ATTENUATING EFFECT OF VITAMIN E ON THE DEFICIT OF LEARNING AND MEMORY OF RATS WITH CHRONIC FLUOROSIS: THE MECHANISM MAY INVOLVE MUSCARINIC ACETYLCHOLINE RECEPTORS

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ABSTRACT: The protective role of vitamin E (Vit E) against neurotoxicity induced by fluorosis was investigated by using Sprague-Dawley (SD) rats fed with 50 ppm fluoride in drinking water for 10 months. Spatial learning and memory of rats were measured by the Morris water maze test; the expressions of M1 and M3 muscarinic acetylcholine receptors (mAChRs) at the protein level in the hippocampus and cortex were detected by immunohistochemistry; and the levels of $O_2^{\bullet-}$ and malondiadehyde (MDA) were evaluated by biochemical methods. The results showed that high fluoride inhibited learning ability and memory of the rats, reduced the protein expressions of both M1 and M3 mAChRs, and elevated the levels of $O_2^{\bullet-}$ and MDA in the rat brains. Interestingly, the treatment of Vit E prevented the increased production of $O_2^{\bullet-}$ and MDA in brains of the rats fed with high fluoride. In addition, Vit E attenuated the decreased learning ability and memory of the rats exposed to high fluoride, and the mechanism for this may involve the recovered expression of mAChRs resulting from the use of the antioxidant.

Key words: Chronic fluorosis; Learning and memory; Muscarinic acetylcholine receptors (mAChRs); Oxidative stress; Rat brain; Vitamin E.

INTRODUCTION

In our early investigations, we indicated that the excessive accumulation of fluoride over a long period can cause the elevation of free radicals and the inhibition of antioxidant defenses, which induces a vast array of symptoms and pathological changes in many tissues and organs, in addition to its well-known effects on the skeleton and teeth.[1-3] Fluoride can cross cell membranes and affect various soft tissues leading to impairment of tissue functions.[4] Chronic fluorosis leads especially to dysfunctions of the central nervous system (CNS), which results in lethargy, insomnia, and deterioration of learning and memory.[5, 6]

Cholinergic neurotransmitter signaling in the CNS is essential to support learning and memory in both humans and animals. The deteriorated learning and memory in rats with chronic fluorosis are associated with alterations in the cholinergic system.[5,7] Acetylcholine receptors play an important role in cholinergic neurotransmitter signaling and include two types, i.e., nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). Our previous studies showed that the decreased capacity in learning and memory was positively correlated with a reduced expression of nAChRs in the adult and offspring rats with chronic fluorosis.[8-10] Similarly, mAChRs have also been obviously implicated in learning and memory.[11-

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[13] It is clear that mAChRs are part of the G-protein-coupled receptor family members with five subtypes (M1-M5). Pharmacological studies suggest that M1 and M3 mAChRs are connected to learning and memory processes.[14-18]

A previous study in our group and other investigations indicated that increased generation of free radicals and enhanced lipid peroxidation have been shown to play an important role in fluorosis.[1,19] Fluoride, an excitotoxin, stimulates over excitation of glutamate and aspartate receptors and leads to free radical generation causing CNS injury.[20,21] The literature suggests that fluoride toxicity induces a high level of oxidative stress which has an immense effect on the levels of neurotransmitters and also on anti-oxidative markers such as glutathione, superoxide dismutase (SOD), and vitamins (C, D, and E).[21] Chronic fluorosis also induces behavioural change, depression, deterioration of short-term spatial memory,[22,23] and neural damage in rodents as well as in humans.[24-26] The protective antioxidants[27] are essential nutrients for animal growth and development. Dietary vitamin E (Vit E) is believed to be a lipid-soluble antioxidant and exists as 8 major compounds (4 tocopherols and 4 tocotrienols) with the most abundant form being α-tocopherol, whose main function is to protect the cell membranes from oxidative stress by decreasing the reactive oxygen species (ROS) resulting from fluoride toxicity.[28,29]

Based on previous research results, we replicated the animal model of chronic fluorosis, and treated the animals with antioxidants to reveal if Vit E can improve the learning and memory ability by reducing the level of oxidative stress and enhancing the expression of M1 and M3 mAChRs in the brains of rats with chronic fluorosis.

