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A relationship in adrenal androgen levels between mothers and their children from a dioxin-exposed region in Vietnam

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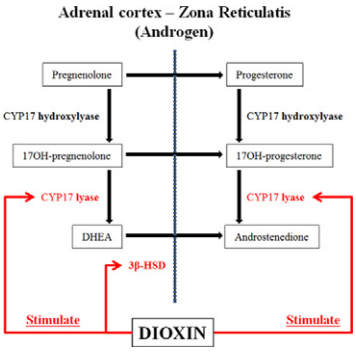
HIGHLIGHTS

- Maternal dioxin levels were 2- to 5-fold higher in the dioxin-contaminated region.
- Salivary DHEA level in children was higher in the dioxin-contaminated region.
- Serum androstenedione level in mothers was higher in the dioxin-contaminated region.
- Salivary DHEA in children related positively with serum androstenedione in mothers.
- Dioxin enhanced androgens biosynthesis in both mother and children.

GRAPHICAL ABSTRACT



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ABSTRACT

Over the past decades, southern Vietnam has been burdened by dioxins from contaminated herbicides sprayed during the Vietnam War. In a previous study, we found that dioxin exposure decreased levels of salivary dehydroepiandrosterone (DHEA), an adrenal androgen, in 3-year-old children. In present study, to assess the relationship between adrenal hormones disruption in lactating mothers and in children, we compared mother-child pairs from dioxin- and nondioxin-contaminated regions. In 2010 and 2011, mother-child pairs from a dioxin hotspot region (n = 37) and a non-contaminated region (n = 47) were recruited and donated breast milk and serum samples for dioxin and steroid hormones determination. Mothers were 20–30 years old and had given birth to their first child between 4 and 16 weeks previously. One year later, saliva samples were collected from the children. Dioxin levels in breast milk were determined by gas chromatography/high-resolution mass spectrometry. Salivary DHEA, cortisol in children and androstenedione (A-dione), estradiol, cortisol, and DHEA in maternal serum were analyzed by liquid chromatography/tandem mass spectrometry. Concentrations of dioxin congeners

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in the hotspot region were 2- to 5-fold higher than in samples from the non-contaminated region. Salivary DHEA levels in children and serum A-dione levels in mothers were significantly higher in the hotspot region; no difference was found in the levels of other hormones. Moreover, there was a significant positive correlation between the elevated hormone levels in mothers and children ($r = 0.62$, $p < 0.001$). Several dioxin congeners exhibited strong significant dose-response relationships with salivary DHEA and serum A-dione levels. Our findings suggest that dioxin disrupts adrenal androgens in mothers and breastfeeding children through the same mechanism.

1. Introduction

The past few decades have seen a growing interest in the health effects posed by endocrine-disrupting chemicals (EDCs), which interfere with the biosynthesis, transport, and metabolism of steroid hormones and are associated with disease and disability (Frye et al., 2012). EDCs may affect exposed individuals and their children, as well as subsequent generations (Schug et al., 2011). Some studies have shown that children are more sensitive than adults to toxic chemicals; therefore, assessment of endocrine effects in children is essential (Dourson et al., 2004; Needham and Sexton, 2000). Numerous studies have addressed the effects of dioxins, which are widespread and persistent toxic chemicals generated as by-products of industrial and agricultural activities (Dwyer and Themelis, 2015; Gilpin et al., 2003; Liberti, 2014; Sappington et al., 2015).

During 1962 to 1971, the United States Air Forces sprayed dioxin-contaminated herbicides over South of Vietnam for the purposes of defoliation and crop destruction. It has been suggested that dioxin have caused teratogenic health effects, cancer, and neurodevelopmental disorders (Sterling and Arundel, 1986; Pham et al., 2015a, 2015b; Tran et al., 2016). Despite natural elimination from the environment after four decades, elevated levels of dioxins are still recorded at some former airbases, where herbicides were spilled or sprayed for security reasons (Minh et al., 2009; Schecter et al., 2001; Stellman et al., 2003). Although current maternal dioxin levels are decreased by around hundred times in comparison to extremely high TCDD level of 1832 parts per trillion in breast milk samples collected in 1970, dioxin concentrations in breast milk and in the blood of adult men in polluted regions are still three to five times higher than those recorded in non-contaminated regions (Hue et al., 2014; Manh et al., 2014; Manh et al., 2015; Schecter et al., 1995).

The three regions most severely polluted with dioxins, Bien Hoa, Phu Cat, and Da Nang, are termed "hotspots" (Dwernychuk, 2005). In addition to direct exposure from the environment, transfer of dioxins through the food chain is the main route of indirect human exposure for Vietnamese living in and around these regions (Mai et al., 2007; Schecter et al., 2006; Schecter et al., 2003). Because of their highly lipophilic properties and long half-life, dioxins accumulate in adipose tissues and are excreted in breast milk (Van den Berg et al., 1994; Ulaszewska et al., 2011). It has been reported that maternal dioxin body burdens decrease on average by 20%–30% during the lactation period (Abraham et al., 1996). Therefore, infants have a high risk of dioxin exposure from breast milk.

To date, multiple studies have focused on health risk assessments of residents at dioxin hotspots (Anh et al., 2014; Pham et al., 2015a, 2015b; Tuyet-Hanh et al., 2015; Van Thuong et al., 2015). However, few studies have looked into the endocrine effects of dioxins in humans. In the past 10 years, our group conducted epidemiological studies in hotspots in Vietnam, to determine the impact of dioxin exposure on the endocrine system, focusing in particular on steroid hormone disruption.

Our recent findings suggest that dioxin exposure leads to a disruption of several sex hormones with age, leading to a higher incidence of prostate cancer in Vietnamese men from a hotspot region (Sun et al., 2017). Furthermore, we previously reported significant associations between dioxin concentrations in breast milk and cortisol or cortisone levels in maternal serum or saliva (Kido et al., 2014; Manh et al., 2013; Nhu et al., 2010). Our group also found that infants born to mothers categorized in high cortisol group, tended to exhibit low birth weight (Van Tung et al., 2016). Regarding endocrine disruption in children, we previously found that salivary dehydroepiandrosterone (DHEA) level, a major adrenal androgen, is lower by approximately 50% in 3-year-old children from a hotspot region, compared with children from a control region. Moreover, the salivary DHEA levels were associated negatively with maternal dioxin concentrations in breast milk (Kido et al., 2016). However, whether dioxin-induced hormone disruption in children correlates with maternal hormone disruption is still unknown. To elucidate the endocrine effects of dioxin exposure on a more immature stage of development, the current study focused on adrenal glucocorticoids and androgens in mother and 1-year-old child pairs.

