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Decreased serum testosterone levels associated with 17β-hydroxysteroid dehydrogenase activity in 7-year-old children from a dioxin-exposed area of Vietnam

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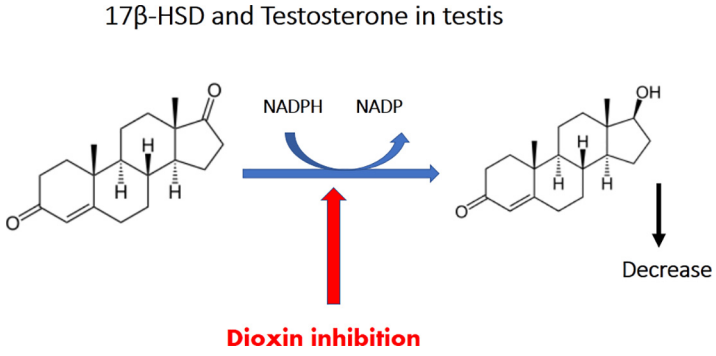
HIGHLIGHTS

- A cohort study has been carried out in a dioxin-sprayed area of Vietnam
- Serum steroid hormones in 7-year-old were estimated by LC-MS/MS
- Testosterone levels and 17β-HSD activity were lower
- DHEA level decreases at ages of 3-and 5 were recovered to normal range by age of 7
- Testosterone levels and 17β-HSD correlated with maternal dioxin levels

GRAPHICAL ABSTRACT



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ABSTRACT

Since 2008, we have conducted epidemiological cohort studies on the relationship between dioxin exposure and disruption with children in the area sprayed with defoliants during the Vietnam War. In a long-term survey of children through the age of five, we observed androgen disruption due to decreased dehydroepiandrosterone (DHEA) and testosterone levels. In this study of 7-year-old, we separately elucidated androgen disruption for

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boys and girls, and discussed with respect to hormone disruption with sex differences on the steroid hormone biosynthesis process.

This follow-up was conducted with 96 mother-child pairs in Vietnam (hotspot area: 45, non-sprayed area: 51). We took a questionnaire, the physical measurement and assayed 7 steroid hormones in their serum by LC-MS/MS. We examined the relationship between the hormone levels in the serum and dioxin levels in the maternal breast milk. The results showed that the serum DHEA level in the 7-year-old children in the hotspot recovered to levels in the non-sprayed area. The testosterone level of 66.5 pg/mL for boys in the non-sprayed area was 1.5 times the girls level of 44.6 pg/mL, a male-dominant effect. The testosterone level in boys and girls from the hotspot were significantly lower than in the non-sprayed area with no sex difference. The 17β-hydroxysteroid dehydrogenase (17β-HSD) activity was significantly higher in boys than in the girls from the non-sprayed area, but was significantly lower in the hotspot boys than in the non-sprayed area boys. Both the testosterone level and 17β-HSD activity in the boys were inversely correlated with the TEQ total PCDD/Fs in the maternal breast milk.

These results indicated that dioxin delayed the expression of the testosterone level and 17β-HSD activity with growth in the 7-year-old boys. The serum DHEA in the 7-year-old children recovered to the levels of the children in the non-sprayed area.

1. Introduction

Polychlorinated dibenzo-p-dioxin and dibenzofuran (PCDD/F) are collectively referred to as dioxin. Dioxin is known as an endocrine disruptor. The compound TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is the most toxic dioxin molecular species. The typical diseases caused by dioxin are known to be carcinogenicity (Chamie et al., 2008; Warner et al., 2011), skin disorders (Young and Young, 2017), teratogenicity (Sterling and Arundel, 1986), reproductive abnormalities (Guo et al., 2000), neurodevelopmental disorders (Tran et al., 2016; Nishijo et al., 2014), and immune abnormalities (Miyashita et al., 2011; Weisglas-Kuperus et al., 2000). Based on cellular toxicity studies, it is known that dioxin or PCB (polychlorinated biphenyl) binds to the aryl hydrocarbon receptor (AhR) and causes reproductive (Hernandez-Ochoa et al., 2009), teratogenic (Mimura et al., 1997), immune system (Nguyen et al., 2010), and hormonal disruptions (Baba et al., 2005).

