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Low-to-moderate fluoride exposure, relative mitochondrial DNA levels, and dental fluorosis in Chinese children

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ABSTRACT

Background: The alteration of mitochondrial DNA (mtDNA) content contributes to many diseases, however, little is known about its effect on the prevalence of dental fluorosis (DF).

Objectives: We conducted a cross-sectional study to investigate the association of low-to-moderate fluoride exposure with relative mtDNA levels in relation to DF in children.

Methods: We recruited 616 resident children, aged 7–13 years, randomly from low-to-moderate fluoride areas in Tianjin, China. We measured the fluoride concentrations in drinking water and urine using the national standardized ion selective electrode method, and determined the relative levels of mtDNA using a quantitative real-time polymerase chain reaction assay. The association among fluoride exposure, relative mtDNA levels, and the prevalence of DF were examined using multivariable linear and logistic regression models. We also performed stratified and mediation analyses.

Results: The relative mtDNA levels of participants in the DF group were significantly lower than in the non-DF group (0.95 ± 0.44 *vs.* 1.12 ± 0.45, $P < 0.001$). In the adjusted models, we found that a 1 mg/L increment in water fluoride concentration was associated with a 0.10-unit decrease in circulating relative mtDNA levels (95% *CI*: −0.14, −0.06) and a 2.85-fold increase (95% *CI*: 2.01, 3.92) in moderate DF prevalence. A 1 mg/L increment in urinary fluoride level was associated with a 0.12-unit decrease in circulating relative mtDNA levels (95% *CI*: −0.14, −0.09) and a 1.85-fold increase (95% *CI*: 1.39, 2.39) in moderate DF prevalence. Stratified analysis indicated a weaker positive association of DF prevalence with fluoride exposure, while a stronger inverse relationship with relative mtDNA levels in boys than in girls. Assuming causality, we estimated that circulating mtDNA levels mediated 13.0% (95% *CI*: 5.2, 28.7%) and 9.6% (95% *CI*: 4.7, 18.5%) of the estimated effect of a 1 mg/L increment in water fluoride and urinary fluoride on prevalence of moderate DF, respectively.

Conclusions: Gender potentially modifies the associations of DF prevalence with relative mtDNA levels and low-to-moderate fluoride exposure. The reduced circulating mtDNA levels may partly mediate the elevated prevalence of moderate DF in children under such exposure.

Abbreviations: DF, dental fluorosis; LBW, low birth weight

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1. Introduction

Fluorine is an extremely active element and widely distributed in air, water and soil in various chemical compound states. Numerous studies have reported a strong association between appropriate fluoride levels and dental caries prevention (Petersen and Phantumvanit, 2012). However, long-term excessive fluoride exposure results in fluorosis, which is characterized by skeletal fluorosis and dental fluorosis (DF) (Onoriobe et al., 2014; Zhang et al., 2017). It is noteworthy that DF has received many concerns during the past decades (Martinez-Mier, 2018). Epidemiologic evidence has revealed strongly positive association between exposure to high fluoride concentration with the prevalence of DF (Lima-Arsati et al., 2018). Due to the implementation of the low-fluoride drinking water supply plan supported by local governments, the area of high fluoride exposure is shrinking, and the areas of low-to-moderate fluoride exposure is increasing. However, the high incidence of DF in children is still a serious social problem (Chen et al., 2012). Although a large number of studies have confirmed the harmful health effects of high fluoride exposure, the evidence on the potentially harmful effects of chronic exposure to low-to-moderate fluoride on children's dental development is relatively insufficient.

Mitochondria are the energy center of cells and essential for many biological processes, such as cell proliferation, apoptosis, calcium storage and metabolism (Asghari et al., 2017; Vakifahmetoglu-Norberg et al., 2017). While the stable function of mitochondria is basic to the maintenance of cell homeostasis properly (Tsuchiya et al., 2008), mitochondrial dysfunction contributes to the decrease in odontoblast layer width and the impairment of enamel secretion from ameloblasts (Couve et al., 2012; Varga et al., 2015). Interestingly, fluoride was reported to reduce the mitochondrial number in ameloblasts (Pergolizzi et al., 1995; Ribeiro et al., 2006) and lead to mitochondrial dysfunction in odontoblasts and ameloblasts (Li et al., 2013; Suzuki et al., 2017) *in vivo* and *in vitro*. Moreover, it is well known that fluoride can interfere with the secretory function of ameloblasts, thus reducing the formation of enamel matrix and further leading to enamel dysplasia, eventually causing DF (Bronckers et al., 2009). These findings suggest that mitochondrial dysfunction may play a role in fluoride-induced DF development. However, there is currently no epidemiological evidence on the relationship between mitochondrial dysfunction and DF.

Mitochondrial DNA (mtDNA) is a circular genome crucial for the maintenance of mitochondrial function and is present in multiple copies in most cell types. However, mtDNA is particularly vulnerable to intracellular or extracellular stimuli and has a high mutation and degradation rate, since the mtDNA repair mechanisms work less efficiently than that of nDNA (Stewart and Chinnery, 2015). This results in an alteration in mtDNA content, which therefore reflects mitochondrial damage and dysfunction (Cha et al., 2015). Importantly, it has been reported that exposures to environmental factors (such as $PM_{2.5}$, NO_2, PAHs) were associated with decreased mtDNA content (Clemente et al., 2016; Wong et al., 2017), which is involved in the development of many diseases (Bersani et al., 2016; Pyle et al., 2016). However, whether fluoride affects human mtDNA content, a good proxy for mitochondrial function, has never been investigated. In the present study, we performed a cross-sectional study to assess the association of low-to-moderate fluoride exposure with relative mtDNA levels in relation to DF in Chinese school-age children.

