DECREASED LEARNING AND MEMORY ABILITY IN RATS WITH FLUOROSIS: INCREASED OXIDATIVE STRESS AND REDUCED CHOLINESTERASE ACTIVITY IN THE BRAIN

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SUMMARY: The aim of this research was to study the mechanism of the decreased learning and memory of rats with chronic fluorosis. Compared with controls, decreased learning and memory ability, lower levels of total antioxidant capacity (T-AOC), and increased content of malondialdehyde (MDA) in brain tissues were observed in both male and female young adult rats after 6 months with either 5 or 50 mg NaF/L in their drinking water. Interestingly, the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in the brain were reduced more in the rats with the lower NaF concentration than in those with the higher concentration, thereby suggesting a paradoxical dose-response effect of F on these enzymes. The results indicate that the reduced learning capacity and memory ability of rats induced by F may be connected with increased oxidative stress and diminished cholinergic nervous system responses.

Keywords: Cholinesterase; Fluorosis in rats; Learning and memory; Malondialdehyde; Total antioxidant capacity.

INTRODUCTION

As shown in earlier research, excessive ingestion of fluoride (F) can exert toxic effects on many tissues and organs, resulting in serious damage and pathological changes.¹⁻³ Indeed, a link between excessive exposure to F and dysfunction of the central nervous system has been established. A number of histopathological changes, including demyelination, a decrease in the number of Purkinje cells, thickening and disappearance of dendrites, swelling of mitochondria, and dilation of endoplasmic reticulum in neurons, have been observed in the brains of experimental animals subjected to fluorosis.¹,⁴,⁵ Accumulation of F has also been observed in the brains of experimental animals exposed to high doses of F for a prolonged period.⁴ The severity of the adverse effects of F on the behavior of rats is directly correlated with the concentrations of F ion in the plasma and in specific regions of the brain.⁶ In addition, the latencies of the pain reaction and conditioned reflex are longer in rats with high concentrations of F in their diets in comparison to control animals.⁷ In humans, exposure to elevated F drinking water in endemic village areas has been found to cause headache, followed by lethargy and insomnia.⁸ Moreover, the levels of mental work capacity of adults with chronic fluorosis and the Intelligence Quotient (IQ) of children born and raised in the areas with endemic fluorosis were found to be lower than normal.⁹⁻¹² These findings are also consistent with animal studies in rats.¹³,¹⁴ Furthermore, disturbances in brain development in offspring rats with chronic fluorosis and a longer latency of the pain reaction and conditioned reflex in rats receiving higher concentrations of F have been observed as compared to control animals.⁴,⁷

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Although the severity of these adverse effects of F on the behavior of rats is directly correlated with its concentration in the plasma and in specific regions of the brain, the biomechanism of the action of F in reducing IQ is not so clear. In previous studies, we determined the effects of chronic exposure to F on selected biochemical parameters and pathology in rat brains and cultural neurons, which indicated that alterations of membrane lipid and reduction in cholinergic receptors might be involved in damage to brains or neurons.

In this study, for further investigation of the mechanisms of brain disorder affected by F, learning and memory ability, total antioxidant capacity (T-AOC), and malondialdehyde (MDA) levels, together with cholinesterase (ChE) activity, were measured in rats with chronic fluorosis.

**MATERIALS AND METHODS**

**Experimental animals:** Seventy-two young Sprague-Dawley rats (half males and half females, weighting 90–120 g) were purchased from the Experimental Animal Center in Guizhou, China. Ethical permission for these experiments was obtained from the regional ethical committee for animal studies in Guizhou. In the animal housing facility the humidity ranged from 30 to 55% with the temperature between 22 and 25°C. Before treatment, the rats were acclimatized for one week and were then randomly divided into three groups: (a) normal control group with less than 0.5 mg NaF/L in their drinking water; (b) a lower F-exposed group with 5 mg NaF (2.26 mg F ion)/L in their drinking water; and (c) a higher F-exposed group with 50 mg NaF (22.6 mg F ion)/L in their drinking water. During the study, four rats per cage (single sex per cage) were housed in stainless-steel cages suspended in stainless-steel racks, and the treated water and food were available ad libitum. At the end of 6 months, the rats were tested for learning and memory and sacrificed for biochemical study of their brain tissue.

**Fluoride level in urine and bones:** At 6 months after establishing the animal model, urine samples were collected over 24-hr periods in plastic tubes by using special metabolic cages that effectively separate urine and faeces. The urine samples were stored at 4°C. At the end of the experiment, the rats were sacrificed by withdrawing blood from the femoral artery, and brain tissues were stored at –80°C until analyzed. F in the urine was measured by the F-ion selective electrode, and the left thigh bones were treated by high temperature combustion and then F in the bones was determined by the F-ion selective electrode.