The health issues caused by dioxin were established in the following three cases, and this damage has been a social problem. First, a defoliant containing dioxin as an impurity was sprayed over much of South Vietnam over a long period (1965–1971) for the purpose of killing tropical rainforests during the Vietnam War (Stellman et al., 2003; Schecter et al., 1995). Second, diseases in Japan and Taiwan (1968) were caused by the ingestion of rice oil that was contaminated with dioxin and PCB, termed Yusho disease (Tsukimori et al., 2013; Sasamoto et al., 2006). Third, an agricultural chemical factory accident in Seveso, Italy (1976) led to dioxin-polluted air causing health issues (Pesatori et al., 2003; Warner et al., 2011). Recently, Shi and Dong et al. reported that dioxin arising from electronic waste recycling produced hormone disruption in human beings. (Dong et al., 2020; Shi et al., 2020). The most severe dioxin damage in Vietnam has been reported in detail, such as the illnesses of local residents where the defoliant was sprayed, and the health issues (14 diseases) of military personnel involved in the spraying (Sterling and Arundel, 1986; Young and Young, 2017; Yamashita and Hayashi, 1985).

Fifty years have passed since the spraying of the defoliant in Vietnam. However, the dioxin levels in the bodily fluids of the residents in the hotspot are still 2–3 times higher than in the non-sprayed area (Manh et al., 2015; Tawara et al., 2011; The Tai et al., 2011; Nghi et al., 2015; Minh et al., 2009).

Therefore, since 2008, we have investigated the relationship between endocrine disruption and health issues for residents of a hotspot area of Vietnam. We tried to record, between generations, what hormone disruptions are caused by dioxin exposure and what diseases are caused by hormone perturbation.

In first-generation men, testosterone, estradiol, and DHT increased with age, and those were reported to be a risk factor for prostate disease (Sun et al., 2017). In the second-generation mothers, only the cortisol and cortisone were increased, with no significant effect on other hormones (Kido et al., 2014). When mothers of this generation gave birth, they had a higher rate of low birth weights (under 2500 g) than mothers in the non-sprayed area. The impact cause of low-weight births has been reported to be related to maternal dioxin exposure and glucocorticoid levels (Van Tung et al., 2016). In the third generation, follow-up studies of children (1- to 5-year-old) revealed an androgen disruption.

We have reported testosterone and DHEA decline in the serum and changes to related key enzyme activity in the 5-year-old from the hotspot (Oanh et al., 2018).

The purpose of this study was to elucidate androgen disruption and physical development in 7-year-old Vietnamese children. We determined seven steroid hormones in the serum using liquid chromatography tandem mass spectrometry (LC-MS/MS), and the activities of key enzymes in androgen biosynthesis (17β-HSD, CYP 17,20-lyase) were calculated on the basis of the estimated hormone levels. Furthermore, we examined their relationships with the dioxin levels in maternal breast milk, separately for boys and girls, and discussed the mechanism of androgen disruption by dioxin with age.

2. Subject and methods

2.1. Study areas

The dioxin-exposed area (hotspot) selected in the study was the Phu Cat District in the Bind Dinh Province. It was formerly a United States airbase, and was used as an herbicide supply facility and for washing aircraft during the Vietnam War (1965–1971). Phu Cat was one of three areas, along with Bien Hoa and Da Nam, known as hotspots in Vietnam.

The Kim Bang District, Ha Nam Province in northern Vietnam (a non-sprayed area) was selected as the control area. This area was not exposed to herbicides during the war. These two areas are rural areas that are not polluted by industrial pollution. (Manh et al., 2014).