2. Materials and methods

2.1. Study population

A village-based cross-sectional study was conducted in 2015 in rural areas of Tianjin, China. According to the annual surveillance data of the Centers for Disease Control and Prevention, the drinking water sources and water fluoride concentrations in each village have remained stable over the past decade. During the investigation, water samples were collected from the water supplies in each water source for fluoride concentration analysis. A representative sample of local children who were permanent residents since birth was selected using a multi-stage random sampling technique stratified by area. All the mothers lived in the local area from prepregnancy. A total of 616 children who aged 7–13 years met the inclusion criteria for this study. The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All subjects and their parents/guardians provided written informed consent in accordance with the requirements of the Institutional Review Board of Huazhong University of Science and Technology and the Ethical Committee of Tianjin Center for Disease Control and Prevention.

2.2. General data collection

Trained investigators conducted a face-to-face interview with the recruited children and their parents to collect demographic data, including demographics (age and gender), socioeconomics (maternal education, paternal education, and family incomes), maternal disease history during pregnancy (gestational diabetes, malnutrition, and anemia), and delivery conditions [hypoxia, dystocia, premature birth, post-term birth, and low birth weight (LBW)]. The development status of the recruited children was further assessed by the calculation of their body mass index (BMI), which was derived from their height and weight.

2.3. Sample collection

Each subject's spot (early-morning) urine sample was collected using a pre-cleaned, labelled polythene tubes (50 mL). Besides, investigators collected water samples from each public supply in the village using a pre-cleaned, labelled polythene tubes (50 mL). All water and urine samples were transported to the laboratory in an icebox, and then stored at −80 °C until analysis. In addition, 5 mL fasting peripheral blood samples were collected from each subject using polypropylene Na-EDTA tubes. Lymphocytes from blood samples were separated (2–4 h after blood collection) by centrifugation 3000 r/min for 15 min and transferred to 1.5 mL EP tubes for DNA extraction. Then lymphocytes were frozen at −80 °C for subsequent analyses. All samples of each child were collected on the same day.

2.4. Measurement of fluoride concentrations

Water and urine samples were analyzed for the concentrations of fluorine ion (mg/L) using the ion selective electrode method (Kazi et al., 2018). Standard fluoride solutions with concentrations of 10.0 and 100.0 mg/L were used to calibrate the measuring device. The calibration graph for F^- was obtained by ion-selective electrode with the calibration solutions range of 0.1–50.0 mg/L (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 mg/L). Prior to measurement, 30 mL of total ionic strength adjustment buffer III and 5 mL sample were added to each test solution, then a fluorine ion selected electrode (INESA, Shanghai) was connected to a fluoride ion meter (INESA, Shanghai). The fluoride concentration (mg/L) was measured while the solution was stirred at room temperature. Two readings for each sample were recorded and the mean value was calculated. The samples were discarded after the analysis was completed. All chemicals were analytical grade purity or were guaranteed reagents.

2.5. Assessment of DF

Dean's classification system was used to estimate the prevalence and severity of DF. Each participant was examined by two qualified experts independently. The examination was performed under natural light after the vestibular surfaces of the teeth were cleaned and dried. A child's DF index was based on the most severe fluorosis form found on

two or more teeth (Onoriobe et al., 2014). The DF degree was graded as normal, very mild, mild, moderate and severe. Children who had teeth with cavities or who had orthodontic appliances during the investigation were excluded from the analysis.

