

Proteome Alterations in Cortex of Mice Exposed to Fluoride and Lead

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Abstract Both fluoride and lead can cross the blood-brain barrier and produce toxic effects on the central neural system, resulting in low learning and memory abilities, especially in children. In order to identify the proteomic pattern in the cortex of young animals, from the beginning of fertilization to the age of postnatal day 56, pregnant female mice and pups were administrated with 150 mg sodium fluoride/L and/or 300 mg lead acetate/L in their drinking water. Two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MS) was applied to identify differently expressed protein spots. Results showed that there were eight proteins in the cortex that significantly changed, whose biological functions were involved in (1) energy metabolism (Ndufs1, Atp5h, Atp6v1b2), (2) cytoskeleton (Spna2, Tubal1a, Tubb2a), (3) glycation repair (Hdhd2), and (4) cell stress response (Hspa8). Based on the previous and current studies, ATPase, Spna2, and Hspa8 were shared by fluoride and lead both as common target molecules.

Keywords Cortex · Fluoride · Lead · Mice · Proteomics

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Introduction

Environmental lead exposure is considered to be a serious public health issue, especially because of its toxic effect on cognitive function. In many countries, stringent measures have been conducted for several decades to protect children from avoidable lead exposure. For example, in China, the 14-year ban of leaded gasoline has dramatically decreased lead pollution, but lead poisonings are still reported in children living around industrial areas. Data provided by the First Hospital of the Datong Coal Mine Group showed that the average blood lead level in children living a near coal mine area was up to 10.143 $\mu\text{g Pb/dL}$, indicating high lead exposure [1]. Recent studies from both China and the USA pointed out that fluoride ingestion could increase the body lead burden [2–4].

Additionally, fluoride itself can induce central nervous system dysfunction [5–8]. The neurological signs in human fluorosis were first reported by Roholm in 1937, which included overtiredness, lethargy, headache, and giddiness [9]. Up to now, a growing number of epidemiological studies, from China [4, 5, 10], India [11–14], Iran [15, 16], and Mexico [17], shows the negative association between fluoride exposure and children's intelligence. Therefore, the toxic effects of the lead and fluoride combination need further studies.

Following this clue, we recently established an animal model and found that fluoride and lead together significantly decreased the learning ability of rats [18]. Subsequently, we used a two-dimensional gel electrophoresis (2-DGE) to investigate the proteomic pattern in the hippocampus of mice and found nine differentially expressed proteins involved in four biological functions [19]. In order to detect the toxic effects on the cortex, another key tissue related to intelligence, the present experiment was conducted to select target proteins in the cortex in mice exposed to fluoride and lead.

Materials and Methods

Animals

Sixty Kunming mice (female:male=2:1), about 20 g in weight, were procured from the experimental animal center of Shanxi Medical University after obtaining approval from the Institutional Animal Care and Use Committee of Shanxi Agricultural University. All animals were kept in plastic cages. Additionally, they had free access to their standard diets (provided by Shanxi Medical University) and were maintained in a controlled environment of temperature 22–25 °C and 12/12-h light/dark cycle. After a week of acclimatization, each pair of two females and one male was put together, and vaginal plugs were checked daily in the morning. When the plug was established, the male was removed from the cage. Pregnant females were randomly divided into four groups of 10 each and orally exposed to fluoride and/or lead as follows: (1) control group (drinking double distilled water), (2) high fluoride (HiF) group (drinking distilled water containing 150 mg/L sodium fluoride), (3) high lead (HiPb) group (drinking distilled water containing 300 mg/L lead acetate), and (4) high fluoride plus high lead (HiF + HiPb) group (drinking distilled water containing 150 mg/L sodium fluoride plus 300 mg/L lead acetate). The day of parturition was considered to the postnatal day (PND) 0. Before PND 14, the pups were fed totally with maternal milk. Then pups gradually began to eat feed and drink water (the same as dams) by themselves, concomitantly with suckling maternal milk. On PND 21, pups were weaned and got the same treatment as their parental generation until PND 56. The doses of fluoride and lead were chosen according to our previous study [19].

