Environmental fluoride exposure and reproductive hormones in male living in endemic fluorosis villages in China

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Abstract: Objective To explore the influence of high fluoride exposure on reproductive hormones in male living in endemic fluorosis villages in China. Methods A cross sectional study was conducted in Tongxu county in Henan Province, China. Endemic fluorosis villages and control villages were selected by random sampling according to fluoride concentration in drinking water and the prevalence of endemic fluorosis. Local male residents aged from 18 to 50 years old who were born and grew up in the investigated villages were recruited as subjects by cluster sampling. Fasting blood and urine samples were collected. The serum level of GnRH was detected using ELISA. The serum level of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E_2), and testosterone (T) were determined by chemiluminesence immunoassay (CLIA). Results The serum levels of FSH were 7.82 mlU/ml, 10.20 mIU/ml and 9.57 mIU/ml in male from defluoridation villages (DFPG), high fluoride villages (HFG) and control villages (CG) respectively. FSH level in male from DFPG was significant lower than that from HFG and CG (P<0.05). The serum levels of E_2 were 33.67 ng/mL, 29.17 ng/mL and 28.99 ng/mL in DPFG, HFG and CG respectively. E_2 level in male from DFPG was significant higher than that from HFG and CG (P<0.05). Serum levels of E_2 in CG were associated with LH (r=0.343, P=0.000), age (r=0.195, P=0.015), and inversely associated with serum FSH (r=-0.237, P=0.003), whereas this correlation was not observed for serum E_2 level in DFPG and HFG. Conclusion Long-term fluoride exposure in drinking water may influence the reproductive hormones in males living in endemic fluorosis villages.
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1.Introduction

Fluorine is a necessary element to human body and plays a key role in the prevention and control of dental caries. However, fluoride has a tendency to accumulate in the organisms if the exposure persists over time, even at low concentration (Kebsch et al., 2007). Dental and skeletal lesions are the major clinical feature of endemic fluorosis, which is an important public health concern worldwide and caused by chronic persistent fluoride exposure through ingestion or inhalation and most commonly occurs as a result of high fluoride levels in drinking water or industrial exposure from fumes or dust. It is currently estimated that approximately 43 million children have dental fluorosis and over 2 million adults have skeletal fluorosis in China (Yang and Heng, 2009). Excessive fluoride intake can also affect hormone secreting and soft tissues such as liver, kidney, brain, reproductive, pancreas and so on (Tiainen et al., 2011; Susheela et al., 2005; Ma et al., 2008; Lu et al., 2010). Many researches have been conducted to explore the relationship between fluoride ingestion and reproductive structure or function since 1980. The reported reproductive toxic effects include increases in numbers of abnormal spermatozoa, loss of spermatogenesis in rats, decreased sperm quality and quantity in rats and mice, and decreased reproductive output in mice. Our previous studies also showed that exposed to fluoride in drinking water suppressed rats reproductive ability by affecting sperm quality and quantity, sperm structure and reducing activity of the enzymes (Jiang et al., 2005; Xu et al., 2005).

The neuroendocrine system of the hypothalamus-pituitary-gonad (HPG) axis regulates reproduction in vertebrates and can be influenced by chemicals, therefore affects the reproductive system. Ma et al. observed in the experiment in male rats that fluoride could affect hormone levels of each layer of the hypothalamus-hypophysis-testis axis, and then may disturb the reproductive endocrine function (Ma et al., 2008). However, most of the studies used

animal models, primarily rodents, and often gave high daily doses for short durations to evaluate structural or functional alterations in the male reproductive tract associated with fluoride (Rico et al., 1991; Raymakers et al., 1990). Fewer studies involved in human populations. We conducted, here, a cross sectional study to explore the relationship between sex hormone levels and fluoride exposure in male residents who lived in the high fluoride areas.

