrathin sections (50-70 nm) were generated from this tissue. Following uranyl acetate and lead citrate double-staining, the sections were viewed under a transmission electron microscope.

2.7. Immunohistochemistry

The staining procedures were performed according to the manufacturer's instructions. The expression of the Aβ1-42 protein in the hippocampus was measured via light microscopy (×200) in a double-blind manner. Three consecutive fields were measured for each slice, and the average staining intensity of the three fields represented the expression level of the Aβ1-42 protein. Then, the average Aβ1-42 protein expression was determined for each group of sections.

2.8. Statistical analysis

SPSS 18.0 software was used for statistical analysis. All data were expressed as the Means ± SD. One-way ANOVA was performed for comparisons among multiple groups, LSD or Dunnett T3 was followed by two groups' comparisons. A $P<0.05$ was considered to be statistically significant.

3. Results

3.1. General characteristics of the animals during toxicant exposure

During the experiment, the amounts of drinking water and food intake of all groups were similar, and there was no significant difference in weight of the rats between four groups ($P>0.05$) (Fig. 1).

3.2. Changes in the teeth of the rats in different groups

The incisors of rats in control and aluminum groups were light yellow or claybank, superficially smooth and glossy, in particular, the lower incisors were semi-transparent, and their edges were sharp and smooth (Fig. 2). In contrast, the upper incisors of rats in fluoride and combined groups were claybank, and the lower incisors were chalky, poorly glossy and rough. The changes of incisors in fluoride group were more severely than that in combined group.

3.3. Concentrations of fluoride in urine and brain

In general, there were significant differences in the concentrations of urinary fluoride between the four groups ($P<0.01$) (Fig. 3A). The concentrations of urinary fluoride in combined and fluoride groups were significantly higher than that in the control group ($P<0.01$). At the same time, the concentrations of urinary fluoride in the fluoride group was obviously higher than that in combined group ($P<0.01$).

Significant differences were seen in the concentrations of fluoride in brain among all four groups (P<0.05) (Fig. 3B). The concentrations of fluoride in brain of the fluoride group was significantly higher than that of the control group (P<0.01). However, compared to the control group, no significant differences were seen in the concentrations of fluoride in brain of combined and aluminum groups(P＞0.05).

3.4. Concentrations of aluminum in urine and brain

There were significant differences in the concentrations of urinary aluminum between four groups (P<0.01) (Fig. 4A). The concentrations of urinary aluminum in aluminum and combined groups were significantly higher than that in the control group (P<0.01), but there was no difference between one another (P＞0.05). There were also significant differences in the concentrations of aluminum in brain among all groups (P<0.05) (Fig. 4B). The concentration of aluminum in brain in the aluminum group was obviously higher than that in the control group (P<0.05). Unlike that in aluminum group , the concentration of aluminum in brain of fluoride and combined groups were slightly higher compared to the control group (P＞0.05).

3.5. Short-term memory ability of rats in different groups

There was no significant difference in the rate of alternation among all four groups (P>0.05) (Fig. 5). Compared to the control group, the rate of alternation in all toxicant-exposed groups decreased, the lowest was seen in fluoride group, followed by combined group, and the highest was observed in aluminum group.

3.6. Learning and memory ability of rats in different groups

No significant difference was displayed in the training latency of day 2 and day 3 (Fig. 6A). Compared to the control group, there was no obvious change in the training latency of day 2 in toxicant-exposed group, but a gradual increasing trend was shown in the training latency of day 3, which indicated that fluoride and aluminum might decrease the learning ability of rats. There was a significant difference in the number of entries into target zone between all four groups (P<0.05) (Fig. 6B). Compared to the control group, the number of entries into target zone decreased in other three groups, the maximum was observed in aluminum group, followed by combined group, and the minimum was shown in fluoride group. Moreover, there was a significant

difference between the aluminum group and the control group (P<0.05). Significant difference were seen in the latency of first entering to target zone among all four groups (P<0.05) (Fig. 6C). Compared to the control group, the latency of first entering into target zone became longer in other three groups, and it was significantly longer in aluminum (P<0.01) and combined groups (P<0.05). The latency of first entering into target zone in combined and aluminum groups was distinctly longer than that in fluoride group. Besides, the latency of first entering into target zone in combined group was longer than that in fluoride group, but shorter than that in aluminum group, and this trend was consistent with the results presented in Fig. 6B.

3.7. Pathologic changes of hippocampus in different groups

In the control group, neurons arranged properly, no hyperplasia was seen in spongiocytes and no obvious increase was observed in particles in the cells (Fig. 7A). In all toxicant-exposed groups, spongiocytes proliferated apparently, neurons decreased markedly, and the particles in the cells increased notably (Fig. 7B, Fig. 7C, Fig. 7D). Compared with the fluoride and combined groups, neurons in aluminum group were irregularly distributed apparently. But in combined group, a few of cells appeared to swell. (Fig. 7D).