2.2. Study subject

Lactating mothers and children (60 from Phu Cat and 63 from Kim Bang) participated in a September 2008 study. Since 2008, children in both areas (the hotspot and the non-sprayed area) have been followed up multiple times (as 1-year-old, 3-year-old, and 5-year-old). This time (2015), 7-year-old from Phuc Cat and from Kim Bang was included in the survey. Breast milk was collected from the mothers 4–16 weeks after delivery, and the mothers were 20–30 years old at the time of delivery. The subjects of the follow up studies are shown as Fig. 1.

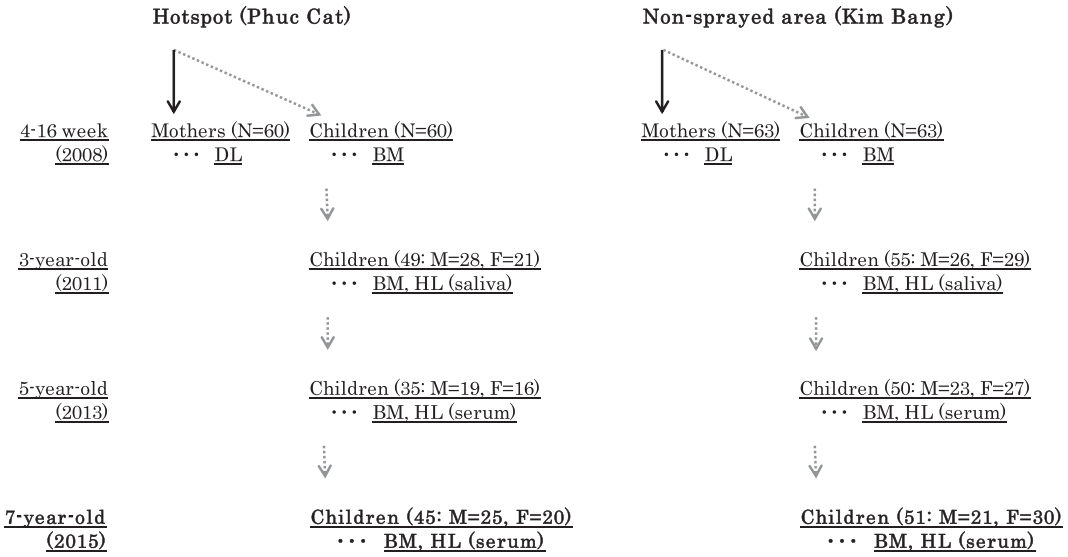

Fig. 1. Study subject M: male child, F: female child. BM: body measurement, HL: hormone level, DL: dioxin levels in maternal milk.

2.3. Sampling

Medical interviews, physical measurements, and blood sampling were performed as follows: the survey in the Phu Cat District was conducted on September 22, 2015, and the survey in the Kim Bang District was conducted on September 19, 2015. The research in both areas was carried out from 8:00 to 10:30 a.m., to escape daily hormone fluctuations. The research staff took body measurements (height, weight, head circumference, and chest circumference) of the 7-year-old. The BMI of each child was calculated based on their height and weight. Blood sampling (2 mL) was performed by the local medical staff. The collected blood was centrifuged, and the obtained serum was stored in a dry ice box and transported to a laboratory in Hanoi. The serum was stored at −70 °C until analysis. When a subject expressed a desire to discontinue blood collection, we respected their request, and told them in advance that there would not be any penalties.

2.4. Analysis of dioxin in breast milk using gas chromatography/high-resolution mass spectrometry (GC-HR-MS)

The dioxin result estimates in breast milk from all participating subjects ($n = 123$) have already been reported in another paper by our group (Kido et al., 2014). Regarding the 7-year-old children ($n = 96$) who participated in this research, they were selected based on dioxin levels in their mothers from previously assayed dioxin data in mother-children pairs using ID numbers.

The polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) were determined using a GC-RH-MS (JMS-700 MS station mass spectrometer, JEOL Ltd., Tokyo, Japan) (Tawara et al., 2011; Kido et al., 2014). Regarding the detection limit of dioxin (LOD), the signal-to-noise ratio was set to 3, and the detection values of the dioxin analogs are shown in Table 2. Concentrations below the detection limit were 50% of the limit value (below LOD = 0.5 X LOD).

The levels of PCDDs/Fs were indicated in pg/g of lipid as the mass concentration and toxic equivalents (TEQs) of the WHO 2005 toxic equivalent factors (Van den Berg et al., 2006).

2.5. Analysis of serum steroid hormones using liquid chromatography tandem mass spectrometry (LC-MS/MS)

The serum hormone estimation was performed by cooperation between the Japanese and Vietnamese researchers using LC-MS/MS at the Centre for Environmental Monitoring in northern Vietnam.

The serum (200 μL) was diluted to 1 mL with purified water, and 3 ng/100 μL of methanol solution containing an internal standard (IS) was added to the serum solution. The stable isotope-labeled compounds used as the IS were as follows: cortisol-11,12,12-$^{2}H_3$, 17-hydroxyprogesterone (17-OH progesterone-2.3.4-$^{13}C_3$), progesterone-2,3,4-$^{13}C_3$, testosterone-2,3,4-$^{13}C_3$, DHEA-4,6,7,7-$^{2}H_4$, estrone-2,3,4-$^{13}C_3$, and estradiol-2,3,4-$^{13}C_3$. After extraction with ethyl acetate according to the previously reported method (Kido et al., 2014), the extract was applied onto a reversed phase solid cartridge column (C18, 60 mg/3 mL) to remove any impurities. The obtained product was converted to a picolinic acid derivative by the acid anhydride method, according to the method of Yamashita et al. (2009). The picolinic acid derivative was purified by a silica gel cartridge column (500 mg/3 mL) to remove excess reagents. After evaporating, the purified derivative was dissolved in 100 μL of 80% acetonitrile/0.1% formic acid solution, and 10 μL of this solution was injected into the LC-MS/MS (model 6430, Agilent, Santa Clara, CA). The column used for the analysis was Zorbax (C18, 1.8 μm, 2.1 × 100 mm), and the elution solvent was a stepwise concentration gradient using a 0.1% formic acid aqueous solution and acetonitrile.

The measuring ions (m/z) for the picolinic acid derivatives (PA) or non-derivatized steroids are as follows: cortisol-PA and cortisol-PA-$^{2}H_3$, 468.2/450 and 471/453; cortisone-PA and cortisol-PA-$^{2}H_3$, 466.2/106 and 471/453; 17-OH progesterone and 17-OH progesterone-$^{13}C_3$, 331/109 and 334/112; progesterone and progesterone-$^{13}C_3$, 315/109 and 318/100.3; DHEA-PA and DHEA-PA-$^{2}H_6$, 394.2/271.2 and 400/277; androstenedione (A-dione) and progesterone-$^{13}C^3$, 287/109 and 318/100.3; testosterone-PA and testosterone-PA-$^{13}C_3$, 394.2/124 and 397/274; estrone-PA and estrone-PA-$^{13}C_3$, 376.1/106 and 379/106; estradiol-2PA and estradiol-2PA-$^{13}C_3$, 483.1/106.0 and 489/106.1. The quantitative value was obtained from each calibration curve and converted into the concentration per serum. The lower limits of quantitation (LLOQ) of cortisol-PA, cortisone-PA, 17-OH progesterone, progesterone, DHEA-PA, A-dione, testosterone-PA, are listed in Table 3. For estrone-PA and estradiol-2PA, it was 0.4, and 0.4 pg per injection, respectively.

