A Dissertation in Partial Fulfillment of the Requirements for the Degree of Master

Studies of Relationships Between the Polymorphism of COMT Gene and Plasma Proteomic Profiling and Children's Intelligence in High Fluoride Areas

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Abstract

Fluoride (F) widely exists in environment with a significant increase in body burden accumulation in recent years. Internal exposure to F due to diet including food, water, fluoride-containing dental products like toothpaste and fluoride supplements, is extensively researched. Additionally, industrial pollution and coal burning have also been reported to be major sources of internal F exposure, especially in China.

It is known that excessive fluoride intake over a long period of time may result in a serious public health problem called fluorosis. In addition to well-known effects of fluorine on skeleton and teeth, the neurotoxicity of F was also confirmed. Roholm et al found that exposure to fluoride can cause bad effect on the central nervous system in rats, generate significant reduction in myelinated nerve fibers, external granular layer in cerebellum, and increase neuronal apoptosis. In addition, alternations in the density of neurons and in the number of undifferentiated neurons were observed in the brains of fetuses therapeutically aborted in an area characterized by endemic fluorosis. It has been indicated that the severity of the adverse effects of fluoride on the behavior of rats is directly correlated to the concentrations of this ion in plasma and in specific regions of the brain. A growing number of epidemiological studies reported that the levels of mental work capacity and the Intelligence Quotient (IQ) for children who were born and raised in the areas with endemic fluorosis were found to be lower than normal.

F concentration in drinking water is long-term stable in years in a certain region. Once absorbed into the blood, fluoride readily distributes throughout the body, with the greatest amount retained in calcium-rich areas such as bone and teeth (dentine and enamel). In infants, about 80-90% of the absorbed fluoride is retained; the other is excreted primarily via the urine. Therefore, urine F and serum F concentrations as the internal exposure index can systematically reflect the burden of F exposure in drinking water. In this study, we measured children's intelligence quotient IQ) using Combined Raven's Test for Rural China (CRT-RC) and determined fluorine concentrations of serum and urine employing

fluorine ion selective electrode, and investigated the correlations between the urine F and serum F concentrations and the children's IQ.

Studies suggested that the neurotransmitter dopamine plays an important role in human cognition. Computational modeling studies indicated that dysfunction in dopamine systems accounts for abnormal cognitive control in the prefrontal cortex. Catechol O-methyltransferase (COMT) is the major mammalian enzyme involved in the metabolic degradation of released dopamine and accounts for more than 60% of the metabolic degradation of dopamine in the frontal cortex. It is therefore plausible that genetic factors that affect COMT function may significantly influence cognition through affecting dopaminergic function. The COMT gene contains a functional polymorphism that codes for a substitution of methionine *(met)* for valine *(vai)* at codon 158. The *met* allele is thermo labile and has one-fourth the enzymatic activity of the *vai* allele. Egan and his colleagues found that there was a relationship between the COMT vall58met polymorphism and cognitive function. However, the relationship between the COMT val158met polymorphism and cognitive function of children in fluoride area has not been reported.

At present, the study of biomarkers of children with mental retardation is rare. Studies have shown that thyroid hormone levels can be used as a sensitive indicator of detection of mental retardation. As direct executor of life function, proteins can directly reflect the physiological process relative to genes. Therefore, we can make use of the proteomics approach to study changes of certain protein or peptide level in the development process of fluorosis to find the abnormal expressed proteins so as to provide certain basis for identifying the biological markers of fluorosis children.

Part I – Effect of high F drinking water on children's IQ

Objective: To investigate the relationships among the children's serum F, urine F, thyroid hormone levels and children's IQ in the high fluoride areas.

Methods: We collected the samples of the drinking water, urine and blood and measured the F concentrations, the levels of thyroid hormone and children's IQ using F ion selective electrode, radioimmunoassay kit (RIA) and the CRT-RC.

Results: The concentrations of urine F and serum F, TSH values and IQ scores were significantly different in children in the high fluoride group compared to those in the control group (P <0.05). Spearman rank correlation analysis showed negative correlations between urine F, serum F concentrations and children's IQ scores (r_S = 0.206, P <0.01, r_S = O.187, P <0.05).

