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Decreased learning ability and low hippocampus glutamate in offspring rats exposed to fluoride and lead

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ABSTRACT

Fluoride (F) and lead (Pb) are two common environmental pollutants which are linked to the lowered intelligence, especially for children. Glutamate, a major excitatory neurotransmitter in the central nervous system, plays an important role in the process of learning and memory. However, the impact of F and Pb alone or in combination on glutamate metabolism in brain is little known. The present study was conducted to assess the glutamate level and the activities of glutamate metabolism related enzymes including asparate aminotransferase (AST), alanine aminotransferase (ALT) and glutamic acid decarboxylase (GAD) in the hippocampus, as well as learning abilities of offspring rat pups at postnatal week 6, 8, 10 and 12 exposed to F and/or Pb. During lactation, the pups ingested F and/or Pb via the maternal milk, whose mothers were exposed to sodium fluoride (150 mg/L in drinking water) and/or lead acetate (300 mg/L in drinking water) from the day of delivery. After weaning at postnatal day 21, the pups were exposed to the same treatments as their mother. Results showed that the learning abilities and hippocampus glutamate levels were significantly decreased by F and Pb individually and the combined interaction of F and Pb. The activities of AST and ALT in treatment groups were significantly inhibited, while the activities of GAD were increased, especially in rats exposed to both F and Pb together. These findings suggested that alteration of hippocampus glutamate by F and/or Pb may in part reduce learning ability in rats.

1. Introduction

Fluoride (F) widely exists in environment with a significant increase in recent years in body burden accumulation (Watanabe et al., 2000; Wang, 2007). Internal exposure to F due to diet including food, water, beverages, tea, fluoride-containing dental products like toothpaste and fluoride supplements, is extensively researched (U.S. Environmental Protection Agency, 2000; Doull et al., 2006). Additionally, industrial pollution and coal burning have also been reported to be major sources of internal F exposure, especially in China and India (Wang et al., 1992; Li et al., 2003; Swarup and Dwivedi, 2002).

It is known that excessive F ingestion over a prolonged period can lead to toxic effects on human and animal health (Wang, 2007). In addition to the well-known effects of fluorosis on the skeleton and teeth, the neurotoxicity of F was also confirmed. A growing number of epidemiological studies reported that F diminished the general cognitive capacities and lead to behavioral problems in children (Li et al., 1995; Zhao et al., 1996; Lu et al., 2000b; Xiang et al., 2003). Animal studies showed that central nervous system function output is also vulnerable to F (Mullenix et al., 1995; Wang et al., 2004; Paul et al., 1998). Significant reduction in myelinated nerve fibers, external granular layer in cerebellum, and increased neuronal apoptosis have been reported in humans, rats, and mice (Sesikeran et al., 1994; Lu et al., 2000a; Trabelsi et al., 2001; Chen et al., 2002). It was also found that F exposure can alter the levels of protein and neurotransmitter, and activities of some enzymes in brain (Bhatnagar et al., 2006; Wu et al., 2006; Chirumari and Reddy, 2007). Nevertheless, there is relatively little known about the effects of F on the glutamate system in hippocampus, which is the major excitatory neurotransmitter in the mammalian central nervous system. Lead (Pb) is a highly neurotoxic metal, and there are numerous reports showing that Pb exposure produces learning impairment in rodents (Cory-Slechta, 1995). In U.S., maximum contaminant level (MCL) of blood Pb was revised downward for five times within more than 30 years after 1970 (Rogan and Ware, 2003). Canfield et al. (2003) reported that the blood Pb level even below the current safety standards still showed a threat to intelligence of children. Interestingly, fluoride, like fluorosilicic acid (H_2SiF_6) and sodium silicofluoride (Na_2SiF_6), has been reported to increase the accumulation of neurotoxicant Pb in the body (Masters and Coplan, 1999; Masters et al., 2000). Another study from China has reported that increasing Pb levels in blood of children has statistically related

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Table 1
Blood fluoride and lead levels in rats treated with high fluoride (HiF), high lead (HiPb) and high fluoride plus high lead (HiF + HiPb) at week 6 (mean ± SE, $n = 8$ rats/group).