The enzyme activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), and cytochrome P450 17,20-lyase (CYP17 lyase) were calculated from the ratio of the serum concentration using the following formula:

$$\text{CYP17 lyase activity}\ (\%) = 100 \times (\text{DHEA} + \text{A-dione} + \text{Testosterone})/17\text{-OH progesterone}$$

Table 1
Characteristics of subjects in the hotspot and the non-sprayed area.

	Parameters	N	Hotspot	N	Non-exposed	*p* value
	Full breastfeeding (weeks)	45	11.0 (10.0–12.0)	51	10.0 (8.0–13.0)	0.832
	Height (cm)	45	118.4 ± 1.1	51	119.4 ± 1.0	0.291[a]
	Weight (kg)	45	20.7 (18.1–24.8)	51	21.2 (19.4–24.0)	0.240
	BMI (kg/m^2)	45	14.3 (13.6–17.3)	51	14.8 (13.9–16.6)	0.436
Total	Head circumference (cm)	45	51.1 ± 1.0	51	51.4 ± 1.0	0.338[a]
Boys&Girls	Chest circumference (cm)	45	56.3 (53.8–60.5)	51	57.7 (55.8–60.6)	0.082
	Full breastfeeding (months)	25	10.8 ± 1.3	21	10.8 ± 1.4	0.959[a]
	Height (cm)	25	121.8 (118.1–124.7)	21	120.8 (118.0–123.4)	0.783
	Weight (kg)	25	22.1 (19.7–27.6)	21	22.2 (20.0–27.1)	0.860
	BMI (kg/m^2)	25	15.3 (13.7–17.7)	21	15.3 (13.8–17.7)	0.921
	Head circumference (cm)	25	51.7 ± 1.0	21	51.6 ± 1.0	0.834[a]
Boys	Chest circumference (cm)	25	57.5 (54.9–64.3)	21	59.6 (56.8–63.8)	0.310
	Full breastfeeding (months)	20	10.2 ± 1.2	30	10.2 ± 1.4	0.959[a]
	Height (cm)	20	115.4 ± 1.0	30	118.5 ± 1.0	0.026[a,*]
	Weight (kg)	20	18.6 (17.1–21.0)	30	20.2 (19.1–23.8)	0.029[*]
	BMI (kg/m^2)	20	14.1 (13.2–15.8)	30	14.7 (14.0–16.2)	0.137
	Head circumference (cm)	20	50.4 ± 1.0	30	51.3 ± 1.0	0.013[a,*]
Girls	Chest circumference (cm)	20	55.4 ± 1.1	30	57.7 ± 1.1	0.086[a]

Data are shown as mean ± standard deviation for a normal distribution and as median (interquartile range) for non-parametric.
[a] Student's *t*-test, Mann-Whitney test.
[*] Significant.

$$3\beta-\text{HSD activity}\ (\%) = 100 \times (\text{A}-\text{dione} + \text{Testosterone})/(\text{DHEA})$$

$$17\beta-\text{HSD activity}\ (\%) = 100 \times (\text{Testosterone})/(\text{A}-\text{dione})$$

2.6. Statistical analysis

The dioxin levels were converted to the common logarithm of log10 and expressed as the geometric mean, geometric standard deviation, or median (interquartile range). The difference of the mean in each indicator was calculated using the Student's *t*-test for the normal distribution and the Mann-Whitney *U* test for the non-normal distribution.

Additionally, depending on the distribution, the Pearson correlation coefficients and Spearman correlation coefficients were calculated between the dioxin congener and hormone levels, as well as the enzyme activity.

A multiple regression analysis was performed to consider the effects of the multiple factors. The Benjamine – Hochburg procedure was applied to adjust the *p*-value for the multiple analysis. For the multiple linear regression analysis, the analysis was done using Bonferroni corrections as a sensitivity for the multiple comparisons which altered the required level of the *p* value for significance to 0.003 or less (0.003 = 0.05/17), and 17 dioxin congeners were finally included in the analyses (Hu et al., 2019). The body measurements (height, weight, and head and chest circumferences) and steroid hormones (DHEA, A-dione, testosterone, progesterone, 17OH Progesterone, cortisol, and cortisone) were selected as dependent variables, while mother's age, residence, full breastfeeding period, BMI, and dioxin congeners were selected as independent variables.