2.6. Measurement of circulating relative mtDNA levels

DNA samples were extracted from lymphocytes using the DNA extraction kit (GK1042, Shanghai Generay Biotech Co., Ltd., Shanghai, China), and quantified using the Nanodrop ND1000 (Thermo scientific, Wilmington, DE, USA) subsequently. The relative level of mtDNA was measured in leukocytes of blood by determining the ratio of two mitochondrial genes [MTF3212/R3319 (mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319) and MTND1 (mitochondrial encoded NADH dehydrogenase 1)] to two single-copy nuclear control genes [RPLP0 (acidic ribosomal phosphoprotein P0) and ACTB (β-actin)] using a quantitative real-time polymerase chain reaction (QPCR) assay (ABI 7900 HT system). Primers and conditions for mtDNA content analysis were performed as reported previously (Clemente et al., 2016; Janssen et al., 2012). The primers for QPCR analysis of MTND1 were: MTND1-F 5'ATG GCC AAC CTC CTA CTC CT3', and MTND1-R 5'CTA CAA CGT TGG GGC CTT T3'. The primers for QPCR analysis of MTF3212/R3319 were: MTF3212/R3319-F 5'CAC CCA AGA ACA GGG TTT GT3', and MTF3212/R3319-R 5'TGG CCA TGG GTA TGT TGT TAA3'. The primers for QPCR analysis of RPLP0 were: RPLP0-F 5'GGA ATG TGG GCT TTG TGT TC3', and RPLP0-R 5'CCC AAT TGT CCC CTT ACC TT3'. The primers for QPCR analysis of ACTB were: ACTB-F 5'ACT CTT CCA GCC TTC CTT CC3', and ACTB-R 5'GGC AGG ACT TAG CTT CCA CA3'. PCR amplification was performed in a 10 μL reaction mixture with the following final concentrations: 5 μL of 2 × Power SYBR Green PCR Master Mix (RR430A, TaKaRa, Japan), 0.5 μL of primer with a concentration of 0.15 μmol/L, and 2.5 μL of DNA template with a concentration of 0.5 ng/μL. PCR conditions were as follows: 1 min at 95 °C for activation of the polymerase enzyme and initial denaturation, followed by 40 cycles of 5 s at 95 °C for denaturation and 30 s at 60 °C for annealing and extension. The raw data produced by qPCR-based technologies are fluorescence signal intensities captured at the end of each amplification cycle. Amplification specificity and absence of primer dimers were confirmed by dissociation curve analysis at the end of each run (15 s at 95 °C, 15 s at 60 °C, 15 s at 95 °C). After diluting the standard DNA (obtained by pooling DNA from 50 participants who were randomly selected from the current population) step by step in a 1:2 ratio, we used standard DNA to generate a seven-point standard curve (range: 12.5–0.195 ng), then we calculated the amplification efficiencies of 4 pairs of primers (the amplification efficiencies of MTF3212/R3319, MTND1, RPLP0 and ACTB were 100.25%, 103.35%, 101.90% and 106.47%). In addition, we used standard DNA as calibrator DNA to adjust the plate effects. Two negative controls (ddH_2O as template) and six inter-run calibrators sample were carried along in each PCR plate. Detailly, mtDNA content of each sample is determined according to the formula of $2^{\wedge}[(Ct_{RPLP0} + Ct_{ACTB})/2 - (Ct_{MTF3212/R3319} + Ct_{MT\text{-}ND1})/2]$ (Janssen et al., 2012). Then the mtDNA content in each sample was normalized to calibrator DNA to standardize the effects among different runs (Janssen et al., 2012; Melkonian et al., 2015; Sun et al., 2014) and defined as the measurement of relative mtDNA level. The coefficient of variation for the mtDNA content in inter-run samples was 4.83%.

2.7. Statistical analysis

Data were presented as numbers (percentages) for categorical variables, mean (with standard deviation) for parametrically distributed variables, and median (with interquartile range) for non-parametrically distributed variables. We first divided the whole population into two groups according to whether they had dental fluorosis. The baseline characteristics and fluoride exposure levels were compared by a student's *t*-test or Mann-Whitney *U* test for continuous variables, whereas a Chi-square test was used for the comparisons of the discrete data between the two groups. We further utilized logistic regression model and general linear regression model to examine the effects of fluoride exposure on DF prevalence and relative mtDNA levels. Besides, the associations of DF prevalence with relative mtDNA levels were estimated using binary logistic regression models or multivariate logistic regression. Estimates of linear trends across increasing tertiles of fluoride exposure and relative mtDNA levels were performed by treating the median of each tertile as a continuous variable. For water fluoride, the tertile ranges were: tertile 1 ($n = 236$), ≤ 0.70 mg/L; tertile 2 ($n = 180$), 0.71–1.50 mg/L; and tertile 3 ($n = 200$), > 1.50 mg/L. And for urinary fluoride, the tertile ranges were: tertile 1 ($n = 204$), ≤ 0.21 mg/L; tertile 2 ($n = 214$), 0.22–2.08 mg/L; and tertile 3 ($n = 198$), > 2.08 mg/L. For relative mtDNA levels, the tertile ranges were: tertile 1 ($n = 206$), ≤ 0.75; tertile 2 ($n = 208$), 0.76–1.10; and tertile 3 ($n = 202$), > 1.10. In addition, we tested the interaction between fluoride exposure and gender on relative mtDNA levels and DF prevalence, and the interaction between relative mtDNA levels and gender on DF prevalence by adding a product interaction term to each respective model. Furthermore, we also tested the interaction between fluoride exposure and relative mtDNA levels on DF prevalence. And the results were presented as odds ratios (*ORs*) or regression coefficients β with their 95% confidence intervals (95% *CIs*). The adjusted variables were age, gender, BMI, maternal education, paternal education, family incomes, LBW. The covariates were selected based on the characteristics of our study population and the existing literature or previous reports.

To further evaluate the role of mtDNA in the association between fluoride exposure and DF prevalence, we used mediation analysis with SAS macro as described by Lin et al. (1997); the method and macro were developed by the Harvard T.H.CHAN School of Public Health and can be found at http://www.hsph.harvard.edu/donna-spiegelman/software/mediate/. Briefly, the method required two regression models: the first model describes the association of fluoride exposure with DF prevalence without adjusting for relative mtDNA levels (the total effect); and the second model describes the association between fluoride exposure and DF prevalence adjusted for relative mtDNA levels (the direct effect) (Vanderweele and Vansteelandt, 2010). The mediation effect of relative mtDNA levels on the association between fluoride exposure and DF prevalence can be obtained by a ratio in which the difference between the total effect and direct effect is divided by the unadjusted excess risk (*i.e.* (total effect-direct effect)/(total effct − 1) ∗100) (Kaufman et al., 2004; Lin et al., 1997).