Sample Preparation

Animals were weighed. Mice were anesthetized with 20 % urethane (ethyl carbamate, $\text{NH}_2\text{COOC}_2\text{H}_5$) solution at the age of PND 56. The cortex was quickly separated from the whole brain and stored at -80°C for future use. Fluoride and lead concentrations in the brain were detected by fluorine ion selective electrode and graphite furnace atomic absorption spectrometry, respectively. For 2-DGE, cortices were solubilized in 1 mL ice-cold lysis buffer containing 30 mM Tris-HCl, 7 M urea, 2 M thiourea, 4 % w/v CHAPS, and a protease inhibitor mixture, and then centrifuged at 3000 rpm for 10 min, incubated for 1 h at room temperature, and centrifuged again at 20,000g for 1 h at 4 °C. Protein concentration was measured by Bradford method.

2-DGE

Protein samples in the cortex from three mice of each group were separated in the first dimension by isoelectric focusing

(IEF). The protein samples of each 350 μL were rehydrated in a rehydrating tray and covered by a pH 4–7, 7 cm immobilized pH gradient (IPG) strip, for 15 h rehydration at room temperature. IEF was running in the following conditions: (1) 250 V for 1 h linear gradient, (2) 500 V for 1 h linear gradient, (3) 4000 V for 3 h linear gradient, (4) 4000 V rapid gradient until reaching total 20,000 Vh, and (5) 500 V rapid gradient for 24 h. Prior to the second dimension, the strip was equilibrated in solution A (50 mM Tris-HCl, pH 8.8 6 M urea, 2 % SDS, 30 % glycerol, and 1 % DTT) for 15 min and in solution B (50 mM Tris-HCl pH 8.8, 6 M urea, 2 % SDS, 30 % glycerol, and 4.5 % iodoacetamide) for another 15 min. Strips were transferred to 12 % SDS-PAGE gel for second-dimension electrophoresis. Electrophoresis run at 10 mA/gel for 30 min and then at 30 mA/gel until bromophenol blue reached the end of the gel. Following electrophoresis, gels were stained with Coomassie blue G-250 overnight, destained with 1 % glacial acetic acid, and washed with Milli-Q H_2O for three times. Gels were scanned by UMAX2100XL. Combining with automatic and subsequent manual procedures, spot detection, spot editing, background subtraction, and spot matching were measured by PDQuest 8.0 image analysis software (Bio-Rad, Hercules, CA). Briefly, three gels in the control group were matched, edited, and produced into a normalized gel for gel analysis. Compared with protein spots in the normalized gel of the control group, the relative quantities of protein spots in treatment groups were calculated. Then spots with the same trend in three treatment groups were chosen for the next mass spectrometry analysis.

In-gel Digestion and Mass Spectrometry Analysis

The selected protein spots were excised from the Coomassie stained gel. The gel piece was destained in a solution of 0.1 M ammonium bicarbonate and 50 % acetonitrile for three times of 30 min each. Subsequently, the gel piece was allowed to dry and rehydrated with the addition of modified trypsin in 4 μL 20 mM NH_4HCO_3 and incubated for 13 h at 37 °C. Peptides were extracted by 8 μL 5 % trifluoroacetic acid (TFA) solution for 1 h at 37 °C followed by the second time with 8 μL 2.5 % TFA for 1 h. The peptide-containing solution was dried and resuspended with 2 μL 0.5 % TFA. Then reconstruction was performed with a saturated solution of 50 % acetonitrile and 0.1 % TFA. After that, reconstructed samples were loaded on a Score 384 target plate. All mass spectra were acquired on a Bruker autoflex matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometer (MS; Bruker, Germany). Peptide mass fingerprints were generated by the reflectron mode with a 337-nm nitrogen laser, with an acceleration voltage of 20 kV and a reflected voltage of 23 kV.

The proteins were identified through MASCOT search engine (<http://www.matrixscience.com>) with the help of the entire National Center for Biotechnology Information (NCBI)

mouse database. The peptide error of the max molecular weight was set as 0.5 Da in the searching process, with the assumption that the peptides were monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. The functions of target proteins were annotated by using Molecule Annotation System 3.0 (MAS 3.0) (<http://bioinfo.capitalbio.com/mas3/>).