3.8. Ultrastructural changes of hippocampus in different groups

In the control group, the morphology of neurons was regular, the membranes were intact, and the mitochondrial quantity was normal (Fig. 8A). In fluoride and aluminum groups, the mitochondria enlarged obviously, and the cristae and stroma were dissolved (Fig. 8B, Fig. 8C). The mitochondrial quantity in aluminum group was decreased markedly (Fig. 8C). In the combined group, the mitochondria enlarged and the mitochondrial quantity decreased (Fig. 8D).

3.9. The expression of $A\beta_{1\text{-}42}$ protein in hippocampus

The expressions of $A\beta_{1\text{-}42}$ protein in all toxicant-exposed groups were significantly higher than that in the control group (P<0.01) (Fig. 9). Among three toxicant-exposed groups, the highest expression was observed in aluminum group, followed by combined group, and the lowest expression was shown in fluoride group, which was consistent with the results of the Morris water maze.

4. Discussion

The focus on the combined toxicity of fluoride and aluminum has grown, as both compounds exert analogous damage to specific organs [21]. The impact of combined treatment of fluoride and aluminum on the learning and memory ability of rats were examined in this study, and the results would provide a new theoretical foundation for further studies of the impact on the nervous system among individuals living in areas in which drinking-tea fluorosis is prevalent.

In this study, an animal model of fluoride, aluminum and combined exposure was successfully established by administering fluoride and aluminum in drinking water. The results indicated that fluoride or aluminum exposure damaged the learning and memory ability of rats, which was consistent with previous reports. It has been reported that 50 mg/L F^- damaged rat learning and memory abilities [22]. By exposing rats to toxicants using 0.2%, 0.4% or 0.6% $AlCl_3$ (equivalent to 40 mg/L Al^{3+}, 80 mg/L Al^{3+} and 120 mg/L Al^{3+}, respectively), it was found that different concentrations of $AlCl_3$ decreased the rats' recognition ability to varying extents[23]. The results of the Morris water maze of this study revealed that the learning and memory ability of rats in all toxicant-exposed decreased in varying degrees. The most severe was seen in the aluminum group, followed by the combined group, and the lightest was shown in the fluoride group. In other word, the damage to the nervous system caused by aluminum intoxication was more severe than that caused by fluoride intoxication. Additionally, as the main component of senile plaques, the expression of $A\beta_{1\text{-}42}$ protein increased in all toxicant-exposed groups, and the expression in the aluminum group was the highest, this result was consistent with the above injury to the learning and memory ability of the rats.

The results also suggested that there may be antagonistic effect between fluoride and aluminum, and the damage to the brain would be alleviated compared with aluminum group when the proportion of fluoride to aluminum was 1:2. Namely, fluoride could partly mitigate the loss of learning and memory ability which caused by aluminum alone. Under the effect of gastrointestinal PH, a kind of difficultly soluble compounds was formed by fluoride and aluminum together, consequently

reduced the absorption of fluoride and aluminum[24]. This mechanism could partly explain the result in present study that the concentrations of fluoride and aluminum in brain in combined group were lower than that in aluminum group. Besides, the compound decreased the accumulation of aluminum in the body, inevitably resulting in incomplete antagonism of injury which caused by aluminum. On the other hand, evidence developed by other researchers reinforced our results from opposite angle, they revealed that aluminum might exert some impact on the absorption of fluoride in the intestine, preventing fluoride from depositing in the body and thus reducing the toxicity of fluoride[24].

Different quantities of decreased neurons and increased spongiocytes in hippocampus of all toxicant-exposed groups were observed, and neurons in aluminum group were irregularly distributed apparently. The change trend of mitochondrial quantity in all toxicant-exposed groups was consistent with the result of learning and memory ability of rats in different experimental groups. The damage to the nervous system caused by fluoride and aluminum might occur via oxygen radicals. It is recognized that mitochondria are main energy-producing organelles which generate 80% of the total oxygen radicals, the generation and elimination of oxygen radicals in normal cells often remains balanced. Chronic fluorosis or aluminum intoxication would lead to mitochondrial swelling and injury in specific brain areas, disrupt the balance of oxygen radicals, thus deteriorate the cognitive function[25-26].

In conclusion, our study suggested that aluminum exerts a more severe damaging effect on the nervous system than fluoride does. And the harmful effect of combined fluoride and aluminum (at a proportion of 1:2) on learning memory ability of rats was less than the effect of aluminum alone, which indicated that the fluoride could alleviate the harmful effect of aluminum on the nervous system, namely there was antagonistic effect between fluoride and aluminum. However, this mechanism requires further test and verify. In future, we would continue to explore the mechanism by which fluoride and aluminum leads to damage of the nervous system by appropriately adjusting the fluoride and aluminum dose and duration of administration and by improving the research methods.