The significance level was set at $p < 0.05$. All the statistical analyses used the SPSS 12.0 Software and the JMP 12 Software package (SAS Institute, Cary, NC, USA) and Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA).

2.7. Ethics committee

This study was approved by the Medical Ethics Committee (No. 455) of Kanazawa University. We also agreed that, for the subjects of the study, the details of the study should be adequately explained to each child's mother or father in writing, and signed upon participation.

3. Results

3.1. Characteristics of study subjects

Table 1 shows the demographic characteristics of the 7-year-old children from the hotspot and the non-sprayed areas. There was no difference in the mother's age or residence period between the two areas. The heights, weights, and head circumferences of the girls were significantly lower in the hotspot than in the non-sprayed area ($p = 0.026$, $p = 0.029$, $p = 0.013$, respectively); however, overall there was no difference in any parameters between the two areas for the children.

Table 2
Comparison of dioxin congener levels in breast milk between the hotspot and the non-sprayed area.

Dioxin congeners	LOD	Breast milk level (pg/g of lipid or TEQ)				Fold	*p* value
		Hotspot ($N = 45$)		Non-exposed ($N = 51$)			
	(pg/g lipid)	GM	GSD	GM	GSD		
2,3,7,8-TeCDD	0.01	1.2	1.8	0.3	2.2	3.5	<0.001[*]
1,2,3,7,8-PeCDD	0.01	3.9	1.5	1.2	1.6	3.4	<0.001[a,*]
1,2,3,4,7,8-HxCDD	0.02	1.8	1.5	0.6	1.6	3.1	<0.001[a,*]
1,2,3,6,7,8-HxCDD	0.02	6.1	1.6	1.3	1.6	4.9	<0.001[a,*]
1,2,3,7,8,9-HxCDD	0.02	2.4	1.5	0.5	1.7	4.3	<0.001[a,*]
1,2,3,4,6,7,8-HpCDD	0.02	11.9	1.6	2.3	1.6	5.1	<0.001[*]
OCDD	0.05	62.5	1.5	10.9	1.6	5.7	<0.001[a,*]
2,3,7,8-TeCDF	0.01	0.6	1.7	0.6	1.7	0.9	0.408
1,2,3,7,8-PeCDF	0.01	1.7	1.8	0.4	1.7	4.0	<0.001[a,*]
2,3,4,7,8-PeCDF	0.01	5.5	1.4	2.9	1.4	1.9	<0.001[a,*]
1,2,3,4,7,8-HxCDF	0.02	12.5	1.8	1.8	1.5	6.9	<0.001[a,*]
1,2,3,6,7,8-HxCDF	0.02	7.5	1.7	1.6	1.5	4.8	<0.001[a,*]
1,2,3,7,8,9-HxCDF	0.02	0.3	1.9	0.1	1.6	2.4	<0.001[*]
2,3,4,6,7,8-HxCDF	0.02	1.3	1.5	0.5	1.6	2.7	<0.001[a,*]
1,2,3,4,6,7,8-HpCDF	0.02	13.1	2.0	1.4	1.8	9.6	<0.001[*]
1,2,3,4,7,8,9-HpCDF	0.02	1.3	2.0	0.2	1.6	7.8	<0.001[a,*]
OCDF	0.05	0.9	2.9	0.3	1.9	3.1	<0.001[*]
TEQ total PCDDs		6.4	1.4	1.8	1.6	3.6	<0.001[a,*]
TEQ total PCDFs		4.2	1.5	1.4	1.4	3.0	<0.001[a,*]
TEQ total PCDDs/Fs		10.8	1.4	3.2	1.5	3.4	<0.001[a,*]

Data are reported as geometric mean (GM) and geometric standard deviation (GSD) Fold = hotspot(GM) / non-sprayed(GM), TEQs; toxic equivalent.
[a] Student's *t*-test, Mann-Whitney test.
[*] Significant.