Conclusions: The long-term intake of excessive fluoride can affect children's IQ.

Part II – The relationship between COMT gene polymorphism and children's IQ

Objective: To explore the relationship between COMT val158met polymorphism and children's IQ.

Methods: Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods were used to discern the relationship between the functional polymorphism of COMT and children's IQ.

Results: Children with the high-activity val/val genotype showed significantly higher IQ scores on CRT-RC Test than those with low-activity genotype.

Conclusions: The val/val genotype may have a protective effect on cognitive performance in children.

Part III – Proteomic analysis of plasma in children chronically exposed to fluoride

Objective: This study was to investigate the differences in plasma proteomic profiling between children of high F exposure and controls.

Methods: 10 children of high fluoride exposure and 10 children from the control group were selected, respectively. Plasma proteomic profiling was analyzed by formatrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-TOF MS) technology.

Results: At the high F group, we found 7 differentially expressed protein spots and give proteins were successfully identified, which were found to be unregulated.

Conclusions: The alterations of the plasma protein expression patterns provide certain basis for seeking sensitive markers of fluorosis children.

Key words: fluorine; children's IQ; COMT gene polymorphism; plasma proteomic; PCR-RFLP; 2-DE; MALDI-TOF-TOF-MS

Part I: Effect of High Fluoride Drinking Water on Children's IQ

Clinical and experimental research has shown that fluoride can cross the blood-brain barrier and collect in brain tissue, resulting in changes to brain morphology and function that influence intellectual development. [24] Exposure to fluoride is related to decline in child intelligence.[25-29] At present, some people believe that levels of the thyroid-stimulating hormone (TSH) can be used as an direct indicator of child intellectual ability. Therefore, this research tests the IQ scores, blood serum and urine fluoride concentrations, and blood serum levels of triiodothyronine (T_3), thyroxine (T_4), and TSH of a high-fluoride group and a control, in order to investigate the relationship between IQ score, fluoride exposure, and levels of thyroid-relevant hormones.

Materials and Method

1 Instruments and Reagents

1.1 Major Instruments
(Omitted)

1.1 Major Reagents
(Omitted)

2. Primary Method

2.1 Research Subjects

The city of Tianjin is fairly representative of fluorosis in China. [30] Controlling for societal, economic, educational, geographical, and environmental factors, we randomly selected 68 Grade 5 students from areas in Tianjin with drinking water fluoride concentrations lower than 1.0 mg/L as our control group, and 55 Grade 5 students from areas with fluoride concentrations higher than 1.0 mg/L as our high fluoride group, for a total of 123 subjects. After informing the parents or guardians with a written notice, we carried out IQ testing and took water, blood, and urine samples.

2.2 Sample Collection
(Omitted)

2.3 IQ Testing

IQ testing was carried out using the *Chinese Combined Raven's Test Picture Book*, and IQ score determined using rural children IQ norms (CRT-C_2).

The rural children IQ norms for the *Chinese Combined Raven's Test* (CRT-C_2) were revised from the original Raven test norms by the technology group headed by Professor Wang Dong in the Institute of Endocrinology at Tianjin Medical University. The Raven test is an easily-applied and economically practical IQ test; it is popular among researchers due to the fact that it can be done quickly, in groups, and is not limited by the subject's culture, race, or language.

The testing was carried out in a quiet classroom. After the testers gave instructions, the children had 40 minutes to individually complete the exercises in the picture book. An IQ score for each child was calculated using the rural children IQ norms. IQ is measured on a single scale which can be adjusted based on age. Based on the CRT-RC instruction manual the standard scale was divided into 7 ranges: intellectually disabled (IQ < 69), borderline (IQ 70-79), below average (IQ 80-89), average (IQ 90-109), above average (110-119), outstanding (IQ 120-129), extremely outstanding (IQ > 130).[31]

2.4 Reagent preparation
(Omitted)

2.5 Method

2.5.1 Drinking Water Fluoride Concentration Testing
(Omitted)

2.5.2 Urine Fluoride Concentration Testing
(Omitted)

2.5.3 Blood Serum Fluoride Concentration Testing
(Omitted)

2.5.4 Blood Serum T_3 Concentration Testing
(Omitted)

2.5.5 Blood Serum T_4 Concentration Testing
(Omitted)

2.5.6 Blood Serum TSH Concentration Testing
(Omitted)

2.6 Statistical Analysis

The SPSS 11.1 software package was used. The two groups were compared using *t*-tests, and the associations between variables were analyzed using linear correlation. P < 0.05 was taken as the standard for statistical significance.