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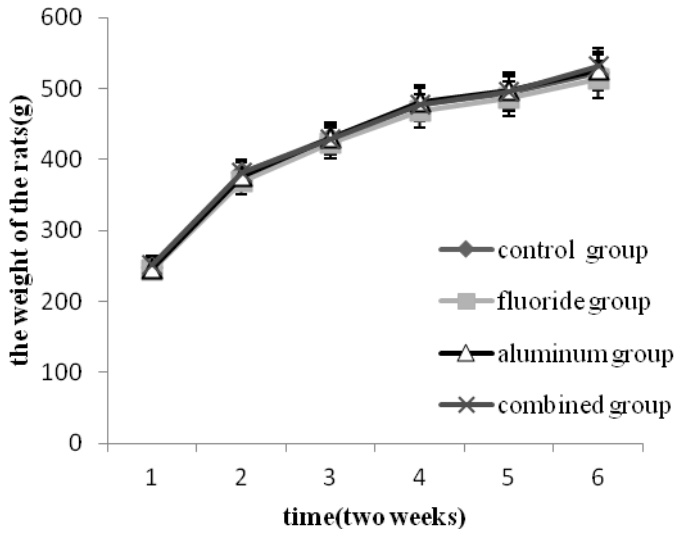


Fig. 1. The weight of rats in different groups during experiment. No significant difference was seen in the weight of rats between the four groups (P>0.05).

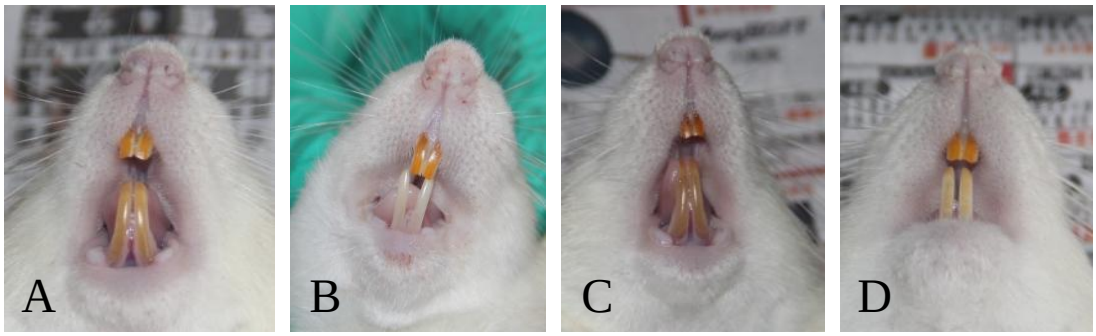


Fig. 2. The changes in teeth of rats in all four groups. (A) control group, (B) fluoride group, (C) aluminum group, (D) combined group.