Sensitivity analyses were conducted by adjusting for different covariates, including demographics (age and gender), development (BMI), socioeconomics (maternal education, paternal education, and family incomes), maternal disease history during pregnancy (gestational diabetes, malnutrition, and anemia), and delivery conditions (hypoxia, dystocia, premature birth, post-term birth, and LBW). Epidata (version 3.0, Epidata Association, Odense, Denmark) was used for database construction. Statistical analyses were accomplished with SAS software package (version 9.4, SAS Institute Inc., Cary, NC, USA). For the interaction term, we relaxed the significance level to $P < 0.10$. The remaining *P* values were two-sided with a significance level of 0.05.

3. Results

3.1. Basic characteristics of the participants

We assigned all participants ($n = 616$) into the non-DF group or the DF group. The characteristics of all the participants are summarized in Table 1. All participants agreed to undergo the standard DF examination, and 376 (61.04%) had DF. The proportions of boys and LBW children were enhanced in the DF group as compared to the non-DF group ($P < 0.05$, respectively). The distributions of age, BMI, family incomes, maternal education and paternal education were comparable between the two groups.

The water fluoride and urinary fluoride concentrations of participants in the DF group were significantly higher than those in the non-

Table 1
Basic characteristics of general population.

Variables	non-DF group	DF group	P[d]
n	240	376	–
Age[a] (years)	9.72 ± 1.06	9.88 ± 1.04	0.054
Gender[b]			0.044
Male	109(45.42%)	202(53.72%)	
Female	131(54.58%)	174(46.38%)	
Height (cm)[a]	142.00 ± 9.10	142.21 ± 8.79	0.775
Weight (kg)[a]	36.25 ± 10.73	36.65 ± 10.75	0.656
Body mass index (kg/m^2)[a]	17.72 ± 3.63	17.84 ± 3.72	0.683
Low birth weight[b]	5(2.08%)	27(7.18%)	0.005
Family incomes (RMB/year)[b]			0.963
< 10,000	18(8.04%)	30(8.55%)	
10,000–30,000	96(42.86%)	152(43.30%)	
> 30,000	110(49.11%)	169(48.15%)	
Maternal education[b]			0.286
Primary and below	192(81.01%)	310(84.47%)	
High school	33(13.92%)	47(12.81%)	
Junior college and above	12(5.6%)	10(2.72%)	
Paternal education[b]			0.515
Primary and below	208(87.39%)	311(85.44%)	
High school	21(8.82%)	42(11.54%)	
Junior college and above	9(3.78%)	11(3.02%)	
Water fluoride (mg/L)[c]	0.70(0.40–0.80)	1.60(1.20–2.60)	< 0.001
Urinary fluoride (mg/L)[c]	0.17(0.09–0.31)	2.11(0.45–2.69)	< 0.001
Relative mtDNA levels[a]	1.12 ± 0.45	0.95 ± 0.44	< 0.001

[a] Data were presented as mean ± standard deviation for continuous variables.
[b] Number (percentage/proportion) for categorical variables.
[c] Data were presented as P_{25}-P_{75} for continuous variables.
[d] Student's *t*-test or Mann-Whitney *U* test was used to compare the difference of continuous variables, and Chi-square test was applied to compare the difference of categorical variables.

DF group ($P < 0.001$, respectively). Besides, the urinary fluoride level presented a significant linear association with the water fluoride concentration ($r_s = 0.683$, $P < 0.001$). Additionally, the relative mtDNA levels of participants in the DF group were significantly lower than those of participants in the non-DF group (0.95 ± 0.44 *vs.* 1.12 ± 0.45, $P < 0.001$).

3.2. Association between low-to-moderate fluoride exposure and relative mtDNA levels

In categorical analyses (see Table 2), water fluoride concentrations were negatively associated with relative mtDNA levels ($P < 0.001$ for trend over tertiles), with adjusted β of −0.24 (95% *CI*: –0.32, −0.15) for children exposed to 0.71–1.50 mg/L water fluoride (tertile 2) and − 0.32 (95% *CI*: –0.39, −0.24) for children exposed to > 1.50 mg/L water fluoride (tertile 3), when compared to the children exposed to < 0.71 mg/L water fluoride (tertile 1). Similarly, the relative mtDNA levels showed a downward trend with increasing urinary fluoride tertiles ($P < 0.001$ for trend over tertiles), with adjusted β of −0.03 (95% *CI*: –0.12, 0.06) for children with 0.22–2.08 mg/L urinary fluoride concentration (tertile 2) and − 0.27 (95% *CI*: –0.35, −0.20) for children with urinary fluoride concentration > 2.08 mg/L (tertile 3), when compared to the children with urinary fluoride concentration ≤ 0.21 mg/L (tertile 1).

In continuous analyses, we observed a reduction of 0.10 (95% *CI*: –0.14, −0.06) of relative mtDNA levels with each 1 mg/L increase in the concentrations of water fluoride in all children after adjusting for potential confounding factors (see Table 2). Besides, we also analyzed the relationship between relative mtDNA levels and urinary fluoride concentrations. After adjusting for potential confounding factors, we observed a reduction of 0.12 (95% *CI*: –0.14, −0.09) in the relative mtDNA levels for every 1 mg/L increment in the urinary fluoride concentrations (see Table 2).

