Letter to the Editor

Fluoride Exposure, Follicle Stimulating Hormone Receptor Gene Polymorphism and Hypothalamus-pituitary-ovarian Axis Hormones in Chinese Women*

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The effects of fluoride exposure on the functions of reproductive and endocrine systems have attracted widespread attention in academic circle nowadays. However, it is unclear whether the gene-environment interaction may modify the secretion and activity of hypothalamus-pituitary-ovarian (HPO) axis hormones. Thus, the aim of this study was to explore the influence of fluoride exposure and follicle stimulating hormone receptor (FSHR) gene polymorphism on reproductive hormones in Chinese women. A cross sectional study was conducted in seven villages of Henan Province, China during 2010-2011. A total of 679 women aged 18-48 years were recruited through cluster sampling and divided into three groups, i.e. endemic fluorosis group (EFG), defluoridation project group (DFPG), and control group (CG) based on the local fluoride concentration in drinking water. The serum levels of gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) were determined respectively and the FSHR polymorphism was detected by real time PCR assay. The results provided the preliminary evidence indicating the gene-environment interaction on HPO axis hormones in women.

Fluorine is an essential trace element for the development of bone and teeth, and even the nervous and reproductive systems. Previous publications indicated that high fluoride exposure could cause the dysfunction of reproductive and endocrine systems, causing a change in sexual hormone activities, and thus influencing the reproductive function[1-2]. However, the number of the previous studies in humans was not only less than the number of the studies in animals, but the results were also inconsistent.

It is possible that genetic changes, such as single nucleotide polymorphisms (SNPs), either by themselves or in combination, modify and finetune endocrine-feedback systems and hormone action[3]. The FSH receptor (FSHR) belongs to the superfamily of G-protein coupled receptors coded by the *FSHR* gene (chr 2p21) and plays a crucial role in the physiology of human reproductive system. Mutation screening of the *FSHR* gene found various SNPs, both in the core promoter and in the coding region[3]. SNP (rs1394205) of *FSHR* in the core promoter (5'-untranslated region) is at nucleotide position-29, resulting in a G/A exchange (-29G>A) in a potential GGAAA binding domain for a c-E-twenty-six specific (c-ETS) transcription factor[3]. There has been studies on its association with ovarian response[4] but not enough, especially in regards to its association with serum hypothalamus-pituitary- ovarian (HPO) axis hormones in women. On the other hand, some researchers believed that fluorosis was related to genetic susceptibility[5], but few publications specifically evaluated the influence of fluoride exposure and genetic susceptibility on changes in reproductive hormones. We conducted this cross sectional study to explore the influence of water fluoride exposure and *FSHR* gene -29G>A polymorphism on HPO axis hormones in adult women.

A cross sectional study was conducted among women selected through cluster random sampling in seven villages in Tongxu County of Henan Province, China during 2011-2012. The seven villages included two endemic fluorosis villages (the average level of fluoride in drinking water >1.0 mg/L according to Chinese water quality standard), two defluoridation

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project villages (endemic fluorosis villages where drinking water defluoridation projects have been implemented by the end of 2008), and three non-endemic fluorosis villages (the average level of fluoride <1.0 mg/L). Women aged 18-48 years, who were born and grew up in the villages or lived there for more than five years, were recruited and divided into 3 groups, i.e. endemic fluorosis group (EFG), defluoridation project group (DFPG), and control group (CG). Upon receiving their written consent, a face to face interview was conducted by using a standardized and structured questionnaire to collect the information about their demographic characteristics, medical conditions, medication use, reproductive history, tobacco use and alcohol consumption, etc. Women who had received drug treatment, such as bisphosphonates, calcitonin, fluoride, or hormone replacement therapy were excluded. A total of 679 women were eligible for this study (86.72%). Fasting blood samples (10 mL) and instant urine samples (50 mL) were collected from the subjects. After centrifugation, serum and white blood cells were separated and frozen at -80 °C for subsequent analyses. All the procedures were approved by the Institutional Review Board of Zhengzhou University in China.

Fluoride levels in urine samples were detected by using fluoride ion selective electrodes. Gonadotropin releasing hormone (GnRH) level in serum was determined with enzyme-linked immunosorbent assay (ELISA) (R&D). Follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) levels in serum were detected by using chemiluminescence immunoassay (CLIA) (Autobio Labtec Instruments Co. Ltd.). Each sample was determined in duplicate and 10% of the samples were retested randomly.

