EFFECTS OF FLUORIDE AND LEAD ON N-METHYL-D-ASPARTATE RECEPTOR 1 EXPRESSION IN THE HIPPOCAMPUS OF OFFSPRING RAT PUPS

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SUMMARY: To investigate whether excitotoxicity is involved in neurotoxicity of fluoride (F) alone and in combination with lead (Pb), the expression levels of the gene and protein N-methyl-D-aspartate receptor 1 (NMDAR1) in the hippocampus of offspring rat pups at postnatal days 14 and 28 exposed to F and/or Pb were determined by quantitative real-time polymerase chain reaction (QRT-PCR) and immunochemistry. 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. At day 14 and at day 28, 6 pups (female:male = 1:1) were chosen randomly from each group for further investigation. Results showed that at postnatal days 14 and 28, the expression levels of NMDAR1 gene were increased by 11% and 26%, respectively, in pups exposed to F compared to the controls, and the protein levels were also significantly enhanced. In pups exposed to Pb alone and to Pb plus F, the levels of the NMDAR1 gene and protein were decreased at postnatal day 14 and then increased at postnatal day 28. These results may provide evidence of a relationship between the excitotoxicity and neurotoxicity of F and/or Pb.

Keywords: Fluoride neurotoxicity; Gene expression; Hippocampus; Lead toxicity; N-methyl-D-aspartate receptor 1; Offspring rats; Protein expression.

INTRODUCTION

A growing number of epidemiological investigations[1-7] as well as clinical[8] and experimental studies[9-13] have demonstrated that F adversely affects the central nervous system (CNS). The neurotoxicity of F may be due, in part, to generation of free radicals and lipid peroxidation.[9,11-13] Blaylock has proposed that these processes may be involved a central mechanism called excitotoxicity,[14-15] which he considers to be a common mechanism in various metal-related neurotoxic reactions, including those of mercury, lead, cadmium, tin, and aluminum.[14-15] However, little is known about the actual relationship between F and excitotoxicity.

In addition, F in the form of sodium fluoride (NaF), fluorosilicic acid (H_2SiF_6), and sodium silicofluoride (Na_2SiF_6) has been found to increase the concentration of neurotoxicant lead (Pb) in water[16-17] and the accumulation of Pb in the body.[18-19] A recent study from China also reported that high blood Pb in children is statistically related to high underground water F.[20]

N-methyl-D-aspartate receptor 1 (NMDAR1) activation is believed to be involved in the process of excitotoxicity.[25] Accordingly, the present study was undertaken to investigate the effect of F and Pb administered alone or in

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combination on the gene and protein expression levels of NMDAR1 in the hippocampus of offspring rat pups.

MATERIALS AND METHODS

Establishment of animal model: Twelve adult Wistar albino rats (male:female = 1:2) were obtained from the Experimental Animal Center of Shanxi Medical University and kept in plastic cages in our laboratory with their standard diets. After one week, one male and two females were placed 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). In this experiment, the rats had free access to food and water under standard temperature (22–25ºC), 12/12-hr light/dark cycle, ventilation, and hygienic conditions. The study design was approved by the Institutional Animal Care and Use Committee of China.

Before postnatal day 14, the rat pups derived their nutrients only from maternal milk. After day 14, they gradually began to eat feed and drink water, concomitantly with suckling maternal milk. At the age of postnatal day 21, the pups ate and drank entirely by themselves. At postnatal day 14, three male and three female offspring rats were randomly selected from each group for testing. The rest of the offspring pups were exposed to F and/or Pb through water and maternal milk for 7 days till weaning at postnatal day 21. Then they were given the same treatments as the adult rats until testing at postnatal day 28.

Tissue preparation: At postnatal days 14 and 28, the rat pups after a 24-hr food-and-water fast were anesthetized with 20% urethane (ethyl carbamate, $NH_2COOC_2H_5$) solution. Blood was collected from the eyeball and stored at –80ºC for blood F and Pb determination by kinetic potentiometry using F ion-selective electrode and GFAAS (graphite furnace atomic absorption spectrometry), respectively. Additionally, to confirm the reliability of the data, the blood F was re-analyzed one year later after collection of the blood. The brains were quickly removed, and the left hemispheres of the hippocampus were separated and stored in liquid nitrogen for the gene expression test. The right hemispheres were fixed, dehydrated, and embedded in paraffin for immunohistochemical examination.