MATERIALS AND METHODS

Materials: Rabbit polyclonal anti-M1, anti-M3 mAChR antibodies (Santa Cruz Biotechnology Inc., USA); Vectastain ABC Kit, peroxidase substrate DAB kit (Vector Lab, USA); kits for $O_2^{\bullet-}$ and measuring malondialdehyde (MDA) (Nanjing Jiancheng Bioengineering Institute, China); sodium fluoride (NaF, analytical reagent), and vitamin E (Vit E) (Sigma, USA) were purchased from the sources indicated.

Animal model with chronic fluorosis: Thirty, one-month-old (half males and half females, and body weight 100–120 g each), Sprague-Dawley (SD) rats, were purchased from the Experimental Animal Center in Guizhou, China, and the protocol was pre-approved by the regional ethical committee there. The humidity ranged from 30–55% and the temperature remained between 22–25ºC. All animals were acclimatized for one week in a housing facility before treatment.

The animals were divided randomly into 3 groups of 10 each, i.e., the untreated control (< 0.5 ppm fluoride in drinking water), the high fluoride (50 ppm fluoride added in drinking water) and the Vit E plus high fluoride (50 ppm fluoride in drinking water + 50 mg Vit E/kg/once a day by intragastric administration). Each group was fed with a normal diet (containing less than 6 ppm fluoride). During the study, the rats were housed in stainless-steel cages suspended in stainless-steel

racks, and the treated water and chow were administered to animal *ad libitum* for 10 months.

Measuring spatial learning ability and memory: Spatial learning and memory of the rats was evaluated by using the Morris water maze test.[30] The maze consists of a circular pool (180 cm in diameter) with dark walls and is filled with tap water colored by dark ink. An escape platform (9 cm in diameter) made of stainless steel with dark walls is submerged 0.5 cm below the surface of the water. Each rat was subjected to four trials each day with a 5- to 7-min interval of rest between the trials for a training period of 4 days. The movement of the rats was monitored with Videotrack software (Viewpoint). During the navigation test, the time required to locate the escape platform (escape latency) was determined, and after locating this platform, the animal was allowed to sit on it for 2 sec. Rats who failed to find the platform within 60 sec were guided to the platform and then allowed to remain on it for 2 sec as well, in which case the escape latency was recorded as 60 sec. The four trials on each individual day were averaged for statistical analysis. Furthermore, on day 5, after the platform was removed, the time to first crossing the site where the platform was originally located was recorded. All of the behavioral tests were conducted in a quiet environment with subdued lighting.

Detection of M1 and M3 mAChRs at protein levels by immunohistochemistry: Immunohistochemical staining for M1 and M3 mAChR proteins in the hippocampus and cortex of rat brains from the three groups were performed. Briefly, the sections were treated with blocking buffer (DAKO) for 30 min at room temperature (RT) and thereafter incubated with anti-M1 or anti-M3 mAChR antibodies (1:100 or 1:300 dilution in PBS), respectively, overnight at 4ºC. Following a thorough rinse in PBS, the sections were incubated with the secondary antibodies, i.e., biotinylated goat anti-rabbit IgG (diluted 1:50 in 10% horse serum) for 30 min at RT. Sections were incubated subsequently with avidin-biotinylated enzyme complex and DAB, dehydrated with increasing concentrations of ethanol, cleared with xylene, and then mounted in Permount. Negative controls for these immunohistochemical procedures were incubated with nonimmune serum instead of the primary antibodies, which resulted in no detectable staining. The optical densities (OD) of M1 and M3 mAChR immunoreactivity (IR) were measured by using a CM-2000B Biomedical Image Analysis System (Beihang University, China). The OD of M1 and M3 mAChRs was analyzed by the microdensitometry of the granule cells of the pyramidal cells of the hippocampus CA3 and the cortices. The OD of the white matter on the same section served as the value of the background. The absolute OD values were obtained by subtracting the OD of the background.