**Spatial learning and memory:** Spatial learning and memory of the rats were evaluated by the Morris Water Maze test. The maze consists of a circular pool (180 cm in diameter) with dark walls and filled with ink-darkened tap-water. An escape platform (9 cm in diameter) made of stainless steel with dark walls is submerged 0.5 cm below the surface of this water. Each rat was subjected to 4 trials each day with a 5- to 7-min interval of rest between trials for a training period of 5 days. The formal evaluation began on the sixth day after training. The movement of the rats was monitored with View Point Videotrack Software. 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 that 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; the escape latency in these cases was recorded as 60 sec. The 4 trials on each individual day were averaged for statistical analysis. In addition, on day 7 in which the platform was removed, the number of central platform crossings by the rats was counted. The rats were also subjected to a 60-sec probe trial in which the animals were allowed to search for the place of the platform, and the time spent in each quadrant of the maze (quadrant search time) was recorded. All these behavioral tests were conducted in a quiet environment with subdued lighting.

**Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) assays:** The brain tissues were homogenized in 1:9 (W/V) 0.9% saline. Activities of AChE and BuChE were determined by an improved Ellman’s colorimetric method employing acetylthiocholine (ATC) and butyrylthiocholine (BTC), respectively, as substrates for the reactions. The hydrolysis of ATC or BTC was monitored at a wavelength of 412 nm by the formation of the yellow 5-thio-2-nitrobenzoate anion resulting from the reaction of 5,5’-dithio(bis-2-nitrobenzoic acid) (DTNB) with thiocholine, released by the enzymatic hydrolysis of ATC or BTC. To distinguish the activity of AChE from BuChE, ethopropazine hydrochloride (100.5 µM) was used as a BuChE inhibitor in control preparations. In contrast, 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (1 µM) was used as an AChE inhibitor to distinguish BuChE from AChE. Activities were calculated by using the following formula:

\[ \text{ChE (U/mg protein)} = \frac{\text{OD (sample-control)}}{\text{OD (standard blank)}} \times 1 \text{ µmol/mL} \]

Data were normalized to the amount of protein measured by the Lowry method, using the Bio-Rad DC protein assay and bovine serum albumin as the standard.

**Total antioxidant capacity (T-AOC):** The brain tissues were homogenized in 1:9 (W/V) of 0.9% saline. T-AOC was determined by chemical colorimetry and was calculated by the following formula:

\[ \text{T-AOC (U/mg protein)} = \frac{\text{OD (control sample)}}{0.01} \]

Data were normalized to the amount of protein measured by the method as mentioned above.

**Detection of lipid peroxidation:** The level of MDA, one of the products of lipid peroxidation, was quantitated employing the thiobarbituric acid-reactive substance assay (TBARS).

**Statistical analysis:** Results are expressed as means ± SD. Statistically significant differences were analyzed by variance ANOVA and two-tailed Student’s t-test employing the SPSS10.0 software.
RESULTS

Fluoride in urine and bone: The significantly increased contents of F in urine and bone in the rats resulting from the two levels of NaF administration are shown in Figures 1A and 1B.

Oxidative stress: As measured by changes in T-AOC and MDA level, increased oxidative stress was seen in the brains of the rats with NaF in their drinking water. A significant decrease in T-AOC occurred with both concentrations of NaF (Figure 2A), but the increase in MDA level was significant only for the higher concentration of NaF (Figure 2B).

Changes in ChE activity: Significant inhibition of the activities of AChE (Figure 3A) and BuChE (Figure 3B) was detected in brain tissues of the rats with NaF in their drinking water as compared to controls. However, the degree of inhibition of ChE activities was less with the higher NaF concentration than with the lower concentration.
Spatial learning and memory: The mean escape latency values are shown in Figure 4A, in which a more prolonged escape latency is seen to have occurred in the rats receiving the higher concentration of NaF in their drinking water as compared with controls. This decrease in spatial learning and memory is also apparent in the smaller number of platform crossings by the rats with the higher NaF concentration, but the difference was not statistically significant (Figure 4B). However, a positive correlation ($r = 0.695; p<0.05$) between the MDA level and the prolonged time of escape latency was found in the rats administered the higher concentration of NaF.