Total RNA extraction and QRT-PCR: The total RNA was extracted from hippocampus using the Trizol reagent (Invitrogen, USA) according to the manufacture's protocol, and was reverse transcribed using a TapMan Reverse Transcription Kit (Takara PrimeScript reagent, China). With Primer 3.0 plus, two pairs of specific primers (Table 1) were designed according to published rat cDNA gene sequences of NMDAR1 and β-actin which were aligned against the mice, rats and human genome with the Basic Local Alignment Search Tool (BLAST) of

the National Center for Biotechnology Information (NCBI). The specificity of the primers was tested by conventional reverse transcription polymerase chain reaction (RT-PCR) before being used for the QRT-PCR test. The expression level of NMDAR1 gene was quantified by real-time amplification of NMDAR1 gene and the house-keeping gene β-actin as a control from the above RNA preparation using the Mx3000P™ QRT-PCR system (Stratagene, USA) and two-Step SYBR® QRT-PCR kit (Takara, China). Thermocycling conditions were as follows: after initial denaturation at 95ºC for 15 sec, 40 PCR cycles were started with thermocycling conditions at 95ºC for 5 sec, 62ºC for 10 sec, and 72ºC for 6 sec, and then followed by the reaction melting curve analysis to verify the specificity of the amplified products. The Mx3000P™ QRT-PCR system provided the results of relative quantification of the QRT-PCR product.

Table 1. Primer sequences with their corresponding PCR product size

Gene	Primers (5'→3')	Primer locations	Product (base pairs)	Genebank accession No.
NMDAR1	ttcacagaagtgcgatctgg	2485-2504	108bp	NM_017010
	atggacagggaaacgttctg	2592-2611		
β-actin	agccatgtacgtagccatcc	471-490	115bp	NM_031144.2
	accctcatagatgggcacag	585-605		

Immunohistochemistry for NMDAR1: By using a rotary microtome, 5-μm sections of the hippocampus recoveries were attached to slides by treatment with APES (3-aminopropyl-triethoxysiloxane). After baking at 37ºC for 24 hr, the sections were stained with NMDAR1 immunohistochemistry kits (Wuhan Boster Bioengineering Co., China). Prior to incubation with the NMDAR1 antibody, the sections were deparaffinated and submitted to microwave treatment in 0.01 M citrate buffer solution (pH 6.0) for 20 min followed by quenching of endogenous peroxidase activity by immersion in 0.3% H_2O_2 for 30 min. Rabbit anti-rat NMDAR1 polyclonal antibody was diluted 1:100, and incubation was conducted overnight at 4ºC. Subsequently, sections were incubated with biotin-conjugated second antibody and streptavidin-peroxidase solution for 1 hr at room temperature. Finally, diaminobenzidine (DAB) solution, as a chromogen, was applied to color the NMDAR1 protein. The images of sections were captured by an upright microscope (model: BX51, OLYMPUS of Japan). Optical densities of the positive cells were determined by Image-Pro® Plus Version 5.1 micrograph analysis software (made in Media Cybernetics Inc. of America), using the relative quantification method of Wan et al.[45]

RESULTS

Blood F and Pb in rat pups at postnatal days 14 and 28: The levels of F and Pb in blood of the rat pups on postnatal days 14 and 28 are given in Table 2. Blood F and Pb showed increasing trends at day 14, but the changes were not statistically significant. On day 28 the blood F level in the HiF and HiF+HiPb groups was significantly higher than in the controls ($p=0.033$, $p=0.01$, respectively). A small increase occurred in the blood Pb level, but it was not statistically significant. Compared to the data on day 14, blood F in the control, HiF, HiPb, and HiF+HiPb

groups was significantly increased on day 28, and the p values were 0.008, 0.015, 0.00005, and 0.00025, respectively.

Table 2. Blood F (μg/mL) and Pb (μg/dL) in offspring rats at postnatal days 14 and 28 (mean±SD, n = 6)

	14th day		28th day	
	Blood F (μg/mL)	Blood Pb (μg/dL)	Blood F (μg/mL)	Blood Pb (μg/dL)
Control group	0.007±0.001	13.50±1.300	0.014±0.003‡	17.26±1.881
HiF group	0.009±0.004	14.55±2.202	0.042±0.025*†	17.69±1.303
HiPb group	0.007±0.001	16.65±3.038	0.020±0.007‡	20.18±3.230
HiF+HiPb group	0.008±0.001	16.32±3.355	0.034±0.014*‡	18.60±2.670

*p<0.05 HiF, HiPb, and HiF+HiPb groups compared with the control group.
†p<0.05, ‡p<0.01 compared with level at day 14.