Results

1. IQ score of children in control and high-fluoride group

The average ages of the children in the control and high-fluoride groups were 11.3 and 11.6, respectively; there is no statistically significant difference between the ages (P >

0.05). The difference between the IQ scores of the two groups is significant ($P < 0.001$), see Table 1-4. With respect to gender, the difference between IQ scores of females in the high-fluoride group and the IQ scores of females in the control group is statistically significant ($P < 0.001$), but the IQ score difference for males is not significant ($P > 0.05$), see Table 1-5. If all the children in both groups are considered, there is no statistically significant difference between the genders with respect to IQ score, see Table 1-6. In the control group, 47.1% of the children have IQ scores which puts them in the outstanding range or above (IQ > 120), whereas this is true for only 21.8% of the high-fluoride group. The peak of the IQ score distribution for the control group is in the 110-119 range (29%), but for the high-fluoride group it is in the 90-109 range (40%). In the high-fluoride group, 9.1% of the children have IQs in the below average range, but this is true for only 2.9% of the children in the control group. See Figure 1-1.

Table 1-4:IQ score of children in control and high-fluoride group

Group	*n*	IQ Score ($x \pm s$)
Control	68	117.75 ± 12.744
High-fluoride	55	108.69 ± 13.514**
Compared with control ** P < 0.001		

Table 1-5: IQ score of children in control and high-fluoride group by gender

	IQ Score ($x \pm s$)	
Group	male	female
Control	115.69 ± 14.573	119.28 ± 11.145
High fluoride	111.63 ± 10.547	105.86 ± 15.529**
Compared with control ** P < 0.001		

Table 1-6: IQ score of children by gender

Group	*n*	IQ Score ($x \pm s$)
male	56	113.73 ± 12.841
female	67	113.67 ± 14.652**
Compared with control ** P < 0.001		

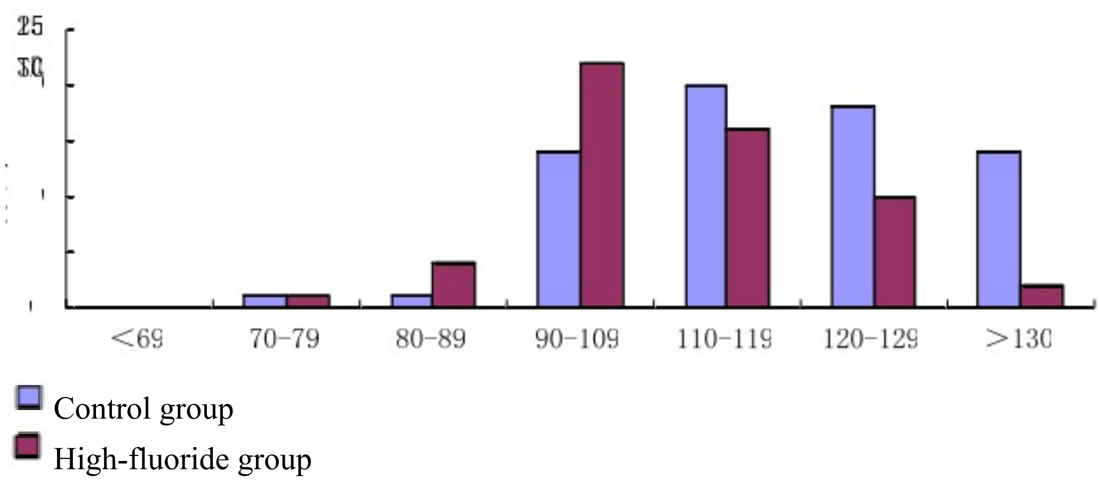

Figure 1-1: IQ distribution

2. Concentration of fluoride in drinking water, urine, and blood serum for high-fluoride and control groups

The drinking water fluoride concentration of the high-fluoride group was 1.40 + 1.30 mg/L, and for the control group it was 0.63 ± 0.07 mg/L, a statistically significant difference ($P < 0.05$).