	Control	HiF	HiPb	HiF + HiPb
Fluoride (mg/L)	0.59 ± 0.18	2.78 ± 0.28**	0.63 ± 0.14	2.90 ± 0.33**
Lead (μg/L)	42.45 ± 2.32	47.49 ± 3.31	169.94 ± 4.42**	132.28 ± 6.39**

See text for details of statistical analyses.
** $p < 0.01$ (compared with the control group in all tables).

with the high underground water F (Zhai et al., 2006). However, our understanding of the combined effects of F and Pb is still in its infancy.

The present study was therefore undertaken to investigate the effect of F and Pb administered alone or in combination on learning ability and glutamate metabolism in brain.

2. Materials and methods

2.1. Animals

Adult *Wistar albino* rats were obtained from the experimental animal center of Shanxi Medical University and then kept in plastic cages in our laboratory with their standard diets. After 1-week quarantine period, one male and two females were put in a cage together for mating. As soon as the vaginal plug was established, the females were separated and moved to separate cages. Beginning with the day of delivery, these females were divided into control and experimental groups as follows: (1) control group: received double distilled water; (2) high fluoride (HiF) group: received sodium fluoride (150 mg/L); (3) high lead (HiPb) group: received lead acetate (300 mg/L); (4) high fluoride plus high lead (HiF + HiPb) group: received sodium fluoride (150 mg/L) and lead acetate (300 mg/L). Before postnatal week 2, the rat pups derived their nutrients only from maternal milk. After week 2, they gradually began to eat feed and drink water, concomitantly with suckling maternal milk. At the age of postnatal week 3, the pups ate and drank entirely by themselves and given the same treatment as their parental generation. All of the offspring had free access to food and water until testing at week 6, 8, 10, and 12, and maintained their normal diets under standard temperature (22–25 °C), 12/12-h light/dark cycle, ventilation, and hygienic conditions. The study design was approved by the Institutional Animal Care and Use Committee of China.

2.2. Learning ability evaluation

At week 6, 8, 10, and 12, learning ability of rats were detected by a three-branch radial maze (Y-maze) (Chen et al., 1990). Each branch of the response box had a signal light at the end. During the experiment, the presence of signaling light indicated a safe, non-electrified area, while the other two branches were electrified, to determine whether the rats could discriminate between the signals and actively avoiding the electric shock. The branch with the light turned on was changed randomly after each run. When the rat actively moved down the lighted branch in order to avoid the electric shock, it was counted as a correct reaction, any other reaction was considered to be an error. The testing was split up into runs of twenties, i.e. each rat was test 20 times, and then another 20 runs were carried out at the same time next day; 18 correct reactions in 20 consecutive runs (90% success rate) was considered the standard for having mastered the test. The error number (EN) of Y-maze runs each rat had before it mastered the test was recorded, lower numbers indicating higher learning ability. Another two kinds of indications for the learning test are the number of days (learning days) it took the rats to meet the learning standard and the total reaction time (TRT) for summing the reaction time each rat spent during per test day.

2.3. Glutamate analysis

After the learning tests were completed, the rats were anesthetized with 20% urethane (ethyl carbamate, $NH_2COOC_2H_5$) solution. The hippocampus was removed quickly, weighed, cooled in ice-cold 50 mM Tris–HCl buffer (pH 7.55) and homogenized. The homogenate was centrifuged for 10 min at 3000 rpm, the supernatant was used for glutamate assay. Glutamate concentration was measured using an A740 reagent kit (Nanjing Jianchen Biological Institute). Using the standard glutamate stock solution to produce standard curve, glutamate levels in the samples were detected by spectrophotometer at 340 nm, and were expressed as μmol/g prot.