In Vietnam, children are breastfed mainly to least 12 months. Thus, breastfeeding was the major route of dioxin exposure for children in the present study. We therefore considered maternal dioxin levels to reflect the dioxin body burden in these children. Moreover, the estimation of multiple hormones in infants is difficult because the levels are low, and preferred sampling methods should be non-invasive. Therefore, we selected saliva as our testing matrix because it can be collected non-invasively by established methods (Kido et al., 2014; Lewis, 2006).

In this study, we firstly measured hormone levels in mother and paired 1-year-old child by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Following the steroid hormone biosynthesis pathway in the adrenal gland, key enzymatic activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 17A1 lyase (CYP17 lyase) were calculated. Finally, we evaluated the hormonal correlation between mother-child pairs and the association between the hormone levels or enzymatic activities and maternal dioxin congeners.

2. Subjects and methods

2.1. Study region

The hotspot region selected for the study was Bien Hoa, an industrial city in Dong Nai Province east of Ho Chi Minh City. Approximately 50% of the most contaminated herbicide (Agent Orange, AO) was stored in the Bien Hoa airbase during the war time, and at least four AO spills occurred there between 1969 and 1970 (Dwernychuk et al., 2002; Young, 2009). Elevated dioxin levels are still found in environmental and human samples collected in and around this region (Huyen et al., 2015; Nghi et al., 2015; Van Thuong et al., 2015). The non-contaminated reference region used in this study was the Kim Bang district, which is located in northern Vietnam, and was therefore not sprayed during herbicide operations. Moreover, as Kim Bang is a rural area not located in an industrial development zone, agriculture is the main activity there, and residents are not affected by industrial chemical pollution (Manh et al., 2014; Nhu et al., 2011).

2.2. Subjects and sample collection

Participants were women aged 20–30 years who had given birth to their first child 4 to 16 weeks previously. We recruited 52 mothers from Bien Hoa (September 2010) and 52 mothers from Kim Bang

(September 2011). Between 8:00 and 10:00 in the morning of enrollment, breast milk and blood samples were collected from participants by local support staff. Participants were also required to complete a questionnaire concerning social characteristics, diseases, and hormonal therapy, and body measurements were obtained for both mothers and children. One year later, about 200–500 mg of saliva were collected from the children between 08:00 and 10:00 in the morning using tweezers and hormone-free cotton swabs. To obtain hormone-free cotton, cotton was washed with hot distilled water and ethanol twice, followed by natural evaporation at room temperature. Afterward, the cotton was dried at 50–60 °C for several hours. DHEA levels in the cotton were below the limit of quantitation (LOQ). Swabs were inserted into the children's mouths and allowed to soak for 1 min. The soaked saliva cotton swabs were then placed into conical tubes; the swabbing was repeated for a total of three replicates. The volume of saliva was calculated by weight. Maternal blood collection and pre-treatment methods at enrollment were outlined in detail previously (Kido et al., 2016; Kido et al., 2014). To observe physical development, each child's body indices were also measured. Some loss to follow-up (migration) and censoring (missing answers in the questionnaire) occurred. Consequently, breast milk, serum, and child saliva samples were available for 37 and 47 mother-child pairs from Bien Hoa and Kim Bang, respectively. All samples were stored cold with dry ice for transportation and kept at −70 °C until analysis in Japan.

This study was approved by the Medical Ethics Committee of Kanazawa University. Prior to sample collection, informed consent ensuring identity masking and commitment to scientific purposes was given by from each participant.

2.3. Instruments

2.3.1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

We used an API-4000 triple-stage quadrupole mass spectrometer (SCIEX, Framingham, MA, USA) combined with an Agilent 1100 liquid chromatography (LC) system (Agilent Technologies, Santa Clara, CA, USA) and CTC Analytics Pal Auto-Sampler System. The ion source was operated in an electrospray ionization mode. The analytical columns for salivary steroid analysis and serum steroid analysis were a Cadenza CD-C18 (250 × 3 mm, 3 μm; Imtakt, Portland, OR, USA) and a Kinetex C18 (150 × 2.1 mm, 1.73 μm; Phenomenex, Torrance, CA, USA), respectively.

2.3.2. Gas chromatography-high resolution mass spectrometry (GC-HRMS)

We used an HP-6980 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) coupled to a JMS700 high-resolution mass spectrometer (HRMS, JEOL, Tokyo, Japan) in the selected ion-monitoring mode at a resolution of 10,000. The column used for GC was an ENV-5MS (30 m × 0.25 mm, 0.25 μm; Kanto Chemical Co., Inc., Tokyo, Japan).

2.4. Analysis of maternal serum hormone levels by LC-MS/MS

Analyses of steroid hormone levels in serum were performed following the protocol reported previously (Kido et al., 2016; Kido et al., 2014). Serum (200 μL) was diluted with purified water to 1 mL and spiked with cortisol-$^{2}H_4$ (1 ng), DHEA-$^{2}H_4$ (100 pg), and estradiol-$^{13}C_4$ (100 pg) as internal standards. After extraction with ethyl acetate, successive purification with cartridge column and derivatization with anhydrous picolinic acid were carried out according to Yamashita et al. (2009). The purified extract was injected to an LC-MS/MS to determine cortisol, DHEA, androstenedione (A-dione), and estradiol (E2) levels. Both the accuracy and precision in inter- and intra-day assays were within ±20% of estimate values at the lowest levels, and both were within ±15% of estimated values for all concentrations other than the lowest level.