DISCUSSION

It has been shown that F can cross the blood brain barrier and accumulate in the brain of rats exposed to high fluoride. Various reports indicate that neurotoxic effects of long-term F exposure include oxidative stress, changes in the cholinergic nervous system, and decreased ability of learning and memory.
F in high doses can induce deterioration of learning and memory capability in animals and in humans with chronic fluorosis,\textsuperscript{6,7,11,24,25} resulting in lower IQ and decreased mental capacity in the children who live in areas of endemic fluorosis.\textsuperscript{9-10,14,26} In the present study, we observed that high F intake induced a decreased capacity in learning and memory of rats, which is consistent with a report of related findings.\textsuperscript{14}

Excessive production of reactive oxygen species (ROS) free radicals also appears to play a role in diminishing cognitive ability processes such as learning and memory. In recent years it has been recognized that higher concentrations of F produce neuronal dysfunction by mechanisms involving elevated levels of such free radicals.\textsuperscript{5,7,27-29} Under normal conditions the level of ROS radical production and the level of chemical elimination of them are kept in a dynamic balance. However, under oxidative stress, high levels of free radicals are viewed as a key pathogenic factor challenging the compensatory role of the antioxidant system. Numerous studies have examined the relationship between F and the production of free radicals.\textsuperscript{28} As a free radical inducer, high F results in an increased level of lipid peroxidation, which may therefore have an important role in the pathogenesis of fluorosis.\textsuperscript{28,30-32} This hypothesis has received emphasis from results of investigations ranging from studies on patients or animal models with chronic fluorosis to cultured cells treated with high concentrations of F that exhibit increased levels of metabolic products such as MDA and 4-hydroxynonenal from lipid peroxidation,\textsuperscript{11,33-35} reflecting reduced activities of antioxidant enzymes and decreased contents of antioxidant compounds.\textsuperscript{5,36}

In our study, we also examined the levels of T-AOC and MDA influenced by fluorosis. T-AOC is a useful index of the combined action of antioxidants in the body and MDA is an indicator for the products of lipid peroxidation.\textsuperscript{37} Lower levels of T-AOC and higher levels of MDA were observed in the brains of rats with the higher concentration of NaF exposure, suggesting that F induced oxidative stress in the rat brains. In addition, decreased levels of T-AOC were also found in the brains of rats fed with lower concentration of NaF, whereas no significant changes in the level of MDA were found thereby indicating mild damage induced by the lower F exposure.

Interestingly, we observed a positive correlation between the content of MDA and the prolonged time of escape latency in the rats fed with high F. The results suggested that higher concentration of NaF can induce a decreased learning and memory capacity in which a possible mechanism might be connected to the high level of oxidative stress resulting from fluorosis. Since excessive amounts of F can damage cellular membrane structure by lipid peroxidation,\textsuperscript{5} alteration in the membrane lipid composition might influence the insertion and/or turnover of neuronal nicotinic acetylcholine receptors, the function of which is involved in memory and cognition,\textsuperscript{15-17,38} thus suggesting a close correlation between increased oxidative stress and altered behavior of the rats with fluorosis.

ChE activity in the brain is also known to be important for maintaining normal brain physiological function for learning and memory ability. Interestingly, both
decreased and increased activities of ChE have been observed in both humans and animals with chronic fluorosis.\textsuperscript{39-44} Decreased activity of ChE in brain tissues of rats with F intoxication, \textsuperscript{39,40} in the blood of the patients with fluorosis,\textsuperscript{41} and in the gastrocnemius muscle and liver of fluorosed mice\textsuperscript{42} has been reported. The decline in ChE activity in the hippocampus of rats with fluorosis was significantly correlated with higher levels of F in the same region of the brain.\textsuperscript{40} On the other hand, it has also been found that severe damage by F may induce an increased activity of ChE in the brain tissues or blood of mice or rats with fluorosis.\textsuperscript{43,44}

In this connection, we observed significantly inhibited activities of AChE and BuChE in the brains of rats fed with the lower concentration of NaF. In addition, we also detected decreased activity of AChE and a tendency for decline in the activity of BuChE in the brains of rats fed with the high concentration of NaF. However, the decreased activities of these enzymes in the treatment with high F did not reach the degree of that which occurred with the lower concentration of NaF. Thus these results exhibit a paradoxical dose-response effect of F.\textsuperscript{45} Importantly, they show that, under certain circumstances, the inhibitory or stimulatory impact of F can actually be greater at a lower level of intake than at higher level.\textsuperscript{45} We propose, therefore, that moderate fluorosis may mostly induce inhibited activity of ChE, whereas severe fluorosis may exert a stimulatory effect on the activity of ChE. However, detailed mechanistic studies of how F affects the activity of ChE and BuChE await further investigation.

In conclusion, long-term exposure to high F has been found to induce a decreased learning and memory capacity in rats, enhance lipid peroxidation, inhibit the activities of antioxidative enzymes, consume nonenzymatic antioxidants, and decrease activities of AChE and BuChE in the brains of rats with fluorosis. The decline in learning and memory appears to be connected with the high level of oxidative stress and inhibited activity of ChE induced by F.

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