Measurement of the levels of $O_2^{\bullet-}$ and MDA in the brain tissues: The brain tissue (0.1 g) was homogenized in 9 mL normal saline. Homogenates were centrifuged to get supernatant. The protein concentrations of supernatants were determined by using the BCA protein assay kit (Thermo scientific, USA). The contents of $O_2^{\bullet-}$ and MDA were measured by using kits for these parameters, respectively (Nanjing Jiancheng Bioengineering Institute, China).

Statistical analysis: The results are expressed as the means±SD of the values from the different groups. These means were examined for statistically significant differences employing the analysis of variance (ANOVA) followed by Student-Newman-Kenl's test or the two-paired Student's t test in the SPSS16.0 software (SPSS Inc., USA).

RESULTS

Spatial learning and memory: In the rats of the high fluoride group, the increased escape latency time (Figure 1A), the decreased number of crossings of the platform site (Figure 1B), and the decreased time of staying on the site of the platform (Figure 1C) were obvious as compared to the controls, suggesting a decreased spatial learning ability and memory in the rats induced by chronic fluorosis. However, the decrease in the learning ability and memory in the rats due to fluoride was attenuated by the treatment with Vit E (Figure 1).

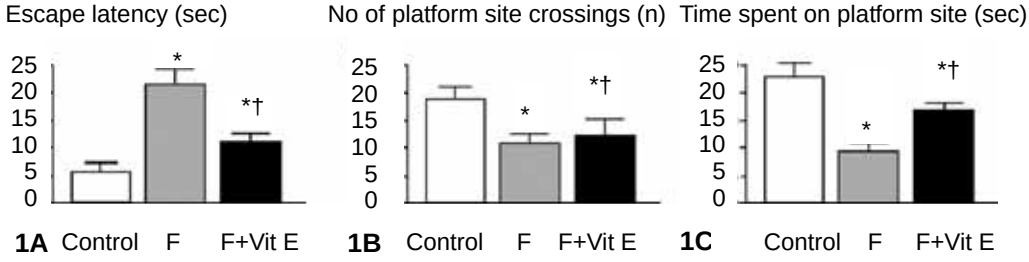
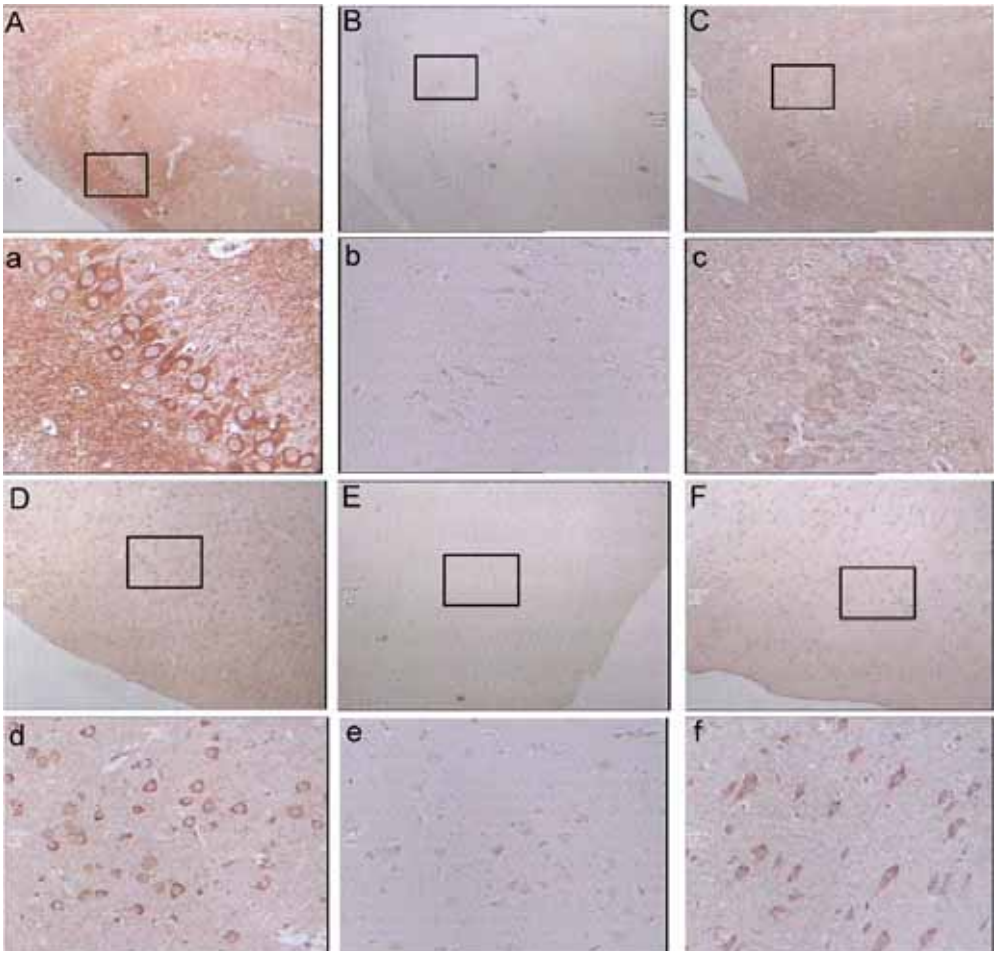
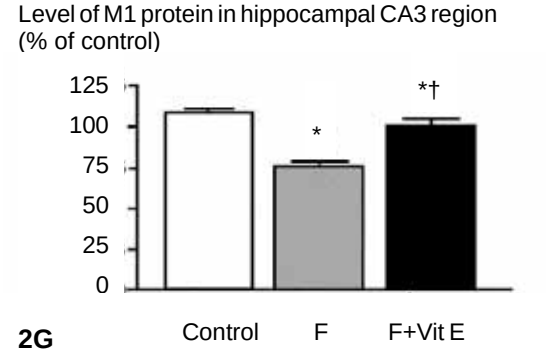