In addition, similar relationship between relative mtDNA levels and fluoride exposure were observed in boys and girls, respectively. Furthermore, we tested the interaction between fluoride exposure and gender on relative mtDNA levels, but did not find any significant effect modification by gender (see Table S1).

3.3. Association between low-to-moderate fluoride exposure and DF prevalence

The association between water fluoride levels and DF prevalence was presented in Table 3 and Table S2. In categorical analyses, the prevalence of DF showed an upward trend with increasing tertiles of fluoride concentrations (all $P < 0.001$ for trend over tertiles) (see Table 3). In continuous analyses, after adjusting for potential confounding factors, we observed significant increases of 47%, 85%, 68% and 2.85-fold in the risk of total DF, very mild DF, mild DF and moderate DF (the *OR*s were 1.47, 1.85, 1.68 and 3.85 respectively) for every 1 mg/L increment in the water fluoride concentrations, respectively (see Table 3).

Besides, we also analyzed the association between urinary fluoride concentrations and DF prevalence. There were significant increases of 39%, 57%, 56% and 1.85-fold in the risk of total DF, very mild DF, mild DF and moderate DF (the *OR*s were 1.39, 1.57, 1.56 and 2.85 respectively) with each 1 mg/L increase in the urinary fluoride, respectively, after adjusting for potential confounding factors (see Table 3).

Similar relationship between fluoride exposure and DF prevalence were observed in boys and girls, respectively. Moreover, we tested the interaction between fluoride exposure and gender on DF prevalence,

Table 2
Associations between fluoride concentrations and relative mtDNA levels.

Fluoride content (mg/L)	Relative mtDNA levels			
	Crude, β (95% *CI*)	*P* value	Adjusted[a], β (95% *CI*)	*P* value
Water fluoride				
Tertile 1 (≤ 0.70)	Reference		Reference	
Tertile 2 (0.71–1.50)	−0.23(−0.31, −0.15)	< 0.001	−0.24(−0.32, −0.15)	0.035
Tertile 3 (> 1.50)	−0.30(−0.37, −0.22)	< 0.001	−0.32(−0.39, −0.24)	< 0.001
Trend test		< 0.001		< 0.001
Increase per 1 mg/L	−0.09(−0.13, −0.05)	< 0.001	−0.10(−0.14, −0.06)	< 0.001
Urinary fluoride				
Tertile 1 (≤ 0.21)	Reference		Reference	
Tertile 2 (0.22–2.08)	−0.05(−0.14, 0.04)	0.273	−0.03(−0.12, 0.06)	0.516
Tertile 3 (> 2.08)	−0.26(−0.34, −0.18)	< 0.001	−0.27(−0.35, −0.20)	< 0.001
Trend test		< 0.001		< 0.001
Increase per 1 mg/L	−0.11(−0.14, −0.09)	< 0.001	−0.12(−0.14, −0.09)	< 0.001

Abbreviation: β, regression coefficient; *CI*, confidence interval.
[a] Adjusted for age, gender, BMI, LBW, maternal education, paternal education, family incomes.

Table 3
Associations between fluoride concentrations and DF prevalence.

DF	Fluoride content (mg/L)	Crude		Adjusted[a]	
		OR (95% *CI*)	*P* value	*OR* (95%*CI*)	*P* value
		Total DF			
	Water fluoride				
	Tertile 1 (≤ 0.70)	Reference		Reference	
	Tertile 2 (0.71–1.50)	2.64(2.08, 3.35)	< 0.001	2.58(2.02, 3.30)	< 0.001
	Tertile 3 (> 1.50)	3.68(2.96, 4.57)	< 0.001	3.64(2.91, 4.55)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per 1 mg/L	1.47(1.40, 1.54)	< 0.001	1.47(1.40, 1.55)	< 0.001
	Urinary fluoride				
	Tertile 1 (≤ 0.21)	Reference		Reference	
	Tertile 2 (0.22–2.08)	1.33(1.14, 1.56)	0.001	1.49(1.26, 1.77)	< 0.001
	Tertile 3 (> 2.08)	3.16(2.56, 3.91)	< 0.001	3.16(2.53, 3.95)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per 1 mg/L	1.39(1.32, 1.45)	< 0.001	1.39(1.32, 1.46)	< 0.001
		Very mild DF			
	Water fluoride				
	Tertile 1 (≤ 0.70)	Reference		Reference	
	Tertile 2 (0.71–1.50)	2.38(1.60, 3.55)	< 0.001	2.33(1.55, 3.51)	< 0.001
	Tertile 3 (> 1.50)	4.66(3.32, 6.54)	< 0.001	4.93(3.48, 6.98)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per 1 mg/L	1.80(1.60, 2.01)	< 0.001	1.85(1.63, 2.11)	< 0.001
	Urinary fluoride				
	Tertile 1 (≤ 0.21)	Reference		Reference	
	Tertile 2 (0.22–2.08)	1.30(0.92, 1.82)	0.133	1.31(0.92, 1.86)	0.135
	Tertile 3 (> 2.08)	3.93(2.79, 5.55)	< 0.001	4.02(2.81, 5.74)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per 1 mg/L	1.56(1.39, 1.75)	< 0.001	1.57(1.41, 1.76)	< 0.001
		Mild DF			
	Water fluoride				
	Tertile 1 (≤ 0.70)	Reference		Reference	
	Tertile 2 (0.71–1.50)	4.28(2.92, 6.26)	< 0.001	4.17(2.80, 6.20)	< 0.001
	Tertile 3 (> 1.50)	6.98(4.91, 9.94)	< 0.001	6.88(4.78, 9.92)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per 1 mg/L	1.69(1.59, 1.79)	< 0.001	1.68(1.57, 1.79)	< 0.001
	Urinary fluoride				
	Tertile 1 (≤ 0.21)	Reference		Reference	
	Tertile 2 (0.22–2.08)	1.52(1.24, 1.86)	< 0.001	1.79(1.44, 2.23)	< 0.001
	Tertile 3 (> 2.08)	5.83(4.11, 8.26)	< 0.001	5.99(4.15, 8.66)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per 1 mg/L	1.55(1.45, 1.66)	< 0.001	1.56(1.45, 1.67)	< 0.001
		Moderate DF			
	Water fluoride[b]				
	Increase per 1 mg/L	3.32(2.72, 4.05)	< 0.001	3.85(3.01, 4.92)	< 0.001
	Urinary fluoride[c]				
	Increase per 1 mg/L	2.43(2.10, 2.82)	< 0.001	2.85(2.39, 3.39)	< 0.001