2.Materials and methods

2.1.Location and population

A corss sectional study was conducted in six villages of Tongxu county in Henan Province, China in 2011 by using simple cluster sampling method. It included three endemic fluorosis villages and three non-endemic fluorosis villages. Endemic fluorosis villages were defined as villages with fluoride levels exceeding 1.0 mg/L (Chinese water quality standard) in drinking water. Two of the three villages were conducted defluoridation project of drinking water at the end of 2008. Non-endemic fluorosis villages were defined as villages with fluoride levels of less than 1.0 mg/L in drinking water. There were no significant differences in the natural environment, socioeconomic status, life styles, dietary habit, and other demographic characteristics among the six villages. A total of 354 male residents who were aged between 18 and 50 years and who were born and grew in the six villages were considered eligible for the study. Among them, 55 male residents were excluded from the study because of working in the cities as migrant workers over five years. A total of 299 participants met the inclusion criteria in this study with participation rate of 84.5%. They were divided into three groups, HFG, DFPG and CG. Upon receiving their written consent, an in-person interview was conducted at the village clinics using a standardized and structured questionnaire to collect information on demographic factors, medical conditions, marriage status, and medication use including supplemental vitamins, reproductive history, smoking and alcohol consumption, the main source of heating and cooking fuel and dietary intakes. Each subject provided two 5 mL fasting blood samples and 50 mL of instant urine sample. Blood was collected in red top vacuum tubes, and placed immediately on ice. After centrifugation, serum and white blood cells were separated and frozen at -70℃ for subsequent analyses. All procedures were approved by the Institutional Review Board at Zhengzhou University, China.

2.2.Detection of fluoride exposure levels

The concentration of environmental fluoride exposure including air, drinking water, soil, vegetables, grain including wheat and corn, as well as fluoride levels in the urine samples were detected by fluoride ion selective electrode (Shanghai Exactitude Instrument Company, China).

2.3.Detection of serum hormone levels

All the serum samples were stored in a -70℃ freezer and had no thaws prior to assay for hormone levels. GnRH was determined by ELISA. LH, FSH, E_2 and T were detected using CLIA. The CLIA test kit and Chemiluminescence Apparatus were provided by Autobio Company (LUmo Luminometer, Autobio Labtec Instruments Co. Ltd., Zhengzhou, China). Each sample was run in duplicate and 10% of total samples were retested randomly. The intra- and inter-assay coefficients of variation were less than 10% for these assays.

2.4.Data analysis

The database was established using Epidata 3.0 software (Epidata 3.0 for windows, Epidata Association Odense, Denmark) and all the data was doubled entered into the database by different people. Kolmogorov-Smirnov test and Levene test were used to inspect the normality and homogeneity of variance of all data. Fluoride level in urine and hormone levels of LH, FSH and T in serum was logarithmically-transformed to achieve approximately normal distribution, and the transformed values were used in data analyses. Differences of fluoride in urine and hormone levels of LH, FSH and T in serum among different groups were examined by analysis of variance (ANOVA). Non-parametric test (Kruskal Wallis) was used to estimate the differences of serum GnRH and E_2 levels among different groups. Separate multivariate linear regression models were used to estimate the mean change in E_2 and T levels in different groups in relation to an increase of one standard deviation in serum levels of GnRH, FSH, LH and various personal characteristics including age, urine fluoride level and so on. . All statistical tests were two-sided with 0.05 as a cutoff for significance. Statistical analyses were performed using the SPSS software, version 12.0 (SPSS In., Chicago, USA).

3.Results

3.1.Distributions of select variables in different groups

As shown in Table 1, there were 299 local male residents were enrolled in the study (41 in HFG, 79 in DFPG and 179 in CG respectively). The mean age was 43.73 years in HFG, 40.94 years in DFPG and 41.03 years in CG. There was no statistical significance among the three groups (P>0.05). We compared the body weight, height, drink alcohol rate, smoking rate, the mean marriage age and the rate of

no pregnant history within one year without taking any contraceptive measures after marriage and no statistical significances were observed among the three groups (P>0.05 respectively). But prevalence of dental fluorosis in HFG and DFPG were higher compared with CG (P<0.05 respectively).

Table 1. Distributions of select variables in different groups

	HFG (n=41)	DFPG (n=79)	CG (n=179)
Age(years)*	43.73.00±9.64	40.94±8.83	41.03±8.74
P value	0.357	0.951	
Body weight*	69.18±10.25	71.41±10.47	70.70±11.32
P value	0.522	0.683	
Height*	167.64±5.85	168.30±6.54	168.39±5.38
P value	0.654	0.047	
Drink alcohol(n)#	44.4%	58.2%	48.6%
Yes	20	46	87
No	25	33	92
P value	0.739	0.177	
Smoking&	73.3%	68.4%	66.5%
Yes	33	54	119
No	12	25	60
P value	0.476	0.886	
Dental fluorosis^	77.8%	65.8%	20.7%
Yes	35	52	37
No	10	27	148
P value	**0.000**	**0.000**	
Marriage age*	22.37±2.56	23.14±3.08	23.43±3.60
P value	0.911	0.471	
No pregnant history within one year after marriage$	35.0%	29.8%	19.4%
Yes	26	52	137
No	14	20	33
P value	0.056	0.174	