3.2. Dioxin congener levels in breast milk

Table 2 shows the levels of 17 dioxin congeners and the TEQs of dioxin equivalents in the maternal breast milk from both areas. The levels of the dioxin congeners, except for 2,3,7,8-TeCDF and 2,3,4,7,8-PeCDF, were significant, 3–10 times higher in the hotspot than in the non-sprayed area.

In particular, the levels of the highly-chlorinated furan-types, 1,2,3,4,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, and 1,2,3,4,7,8,9-HpCDF, in the hotspot were 6.5–9.4 times higher than in the non-sprayed area. The total PCDD, PCDF, and TEQ total PCDD/Fs were 3.0–3.6 times higher in the hotspot area.

3.3. Steroid hormones and enzyme activity in children

Table 3 shows the concentrations of the serum steroid hormones and the activity of the enzymes closely related to androgen biosynthesis in the 7-year-old children from both areas. Fig. 2 shows the serum hormone levels and enzyme activities in the 5- and 7-year-old children in both areas by sex. Oanh et al. (2018) has already reported all of the collective data about the hormonal and enzyme activities in the 5-year-old children. In this study of the 7-year-old children, these data were separately reclassified into boys and girls to elucidate changes with age.

The testosterone concentrations in the 7-year-old boys and girls in the hotspot were 22.1 pg/mL and 24.2 pg/mL, respectively, and the suppression rates were equivalent to 66.7% and 45.7% of those in the non-sprayed area (Table 3). The suppression of testosterone by dioxin was more significantly observed in the boys than in the girls. In the non-sprayed area, the serum testosterone concentrations in the boys and girls were 66.5 pg/mL (5y: 54.5 pg/mL) and 44.6 pg/mL (5y: 43.0 pg/mL), respectively. The boys showed a significantly greater increase with growth (boys 18%, girls 3.6%).

The 17β-HSD activity in the 7-year-old boys in the hotspot was significantly lower than in the non-sprayed area, as well as that in the 5-year-old boys ($p < 0.001$). However, there was no significant difference seen between the two areas for the girls ($p < 0.119$).

The DHEA concentrations in the 7-year-old boys and girls from the hotspot area were 826.5 pg/mL and 659.5 pg/mL, respectively. The DHEA concentrations in the hotspot were equal to, or higher than those of children in the non-sprayed area (Table 3). In Fig. 2(C), the DHEA concentrations of the 5-year-old boys and girls in the hotspot were as low as 43.7% and 58.8%, respectively, compared to the 5-year-old in the non-sprayed area.

The serum cortisol, cortisone and 17-OH progesterone levels in the 7-year-old children showed no significant differences between the two areas ($p = 0.818$, $p = 0.115$, $p = 0.056$, respectively). The serum estradiol in the 7-year-old boys and girls could not be sufficiently measured by this method.

At the same time, the CYP17 lyase activity in the hotspot for the boys and girls was equal to, or higher than that in the non-sprayed area (Fig. 4, Table 3).

As shown in Fig. 5(A), there was a strong positive correlation between the testosterone concentration and 17β-HSD activity in the boys from both areas ($r = 0.758$, $p < 0.001$). It was clearly exhibited that the 17β-HSD activity was inhibited by the dioxin.

Table 3
Comparison of hormone levels in breast milk between the hotspot and the non-sprayed area.