Abbreviation: *OR*, odds ratio; *CI*, confidence interval.
[a] Adjusted for age, gender, BMI, LBW, maternal education, paternal education, family incomes.
[b] When the water fluoride concentration is < 0.7 mg/L (tertile 1), no children have moderate dental fluorosis.
[c] When the urinary fluoride concentration is < 0.21 mg/L (tertile 1), no children have moderate dental fluorosis.

and then observed significant effect modification by gender for the prevalence of mild DF and moderate DF (see Table S2).

3.4. Association between relative mtDNA levels and DF prevalence

After analyzing the relationship between relative mtDNA levels and DF prevalence, we observed a downward trend of the prevalence of total DF with increasing tertiles of relative mtDNA levels in categorical analyses ($P < 0.001$ for trend over tertiles) (see Table 4).

In continuous analyses, when adjusting for potential confounding factors, there were statistically significant reductions of 35%, 43%, 42% and 94% in the risk of total DF, very mild DF, mild DF and moderate DF (the crude *OR*s were 0.65, 0.57, 0.58 and 0.06 respectively) with each one-unit increase in the relative mtDNA levels, respectively (see Table 4). Similar relationship between fluoride exposure and DF prevalence were observed in boys. However, the significant association between relative mtDNA levels and DF prevalence was not observed in girls. Furthermore, we tested the interaction between relative mtDNA levels and gender on DF prevalence, and then observed significant effect modification by gender for the prevalence of total DF and mild DF (see Table S3).

3.5. Mediation analysis

A mediation analysis of relative mtDNA levels in the association between water fluoride and the prevalence of DF is presented in Table S5. Significant mediation by relative mtDNA levels was observed in the models associating water fluoride with the prevalence of total DF and moderate DF. And the proportions of the association between water fluoride and the prevalence of total DF and moderate DF significantly mediated in part by relative mtDNA levels were 3.3% (95% *CI*: 1.2, 34.5) (see Table S5) and 13.0% (95% *CI*: 5.2, 28.7) (Fig. 1A), respectively. However, we found that the association between water fluoride and the prevalence of very mild DF and mild DF was not mediated by relative mtDNA levels significantly.

Table 4
Association between relative mtDNA levels and DF prevalence.

DF	Relative mtDNA levels	Crude		Adjusted[a]	
		OR (95% *CI*)	*P* value	*OR* (95% *CI*)	*P* value
		Total DF			
	Tertile 1 (≤ 0.75)	Reference		Reference	
	Tertile 2 (0.76–1.10)	0.83(0.73, 0.95)	0.006	0.86(0.75, 0.99)	0.035
	Tertile 3 (> 1.10)	0.66(0.56, 0.78)	< 0.001	0.63(0.53, 0.76)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per unit	0.65(0.54, 0.77)	< 0.001	0.65(0.54, 0.80)	< 0.001
		Very mild DF			
	Tertile 1 (≤ 0.75)	Reference		Reference	
	Tertile 2 (0.76–1.10)	0.79(0.52, 1.19)	0.259	0.79(0.52, 1.20)	0.268
	Tertile 3 (> 1.10)	0.65(0.43, 0.99)	0.044	0.66(0.42, 1.03)	0.066
	Trend test		0.079		0.093
	Increase per unit	0.56(0.36, 0.88)	0.012	0.57(0.36, 0.89)	0.015
		Mild DF			
	Tertile 1 (≤ 0.75)	Reference		Reference	
	Tertile 2 (0.76–1.10)	0.79(0.66, 0.94)	0.010	0.82(0.68, 0.99)	0.042
	Tertile 3 (> 1.10)	0.56(0.45, 0.71)	< 0.001	0.52(0.40, 0.67)	< 0.001
	Trend test		< 0.001		< 0.001
	Increase per unit	0.58(0.45, 0.75)	< 0.001	0.58(0.44, 0.78)	< 0.001
		Moderate DF			
	Tertile 1 (≤ 0.75)	Reference		Reference	
	Tertile 2 (0.76–1.10)	0.37(0.18, 0.80)	0.011	0.36(0.17, 0.76)	0.008
	Tertile 3 (> 1.10)	0.24(0.10, 0.59)	0.002	0.15(0.05, 0.39)	< 0.001
	Trend test		0.010		0.079
	Increase per unit	0.12(0.04, 0.40)	0.001	0.06(0.02, 0.20)	< 0.001

Abbreviation: *OR*, odds ratio; *CI*, confidence interval.
[a] Adjusted for age, gender, BMI, LBW, maternal education, paternal education, family incomes.