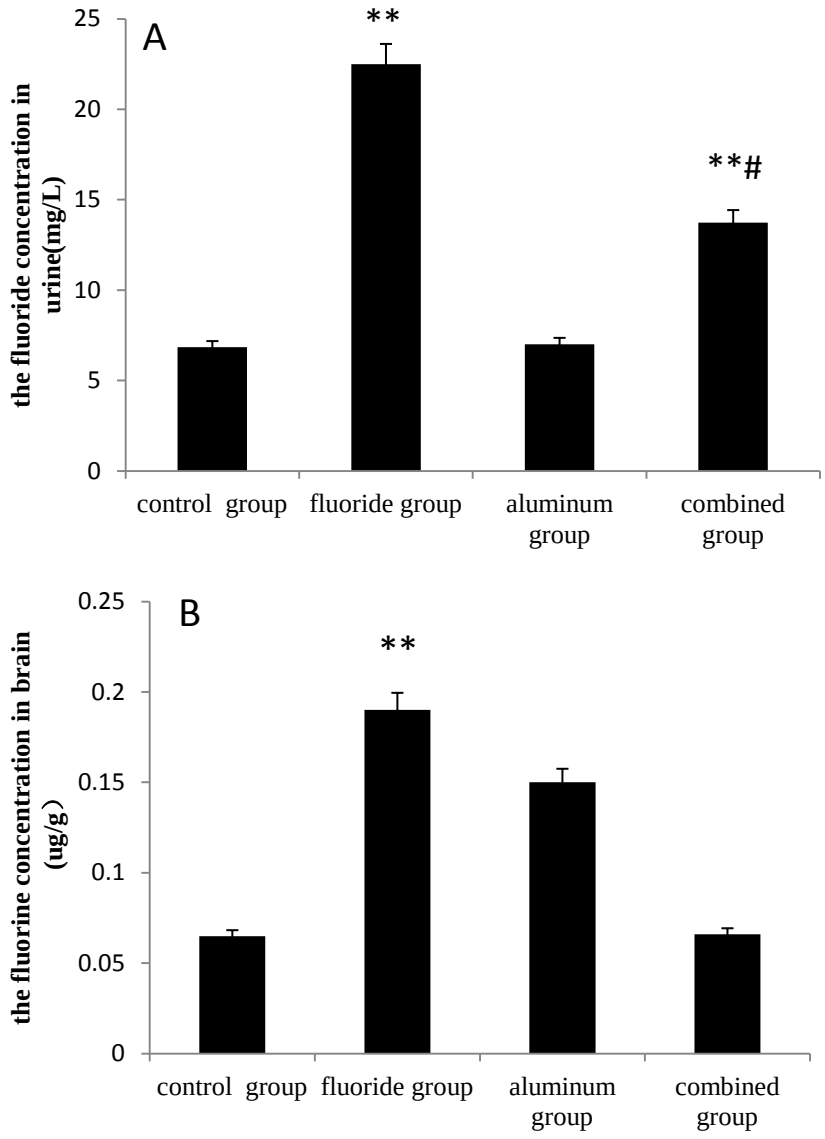


Fig. 3. The concentrations of fluoride in urine (A) and brain (B) of different groups. The concentration of urinary fluoride in fluoride and combined groups was significantly higher than that in control group, and that in fluoride group was also significantly higher than that in combined group (A). The concentration of fluoride in brain in the fluoride group was significantly higher than that in the control group (B) (** $P<0.01$ compared to the control group, #$P<0.01$ compared to the fluoride group).

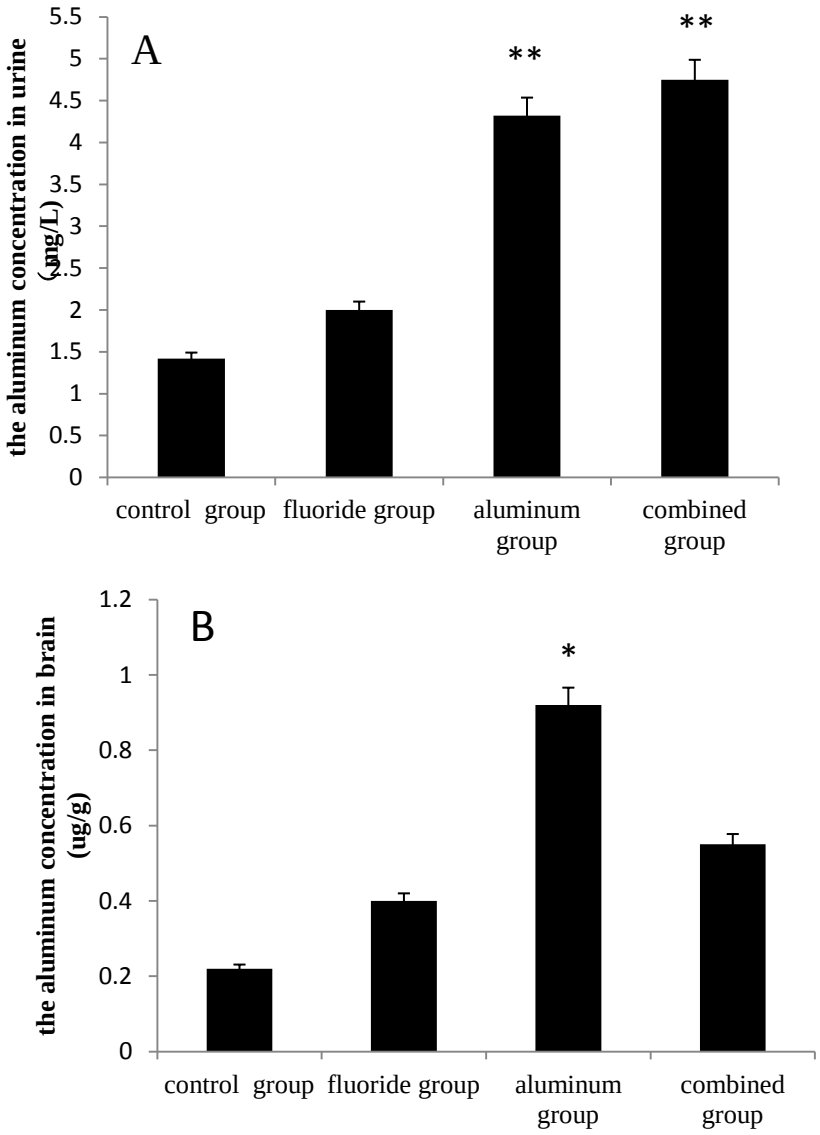


Fig. 4. The concentration of aluminum in urine (A) and brain (B) of the different groups. The concentrations of urinary aluminum in aluminum and combined groups were significantly higher than that in control group (A). The concentration of aluminum in brain of aluminum group was significantly higher than that of the control group (B) ($\bar{x} \pm s$, *P<0.05 and **P<0.01 compared to the control group).