Statistical Analysis

By using GraphPad Prism5 software (GraphPad Software Inc., San Diego, USA), differences between groups were evaluated by one-way ANOVA with Dunnett as the post test. Experimental data was expressed as mean±SEM. Statistical significance was considered when $P<0.05$ and $P<0.01$.

Results

The Body Weight and the Index of Brain

In the HiPb and HiF + HiPb groups, the body weights of mice were significantly decreased compared with the controls. Fluoride alone reduced the body weight of mice; however, no statistical difference was observed (Fig. 1a).

The index of cortex was calculated by the ratio of cortex weight to body weight. Data in Fig. 1b showed that no significant difference was observed in the indexes of the cortex between the control and treatment groups.

Fluoride and Lead Contents in Brain

Figure 2 presented the contents of fluoride and lead in the brain of offspring mice exposed to the fluoride and/or lead for 56 days. Compared with the control, fluoride levels in the HiF ($P<0.01$) and HiF + HiPb ($P<0.01$) groups were significantly increased, and significant higher lead levels were found in lead-exposed group ($P<0.01$) and both together ($P<0.01$).

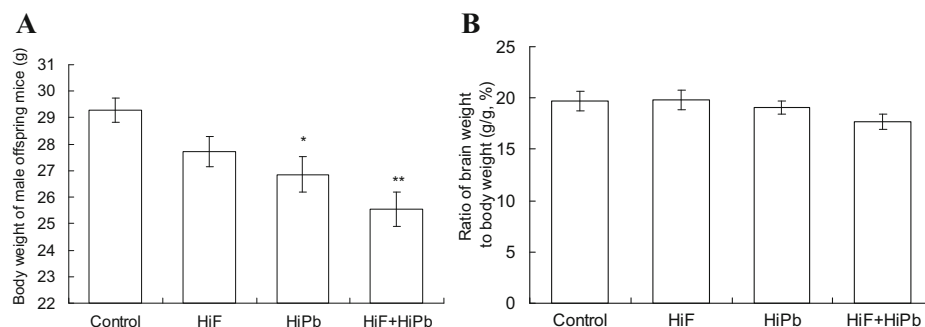


Fig. 1 **a** The body weight of mice in the control group, HiF group, HiPb group, and HiF + HiPb group. **b** The ratio of brain weight to body weight in the control group, HiF group, HiPb group, and HiF + HiPb group. Each

Protein Fractionation and Identification

To identify differently expressed proteins that responded to fluoride and/or lead, the proteome in the cortex of offspring male mice treated with fluoride and/or lead was compared with that in controls. The representative 2D gels of each group were showed in Fig. 3. The parameters of protein spots were analyzed by PDQuest software package. The spots in each group were well matched to the reference gel, and eight spots changed at least two folds with the same trend in the treatment groups compared to the controls. These eight spots were selected for the following mass spectrometry analysis.

Four of eight spots were significantly down-regulated, and the remaining four spots were dramatically up-regulated. The differentially expressed proteins were identified as spectrin alpha chain 2 (Spna2), NADH-ubiquinone oxidoreductase 75 kDa subunit (Ndufs1), heat shock cognate 71 kDa protein (Hspa8), ATP synthase subunit d (Atp5h), V-type proton ATPase subunit b (Atp6v1b2), haloacid dehalogenase-like hydrolase (Hdhd2), tubulin alpha-1A (Tuba1a), and tubulin beta-2A (Tubb2a). The isoelectric point and the molecular weight of all the identified protein spots in Table 1 were consistent with the spot position on the gel, as seen in Fig. 3.