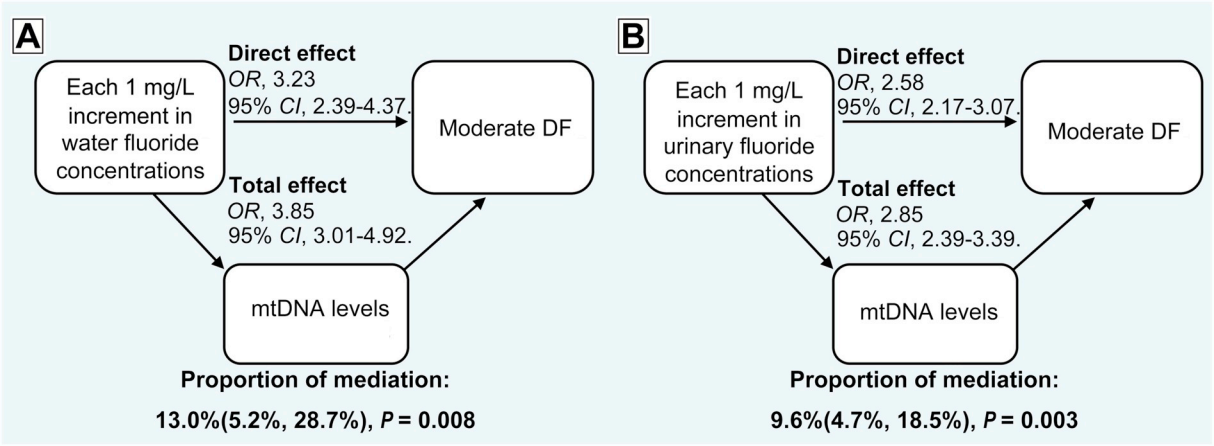

Fig. 1. Mediation analysis of the estimated effect (95% *CIs*) of fluoride exposure (mg/L) on moderate DF through circulating relative mtDNA levels. (A) Mediation analysis suggested circulating mtDNA levels mediated 13.0% (95% *CI*: 5.2, 28.7) of the association between water fluoride and the prevalence of moderate DF. (B) Mediation analysis suggested circulating relative mtDNA levels mediated 9.6% (95% *CI*: 4.7, 18.5) of the association between urinary fluoride and the prevalence of moderate DF. Models were adjusted for age, gender, BMI, LBW, maternal education, paternal education, family incomes.

Subsequently, we analyzed the mediation effect of relative mtDNA levels on the association between urinary fluoride and the prevalence of DF. We found that relative mtDNA levels significantly mediated 9.6% (95% *CI*: 4.7, 18.5) of the association between urinary fluoride and moderate DF prevalence (Fig. 1B), but mediation was not statistically significant for the prevalence of total DF, very mild DF and mild DF (see Table S5).

3.6. Sensitivity analysis

We conducted sensitivity analyses for the effects of fluoride exposure on relative mtDNA levels and DF prevalence (see Tables S6 and S7), and the effect of relative mtDNA levels on DF (see Table S8), adjusting for the following covariates: demographics (age and gender), development (BMI), socioeconomics (maternal education, paternal education, and family incomes), maternal disease history during pregnancy (gestational diabetes, malnutrition, and anemia), and delivery conditions (hypoxia, dystocia, premature birth, post-term birth, and LBW). The results were similar to those observed in our primary analyses.

4. Discussion

To accurately assess the impact of fluoride exposure on children, we selected both water fluoride and urinary fluoride as external and internal exposure indicators, respectively, and observed that the levels of both were positively associated with the prevalence of DF, especially with the prevalence of moderate DF. These findings are consistent with

previous epidemiological and animal studies (Chen et al., 2012; Li et al., 2017). The water fluoride level of 2.0 mg/L is reported to be the threshold that caused severe DF in US children (Selwitz et al., 1998), whereas Rango et al. (2014) found that the children barely had severe DF with water fluoride concentrations < 4.0 mg/L in Ethiopian. In addition, under the same fluoride exposure conditions, the prevalence of severe DF in children with good nutritional status is significantly lower than those of malnourished children (Del Carmen et al., 2016). In this study, we did not observe severe DF in children exposed to water fluoride up to a level of 3.90 mg/L (the mean concentration of water fluoride is 1.40 mg/L, and the 95% *CI* is 1.32–1.48 mg/L). This may be explained by differences in the levels of fluoride exposure, nutritional status and population susceptibility.