* Values are means±SD

Drink alcohol means drinking at least once a week on the average

& Smoking means at least once a day and continuous smoking a month or more

^ Dental fluorosis were examined using Dean's method

$ The rate that one's wife was not pregnant when living together without taking any contraceptive measures

The two *P* values in each line represent HFG and DPFG compared with CG respectively

3.2.Determination result of environmental fluoride

The results of fluoride concentration in drinking water was shown in Table 2, which were 2.44 mg/L, 0.36 mg/L and 0.37 mg/L in HFG, DFPG and CG respectively, it was higher in HFG than that in DFPG and CG (P<0.05). No significant differences were observed between DFPG and CG (P>0.05). We also assayed environment fluoride include air, soil, vegetables and grain. No statistical significances were observed among the three groups (P>0.05 respectively).

Table 2. Water fluoride concentration (mg/L) of different areas

Group	n	$\bar{x}\pm S$	t	*P*
HFG	13	2.44±1.88		
DFPG	8	0.36±0.30	4.75	0.000**
CG	16	0.37±0.15	4.96	0.000*

*：HFG Vs CG；**：HFG Vs DFPG

3.3.Results of reproductive hormones and urine fluoride

Fluoride level in urine in different groups was: HFG> DFPG> CG, and there was significant difference in every two groups (P<0.05 respectively) (Tables 3-2). We compared levels of serum GnRH, FSH, LH, E_2, and T in all the participants from the six villages (Tables 3-1 and Tables 3-2). Compared with HFG and CG, the serum level of FSH was significantly lower in DFPG although all of them were within the ranges considered normal (P<0.05 respectively) (Tables 3-2). However, no statistical significance was observed between HFG and CG (P>0.05). Serum level of E_2 in DFPG was slightly higher compared with HFG and CG (P<0.05 respectively). No significant differences were observed among HFG, DFPG and CG for serum GnRH, LH, or T (P>0.05 respectively) (Tables 3-1 and Tables 3-2).

Tables 3-1. Reproductive hormone levels in individuals (Median (P_{25},P_{75})) in DFPG, HFG and CG

Group	n	GnRH (ng/mL)	E_2 (ng/ml)
CG	179	23.51(20.24, 27.21)	28.99(20.24, 33.92)
DFPG	79	21.45(24.07, 26.62)	33.67(29.25, 42.98)
HFG	41	23.58(21.72, 26.85)	29.17(20.01, 44.53)
x^2		1.039	9.611
P		0.595	**0.008**

Tables 3-2. Urine fluoride and reproductive hormone levels in individuals ($\bar{x}\pm S$) from DFPG, HFG and CG

Group	n	Urine F (mg/L)	LH (mIU/ml)	FSH (mIU/ml)	T (ng/ml)
CG	179	1.00±0.48	6.51±4.95	9.57±6.27	4.29±1.30
DFPG	79	1.37±0.67	6.48±5.14	7.82±7.29	4.41±1.36
HFG	41	2.64±1.40	6.37±4.87	10.20±9.68	4.45±1.21
t		104.318	0.048	4.857	0.396
P		**0.000**	0.954	**0.008**	0.674

3.4.Association of E_2, T with serum GnRH, FSH, LH and personal characteristics

Serum levels of E_2 in CG were associated with LH (r=0.343, P=0.000), age (r=0.195, P=0.015), and inversely associated with serum FSH (r=-0.237, P=0.003) (Table 4), whereas no significant association was observed for serum E_2 level in DFPG and HFG (Table 5 and Table 6). However, serum T in HFG was inversely associated with serum FSH level (r=-0.503, P=0.047) (Table 6).