Figure 1. Escape latency (sec), the number of crossings of the original site of the platform (n), and the time (sec) spent staying on the original site of the platform of the rats in the different groups (Control, F=fluoride group; F+Vit E= fluoride+Vit E group). Spatial learning and memory were tested in a Morris water maze system involving a circular pool 180 cm in diameter. 1A: The escape latency (sec) of each group during the training sessions held 4 times a day for 4 days and also, on the 5th day after the platform was removed, the time to the first crossing of the original platform site; 1B: The number of times (n) the original site of the central platform was crossed after the platform was removed on the 5th day; C: The time (sec) spent on the original site of the platform on the 5th day. Values in the bar graphs are means±SD of 10 rats. *$p<0.05$ in comparison to the control group and †$p<0.05$ in comparison to the fluorosis group as determined by the analysis of variance (ANOVA), followed by the Student-Newman-Keul's test.

The expressions of M1 and M3 mAChRs in the brains of each group measured by immunohistochemistry: The M1 (Figure 2) and M3 (Figure 3) mAChRs were widely localized in the dendrites and soma of neuronal cells in the hippocampal and cortex. The significantly decreased expressions of the M1 (Figure 2) and M3 (Figure 3) mAChRs detected by immunohistochemistry in the high fluoride group were observed both in the hippocampus and cortex of the rats in comparison with the control group. Furthermore, the inhibited protein expressions of the M1 (Figure 2) and M3 (Figure 3) mAChRs resulted from the exposure of high fluoride were significantly attenuated by the treatment with Vit E. The IR OD values of the M1 (Figure 2G and 2H) and M3 (Figure 3G and 3H) mAChRs were calculated in brain regions of each group.