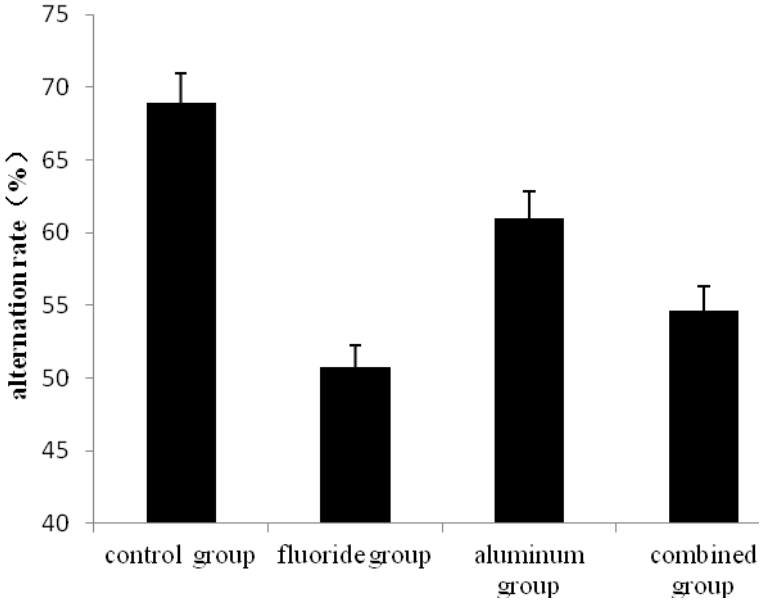
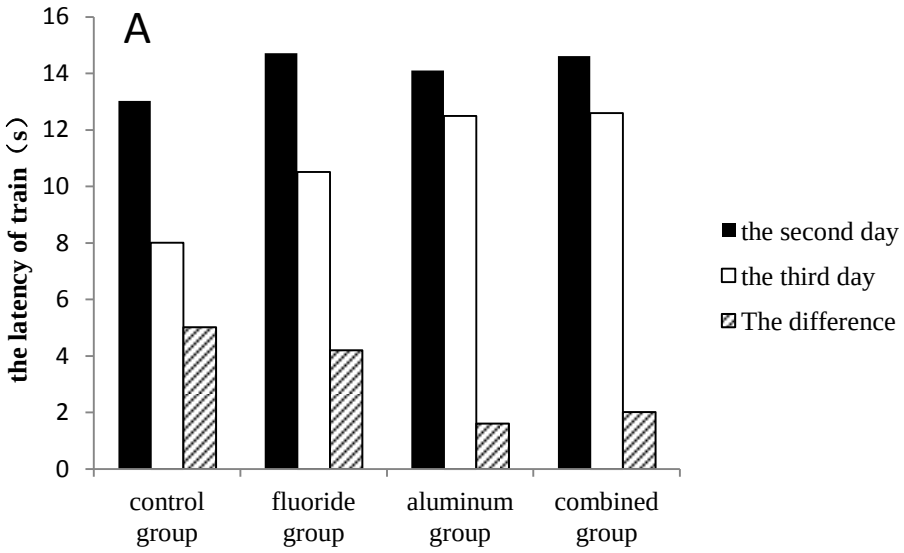
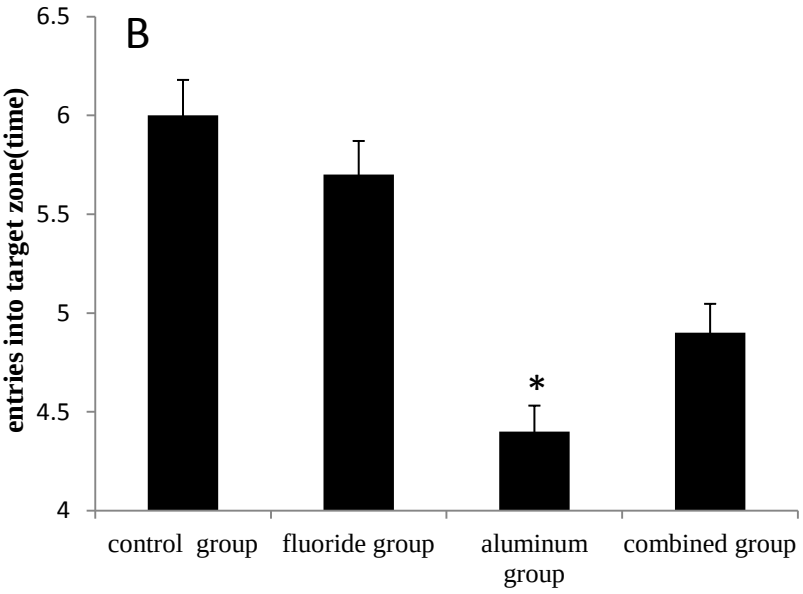
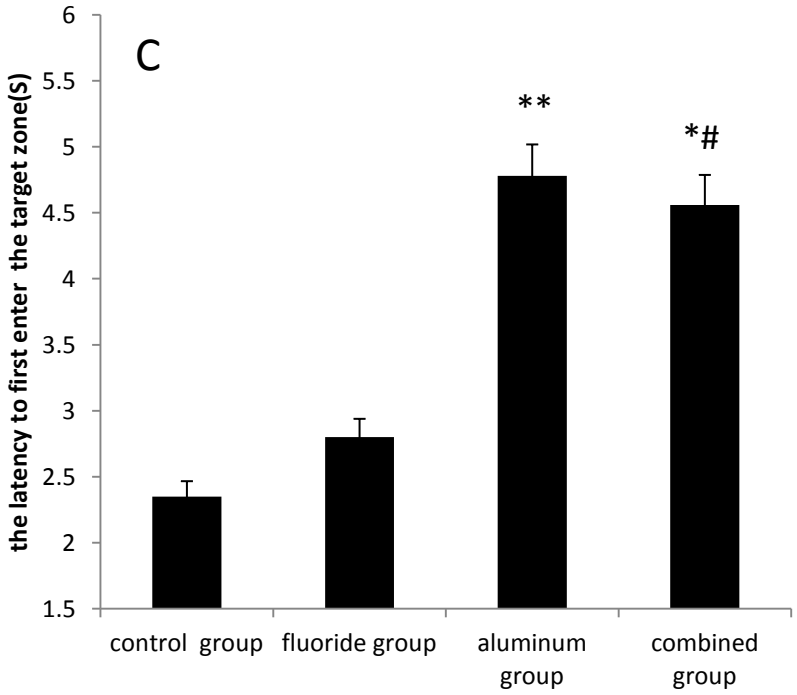