Quantification of NMDAR1 gene expression: Figure 1 shows the standard curves of the NMDAR1 and β-actin genes, which were obtained by correlation of the Ct values (threshold cycles) with the dilution series of the two genes exhibiting a relatively low intra-assay variation. The linear regression equation, correlation coefficient, and amplification efficiencies of the NMDAR1 and β-actin genes are recorded in Figure 1.

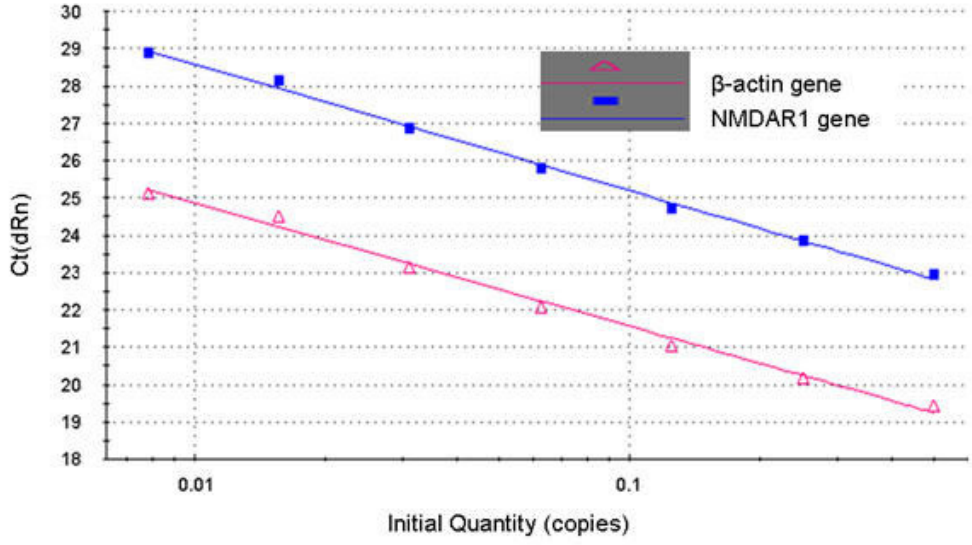

Figure 1. Standard curves for NMDAR1 and β-actin genes obtained by the correlation of the Ct values with the dilution series of the NMDAR1 and β-actin gene. Standard Curve of NMDAR1: Logfit values; SYBR Standards, RSq:0.996; SYBR, Y=-3.385*LOG(X) + 21.81, Eff.=97.4%. Standard Curve of β-actin: Logfit values; SYBR Standards, RSq:0.993; SYBR, Y=-3.313*LOG(X) + 18.25, Eff.=100.4%.

Specificity of QRT-PCR amplification was verified by the production of a single peak in melting curve analysis (Figure 2)

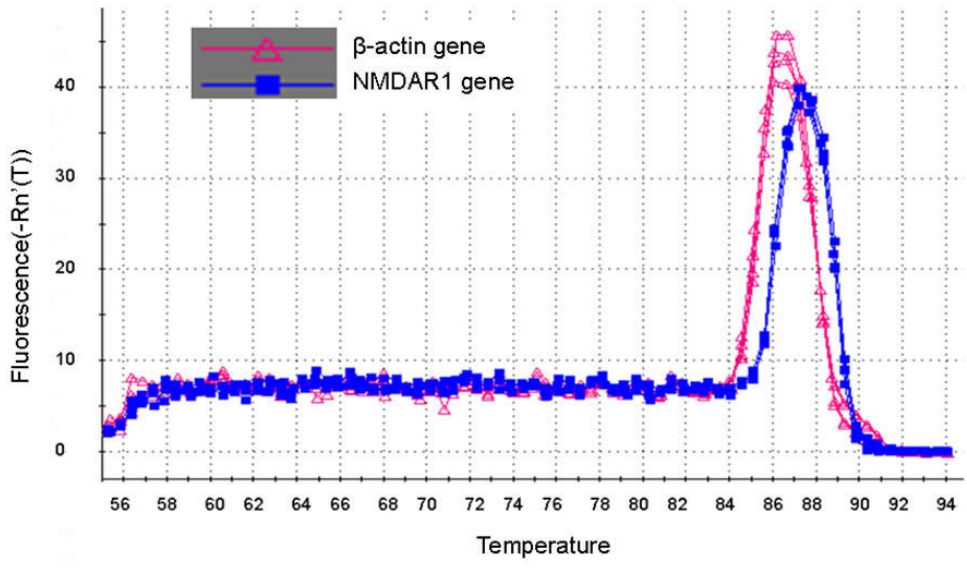

Figure 2. Melting curves for NMDAR1 and β-actin genes. The single peak at 87°C for NMDAR1 (second peak) and β-actin at 86°C (first peak) indicates that no other transcripts were amplified in the QRT-PCR.