Table 4. Separate multivariate linear regression models of E_2 and T with HPG hormones, Age and Urine F in CG

		β	t	*P*
E_2				
	GnRH	-0.063	-0.902	0.368
	LH	0.343	4.655	**0.000**
	FSH	-0.237	-2.976	**0.003**
	T	0.012	0.165	0.869
	Age	0.195	2.459	**0.015**
	Urine F	-0.101	-1.429	0.155
T				
	GnRH	0.056	0.754	0.452
	LH	-0.049	-0.592	0.554
	FSH	-0.123	-1.430	0.155
	E_2	0.013	0.165	0.869
	Age	-0.153	-1.805	0.073
	Urine F	0.007	0.091	0.928

Table 5. Separate multivariate linear regression models of E_2 and T with HPG hormones, Age and Urine F in DFPG

		β	t	*P*
E_2				
	GnRH	0.065	0.522	0.603
	LH	-0.085	-0.531	0.597
	FSH	-0.068	-0.432	0.667
	T	0.093	0.713	0.478
	Age	0.101	0.793	0.430
	Urine F	-0.017	-0.144	0.886
T				
	GnRH	-0.154	-1.368	0.176
	LH	-0.280	-1.933	0.057
	FSH	0.000	0.001	0.999
	E_2	0.079	0.713	0.478
	Age	-0.227	-1.976	0.052
	Urine F	0.072	0.656	0.514

Table 6. Separate multivariate linear regression models of E_2 and T with HPG hormones, age and urine F in HFG

		β	t	*P*
E_2				
	GnRH	-0.011	-0.065	0.948
	LH	0.007	0.027	0.978
	FSH	0.161	0.607	0.548
	T	-0.095	-0.541	0.592
	Age	0.229	1.326	0.194
	Urine F	-0.098	-0.595	0.556
T				
	GnRH	-0.144	-0.926	0.361
	LH	0.253	1.019	0.315
	FSH	-0.503	-2.062	**0.047**
	E_2	-0.090	-0.541	0.592
	Age	-0.121	-0.709	0.483
	Urine F	-0.092	-0.579	0.567

4.Discussion

Endemic fluorosis is a major public health concern in China due to the excessive consumption of fluoride through drinking water, brick tea, and coal-burning. Most of the fluorosis cases in Henan province are caused by the high concentration of fluoride in drinking water. In view of the influence of tea and coal consumption against chronic fluorosis, the fluoride levels in indoor and outdoor air, vegetables and crops were determined in the six villages. We found that most of the families cooked the meal using wheat straw and cornstalk as the sources of energy both in endemic fluorosis villages and control villages because of relatively undeveloped economy. The fluoride levels in indoor and outdoor air were all lower than the national standard and also no differences were found in different villages. Fluoride level in grain, vegetables also had no significant differences in all six villages. The results above showed that consumption of fluoride in drinking water is the major exposure pathway in the investigated villages. As for the fluoride concentration in urine, it was lower in male from DFPG compared with that form HFG, but higher compared with that form CG (P<0.05 respectively). It suggested that body fluoride level still remains relatively higher even if the defluoridation project has been implemented for two years. As we know, about half of the absorbed fluoride is quickly incorporated into developing bone and teeth, where nearly all of the body's fluoride is found (Padula and Macmillan, 2005). The absorbed fluoride by the skeleton is most efficient in children and decreases with age (Maudsley et al., 2004), but this process can continue up to age 55 (Merviel et al., 2005). Considering that endemic fluorosis is a public health issue in China and that the weight of the evidence in human beings and animals associates fluoride exposure with reproductive effects, we decided to assess these effects in male residents living in endemic fluorosis villages including villages which have been implemented defluoridation project for two years. Moreover, the present study was performed in a population exposed to fluoride levels lower than those previously reported in experimental or epidemiological investigations.

The activity of the hypothalamic-pituitary-gonadal (HPG) axis is controlled by GnRH (Filicori et al., 1984). GnRH is a hypothalamic neuronal secretory decapeptide that plays a pivotal role in mammalian reproduction (Ramakrishnappa et al., 2005). The result showed that there was no

significant difference in serum GnRH level in each group, similar to the previous study result (Ma et al., 2008). Because the process of hormone regulation is very complicated, GnRH aslo widely exists in reproductive tissues which extra-hypothalamic-pituitary axis such as didymus, prostate, ovary, mucous membrane, placenta and so on (Xu et al., 2010). Thus, GnRH may be maintained the normal level in serum by some pathways of regulation.