Discussion

Fluoride and lead are two common environmental pollutants and neurotoxicants which are linked to the lowered intelligence, especially for children. It is well documented that both fluoride and lead can penetrate the blood-brain barrier and accumulate in the brain resulting in biochemical and functional changes in the nervous system [20, 21]. In this study, the levels of fluoride and lead in the brain were significantly higher in correspond treatment groups than those in controls. A large body of data from epidemiological and laboratory investigations supported that high fluoride exposure affected the growth of children and young animals. For example,

bar represents the mean±SEM ($n=6$). * $p<0.05$; ** $p<0.01$, significant differences compared with the control

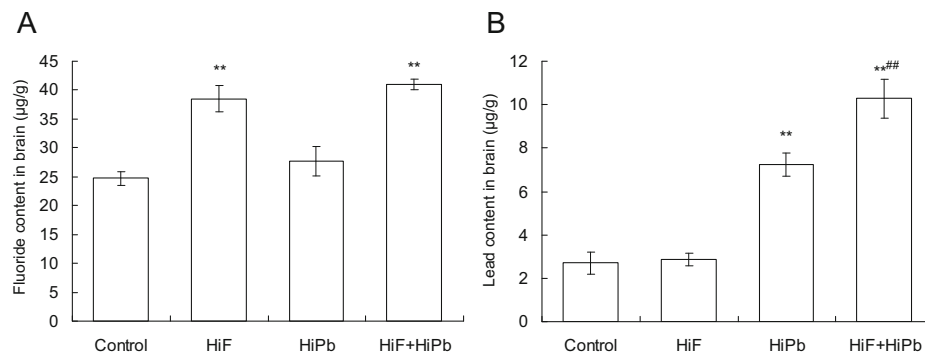


Fig. 2 **a** Fluoride content in the brain of mice from the control group, HiF group, HiPb group, and HiF + HiPb group. **b** Lead content in the brain of mice from the control group, HiF group, HiPb group, and HiF + HiPb

group. Each bar represents the mean±SEM ($n=6$), ** $p<0.01$, significant differences compared with the control; ## $p<0.01$, significant differences compared with lead group

children living in a village with 8.3 mg/L fluoride in ground water exhibited short height compared with children exposed to 0.5 mg/L fluoride [22]. Studies in mice [23], rats [24], and rabbit [25] reported the significant decrease in body weight. The inhibition in growth was also demonstrated by experiments focusing on lead toxicity [20]. Here, we observed that the ingestion of fluoride and lead significantly reduced the body weight of offspring mice, which is consistent with the

results of studies mentioned above. However, the ratio of brain weight to body weight presented no change between control and treatment groups, which was in contrast with the data of Wang et al. [26] who found an increase in rats at postnatal day 10 in the fluoride group. The different results might be due to variations in fluoride exposure time, dose, and animal.

With an attempt to identify the specific biochemical markers of fluoride and lead toxicity, 2D-PAGE combined

Fig. 3 Two-dimensional electrophoresis representative gels of cortex proteins from the control (a), HiF group (b), HiPb group (c), and HiF + HiPb group (d). Proteins were separated on 7 cm pH 4–7 IEF strips followed by SDS-PAGE and Coomassie blue G-250 staining. The proteins were then detected and compared using the PDQuest software package. Selected spots represented the cortex proteins with different levels (two folds) compared with the controls