The ratios of (androgen + estrogen) to cortisol and the ratios of A-dione to DHEA were calculated from individual serum levels using the following equations, and were defined as CYP17 lyase activity and 3β-HSD activity, respectively (Li and Wang, 2005; Sun et al., 2017).

$$(\text{Androgen} + \text{estrogen}) : \text{cortisol ratio as CYP17 lyase activity } (\%) = 100 \times (\text{DHEA} + \text{A-dione} + \text{E2 levels})/(\text{cortisol level})$$

$$\text{A-dione} : \text{DHEA ratio as 3}\beta\text{-HSD activity } (\%) = 100 \times (\text{A-dione level})/(\text{DHEA level})$$

2.5. Child salivary hormone analysis

After extracting the saliva-soaked cotton swabs with ethanol three times, the obtained solution was spiked with 1 ng cortisol-$^{2}H_4$ and 100 pg DHEA-$^{2}H_4$ as internal standards. The mixture was evaporated in a centrifugal evaporator at 40 °C. Following dilution with water, the solution was extracted by ethyl acetate and the organic layer was evaporated. The dried extract was purified on a cartridge column (Bond Elut C-18; Agilent Technologies). Purified samples were derivatized with anhydrous picolinic acid. DHEA and cortisol derivatives were analyzed by LC-MS/MS, as described in Section 2.4. The lowest estimated levels, accuracy, and precision were the same as those described in Section 2.4. We did not determine testosterone and A-dione levels in children, as they were too low to detect in the children's saliva samples.

The androgen:cortisol ratio was calculated from DHEA and cortisol levels in saliva and defined as CYP17 lyase activity (Li and Wang, 2005; Sun et al., 2017).

$$\text{Androgen} : \text{cortisol ratio as CYP17 lyase activity } (\%) = 100 \times (\text{DHEA level})/(\text{cortisol level}).$$

2.6. Dioxin analysis by GC-HRMS

Dioxins in breast milk samples were quantified according to a previously reported procedure (Tawara et al., 2011; The Tai et al., 2011). After a series of sample treatments, including extraction, clean-up, purification, internal standard spike (40–80 pg of each 2,3,7,8-substituted ^{13}C-labeled dioxins/furans), and nitrogen flow evaporation, the obtained extracts were reconstituted with 20 μL of nonane containing 40 pg of each ^{13}C-labeled 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (TCDD) and ^{13}C-labeled 1,2,7,8-tetrachlorodibenzofuran (TCDF), followed by analysis with GC-HRMS. Ten congeners of polychlorinated dibenzo-p-dioxins (PCDDs) and seven congeners of polychlorinated dibenzofurans (PCDFs) were quantified. The limit of detection (LOD) was set at a signal-to-noise ratio of 3, and concentrations below the LOD were assigned a value equal to half of the LOD. The estimated concentrations are shown as pg/g lipid. The toxic equivalency (TEQ) values of 17 congeners were obtained by multiplying the concentration (pg/g lipid in milk) by the updated World Health Organization toxic equivalency factors (TEF) (Van den Berg et al., 2006).

In addition, in present study, dioxin daily intake (DDI) of children was calculated using the following equation from our previous study (Manh et al., 2015).

$$\text{DDI (pg-TEQ/kg bw/day)} = (800 \times \text{L} \times \text{TEQ})/\text{W}$$

L: lipid content of breast milk sample (%); TEQ: dioxin TEQ concentration (pg-TEQ/g lipid); W: body weight of child (kg).

2.7. Statistical analysis

All variables were transformed into log10 form to improve normality. Distributions of variables were checked using the Shapiro-Wilk test, and appropriate methods were then applied for data analysis. Chi-squared, Student's *t*-test, or the Wilcoxon test were used for

comparisons, depending on the type and distribution of variables. Since anthropometric values increase with age, body measurements of mothers and children were adjusted for age for analysis of covariance (ANCOVA). Hormonal associations between mothers and children were expressed by Pearson correlation coefficient for pairs of normal-distribution variables and by Spearman's correlation coefficient if one of the variables was not distributed normally. For children, multiple regression analysis was performed with salivary DHEA or CYP17 lyase activity as dependent variables and maternal dioxin levels and children's age, body mass index (BMI), and sex as independent variables. In another multiple regression analysis for mothers, serum A-dione or 3β-HSD activity was used as the dependent variable, and maternal dioxin levels, age, and BMI as independent variables. Statistical significance was determined at $p < 0.05$. All statistical analyses were performed using the JMP statistical discovery software version 9.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Characteristics of study subjects

Table 1 displays the demographic characteristics of the study participants. Mothers from the hotspot region were older than mothers from the non-contaminated region. Income was also significantly higher in the hotspot region, because it is located in an industrial zone. Children from the hotspot region were older than children from the non-contaminated region. After adjustment for age, measures of children from the hotspot region displayed significantly greater height, weight, BMI, and head circumference than those from the non-contaminated region after age adjustment.

Table 1
Characteristics of study subjects.

Subjects	Parameters	Estimation values		p
		Hotspot (n = 37)	Non-contaminated (n = 47)	
Mothers	Year old (years)	26 (24–27)	22 (20–26)	**,a
	Height (cm)	150.9 ± 4.5	150.6 ± 4.8	n.s.[c]
	Weight (kg)	48.6 ± 6.1	48.3 ± 5.3	n.s.[c]
	BMI (kg/m^2)	21.7 (19.2–23.1)	20.7 ± 2.0	n.s.[c]
	Residency (years)	13 (4–26)	20 (18–24)	n.s.[a]
	Income (10^6 VND)	5.2 ± 3.4	3.5 (2.5–4.8)	*,a
Children	Gender	Boy (n = 25) Girl (n = 12)	Boy (n = 25) Girl (n = 22)	n.s.[b]
	Month old (months)	15 (14–16)	14 (14–15)	**,a
	Height (cm)	80.2 ± 3.2	76.3 ± 3.0	***,d
	Weight (kg)	10 (9–11)	9.1 ± 1.2	**,d
	BMI (kg/m^2)	15.6 (15.2–17.0)	15.4 (14.4–16.2)	*,d
	Head (cm)	46.3 (44.8–47.8)	44.9 ± 1.0	**,d
	Chest (cm)	46.8 (45.4–48.6)	45.8 (44.0–47.0)	n.s.[d]

Data are reported as mean ± standard deviation for a normal distribution and as median (interquartile range) for data that were not distributed normally.
BMI: body mass index.
n.s. not significant.
[a] Wilcoxon test.
[b] Chi-squared test.
[c] Analysis of covariance (ANCOVA) was adjusted for maternal age.
[d] ANCOVA was adjusted for children's age.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

3.2. Maternal levels of dioxin congeners

Table 2 shows the LOD of dioxin analysis and the concentrations of 17 dioxin and furan congeners, TEQs and DDI levels in maternal breast milk. Most levels measured were significantly (2- to 5-fold) higher in the hotspot group than in the non-contaminated group, except for levels of the furan congeners 2,3,7,8-TCDF, 1,2,3,7,8-pentachlorodibenzofuran (PeCDF), 1,2,3,7,8,9- and 2,3,4,6,7,8-hexachlorodibenzofuran (HxCDF), and octachlorodibenzofuran (OCDF). The TEQs of PCDDs and PCDFs, along with the values of total TEQs of PCDDs and PCDFs, were 2- to 3-fold higher in the hotspot region than in the non-contaminated region. The DDI of total TEQs was significantly higher in the hotspot region than in the non-contaminated region.