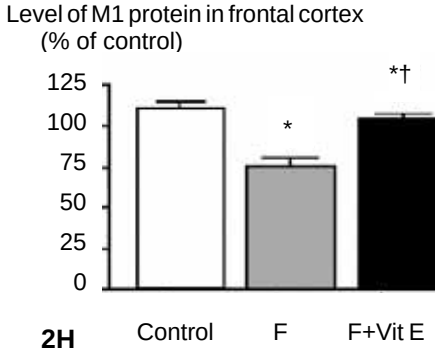
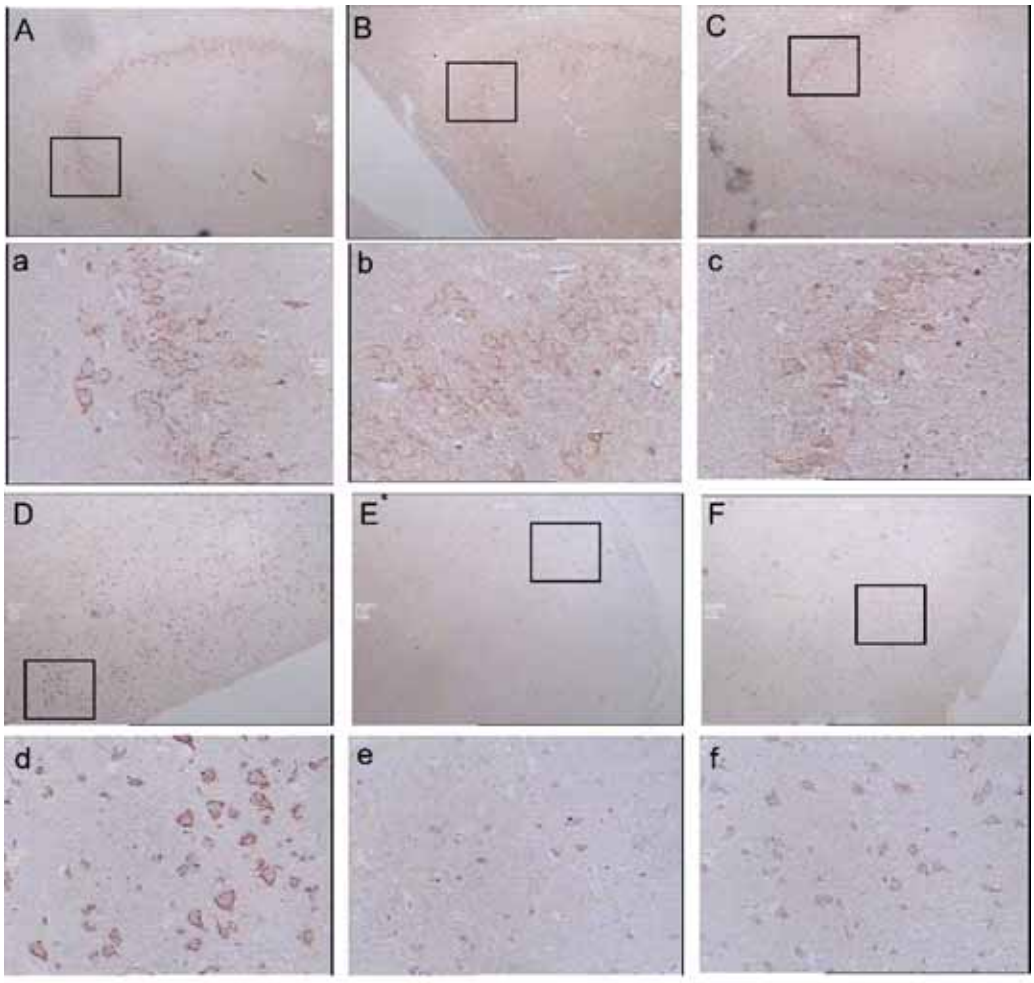


Figure 2. M1 mAChR in the brains of each group (Control, F=fluoride group; F+Vit E= fluoride+Vit E group) detected by immunohistochemistry. A-F (×40) and a-f (×400) showed M1 mAChR: A, a (control); B, b (fluorosis) and C, c (Vit E) in the pyramidal cell layer of the hippocampal CA3 region; D, d (control); E, e (fluorosis) and F, f (Vit E) in the neuronal cells of the frontal cortex. Levels (OD) of M1 mAChR in the hippocampal CA3 region (2G) and the frontal cortex (2H) of each group with 10 rats were quantified. *p<0.05 in comparison to the control group and †p<0.05 in comparison to the fluorosis group as determined by the analysis of variance (ANOVA), followed by the Student-Newman-Keul's test.