2.4. Enzymes assay

Asparate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined according to the method of Reitman and Frankel (Reitman and Frankel, 1957). The tissue homogenate was centrifuged for 10 min at 2500 rpm and 3000 rpm, respectively, and then detected by spectrophotometer at 505 nm. These two enzyme activities were calculated from a standard graph and expressed as U/mg protein.

Glutamic acid decarboxylase (GAD) activity was measured by combining the enzyme extract and appropriate L-sodium glutamate (L-MSG), incubating the mixture at 37 °C for 60 min and terminating the reaction by boiling for 5 min. Then the samples were centrifuged and the produced GABA was analyzed by high performance liquid chromatography (Márquez et al., 1986). The activity was expressed as U/mg protein.

2.5. Statistical analyses

EN and learning days in the behavioral data were analyzed by nonparametric test followed by Chi-square test. The rest data were evaluated by one-way ANOVA followed by the least significant difference (LSD) test. The results were expressed as mean ± SE. Statistical significance was considered when $P < 0.05$ and $P < 0.01$.

3. Results

3.1. Blood F and Pb level

The levels of F and Pb in blood of offspring rats at week 6 were given in Table 1 with unit μg/dL. Fluoride levels in rats exposed to F and F plus Pb were up to 2.78 ± 0.28 μg/L ($P < 0.01$) and 2.90 ± 0.33 μg/L ($P < 0.01$), respectively, which markedly higher than that in control group. Moreover, significantly higher blood Pb levels were found in HiPb group (169.94 ± 4.42 μg/L, $P < 0.01$) and both together (132.28 ± 6.39 μg/L, $P < 0.01$) compared to control. These data evidently verified the establishment of the animal model in the present study.

3.2. Learning ability

F and Pb highly prolonged the learning time (Table 2). Rats in HiF group spent more than 2 days meeting the learning standard. The same result was found in HiPb group, while it required much more time for the animals exposed to both F and Pb. As for the

Table 2
Results of learning days, error number (EN) and total reaction time (TRT) of rats treated with high fluoride (HiF), high lead (HiPb) and high fluoride plus high lead (HiF + HiPb) (mean ± SE, $n = 8$ rats/group).

Week	Control	HiF	HiPb	HiF + HiPb
Learning day (day)				
6	2.33 ± 0.42	2.67 ± 0.56	3.17 ± 0.65	3.50 ± 0.50
8	1.33 ± 0.33	3.00 ± 0.68*	3.17 ± 0.48*	2.50 ± 0.50
10	1.17 ± 0.17	3.11 ± 0.39**	2.14 ± 0.26*	2.67 ± 0.21*
12	1.67 ± 0.33	2.75 ± 0.75*	2.40 ± 1.17*	2.43 ± 0.30*
EN (times)				
6	5.21 ± 0.94	5.25 ± 0.72	6.33 ± 0.86	5.33 ± 0.73
8	1.50 ± 0.67	5.00 ± 0.64**	4.83 ± 0.87*	4.54 ± 0.71*
10	2.71 ± 0.72	5.73 ± 0.60*	3.77 ± 0.65	5.52 ± 0.57*
12	4.39 ± 0.87	4.46 ± 0.81	5.24 ± 0.79	4.00 ± 0.90
TRT (s)				
6	120.68 ± 7.73	150.45 ± 10.62*	131.15 ± 9.26	164.86 ± 4.31**
8	94.91 ± 5.39	107.27 ± 9.67	97.25 ± 9.04	115.83 ± 7.85
10	105.80 ± 5.55	138.26 ± 6.94*	120.23 ± 6.63*	145.75 ± 4.00**
12	118.62 ± 5.87	123.04 ± 8.45	120.64 ± 7.83	135.41 ± 6.02*

Learning days mean the number of days (learning days) it took the rats to meet the learning standard. Error number is the number of wrong reactions, and described as times. Total reaction time refers to summing the reaction time each rat spent during per test day, and the unit is s.
* $p < 0.05$ (compared with the control group in all tables).
** $p < 0.01$ (compared with the control group in all tables).