3.3. Hormone levels in mother-child pairs

Table 3 and Fig. 1 display the LOQ of hormone analysis, the hormone levels in mother-child pairs and the biosynthesis pathway of steroids in the adrenal gland. There were significant differences between the groups, with higher A-dione levels, CYP17 lyase activity, and 3β-HSD activity in mothers from the hotspot region. In contrast, DHEA, cortisol, and E2 levels did not differ significantly between the two groups. In children, salivary DHEA levels and CYP17 lyase activity in subjects from the hotspot region were significantly higher than those of subjects from the non-contaminated region, but cortisol levels did not differ. We did not observe any differences in hormone levels between male and female children from the hotspot region, the non-contaminated region, or the combined cohort (data not shown).

Table 2
Dioxin congener levels in maternal breast milk samples.

Dioxin congeners	LOD (pg/g lipid)	Dioxin concentrations (pg/g lipid)				Ratio[a]	p
		Hotspot (n = 37)		Non-contaminated (n = 47)			
		GM	GSD	GM	GSD		
2,3,7,8-TeCDD	0.01	2.04	2.47	0.56	1.77	3.6	***,b
1,2,3,7,8-PeCDD	0.01	2.82	1.63	1.04	1.80	2.7	***,b
1,2,3,4,7,8-HxCDD	0.02	1.45	1.58	0.79	1.83	1.8	***,b
1,2,3,6,7,8-HxCDD	0.02	4.79	1.86	1.23	1.68	3.9	***,b
1,2,3,7,8,9-HxCDD	0.02	1.66	1.80	0.58	1.76	2.9	***,b
1,2,3,4,6,7,8-HpCDD	0.02	9.37	1.76	2.40	1.93	3.9	***,c
OCDD	0.05	60.61	1.68	16.14	1.78	3.8	***,b
2,3,7,8-TeCDF	0.01	0.46	1.82	0.64	1.62	0.7	*,b
1,2,3,7,8-PeCDF	0.01	0.48	1.95	0.38	2.04	1.3	n.s.[c]
2,3,4,7,8-PeCDF	0.01	4.33	1.52	3.03	1.42	1.4	***,b
1,2,3,4,7,8-HxCDF	0.02	7.19	1.89	1.56	1.59	4.6	***,c
1,2,3,6,7,8-HxCDF	0.02	4.27	1.83	1.30	1.45	3.3	***,c
1,2,3,7,8,9-HxCDF	0.02	0.36	2.00	0.33	1.60	1.1	n.s.[b]
2,3,4,6,7,8-HxCDF	0.02	0.67	1.87	0.53	1.72	1.3	n.s.[b]
1,2,3,4,6,7,8-HpCDF	0.02	4.75	1.85	1.01	2.10	4.7	***,c
1,2,3,4,7,8,9-HpCDF	0.02	0.67	2.31	0.22	1.64	3.0	***,b
OCDF	0.05	0.62	1.70	0.64	1.64	1.0	n.s.[b]
TEQs PCDDs		6.18	1.84	2.00	1.56	3.1	***,b
TEQs PCDFs		2.75	1.61	1.41	1.34	2.0	***,c
Total TEQs		9.19	1.71	3.48	1.39	2.6	***,b
DDI of total TEQs (pg-TEQ/kg bw/day)		22.28	2.40	7.00	1.72	3.2	***,b

Data are reported as GM and geometric standard deviation (GSD).
LOD: limit of detection, TEQs: the toxic equivalency values, DDI: dioxin daily intake of children.
Total TEQs: sum of TEQs of polychlorinated dibenzo-*p*-dioxins (PCDDs) and of polychlorinated dibenzofurans (PCDFs).
n.s. not significant.
[a] Ratios of dioxin geometric mean (GM) in the hotspot region and the non-contaminated region.
[b] Student's *t*-test.
[c] Wilcoxon test.
* $p < 0.05$.
*** $p < 0.001$.

Table 3
Hormone levels in mother-child pairs.

Parameters	LOQ (ng/assay)	Hormone levels or % (ng/ml or %)		p
		Hotspot (n = 37)	Non-contaminated (n = 47)	
In mothers' serum				
DHEA	0.005	3.28 ± 1.24	2.82 (2.33–3.92)	n.s.[b]
A-dione	0.01	1.91 ± 1.00	0.61 ± 0.27	***,a
Estradiol	0.0005	0.02 (0.01–0.03)	0.01 (0.004–0.02)	n.s.[b]
Cortisol	0.05	104.25 ± 50.94	91.20 ± 36.53	n.s.[a]
CYP17 lyase activity (%)		5.39 (4.45–6.48)	4.68 ± 2.23	*,b
3β-HSD activity (%)		63.08 ± 32.37	19.58 ± 6.60	***,a
In children's saliva				
DHEA	0.005	0.13 ± 0.08	0.04 ± 0.02	***,a
Cortisol	0.05	1.14 ± 0.83	0.81 (0.58–1.16)	n.s.[b]
CYP17 lyase activity (%)		13.88 ± 8.33	4.79 ± 4.45	***,a

Data are reported as mean ± standard deviation for a normal distribution and as median (interquartile range) for data that were not distributed normally.
CYP17 lyase activity in mothers' serum (%) = 100 × (DHEA + A-dione + E2 levels) / (cortisol level).
CYP17 lyase activity in children's saliva (%) = 100 × (DHEA level) / (cortisol level).
3β-Hydroxysteroid dehydrogenase (3β-HSD) activity (%) = 100 × (A-dione level) / (DHEA level).
LOQ: limit of quantitation (lowest estimated level), DHEA: dehydroepiandrosterone, A-dione: androstenedione.
n.s. not significant.
[a] Student's *t*-test.
[b] Wilcoxon test.
* $p < 0.05$.
*** $p < 0.001$.