For the high-fluoride group, the urine fluoride concentration (2.19 ± 0.93 mg/L) and blood serum fluoride concentration (0.20 + 0.09 mg/L) are clearly higher than the control ($P < 0.001$).

Table 1-7: Concentration of fluoride in drinking water, urine, and blood serum for high-fluoride and control groups (x ± s, mg/L)				
Group	*n*	Drinking Water	Urine	Blood Serum
Control	68	0.63 ± 0.07	0.80 ± 0.29	0.04 + 0.04
High fluoride	55	1.40 ± 1.30*	2.19 ± 0.93**	0.20 + 0.09**
Compared with control, *P < 0.05, **P < 0.001				

3. Concentration of blood serum T_3, T_4, and TSH for high-fluoride and control groups

The difference in blood serum TSH between the high-fluoride (2.67 ± 0.67 mg/L) and control groups (3.7 ± 2.12 mg/L) is statistically significant, but the differences in blood serum T_3 (2.22 ± 0.37 mg/L vs. 2.15 ± 0.43 mg/L) and T_4 (85.7 ± 17.11 vs. 90.8 ± 11.92) between the two groups is not statistically significant, see Table 1-8.

Table 1-8: Concentration of blood serum T_3, T_4, and TSH for high-fluoride and control groups (x ± s, ng/L)			
Group	T_3	T_4	TSH
Control	2.15 ± 0.43	90.8 ± 11.92	2.67 ± 0.67
High fluoride	2.22 ± 0.37	85.7 ± 17.11	3.7 ± 2.12*
Compared with control, * P <0.05			

4 Correlation analysis

A Spearman rank correlation analysis reveals a positive correlation between drinking water, urine, and blood serum fluoride concentrations (r_S=0.372, $P<0.05$; r_S=0.2378, $P<0.05$). There is a clear negative correlation between IQ score and urine and blood serum fluoride concentrations (r_S=-0.155, $P<0.05$; r_S=-0.154, $P<0.05$).

Conclusion

A growing body of research shows that exposure to high levels of fluoride has toxic effect on the child nerve development. A search on PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) looking for work on the relationship between fluoride and intelligence reveals that relevant research is only taking place in China; it is very rare in Western countries.

This study found that the IQ of children living in high fluoride areas is markedly lower than those in normal areas, which is consistent with the results of Trivedi MH et al.[34] An analysis of the data from these two areas indicates that both blood serum fluoride concentrations and urine fluoride concentrations are related to childhood IQ; that is, as fluoride in the blood serum and urine increases, child IQ decreases. Although some researchers have claimed that thyroid hormone levels can be used as indicators of IQ, in our research we found no significant differences between the high fluoride group and the control group with respect to blood serum T_3 and T_4 levels. While there were significant differences with respect to blood serum TSH, both groups were within the normal range; this effect could be due to inhibition of synthesis and secretion of thyroid peroxidase and thyroid hormones in the high-fluoride group, which, via a negative feedback mechanism, promotes secretion of thyrotropin-releasing hormone (TRH), which also increases secretion of TSH. Fluoride can pass through the blood-brain barrier, affecting the nervous system of infants and children.[35-37] Guan and others have found that fluoride ingested by pregnant mothers can damage the nervous system of the fetus.[38-39] Wang et al [40] tested 4-7 year old children with the WPPSI (Wechsler Preschool and Primary Scale of Intelligence) test and found a relationship between high fluoride and behavioral intelligence but not verbal intelligence. The CRT-RC tests attention and logical thinking; it does not test verbal ability. [41-42] Our research indicates the blood serum fluoride concentration influences behavioral intelligence, but we cannot speak to the question of verbal intelligence; as such, our results are consistent with previous research such as that of Wang and Guo.[40,43]

In summary, we found that negative correlations exist between child IQ and both blood serum and urine fluoride concentrations in areas with fluoride poisoning. Further research should be carried out in other populations in order to confirm that the relationship between fluoride intake and IQ exists independent of effects such as household income or parental educational level. Also, we also need to expand the sample size to ensure that our results are accurate.

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