The LH and FSH, secreted by the pituitary gonadotropes, are dimeric protein hormones and act on the gonad in a sequential and synergistic manner to initiate sexual maturation and maintain cyclic reproductive function (Crawford et al., 2009; Wu et al., 1991). Serum FSH and LH were determined in all of the subjects in this study. It is interesting that FSH serum level of individuals in DFPG was significantly lower than that in HFG and CG, while no statistical significance was found between HFG and CG. Considering the government gave priority to the villages with high fluoride level in drinking water (most exceeding 4.0 mg/L), fluoride has been accumulated in vivo for several decades and the impact of fluoride on human body may still exist although the urine fluoride concentration was slightly lower compared with HFG after implementation of defluoridation project. The higher prevalence of dental fluorosis in DFPG also suggested that the higher fluoride concentration in drinking water before implement of defluoridation oproject. Chen et al. believed that testicle injury appears firstly the change of FSH level and then implicate interstitial cell and subsequently make the change of LH level (Chen et al., 2002). But we did not find any significant difference in serum LH level among of different groups. Regulation of LH and FSH hormones is facilitated through a complex interplay of multiple mechanisms not only including a direct action of hypothalamic GnRH, but also direct and indirect actions of gonadal-derived steroids and peptides (Wu et al., 1991), the change of FSH in DFPG may be not enough to influence the LH level under that condition with fluoride concentration in drinking water around the level of this study. In contrast, Deogracias et al. observed the increase of serum FSH in high-fluoride-exposed group (HFEG) (Ortiz et al., 2003). But in his study, HFEG was defined as working in a fluoride industry that produced fluorhydric acid and aluminum fluoride besides exposed to fluoride in drinking water (3.0 mg/L). It is more easily to absorb the gaseous fluoride by respiratory system, so different exposure way and the higher exposure concentration may be responsible for the different result. Similar to pituitary hormones FSH in this study, serum levels of gonadal steroid hormones E_2 in DFPG individuals were significant difference compared with HFG and CG, while no statistical significance was found between HFG and CG. But E_2, is exactly opposite to FSH, the concentration was increased in DFPG. It suggests that E_2 can feedback negatively on the pituitary hormone, such as FSH. PAK et al. believed that gonadal steroid hormones, such as T and E_2, can feedback negatively on the hypothalamus and/or anterior pituitary to regulate reproduction in mammals (Pak et al., 2001). Melnyk et al. also observed in their animal experiment that E_2 treatments were associated with decreases in mean FSH concentration (Melnyk et al., 1992). The findings of Kemeter et al. suggested that not only the E_2 but also other steroids and/or nonsteroidal ovarian inhibiting factors could inhibit FSH and LH and both FSH and LH are negatively correlated with E_2 in female monkeys (Kemeter et al., 1984). But if the effect of fluoride on male reproductive hormones is as endocrine disruptor, it is unclear so far. Both Deogracias (Ortiz et al., 2003) and our results suggest the existence of a fluoride-induced endocrine disruption over the hypothalamic- pituitary-testis axis.

In this study, we found Serum levels of E_2 in CG were associated with LH, age, and negatively associated with FSH, whereas no significant association was observed for serum E_2 in DFPG and HFG. It suggests that the normal regulatory relationship between gonadal steroid hormone and anterior pituitary hormone may be affected when long-term excessive intake fluoride in drinking water even if the fluoride concentration in drinking water has decreased to normal (<1.0 mg/L) by defluoridation project for two years. We did not observe any correlation between urine fluoride and any of all the selected reproductive hormones after controlling for age. Deogracias et al. also did not find any correlation between urine fluoride and same hormones in the individuals exposed to fluoride in drinking water and individuals exposed to fluoride both in drinking water and in occupational environment (Ortiz et al., 2003). The results above suggest that long-term exposure to fluoride in drinking water may modify the relations of reproductive hormones of hypothalamic-pituitary-testis axis and this modification may not be directly correlated with urine fluoride level.

5.Conclusion

This study provide the preliminary evidence that long-term exposure to fluoride in drinking water may induce endocrine disruption over the hypothalamic-pituitary-testis axis by affecting regulation of reproductive hormone levels. There has some limitations in this study, such as relative fewer

samples in the HFG, thus chance finding cannot be excluded. Cluster sampling method and detailed questionnaire were used to make up and avoid the chance finding. Further limitation of this study is we did not have enough power to detect dose-response/or effect relationship between fluoride level and reproductive hormones. Further studies should be based on larger samples, and should explore in greater detail the dose-response/or effect relationship between fluoride concentration in drinking water and reproductive hormones in male living in endemic fluoride areas. We have conducted a population-based study in several endemic fluorosis villages with different fluoride concentration in drinking water under the support by national natural science foundation of China and it is still under study.

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