3.4. Correlation between maternal serum hormone levels and child salivary hormone levels

Table 4 shows the relationship between hormone levels in mother-child pairs. Mothers' A-dione levels were associated with DHEA levels in their children (Fig. 2; r = 0.62, $p < 0.001$). No significant correlation was found between child DHEA or cortisol levels and other maternal hormone levels.

3.5. Correlation between maternal and child hormone levels and concentrations of dioxin/furan congeners in maternal breast milk

Significant associations were found between most dioxin/furan congeners and children's salivary DHEA levels and CYP17 lyase activity or mothers' serum A-dione levels and 3β-HSD activity. Fig. 3 shows the associations with the highest correlation coefficient values. In children, we found a significant correlation between 1,2,3,4,7,8-HxCDF and salivary DHEA levels (r = 0.59, $p < 0.001$) and CYP17 lyase activity (r = 0.45, $p < 0.001$), as well as between total TEQs and salivary DHEA levels (r = 0.47, $p < 0.001$). In mothers, we found a significant correlation between 1,2,3,4,7,8-HxCDF and serum A-dione levels (r = 0.71, $p < 0.001$), 1,2,3,6,7,8-HxCDF and 3β-HSD activity (r = 0.75, $p < 0.001$), and total TEQs and serum A-dione levels (r = 0.62, $p < 0.001$).

3.6. Correlation between maternal and child hormone levels and dioxin congener levels using multiple regression analysis

Table 5 shows the results of multiple regression analysis using salivary DHEA levels and CYP17 lyase activity in children or A-dione levels and 3β-HSD activity in mothers as dependent variables. We found that two among the 17 congeners, 1,2,3,4,7,8- and 1,2,3,6,7,8-HxCDF, showed a strong correlation with hormone levels and enzyme activities (β = 0.55 and 0.52, respectively, for salivary DHEA levels in children; β = 0.42 and 0.40, respectively, for CYP17 lyase activity in children; β = 0.74 and 0.71, respectively, for A-dione levels in mothers; β = 0.76 and 0.78, respectively, for 3β-HSD activity in mothers).

4. Discussion

Although dioxins as a group are suspected to cause long-term endocrine disruption, few epidemiological studies have investigated dioxin exposure in infants (Patisaul and Adewale, 2009).

The salivary concentration represents the free form of a serum hormone and its quantity is <10% of serum levels. As salivary hormones

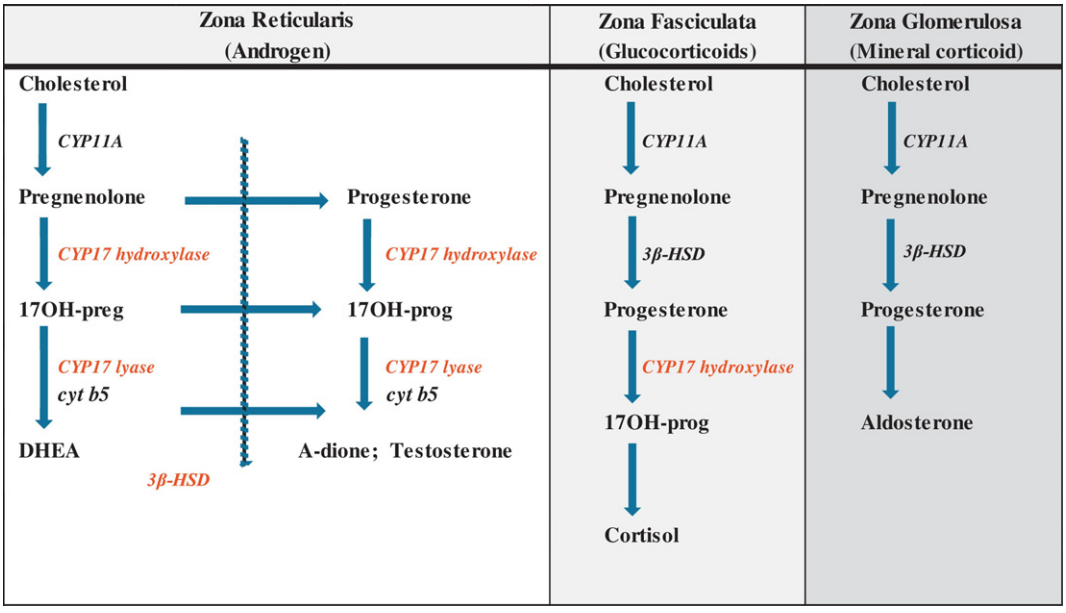

Fig. 1. The steroid biosynthesis pathway in the adrenal gland. 3β-HSD: 3β-hydroxysteroid dehydrogenase, 17OH-preg: 17α-hydroxypregnenolone, 17OH-prog: 17α-hydroxyprogesterone, A-dione: androstenedione, CYP11A: P450scc cholesterol 20,22 side-chain cleavage, CYP17 hydroxylase: p450 17α-hydroxylase, CYP17 lyase: p450 17,20-lyase, cyt b5: cytochrome *b*5.
Bhatt et al. (2016); Li and Wang (2005); Voutilainen and Jääskeläinen (2015); Yoshimoto and Auchus (2015).

Table 4
Correlation between serum hormone levels in mothers and salivary hormone levels in paired children.

Hormones	Subjects (n = 84)			
	Children DHEA (pg/ml)		Children cortisol (pg/ml)	
	r	*p*-Value	r	*p*-Value
Mothers DHEA (ng/ml)	0.06[b]	n.s.	−0.06[b]	n.s.
Mothers A-dione (ng/ml)	0.62[a]	***	0.09[b]	n.s.
Mothers estradiol (pg/ml)	0.09[b]	n.s.	0.04[b]	n.s.
Mothers cortisol (ng/ml)	0.12[a]	n.s.	−0.05[b]	n.s.

n.s. not significant.
[a] Pearson correlation coefficient.
[b] Spearman's correlation coefficient.
*** $p < 0.001$.

are known to be strongly associated with serum hormones, many researchers made the most of salivary hormone analysis in their epidemiology and clinical studies (Kido et al., 2014; Adam and Kumari, 2009; Inder et al., 2012). The hormone estimation by LC-MS/MS has considerably higher specificity and sensitivity than usual immunoassay method. In this study of Vietnamese mother-child pairs, levels of serum A-dione in mothers and salivary DHEA in children were significantly higher (nearly 3-fold) in the hotspot region than in the non-contaminated region. In contrast, levels of other adrenal hormones in either children or mothers did not differ between the two regions (Table 3).