The expression levels of NMDAR1 gene in the hippocampus of offspring rats at postnatal days 14 and 28 exposed to HiF, HiPb and HiF+HiPb are shown in Figures 3 and 4. The results indicated that at postnatal days 14 and 28 the NMDAR1 gene expression levels increased by 11% and 26% in the HiF group in comparison to the control. In the HiPb and HiF+HiPb groups, compared with controls, the gene expressions of NMDAR1 decreased by 13% and 15% at postnatal day 14, then increased by 72% and 22%, respectively, at postnatal day 28.

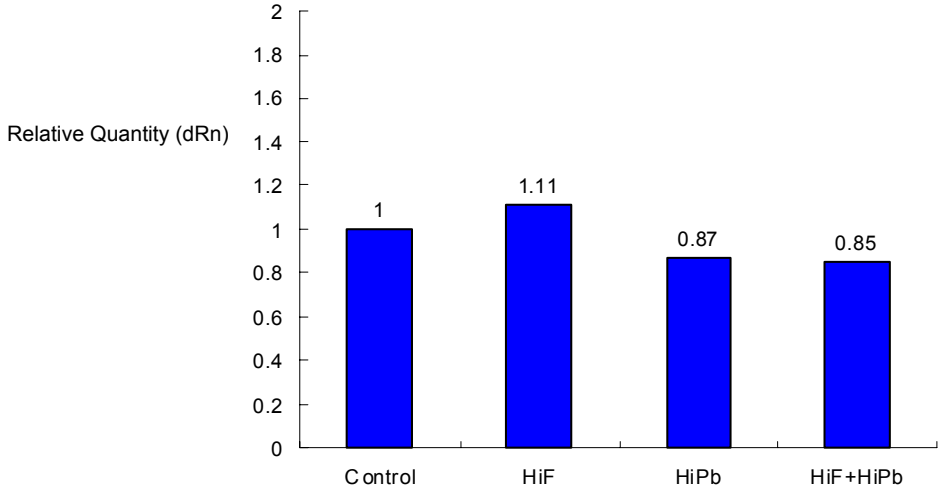

Figure 3. The gene expression of NMDAR1 in hippocampus of offspring rats at postnatal day 14 in control, HiF, HiPb and HiF+HiPb

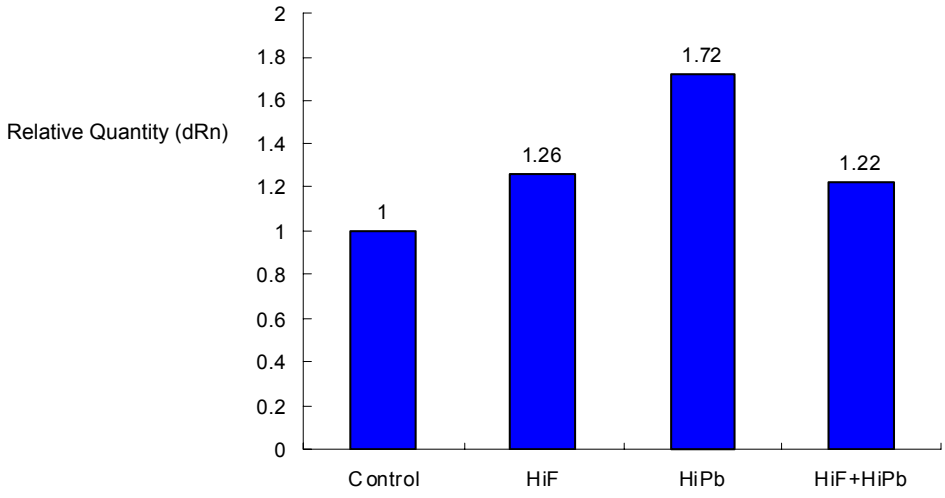

Figure 4. The gene expression of NMDAR1 in hippocampus of offspring rats at postnatal day 28 in control, HiF, HiPb and HiF+HiPb groups.