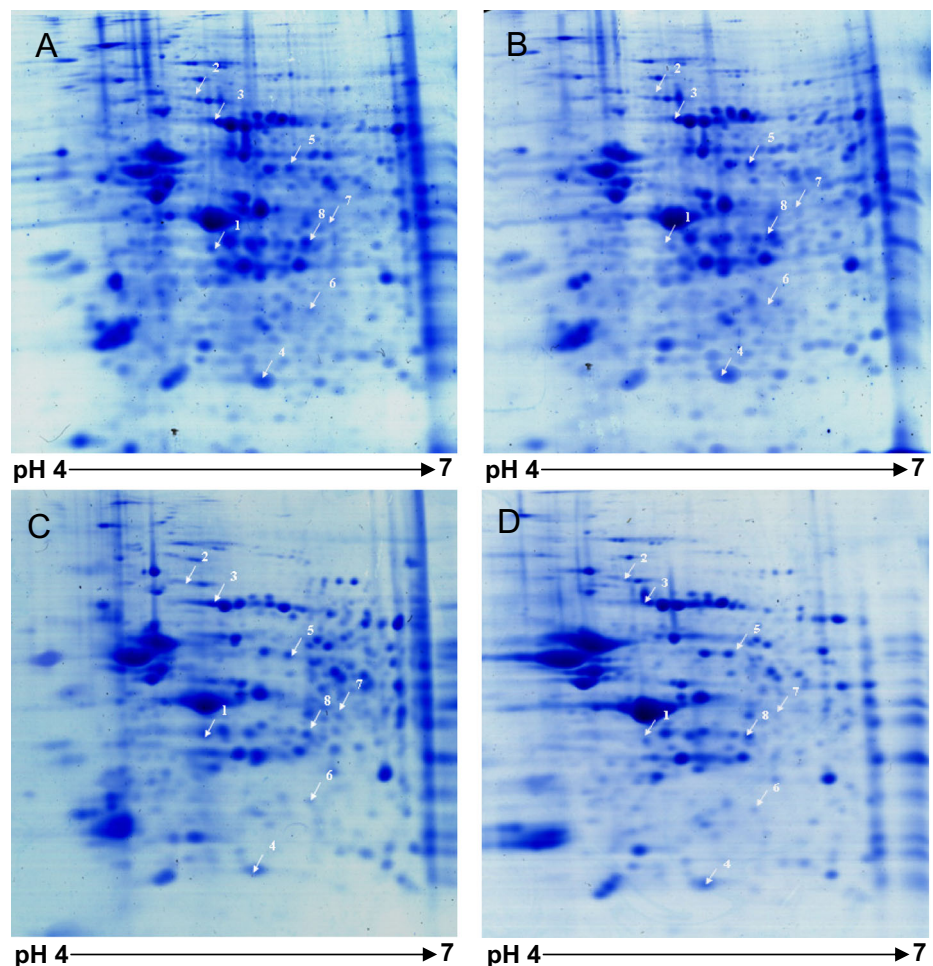


Table 1 Proteomics characteristics of identified protein spots differentially expressed between the control, HiF, HiPb, and HiF + HiPb groups using MALDI-TOF MS

Protein spots	GI accession no.	Molecular weight, isoelectric point	Pep. count	Protein score	Protein name
1	223462890	285,220.6, 5.2	33	715	Spna2 (spectrin alpha chain)
2	229892322	80,752, 5.51	16	229	Ndufs1 (NADH-ubiquinone oxidoreductase)
3	31981690	71,055.3, 5.37	19	547	Hspa8 (heat shock cognate 71 kDa protein)
4	21313679	18,794.6, 5.52	12	454	Atp5h (ATP synthase subunit d)
5	19705578	56,857, 5.57	25	971	Atp6v1b2 (V-type proton ATPase subunit b)
6	34849757	28,883.1, 5.7	9	534	Hdhd2 (haloacid dehalogenase-like hydrolase)
7	6755901	50,787.9, 4.94	13	413	Tuba1a (tubulin alpha-1A chain)
8	33859488	50,274.1, 4.078	23	884	Tubb2a (tubulin beta-2A chain)

with mass spectrometry was applied, and eight differential proteins were selected. They were classified into four categories including energy metabolism (Ndufs1, Atp5h, Atp6v1b2), cytoskeleton development (Spna2, Tuba1a, Tubb2a), glycation repair (Hdhd2), and cell stress response/chaperones (Hspa8), shown in Table 2.

Fluoride and Lead Induced Protein Changes in Energy Metabolism

ATP synthase is a complicated multisubunit protein and consists of two dimmers, a soluble globular F_1 catalytic sector and a membrane-bound F_0 proton-translocating sector [27]. In mitochondria, the two dimmers row in line and pump protons to produce ATP in living cells [28]. The impairment of functional F_0F_1 ATP synthase disturbs the ADP/ATP balance [29]. It had been reported that at least 25 different disease conditions, including severe

neurodegenerative diseases, were related to dysfunction of ATP synthase [30]. In this study, Atp5h, one of the subunits of F_0 [27], was significantly up-regulated by the fluoride and/or lead, while no increase in other subunit expression occurred, indicating an unbalance in the structure of ATP synthase. Ndufs1 is also known as sodium-pumping NADH: NADH-ubiquinone oxidoreductase. It is one of the main ion transporters in cell and the first enzyme in the respiratory chain, which pumps ion from NADH to ubiquinone to create a sodium gradient that is useful for ATP synthesis and other essential processes like nutrient transport and ionic homeostasis [31, 32]. In the present study, we observed a significant decrease in Ndufs1 in fluoride and/or lead groups. It was easy for us to presume that the abnormal ATP synthase and decreased Ndufs1 expression induced by fluoride and lead may result in a low ATP synthesis. Our previous proteomic study on the hippocampus also found the elevated ATPase