Genomic DNA was extracted by using whole blood genomic DNA miniprep kits (Axygen). The genetic analysis of *FSHR* -29G>A polymorphism was carried out with predesigned TaqMan primer and Taqman MGB probe sets (5'-TAT GCA TCC ATC CAC CTG ATT TCT T[C/T] CTG CAT TTG CAG AGA AAA ACC TCCA-3') (Applied Biosystems). PCR conditions comprised of an initial cycle at 95 °C for 10 min, followed by 40 cycles of 92 °C for 15 s and 60 °C for 60 s (MX3000P, Stratagene). Each genotyping plate contained positive and negative controls.

Statistical analyses were performed with software SPSS. The differences in age, menstrual cycle, and fluoride levels in urine among different groups were examined with one-way analysis of variance (ANOVA). A non-parametric test was used to estimate the differences in serum GnRH, FSH, LH, and E_2 levels among different groups. The differences in passive smoking rate, menstrual disorder rate and genotype distribution of *FSHR* were examined with chi square test. The chi square test was also used to test the departures from the Hardy-Weinberg equilibrium. The *FSHR* gene -29G>A polymorphism frequencies in EFG, DFPG, and CG subjects were consistent with the Hardy-Weinberg equilibrium (χ^2=0.880, P=0.348; χ^2=0.396, P=0.529; χ^2=3.172, P=0.075 respectively). A P-value of less than 0.05 was considered statistically significant.

As shown in Table 1, we compared age, menstrual cycle, menstrual disorder rate among the three groups, but the differences had no statistical significances (P>0.05 respectively). The passive smoking rate in EFG was significantly higher than that in CG (P<0.05). However, there was no difference in serum hormone levels between the passive smoking group and non-passive smoking group in CG, DFPG, and EFG respectively (data not shown), so there is no need to consider passive smoking as a confounding factor. The urine fluoride level in EFG was significantly higher than that in DFPG and CG (P<0.001 respectively). The urine fluoride level in DFPG was also significantly higher than that in CG (P<0.001). Urine fluoride level represents the body burden of individuals exposed to fluoride. These results suggested that fluoride could be accumulated in the body due to long-term exposure to fluoride in drinking water and the concentration would be decreased gradually after the removal of fluoride exposure.

Serum GnRH and E_2 levels in women in EFG were significantly lower than those in women in DFPG and CG (P<0.05 respectively). Contradictory findings on the influence of fluoride on serum GnRH have been reported by previous studies[2,6]. Sun et al. demonstrated in male rats that the level of GnRH declined significantly in the exposed group and this decline showed a dose-dependent relationship[6]. However, Hao et al. did not find a difference in GnRH levels in serum between fluoride exposure residents and control group[2]. Different species and different exposure doses might explain the different results. E_2 both inhibits and excites GnRH neurons via presynaptic as well as postsynaptic mechanisms[7]. Thus, it is necessary to explore whether the change in E_2 level was due to the changed GnRH regulation or a result of direct action of fluoride on it. On the other hand, no significant differences were observed

in FSH and LH levels among different fluoride exposure groups, which are consistent with the previous results[2].

The association between *FSHR* -29G>A and ovarian function or related diseases has attracted more attention nowadays. In the present study, genotyping data was available for 679 subjects. We did not find the association between this SNP and serum hormones levels, not only including GnRH, but also FSH, LH, and E_2 in women in the control group (Table 2). However, serum GnRH levels in women in EFG were significantly lower compared with those in women in DFPG and CG, regardless of whether they were carrying the AA, AG, or GG genotype (P<0.05) (Table 3). These results suggested that the relatively lower serum GnRH concentration in women in EFG might be mainly associated with fluoride exposure, but not *FSHR* gene -29G>A polymorphism.