Secretion of both DHEA and cortisol from individual cells in the adrenal gland is regulated by the adrenocorticotropic hormone (ACTH) (Rege et al., 2013). As shown in Table 3, the levels of salivary DHEA in children or serum A-dione in mothers were higher in the hotspot region; however, cortisol levels were not. We therefore speculate that dioxins may directly influence steroid hormone biosynthesis in the adrenal gland.

The human adrenal gland is composed of the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR). Each layer is responsible for the production of specific hormones: Mineralocorticoids in the ZG, glucocorticoids in the ZF, and precursors of active androgen in the ZR (Voutilainen and Jääskeläinen, 2015). As shown in Fig. 1, DHEA and cortisol are synthesized in different zones from pregnenolone in a process catalyzed by cytochrome P450 17A1 (CYP17A1) enzyme, which includes the 17α-hydroxylase and the 17,20-lyase catalytic activity. With the appearance of both activities in the ZR, 17α-hydroxypregnenolone is converted exclusively from pregnenolone by 17α-hydroxylase, followed by production of DHEA, catalyzed by 17,20-lyase (Yoshimoto and Auchus, 2015). In contrast, in the presence

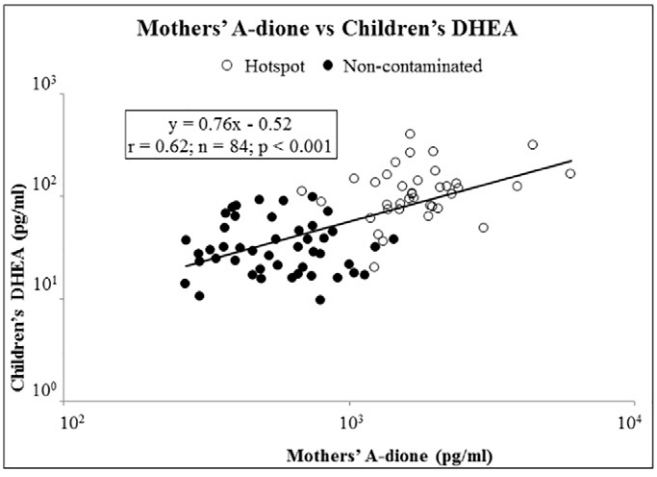

Fig. 2. Correlation between serum A-dione levels in mothers and salivary dehydroepiandrosterone (DHEA) levels in paired children.

of only 17α-hydroxylase but without 17,20-lyase activity in the ZF, 17α-hydroxyprogesterone is produced from progesterone, leading to generation of cortisol (Li and Wang, 2005). In infants, adrenal cortical cells contain an arrangement known as the fetal zona reticularis (ZRF) (Miller, 2009; Rege and Rainey, 2012). High levels of CYP17 and cytochrome *b*5 (cyt b5) are present in the ZRF, unlike 3β-HSD (Voutilainen and Jääskeläinen, 2015).

In the present study, we evaluated CYP17 lyase activity in mother-child pairs and 3β-HSD activity in mothers using ratios of hormone levels as described methods. CYP17 lyase activity was significantly higher in children from the dioxin hotspot indicating 17,20-lyase activity in the adrenal gland had been promoted. We therefore speculated that dioxins may influence steroid hormone biosynthesis in the adrenal gland through enzymatic activities. During pregnancy, a larger quantity of DHEA is produced from maternal pregnenolone in the fetal zone of the fetal adrenal cortex, and 80% of produced DHEA is converted into placental estriol (Kaludjerovic and Ward, 2012). Within several weeks of birth, DHEA levels decrease considerably, along with levels of cyt b5, and remain low until the beginning of adrenarche formation (Rege and Rainey, 2012). The CYP17 activities are modulated by cyt b5 (Bhatt et al., 2016). Therefore, we also hypothesized that dioxin may influence the allosteric regulator function of cyt b5, leading to changes in 17,20-lyase activity in DHEA production in children's adrenal glands.

Between the ages of one and three, DHEA levels decrease. Beyond that stage, DHEA levels increase gradually and reach their highest point in the mid-twenties, then decrease (Turcu et al., 2014; Voutilainen and Jääskeläinen, 2015). Previously, we found lower levels of DHEA in the saliva of 3-year-old children from a dioxin hotspot region, suggesting a dioxin-induced delay in DHEA increases (Kido et al., 2016). In fact, DHEA decreases considerably after birth due to the disappearance of the fetal zone in the adrenal cortex. After one year, the adrenal cortex is differentiated into 3 layers clearly and DHEA is produced in new generated ZR. In the present study, we assumed that dioxin exposure might delay the change of the fetal zone leading to significantly higher levels of DHEA in saliva of 1-year-old children from the dioxin hotspot region.

As shown in Table 3 comparisons of steroid hormone levels showed that dioxin may alter the steroid biosynthesis pathway in the maternal adrenal gland as well. Significantly higher A-dione levels were found in maternal serum samples from the hotspot region. A-dione, which is converted from DHEA, is produced largely through the action of 3β-HSD. The CYP17 lyase activity in subjects from the hotspot region was significantly higher than that in subjects in the non-contaminated region ($p < 0.05$). We suggest that the levels of 17,20-lyase and 3β-HSD activities were both stimulated, leading to promotion of serum A-dione in mothers instead of cortisol or other C19 steroid hormones. High CYP17 lyase activity in mothers from the hotspot region was determined by maternal A-dione production rather than by DHEA (Table 3). These findings highlighted the functional difference between the ZR in mothers and the ZRF in children, which corresponded to 3β-HSD activities. Baba et al. reported that 3β-HSD was induced by dioxin via the aryl hydrocarbon receptor (AhR) in mice (Baba et al., 2008); our epidemiological findings are in accord with their biomolecular measurements. Furthermore, Baba et al. suggested that AhR plays a role in female and male reproduction and in modulation of steroid-regulating enzymes such as CYP19 (aromatase) and steroid acute regulatory protein (Baba et al., 2005). However, the factor implicated in the regulation of CYP17 remains unclear. AhR, which is widely conserved among animal species, is essential in maintaining normal physiological functions. Moreover, AhR is a ligand-activated, transportable factor that mediates diverse dioxin toxicities (Mimura et al., 1997).