Fig. 5. The effect of different treatments on the short-term memory ability of rats based on Y maze test. There was no significant difference in the rate of alternation between the four groups (P>0.05).

Fig. 6. The effect of different treatments on the learning and memory ability of rats based on the Morris water maze test. A gradual increasing trend in the training latency was observed among all groups (A). There was a significant difference in the number of entries into target zone between the aluminum group and the control group (B). The latency of first entering into target zone in aluminum and combined groups was significantly longer than that in the control group, and that in fluoride group was significantly shorter than that in combined group (C) ($x \pm s$, *P<0.05 and **P<0.01 compared to the control group, #P<0.05 compared to the fluoride group).







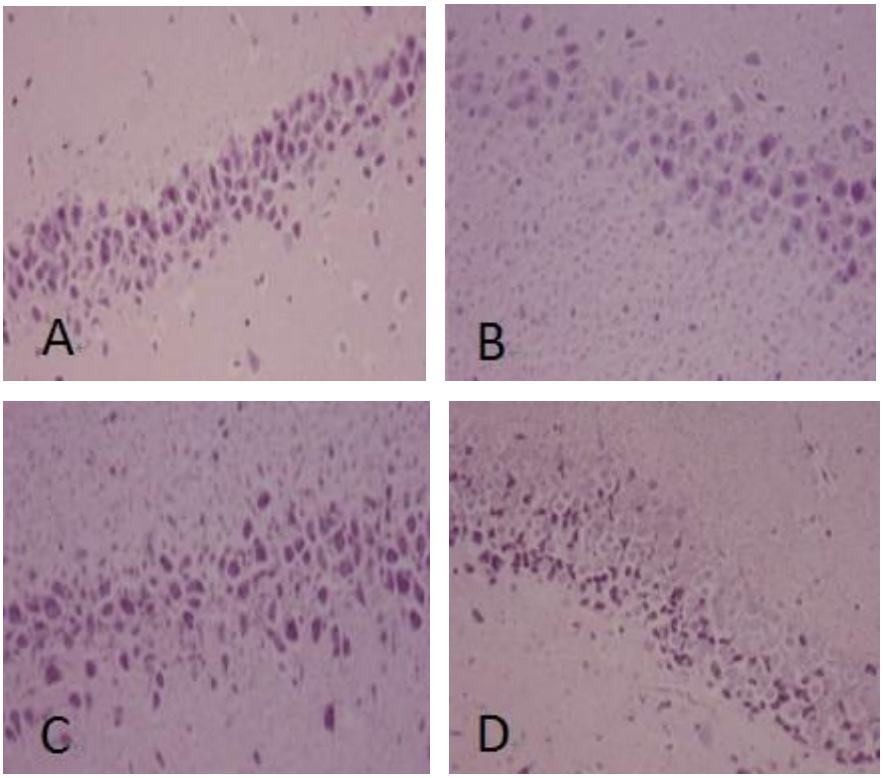


Fig. 7. The pathologic changes of hippocampus in different groups. (A) control group, (B) fluoride group, (C) aluminum group, (D) combined group.

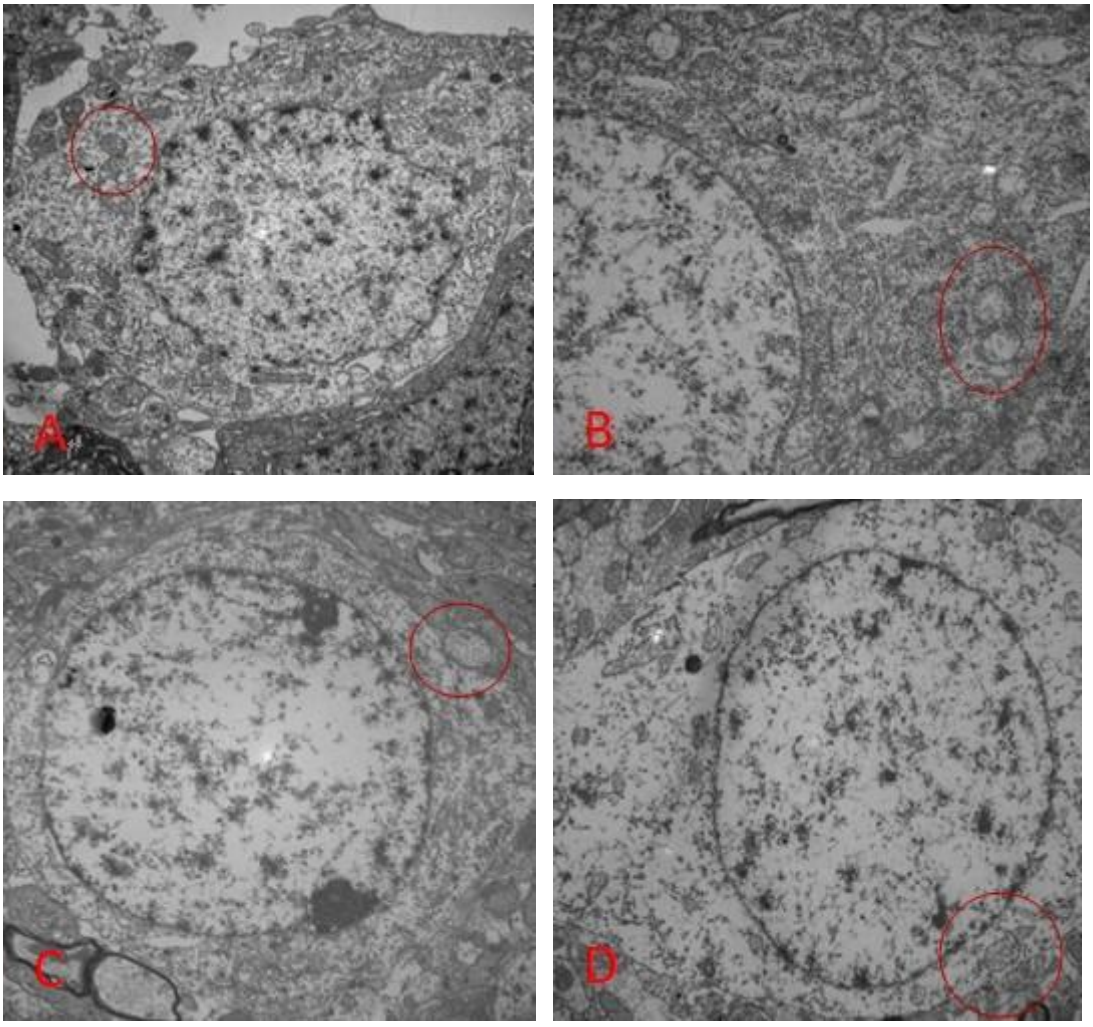
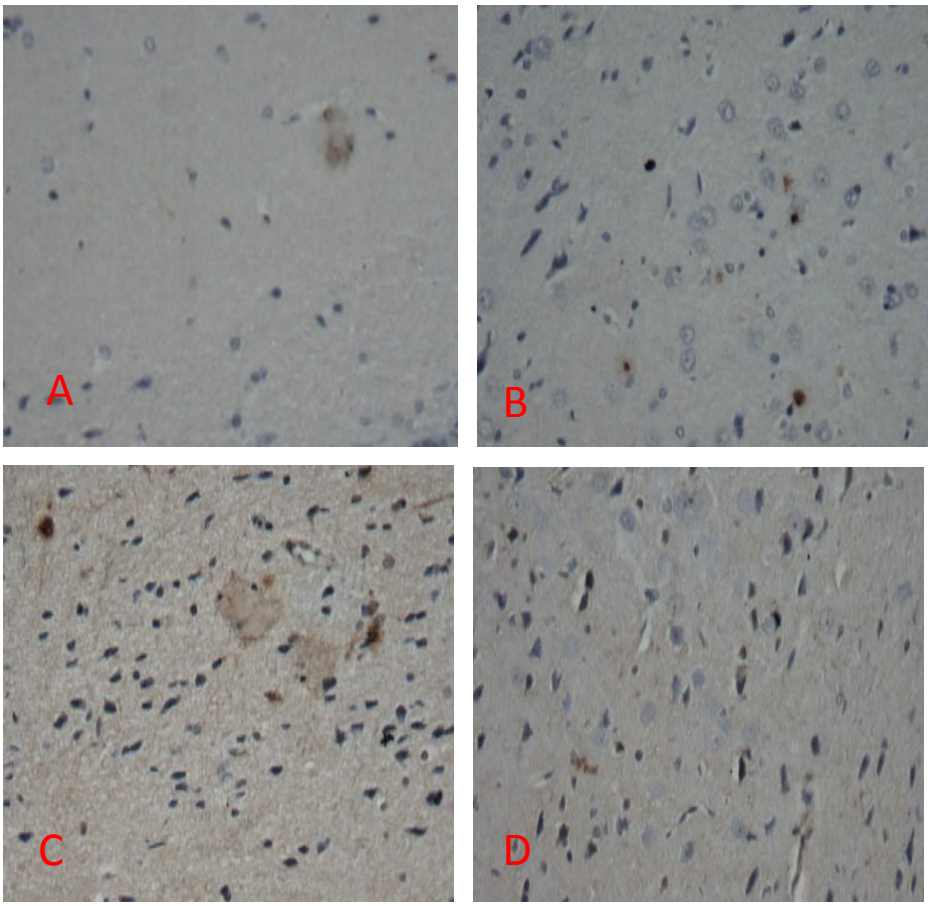