Table 2 Functionalities of identified proteins in the cortex of mice in the control, HiF, HiPb, and HiF + HiPb groups

Function	Proteins	Biological process	Molecular functions	Expression different
Energy metabolism	Ndufs1	ATP synthesis; nutrient transport; ionic homeostasis	Maintaining a sodium gradient	Down-regulated
	Atp5h	ATP synthesis coupled proton transport	Hydrogen ion transporter activity	Up-regulated
	Atp6v1b2	Protein catabolism; ATP metabolism; ion transport	Hydrolase activity; protein binding	Up-regulated
Cell cycle/chaperones	Hspa8	Protein folding; chaperone cofactor-dependent protein folding; regulation of cell cycle; response to stress	Unfolded protein binding; ATPase activity	Up-regulated
Cytoskeleton development	Spna2	Neuronal membrane integrity	Protein binding	Up-regulated
	Tuba1a	Microtubule-based movement; polymerization	Structural molecule activity; GTP binding	Down-regulated
	Tubb2a	Microtubule-based movement; polymerization	Protein binding	Down-regulated
Glycation repair	Hdhd2	Phosphatase activity	Hydrolyze β -D-glucose-1-P	Down-regulated

level and the adversely affected energy metabolism caused by fluoride and lead [19], implying that energy imbalance may be shared by the poisoning of these two elements.

Fluoride and Lead Induced Protein Changes in Cytoskeleton

Tubulin proteins, which form the microtubule cytoskeleton, are related to neuronal differentiation, migration, and axon guidance. Two dimmers, α -tubulin and β -tubulin, compose the polymer of tubulin [33]. Clinical investigations reported that patients with *Tuba1a* mutation clinically presented severe motor and intellectual disabilities and seizures and exhibited the complete loss of gyri and sulci [34]. It was also reported that *Tuba1a* mutation resulted in 1–4 % of classic lissencephaly among which 30 % with cerebellar hypoplasia [35]. In this study, fluoride and/or lead significantly reduced the expression of *Tuba1a* and *Tubb2a*, which can provide a strong evidence for explaining the decreased learning abilities and abnormal locomotor behaviors in mice [19] and rats [18] exposed to both fluoride and lead in our previous studies. Additionally, another cytoskeletal protein, *Spna2*, which is a protein that functions in maintaining neuronal membrane integrity, was found to be enhanced in both the current study and our previous proteomic one on hippocampus [19]. So far as we know, there are four proteins (*Dpysl2*, *Spna2*, *Tuba1a*, and *Tubb2a*) related to cytoskeleton found to be the potential molecular targets of fluoride and lead.

Fluoride and Lead Induced Protein Changes in Glycation Repair

Haloacid dehalogenase-like hydrolases are a large superfamily of small-molecule enzymes existing in diverse organisms, including bacteria, archaea, and eukaryotes [36]. Up to now, there are 6805 proteins belonging to this superfamily in databases [37]. The functions of most of these proteins have not been characterized, and only a few of them are involved in phosphatase, β -phosphoglucuronidase, phosphonate, and dehalogenase activities [38]. *Hdhd2* is one of the members in this superfamily and functions in glycation repair by hydrolyzing β -D-glucose-1-P [37]. Here, we found that fluoride and lead significantly reduced the expression of *Hdhd2*, suggesting a low defensive ability to prevent the deleterious effects of phosphorylated aldoses.

In conclusion, the proteomic analysis of both cortex in this study and hippocampus in our previous study [19] suggested that ATPase, *Spna2*, and *Hspa8* were target molecules shared by fluoride and lead, which are involved in energy metabolism, cytoskeleton, and cell stress. There is no doubt that further investigations are required to detect the detail

mechanisms of these molecules in the pathological process of fluoride and lead poisoning.

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