Immunohistochemistry for NMDAR1: Table 3 shows the protein expression levels of NMDAR1 in the hippocampus of the offspring rats. In comparison with the control group, NMDAR1 expressions were significantly increased in the HiF group ($p<0.05$) and decreased in HiF+HiPb groups ($p<0.01$), while no significance was observed in HiPb group at postnatal day 14. NMDAR1 levels were significantly increased in the HiF ($p<0.05$) and HiPb ($p<0.01$) groups at postnatal day 28. In the HiF+HiPb group the level of NMDAR1 was also increased, but it did not reach statistical significance.

Table 3. NMDAR1 expression measured as optical density in the hippocampus of offspring rats at postnatal days 14 and 28 (mean±SD, n = 6)

Day	Control group	HiF group	HiPb group	HiF+HiPb group
14	0.1803±0.0291	0.1925±0.0256*	0.1723±0.0323	0.1594±0.0121†
28	0.2498±0.0202	0.2774±0.0224*	0.3177±0.2088†	0.2645±0.0259

*$p<0.05$, † $p<0.01$ compared with the control group.

The immunohistochemistry images in Figure 5 illustrate the NMDAR1 expression in the CA3 region of the offspring rats at postnatal day 28 in different groups. The intensity of the staining reflects the expression level of protein for NMDAR1. By visual inspection, the staining intensity increased in the order: HiPb, HiF, and HiF+HiPb compared to the control group. The intensity of the staining is also indicated by the optical density data in Table 3.

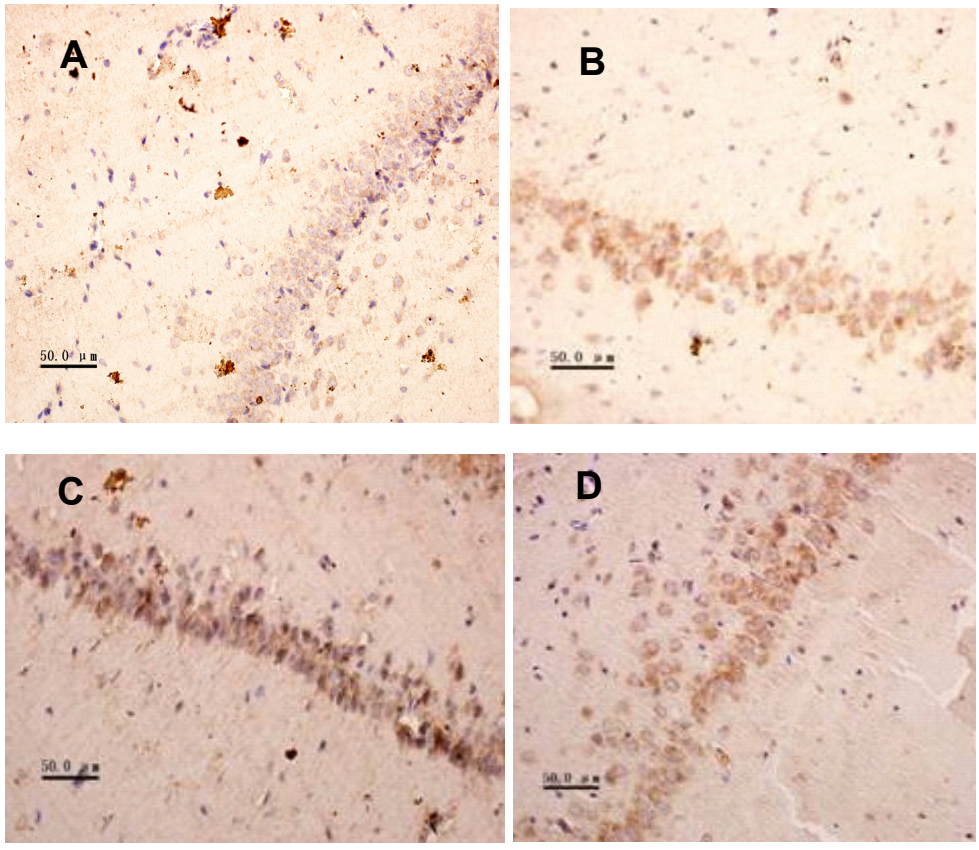

Figure 5. The photomicrograph (×400) shows the NMDAR1 expression in the CA3 region in hippocampus of offspring rats at postnatal day 28, and the brown granules are regarded as th protein expression of NMDAR1. Compared with control group (A), HiPb group (C), HiF grou (B), and HiF+HiLPb group (D) exhibit stronger staining intensity, accounting for the increase i NMDAR1 expression.