In the present study, we analyzed 17 dioxin congeners in breast milk samples. Among these compounds, 2,3,7,8-TCDD is well-known as a toxic chemical with a TEF of 1.0, and considered a reference chemical to which the toxicity of other congeners is compared (Van den Berg et al., 2006). Levels of most dioxin congeners were 2–5 times higher in

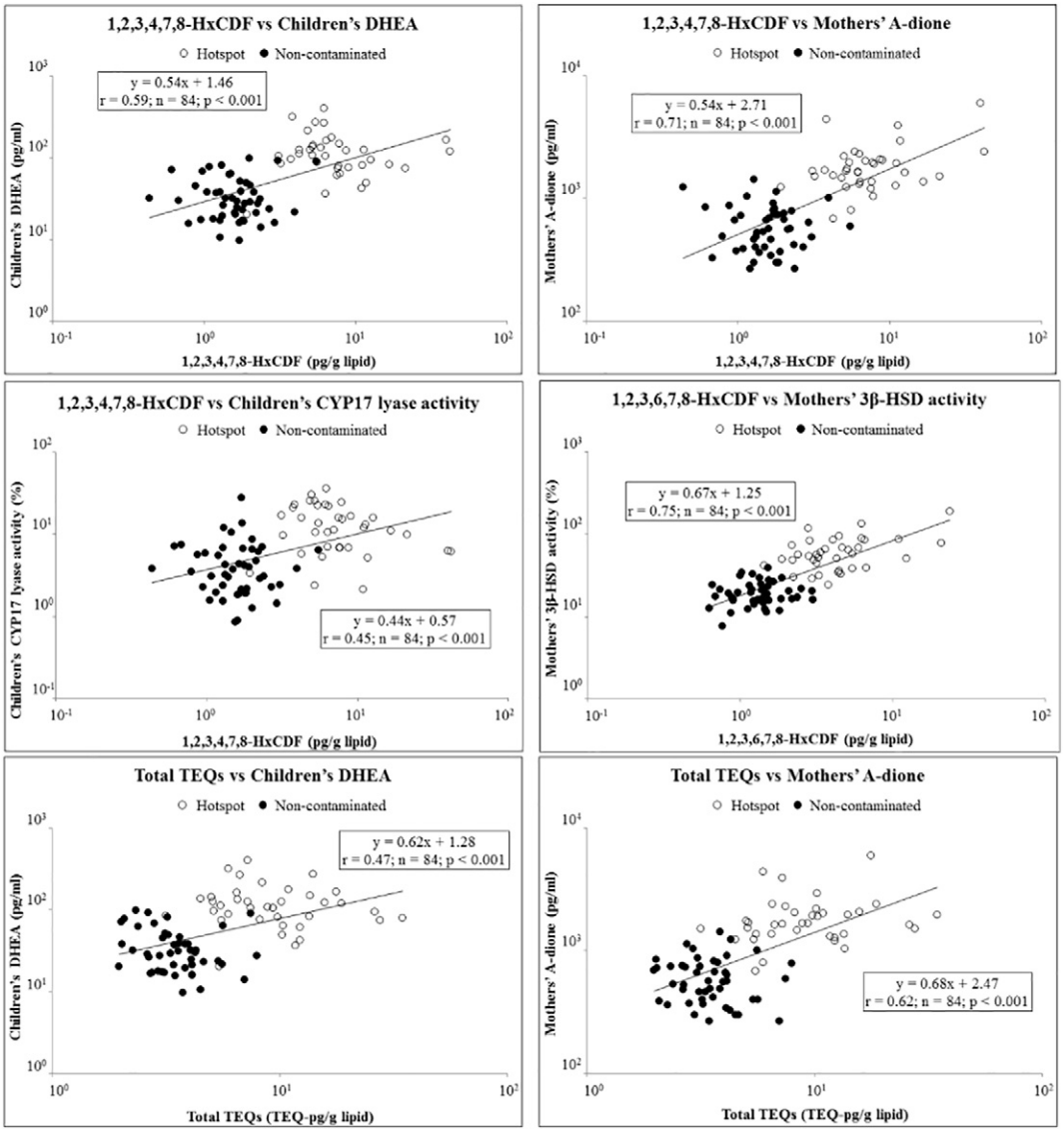

Fig. 3. Correlation between maternal serum hormone levels or children's salivary hormone levels and concentrations of highly chlorinated furan congeners and Total TEQs in maternal breast milk.

the hotspot region than in the non-contaminated region (Table 2); five furan congeners were the exception. Subjects from the non-contaminated region may have been exposed to furan congeners from agricultural herbicides or from combustion of household garbage (Lemieux et al., 2000; Zhang et al., 2011).

Some highly chlorinated congeners, including 1,2,3,4,6,7,8-heptachlorodibenzodioxin (HpCDD), octachlorodibenzodioxin (OCDD), 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF and 1,2,3,4,6,7,8-heptachlorodibenzofuran (HpCDF), were correlated with the levels of DHEA in children and A-dione in mothers, suggesting that they acted as endocrine disruptors in both mothers and children. 3β-HSD activity was closely and specifically related to 1,2,3,4,7,8-HxCDF and 1,2,3,6,7,8-HxCDF (Table 5). R-squared values calculated with these congeners as independent variables were relatively higher than values calculated for TCDD. There were strong dose-response relationships between these congeners and steroid hormone levels as determined by linearity correlation coefficient analysis (data not shown). In Japan, Tsukimori et al. identified that 1,2,3,6,7,8-HxCDF was the strongest congener that affected fetal development in Yusho disease which was caused by contaminated rice with non-*ortho* polychlorinated biphenyl (PCBs), PCDF and PCDD (Tsukimori et al., 2013a, 2013b). In our previous study, 1,2,3,4,7,8-hexachlorodibenzodioxin (HxCDD), 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, and 1,2,3,4,7,8,9-HpCDF exhibited stronger significant dose-response relationships with mothers' serum cortisol and children's salivary DHEA levels (Kido et al., 2016). This implies that highly chlorinated dioxins and furans will tend to accumulate more efficiently in adipose tissues and the adrenal gland because of their lipophilicity and 8–10 year half-life. It is therefore necessary to consider the toxicities of some highly chlorinated congeners that have much lower TEF values but can cause a significant response in exposed subjects.