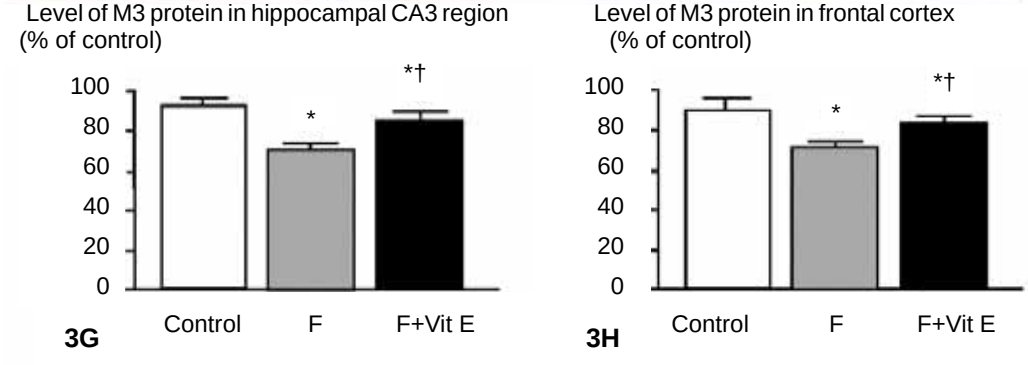


Figure 3. M3 mAChR in the brains of each group (Control, F=fluoride group; F+Vit E= fluoride+Vit E group) detected by immunohistochemistry. A-F (×40) and a-f (×400) showed M3 mAChR: A, a (control); B, b (fluorosis) and C, c (Vit E) in the pyramidal cell layer of the hippocampal CA3 region; D, d (control); E, e (fluorosis) and F, f (Vit E) in the neuronal cells of the frontal cortex. Levels (OD) of M3 mAChR in the hippocampal CA3 region (G) and the frontal cortex (H) of each group with 10 rats were quantified. *$p<0.05$ in comparison to the control group and $^{\dagger}p<0.05$ in comparison to the fluorosis group as determined by the analysis of variance (ANOVA), followed by the Student-Newman-Keul's test.

The levels of $O_2^{\bullet-}$ and MDA in the brain tissues: The levels of $O_2^{\bullet-}$ (Figure 4A) and MDA (Figure 4B) in the brain tissues of rats with the exposure of high fluoride were significantly higher than those in the control group. In addition, the increased levels of $O_2^{\bullet-}$ and MDA in the rat brains induced by high fluoride were significantly attenuated by the treatment of Vit E (Figure 4).

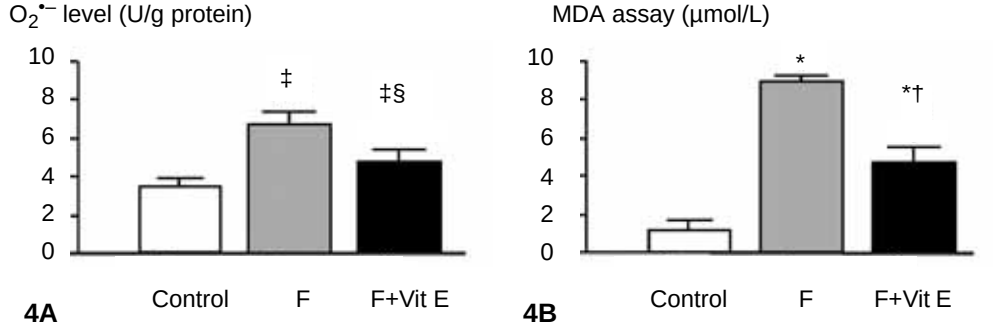


Figure 4. The activities of $O_2^{\bullet-}$ (4A) and the levels of MDA (4B) in the brain tissues of each group (Control, F=fluoride group; F+Vit E= fluoride+Vit E group). The activities of $O_2^{\bullet-}$ and the levels of MDA were measured by biochemical methods. The values are shown as the means±SD of 10 rats. *p<0.05 and ‡p<0.01 in comparison to the control group, †p<0.05 and §p<0.01 in comparison to the fluorosis group as determined by the analysis of variance (ANOVA), followed by the Student-Newman-Keul's test.