DISCUSSION

Animal model: In the present study, compared to controls, blood F in the experimental offspring rat groups increased slowly up to day 14 and significantly afterward at day 28, apparently because the ingestion pathway and exposure time were different. F was ingested by the rat pups exclusively from maternal milk through day 14 and additionally from drinking water beginning around day 21. As a result of drinking water containing 150 mg NaF/L and animal feed containing 11.5 mg F/kg, the blood F levels in the offspring rats at day 28 were markedly increased compared with the levels at day 14. In general, most F or Pb is excreted out of the body or deposited in organs such as bone and hair after entering into body.[21] Blood F and Pb to some extent reflect the temporary F and Pb body burden. In the present short-term study, although the changes in blood F exhibited statistical significance in the experimental groups, blood Pb only showed an increasing trend which may need more exposure time to reach significance level.

Effects of fluoride and/or lead on the gene expression of NMDAR1: The NMDA receptor is a very complex, ligand-gated receptor for glutamate neurotransmission.

This receptor, as an important membrane protein, is involved in physiological mechanisms, including synaptic plasticity and learning and memory,[22-24] as well as pathological conditions, such as excitotoxicity and epileptogenesis.[25] Native NMDA receptor has been reported to have two subunits which are NMDAR1 and NMDAR2A-D.[26-29] The NMDAR1 subunit is present in all NMDA receptors and is linked to the glycine activating subunit. Varying expressions of NMDAR2 subunit are necessary for glutamate recognition and can determine the strength of the NMDA receptor.[26,28,30-33] Additionally, over stimulation of the NMDA receptor may cause a series of intracellular signaling events resulting in activation of the excitotoxicity mechanism, which includes cell death, either through necrosis or apoptosis.[14-15]

In the present study, offspring rats exposed to F showed increased NMDAR1 mRNA levels at postnatal days 14 and 28 compared with those in control groups. A number of previous investigators have found that fluoride can induce cell apoptosis in brain,[34] lung,[35] kidney,[36] liver,[37-38] and bone tissues.[39-40] Furthermore, F is also closely associated with excitotoxicity-related processes, such as free radical production, lipid peroxidation, and microglial activation. Hence, speculatively, based on our results of activation of NMDAR1, F neurotoxicity may be implemented through excitotoxicity. In offspring rats exposed to Pb, the gene expression level of NMDAR1 at postnatal day 28 was also up-regulated, which is consistent with other reports.[41-42]

An important requirement of toxicology is the need to determine whether the components of a mixture share 'toxicologic similarity'. According to data from the HiF and HiPb groups, the neurotoxicity of both F and Pb may share the same mechanism, and such a hypothesis is also supported by our data from the HiF plus HiPb group.

Additionally, compared to the controls, the NMDAR1 gene expressions in the HiPb and HiF+HiPb groups were decreased by 13% and 15% at postnatal day 14 and then increased by 72% and 22% at postnatal day 28, respectively. In our view, the fluctuation of the data at different developmental stages may be due to the different pathway of Pb ingestion. At the age of postnatal day 14, rats absorbed F and Pb in limited amounts only from maternal milk, while at day 28 they ingested these neurotoxicants through maternal milk and drinking water for another 7 days before weaning and drinking water alone for 7 days after weaning.

Effects of fluoride and/or lead on the protein expression of NMDAR1: Although previous reports indicate that the amounts of mRNA for NMDAR1 do not seem to correlate well with the amounts of protein,[43] the changes of NMDAR1 gene expressions observed here are consistent with those of protein expressions. Moreover, no matter if NMDAR1 receptor is up-regulated or down-regulated, the changes in NMDAR1 expression could alter the proportion of subunit proteins and the functional properties of excitatory amino acid receptors, such as the NMDA and AMPA (alpha-amino-3-N-hydroxy-5-methylisoxazole-4-propionic acid) receptors.[44] Therefore our findings imply that F and Pb in drinking water, either individually or in combination, ingested from birth to postnatal days 14 and 28,

can adversely affect the expression of NMDAR1 in the hippocampus, thus contributing to central nervous system dysfunction.

In conclusion, the elevated expression levels of NMDAR1 on both genes and proteins indicate that the neurotoxicity mechanism of F and/or Pb may involve excitotoxicity. Additionally, more experimental studies are still necessary to provide further evidence for F-induced excitotoxicity.

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