We observed significantly higher salivary DHEA levels and CYP17 lyase activity in 1-year-old children from the hotspot region than those from the non-contaminated region, but cortisol levels did not differ between the two regions. In mothers, we observed increases in serum A-dione levels and CYP17 lyase activity. As shown in Fig. 2 and Table 4, a significant positive relationship was found between the two increased hormone levels in mothers and paired children. The levels of dioxin congeners in the umbilical cords obtained from babies who were born to mothers with Yusho poisoning disease were 2.5-fold higher than those of healthy babies (Nagayama et al., 2010). Tsukimori et al. demonstrated the dioxin level in cord blood approximately half of that in maternal blood (Tsukimori et al., 2013a, 2013b). In present study, dioxin in infants is transmitted by umbilical blood and breastmilk

Table 5
Correlations between hormone levels in mothers or children and dioxin congeners using multiple regression analysis.

Congener	Children (n = 84)						Mothers (n = 84)					
	DHEA			CYP17 lyase activity			A-dione			3β-HSD activity		
	β	*p*	r^2	β	*p*	r^2	β	*p*	r^2	β	*p*	r^2
2,3,7,8-TeCDD	0.35	**	0.21	0.38	**	0.19	0.57	***	0.28	0.54	***	0.25
1,2,3,7,8-PeCDD	0.37	**	0.23	0.39	**	0.19	0.58	***	0.33	0.65	***	0.41
1,2,3,4,7,8-HxCDD	0.20	*	0.14	0.09	n.s.	0.06	0.40	**	0.15	0.40	**	0.16
1,2,3,6,7,8-HxCDD	0.46	***	0.30	0.39	**	0.20	0.62	***	0.37	0.66	***	0.42
1,2,3,7,8,9-HxCDD	0.41	**	0.26	0.34	**	0.16	0.58	***	0.33	0.61	***	0.37
1,2,3,4,6,7,8-HpCDD	0.53	***	0.35	0.42	**	0.22	0.60	***	0.35	0.63	***	0.38
OCDD	0.53	***	0.34	0.42	**	0.21	0.63	***	0.39	0.65	***	0.41
2,3,7,8-TeCDF	−0.16	n.s.	0.12	−0.19	n.s.	0.09	−0.34	**	0.13	−0.20	n.s.	0.06
1,2,3,7,8-PeCDF	0.03	n.s.	0.10	0.11	n.s.	0.07	0.15	n.s.	0.04	0.27	*	0.09
2,3,4,7,8-PeCDF	0.21	*	0.14	0.21	*	0.10	0.38	**	0.15	0.40	**	0.17
1,2,3,4,7,8-HxCDF	0.55	***	0.37	0.42	**	0.21	0.74	***	0.52	0.76	***	0.56
1,2,3,6,7,8-HxCDF	0.52	***	0.33	0.40	**	0.20	0.71	***	0.48	0.78	***	0.57
1,2,3,7,8,9-HxCDF	−0.05	n.s.	0.10	−0.06	n.s.	0.06	0.04	n.s.	0.02	0.11	n.s.	0.03
2,3,4,6,7,8-HxCDF	0.002	n.s.	0.10	−0.04	n.s.	0.05	0.12	n.s.	0.03	0.19	n.s.	0.05
1,2,3,4,6,7,8-HpCDF	0.51	***	0.32	0.33	**	0.14	0.62	***	0.37	0.66	***	0.42
1,2,3,4,7,8,9-HpCDF	0.35	**	0.21	0.33	**	0.15	0.54	***	0.30	0.61	***	0.37
OCDF	−0.08	n.s.	0.10	−0.03	n.s.	0.05	−0.11	n.s.	0.03	−0.04	n.s.	0.02
TEQs PCDDs	0.40	**	0.25	0.41	**	0.21	0.63	***	0.36	0.66	***	0.40
TEQs PCDFs	0.38	**	0.23	0.30	**	0.14	0.59	***	0.34	0.65	***	0.41
Total TEQs	0.42	***	0.26	0.40	**	0.20	0.65	***	0.40	0.69	***	0.44

β: standardized beta coefficient; n.s. not significant.
For multiple regression analysis in children: dehydroepiandrosterone (DHEA) or CYP17 lyase activity was used as the dependent variable; dioxin congener levels, age, body mass index, and sex were used as independent variables.
For multiple regression analysis in mothers: androstenedione (A-dione) or 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was used as the dependent variable; dioxin congener levels, age, and body mass index were used as independent variables.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

as represented by the higher DDI of total TEQs in Table 2. However, the quantitative and qualitative distinction between the effects of dioxin exposure by these two routes becomes complex. In accordance with enzymatic activities of the steroid pathway in the adrenal gland, results from our study indicated that dioxin exposure might increase DHEA in children and A-dione in their mother through the same mechanism.

In present study, there are several limitations should be considered. Firstly, the evaluation of dioxins in children would access more accurately the influence of dioxins exposure on hormone levels. Secondly, to associate hormone disruption in mothers and paired children, estimation of maternal steroid hormones during pregnancy along with steroid hormones biosynthesized in fetal adrenal gland or the salivary hormone in baby after birth should be conducted. In accordance with scope and design of the present study, we could not collect those samples from pregnant mothers or from such young children.

To our knowledge, present study is the first evaluation of the endocrine impacts in the 1-year-old children from the dioxin hotspot region in Vietnam. Since endocrine disruptions sometimes could not be determined until onset of adrenarche, it is essential to follow-up those children for more clearly observation of impaired endocrine system and sexual abnormality in particular.

5. Conclusion

Our epidemiological findings on hormone disruption illustrate a relationship between impacts of dioxin exposure on the biosynthesis of adrenal androgen in mothers and their 1-year-old children. We intend to follow up children in present study until age of 10 to access more accurately the endocrine impacts of dioxin exposure in pre- and neonatal period.

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