The correlations between spatial learning ability and memory and the level of mAChRs of the rats in the Vit E plus high fluoride group: Correlation analysis in the group exposed to high fluoride but treated with Vit E showed that the abilities of learning ability and memory of the rats was positively correlated with elevated protein levels of M1 or M3 mAChR (Figure 5).

DISCUSSION

In early studies, most researchers gave more attention to the skeletal or dental changes induced by chronic fluorosis.[11] At present, the non-skeletal effects of fluorosis that affect the soft tissues and organs of the body have been confirmed by a large number of studies, especially for the damage in the central nervous system (CNS).[12-14] Many studies have shown that the cognitive functioning of patients and the learning and memory abilities of animals are significantly decreased in chronic fluorosis.[3,31] In the present study, with its navigation test, we observed that the rats exposed to high fluoride performed more poorly than the controls, indicating that the spatial learning ability of the animals with chronic fluorosis was decreased, which is consistent with the previous reports.[3,12]

It has been found that mAChRs are involved in memory processes in which the cortex and hippocampus interact.[12] In our previous results, we found that the deficit in learning and memory of rats with chronic fluorosis was positively correlated with the decreased expressions of M1 and M3 mAChRs.[32] In the present study, by employing the immunohistochemistry method, we found that M1

and M3 mAChRs were localized in the dendrites and soma of neuronal cells and widely expressed in the granule cell layer of the dentate gyrus, the pyramidal cell layer of the hippocampal CA1-4 area, and in the neurons of all layers of the cortex.