Mounting studies have showed that mtDNA encodes a variety of proteins, which is required for maintaining the normal function of mitochondria (Falkenberg et al., 2007; Malik and Czajka, 2013), while abnormal mtDNA results in mitochondrial dysfunction (Gadaleta et al., 1992; Malik et al. 2013). In addition, mtDNA are particularly sensitive to environmental factors because of their lack of repair capacity (Stewart and Chinnery, 2015). Studies have found that a variety of environmental factors (such as $PM_{2.5}$, NO_2, PAHs) could decrease the mtDNA content (Clemente et al., 2016; Wong et al., 2017). Moreover, *in vivo* evidence revealed that fluoride exposure could lead to mitochondrial structural damage and dysfunction in neurons and spermatozoa of rats (Barbier et al., 2010; Izquierdo-Vega et al., 2008). In the present study, we further evaluated the relationship between fluoride exposure and circulating mtDNA levels in children, and found that the concentrations of water fluoride and urinary fluoride were negatively associated with relative mtDNA levels. These alterations may be due to the DNA damage induced by fluoride and the insufficient repair capacity of mtDNA. However, Sun et al. (2017) revealed that fluoride (100 mg/L NaF) reduces nDNA integrity and increases the mtDNA content in sperm of mice. Contrasting findings between studies may result from differences in species specificity and exposure concentrations.

A large body of research has investigated mechanisms by which fluoride affects ameloblasts and enamel formation during the past decades, and found that numerous biological processes (such as DNA damage, oxidative stress, autophagy and apoptosis) might contribute to DF development (Li et al., 2017; Suzuki et al., 2015). Mitochondria, the energy producers of eukaryotic cells, is considered to play a crucial role in the toxic effects of fluoride (Couve et al., 2012). In the present study, we found that the circulating mtDNA content, a proxy of mitochondrial damage, is negatively associated with the prevalence of DF (especially with moderate DF). Although fluoride was reported to lead to mitochondrial dysfunction contributing to decrease in the odontoblast layer width and the impairment of enamel secretion from ameloblasts (Li et al., 2013; Suzuki et al., 2017), our study provides first epidemiological evidence that mtDNA levels are negatively associated with the prevalence of DF.

Stratified analysis indicated a weaker positive association between DF prevalence and fluoride exposure (see Table S2), and a stronger inverse association between DF prevalence and relative mtDNA levels in boys than in girls (see Table S3), indicating that girls are more susceptible to dental fluorosis than boys when exposed to the same concentration of fluoride. However, our results showed that the prevalence of DF in boys is higher than that in girls (see Table 1). Studies revealed that boys drink more water than girls (Forshee and Storey, 2003; Vieux et al., 2016). In addition, our results showed that the water fluoride concentrations in boys (median and interquartile are 1.20 and 0.70–2.20 mg/L, respectively) are significantly higher than in girls (median and interquartile are 1.00 and 0.50–1.70 mg/L, respectively). Taken together, girls seem to be more susceptible to DF, but the prevalence of DF in girls is lower than that in boys in the present study, perhaps due to lower fluoride intake in girls.

Fluoride is able to enhance oxidative stress, thus inducing apoptosis of ameloblasts LS8 cells and increasing the prevalence of DF in rats, while the attenuation of oxidative stress could significantly combat NaF-induced these detrimental effects (Li et al., 2017). After examining the interaction between fluoride exposure and relative mtDNA levels on the DF prevalence, we did not find a stable effect modification by relative mtDNA levels for DF prevalence (see Table S4). Given the close relationships among oxidative stress, mitochondrial dysfunction and relative mtDNA levels, we hypothesized that the reduced mtDNA content may be a causal intermediate in biological mechanisms linking fluoride exposure to DF. As expected, after grading the DF degree as normal, total DF, very mild DF, mild DF and moderate DF, we found that the associations between fluoride concentrations (water fluoride and urinary fluoride) and moderate DF are partly mediated by circulating mtDNA levels.

Our study has several strengths. We investigated the relationships between low-to-moderate fluoride exposure, relative mtDNA levels and DF, and the mediation effect of relative mtDNA levels on the association between fluoride exposure and the prevalence of DF. Besides, our research was the first to examine the relationship between mtDNA and DF in children. Compared to most previous studies, our research provided more information on the health effects of low-to-moderate fluoride exposure, which could further complete the epidemiological evidence on the biological disadvantages of fluoride across different levels. In addition, the potential confounders of our study participants, including education levels, socio-economic condition and maternal disease history, as well as delivery conditions were relatively homogeneous, making our findings more reliable.

Our study also has some limitations. Due to the cross-sectional design, our research has lesser power in terms of the causal inference of the associations between fluoride exposure and health effects. According to the annual surveillance data from the local CDC, the fluoride concentrations maintained at stable levels in the villages in which our study participants resided, which made the observed health effects associated with fluoride more reliable. However, further prospective studies are essential to validate our findings. Secondly, although morning spot urine was used as a representative of 24-h urine in many environmental epidemiology studies, monitoring of the urinary fluoride concentration throughout the day could be more reliable. Furthermore, the determination of mtDNA copy number requires absolute quantification, while our method provides relative values and therefore the data remain to be confirmed using a more robust assay, as reported by Ajaz et al. (2015). Finally, the assessment of drinking water fluoride levels at the village level may not be satisfactory in reflecting the personal external exposure, and further studies are needed to overcome this limitation.

5. Conclusions

In conclusion, we have showed that low-to-moderate concentrations of water fluoride and urinary fluoride were positively associated with DF prevalence, while inversely associated with circulating mtDNA levels. Additionally, our study indicates that the gender potentially modifies the associations of DF prevalence with relative mtDNA levels and low-to-moderate fluoride exposure, and that the reduced mtDNA levels may partly mediate the elevated prevalence of moderate DF in children under such exposure.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.03.033.

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