Table 1. Distributions of Selected Variables in Different Groups

Groups		EFG	DFPG	CG	F/χ^2	*P*
n		214	162	303		
Age (years)		39.17±7.59	38.93±7.58	37.77±8.18	2.306	0.100
Menstrual cycle (days)		29.89±4.71	29.46±6.43	29.42±3.26	0.629	0.533
Menstrual disorders rate*		30.81% (65/211)	27.16% (44/162)	21.85% (66/302)	5.352	0.069
Passive smoking rate		61.2% (131/214)	58.6% (95/162)	48.8% (148/303)	3.172	0.013[a]
Urine fluoride (mg/L)		2.69±1.58	1.41±1.08	0.94±0.50	164.637	<0.001[b]
GnRH (ng/mL)		19.77 (6.46, 25.35)	24.05 (21.46, 26.42)	24.04 (20.19, 28.89)	52.761	<0.001[c]
FSH (mIU/mL)		7.83 (4.91, 17.95)	7.25 (4.62, 12.56)	7.07 (4.59, 12.62)	4.318	0.115
LH (mIU/mL)		6.28 (3.71, 17.95)	7.90 (4.90, 15.13)	6.95 (3.97, 12.63)	4.212	0.122
E_2 (pg/mL)		47.90 (30.98, 90.50)	61.60 (39.67, 88.28)	58.86(38.41, 90.44)	10.060	0.007[d]
FSHR genotype Number (%)	AA	61 (28.5%)	38 (23.5%)	68 (22.4%)		
	AG	100 (46.7%)	85 (52.5%)	167 (55.1%)	4.015	0.404
	GG	53 (24.8%)	39 (24.1%)	68 (22.4%)		

Note. *menstrual disorders included dysmenorrhea, irregular menses, abnormal leukorrhea, etc. [a]EFG *vs*. CG: P=0.006; [b]EFG *vs*. DFPG and CG: P<0.001 respectively. [c]EFG *vs*. DFPG and CG: P<0.001 respectively. [d]EFG *vs*. DFPG and CG: P=0.007, P=0.005 respectively.

Table 2. Association between Serum Hormone Levels and *FSHR* -29G>A [Median (P_{25}, P_{75})] in Women

Groups		*n*	GnRH (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)	E_2 (pg/mL)
EFG	AA	61	18.42 (5.50, 23.55)	8.42 (5.24, 20.40)	6.65 (4.10, 17.07)	57.00 (37.55, 109.76)
	AG	100	20.01 (6.39, 26.78)	7.38 (4.59, 14.85)	5.68 (3.33, 11.40)	44.02 (28.85, 78.71)
	GG	53	20.49 (8.65, 25.72)	8.39 (5.12, 26.94)	7.77 (3.56, 20.18)	46.49 (31.03, 93.25)
	H		3.384	1.337	2.860	2.893
	P		0.184	0.512	0.239	0.235
DFPG	AA	38	23.97 (20.60, 26.40)	7.23 (4.92, 12.86)	7.13 (4.27, 11.71)	53.86 (39.53, 76.67)
	AG	85	23.76 (21.86, 25.96)	6.96 (4.22, 13.49)	7.89 (4.93, 15.02)	61.94 (41.21, 88.64)
	GG	39	24.82 (20.27, 28.29)	8.16 (5.56, 12.41)	11.35 (5.07, 19.29)	71.10 (38.14, 99.26)
	H		0.780	1.129	3.498	2.028
	P		0.677	0.569	0.174	0.363
CG	AA	68	24.31 (21.13, 29.35)	7.07 (4.39, 11.87)	6.81 (3.99, 13.85)	57.45 (37.53, 97.03)
	AG	167	23.86 (19.94, 28.85)	6.98 (4.78, 12.22)	6.95 (3.70, 11.03)	56.87 (38.38, 87.04)
	GG	68	24.33 (19.68, 28.99)	7.68 (4.18, 14.17)	7.45 (4.21, 14.30)	62.25 (39.35, 103.41)
	H		0.484	0.202	1.422	1.052
	P		0.785	0.904	0.491	0.591

In addition, Table 2 also shows that this polymorphism did not correlate with serum levels of FSH and LH, consistent with the previous study[3]. Therefore, the present in vivo data confirmed the in vitro observation that the SNP at position -29 of FSHR was unlikely to influence FSH activity directly when considered alone[3], or in combination with fluoride.

Serum E_2 level in women in EFG was significantly lower than those in women in DFPG and CG when carrying AG genotype (P<0.05) and a similar phenomenon was observed in women with GG genotype. Moreover, serum E_2 levels was significantly lower in women in EFG compared with those in women in DFPG and CG when carrying AG+GG genotype (P<0.05) (Table 3). The above results suggested that women with G allele may be more susceptible to fluoride exposure to influence serum E_2 level. Considering that serum hormone levels of the HPO axis are significantly different in ovulatory and non-ovulatory periods, we compared the distribution of different menstrual cycle, including ovulatory period, follicular phase and luteal phase, among the three groups; no statistical differences were observed in the distribution of menstrual cycle among the three groups.