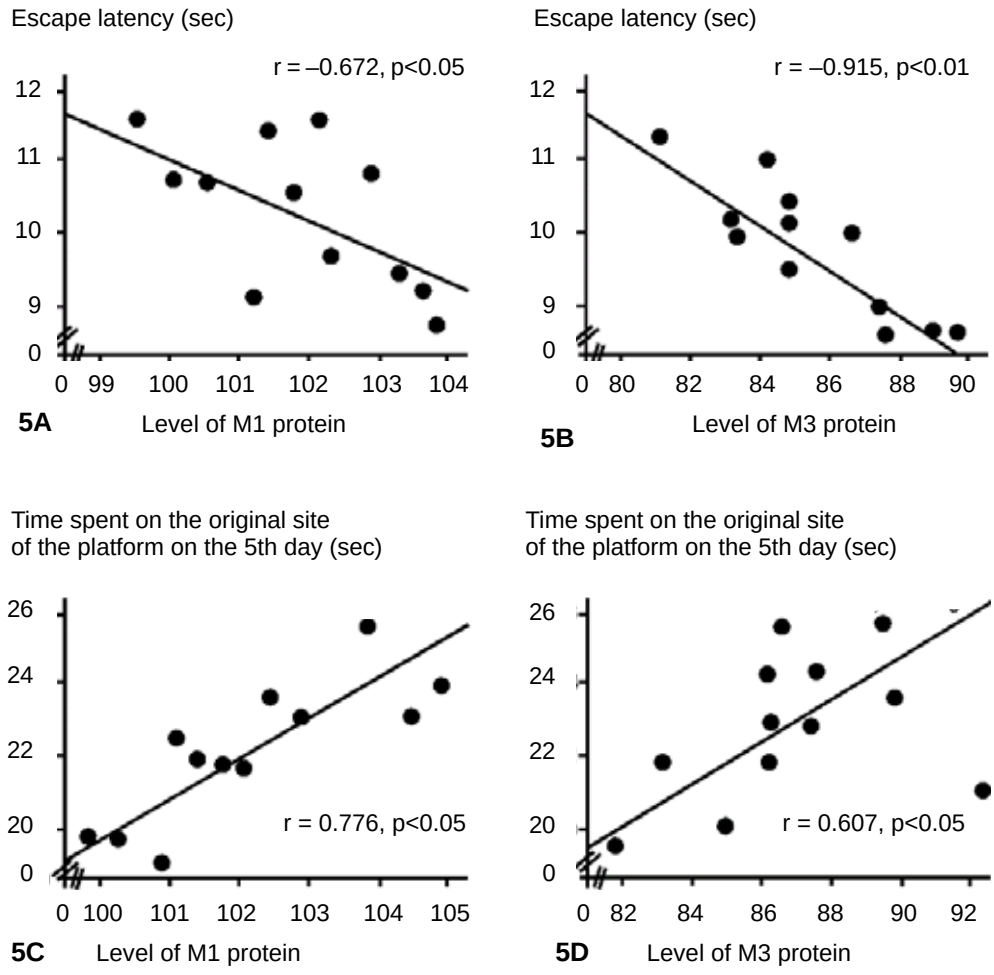


Figure 5. Correlation between the spatial learning ability and memory of rats and the expression of mAChRs in the rat brains with the treatment of high fluoride and Vit E. The values are shown as the means±SD of 10 rats. There were significant negative-linear correlations between the escape latency and the levels of M1 mAChR (5A) and M3 mAChR (5B) and positive-linear correlations between the time spent on the original site of the platform on the 5th day and the levels of M1 mAChR (5C) and M3 mAChR (5D) as revealed by the Pearson correlation test ($p<0.05$ or $p<0.01$).

The significant decreases in the expression of M1 and M3 mAChRs were observed in the hippocampus and cortex of the rats exposed to high fluoride as compared to the controls, were consistent with the changes in the expression of mAChRs detected by Western blotting in our previous investigation.[32]

More evidence for the occurrence of a high level of oxidative stress in chronic fluorosis is emerging, and it is believed that oxidative stress plays an important role in the pathogenesis of the disorder.[1-3,33,34] In the present study, we found higher levels of $O_2^{\bullet-}$ and MDA in the brain tissues of the rats with chronic fluorosis.

Many investigations have been concerned with interventions using anti-oxidative stress compounds or drugs to fight against chronic fluorosis, and the results obtained indicate that using different types of vitamins can inhibit the toxicity induced by high fluoride through attenuating the level of oxidative stress.[35] It is well known that Vit E (a potent peroxyl radical scavenger) is a chain-breaking antioxidant that prevents the propagation of free radicals in membranes and plasma lipoproteins,[36] and Vit E has been found to be important in the early stages of development of the CNS.[37,38] When peroxyl radicals (ROO•) are formed in the CNS, these can react 1000-times faster with Vit E than with polyunsaturated fatty acids.[39] The hydroxyl group of tocopherol reacts with the peroxyl radical to form the corresponding lipid hydroperoxide and the tocopherol radical (Vit E-O•). The Vit E-O• can react with Vit C (or other hydrogen donors) and thereby oxidize the latter, and finally return to the reduced state of Vit E.[40] Our results here support the previous findings, in which the levels of $O_2^{\bullet-}$ and the related MDA in the brains of rats exposed to high fluoride were clearly higher than those of controls, and Vit E decreased the production of $O_2^{\bullet-}$ and the related MDA.

Interestingly, our present results showed that the treatment of Vit E attenuated both the deficit of learning and memory and the decreased level of mAChRs in the rats induced by high fluoride. Learning ability and memory have been confirmed to be significantly connected to the expression of mAChRs.[12] Here we found that there was a significant positive correlation between the learning ability and memory of the rats and the expression of mAChRs in the rat brains in the group with the treatment of Vit E plus high fluoride. Therefore, we believe that the treatment with Vit E of the rats fed with high fluoride not only inhibited the production in the brain of $O_2^{\bullet-}$, and thereafter MDA, but also raised the expression of M1 and M3 mAChRs in the different brain regions, and that this may have attenuated the cognitive deficits.

CONCLUSIONS

In conclusion, the exposure of high fluoride inhibited the learning ability and memory of rats, reduced the protein expressions of both M1 and M3 mAChRs, and elevated the levels of $O_2^{\bullet-}$ and MDA in rat brains. The treatment of Vit E prevented the increased production of $O_2^{\bullet-}$ and MDA in the brains of rats fed with high fluoride. Interestingly, Vit E attenuated the decreased learning ability and memory of the rats fed with high fluoride and the mechanism for this may involve the antioxidant allowing the expression of mAChRs to be recovered.

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