Fig. 8. The ultrastructural changes of hippocampus in different groups. (A) control group, (B) fluoride group, (C) aluminum group, (D) combined group.



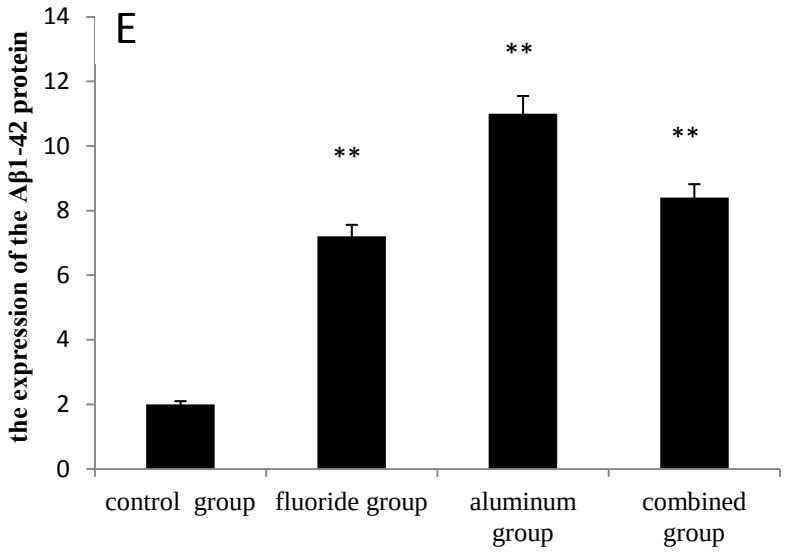


Fig. 9. The expression of $A\beta_{1\text{-}42}$ protein in different groups. (A) control group, (B) fluoride group, (C) aluminum group, (D) combined group, (E) the expression of the $A\beta_{1\text{-}42}$ protein in all groups. The expressions of $A\beta_{1\text{-}42}$ protein in all toxicant-exposed groups were significantly higher than that in the control group ($x \pm s$, **$P<0.01$ compared to the control group).