Rendina et al.[8] did not observe the difference in E_2 levels in postmenopausal women with different -29G>A genotypes. However, Nakayama et al. observed in the study of essential hypertension that the serum E_2 level was significantly lower in postmenopausal women with AA genotype than those without AA genotype[9]. Previous results mentioned above indicated that serum E_2 level might be related to genetic factors, age, and even the health status. In the control subjects of the present study, we did not find the difference in serum E_2 levels in women with different -29G>A genotypes. Therefore, it is necessary to consider if serum E_2 level would decrease more quickly in menopausal women with AA genotype of *FSHR* in the further study.

Table 3. Association between Serum Hormone Levels and *FSHR* -29G>A [Median (P_{25}, P_{75})] in Women with Different Genotypes

Groups		*n*	GnRH (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)	E_2 (pg/mL)
AA	EFG	61	18.42 (5.50, 23.55)	8.42 (5.24, 20.40)	6.65 (4.10, 17.07)	57.00 (37.55, 109.76)
	DFPG	38	23.97 (20.60, 26.40)	7.23 (4.92, 12.86)	7.13 (4.27, 11.71)	53.86 (39.53, 76.67)
	CG	68	24.31 (21.13, 29.35)	7.07 (4.39, 11.87)	6.81 (3.99, 13.85)	57.45 (37.53, 97.03)
	H		26.740	2.474	0.109	0.646
	P		<0.001[a]	0.290	0.947	0.724
AG	EFG	100	20.01 (6.39, 26.78)	7.38 (4.59, 14.85)	5.68 (3.33, 11.40)	44.02 (28.85, 78.71)
	DFPG	85	23.76 (21.86, 25.96)	6.96 (4.22, 13.49)	7.89 (4.93, 15.02)	61.94 (41.21, 88.64)
	CG	167	23.86 (19.94, 28.85)	6.98 (4.78, 12.22)	6.95 (3.70, 11.03)	56.87 (38.38, 87.04)
	H		18.984	1.084	5.310	10.226
	P		<0.001[a]	0.582	0.070	0.006[d]
GG	EFG	53	20.49 (8.65, 25.72)	8.39 (5.12,26.94)	7.77 (3.56,20.18)	46.49 (31.03,93.25)
	DFPG	39	24.82 (20.27, 28.29)	8.16 (5.56,12.41)	11.35 (5.07,19.29)	71.10 (38.14,99.26)
	CG	68	24.33 (19.68, 28.99)	7.68 (4.18,14.17)	7.45 (4.21,14.30)	62.25 (39.35,103.41)
	H		9.701	1.192	1.398	3.403
	P		0.008[b]	0.551	0.497	0.182
AG+ GG	EFG	153	20.08 (6.84,26.16)	7.77 (4.78, 16.88)	6.05 (3.38, 16.87)	44.71 (29.86, 84.87)
	DFPG	124	24.17 (21.55,26.43)	7.25 (4.54, 12.54)	8.28 (4.95, 16.12)	63.71 (39.73, 63.71)
	CG	235	23.98 (19.90,28.85)	7.06 (4.62, 12.69)	6.96 (3.82, 12.21)	59.61 (38.49, 87.51)
	H		28.187	2.054	5.904	12.764
	P		<0.001[c]	0.358	0.052	0.002[e]

Note. [a]EFG *vs.* DFPG and CG: P=0.001, P<0.001 respectively; [b]EFG *vs.* DFPG and CG: P=0.009, P=0.006 respectively; [c]EFG *vs.* DFPG and CG: P<0.001 respectively; [d]EFG *vs.* DFPG and CG: P=0.003, P=0.009 respectively; [e]EFG *vs.* DFPG and CG: P=0.001, P=0.003 respectively.

In summary, this study provided the preliminary evidence that the gene-environment interaction may modify the secretion and efficiency of HPO axis hormones. Serum GnRH and E_2 levels of the HPO axis in women may be primarily affected by chronic fluoride exposure through drinking water, and the women with G allele of -29G>A in *FSHR* gene may be more susceptible to fluoride exposure to influence serum E_2 level.

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