Sex	Hormones/enzymes	LLOQ (pg/injection)	Hotspot			Non-exposed			*p* value
			N	GM	GSD	N	GM	GSD	
Total	Cortisol (ng/mL)	5	45	47.9	1.6	51	46.8	1.7	0.815[a]
Boys&Girls	Cortisone (ng/mL)	5	45	15.3	1.4	51	17.4	1.8	0.115[a]
	17-OH-P4 (pg/mL)	0.2	44	321.0	1.8	50	390.7	1.6	0.056[a]
	Progesterone (pg/mL)	0.5	45	266.4	1.3	51	176.7	1.4	0.000***
	DHEA (pg/mL)	1.5	45	747.6	1.4	51	560.3	1.6	0.002**,[a]
	Androstenedione (pg/mL)	0.15	44	67.4	1.8	51	90.5	1.7	0.011*,[a]
	Testosterone (pg/mL)	0.15	45	23.0	1.6	49	52.1	2.1	0.000***
	3β-HSD (%)		44	12.0	1.7	49	27.6	1.8	0.000***,[a]
	17β-HSD (%)		44	32.6	1.6	49	56.5	2.3	0.018*,[a]
	CYP17 lyase (%)		43	272.5	1.8	48	186.7	1.9	0.013*
Boys	Cortisol (ng/mL)	5	25	46.4	1.7	21	44.2	1.7	0.674[a]
	Cortisone (ng/mL)	5	25	15.0	1.4	21	17.7	1.8	0.072[a]
	17-OH-P4 (pg/mL)	0.2	24	294.2	1.8	21	394.5	1.8	0.085[a]
	Progesterone (pg/mL)	0.5	25	269.0	1.2	21	186.3	1.6	0.000***
	DHEA (pg/mL)	1.5	25	826.5	1.4	21	571.1	1.5	0.003**,[a]
	Androstenedione (pg/mL)	0.15	25	64.6	1.6	21	69.9	1.5	0.602[a]
	Testosterone (pg/mL)	0.15	25	22.1	1.4	19	66.5	1.9	0.000***,[a]
	3β-HSD (%)		25	10.7	1.5	19	25.2	1.6	0.000***,[a]
	17β-HSD (%)		25	34.3	1.5	19	93.2	2.1	0.000***,[a]
	CYP17 lyase (%)		24	310.9	2.0	19	177.2	2.1	0.035*,[a]
Girls	Cortisol (ng/mL)	5	20	49.8	1.5	30	48.6	1.7	0.859[a]
	Cortisone (ng/mL)	5	20	15.7	1.4	30	17.2	1.8	0.530[a]
	17-OH-P4 (pg/mL)	0.2	20	356.3	1.7	29	387.9	1.6	0.791[a]
	Progesterone (pg/mL)	0.5	20	263.3	1.5	30	170.3	1.3	0.000***
	DHEA (pg/mL)	1.5	20	659.5	1.3	30	552.8	1.7	0.303[a]
	Androstenedione (pg/mL)	0.15	19	71.2	2.1	30	108.4	1.6	0.049*,[a]
	Testosterone (pg/mL)	0.15	20	24.2	1.9	30	44.6	2.2	0.004**
	3β-HSD (%)		19	14.1	1.8	30	29.3	1.9	0.000***,[a]
	17β-HSD (%)		19	30.5	1.8	30	41.2	2.1	0.119[a]
	CYP17 lyase (%)		19	230.7	1.5	29	193.2	1.9	0.287[a]

Data are reported as geometric mean (GM) and geometric standard deviation(GSD).
LLOQ: lower limit of quantification, 3β-HSD; 3β-Hydroxysteroid dehydrogenase.
17β-HSD; 17β-Hydroxysteroid dehydrogenase, CYP17 lyase; Cytochrome P450 17,20 lyase.
3β-HSD (%) = 100 × (androstenedione + testosterone) / DHEA, 17β-HSD (%) = 100 × (Testosterone/A-dione).
CYP17 lyase (%) = 100 × (Androstenedione + Testosterone + DHEA) / 17-OH-P4.
[a] Student's *t*-test, Mann-Whitney test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

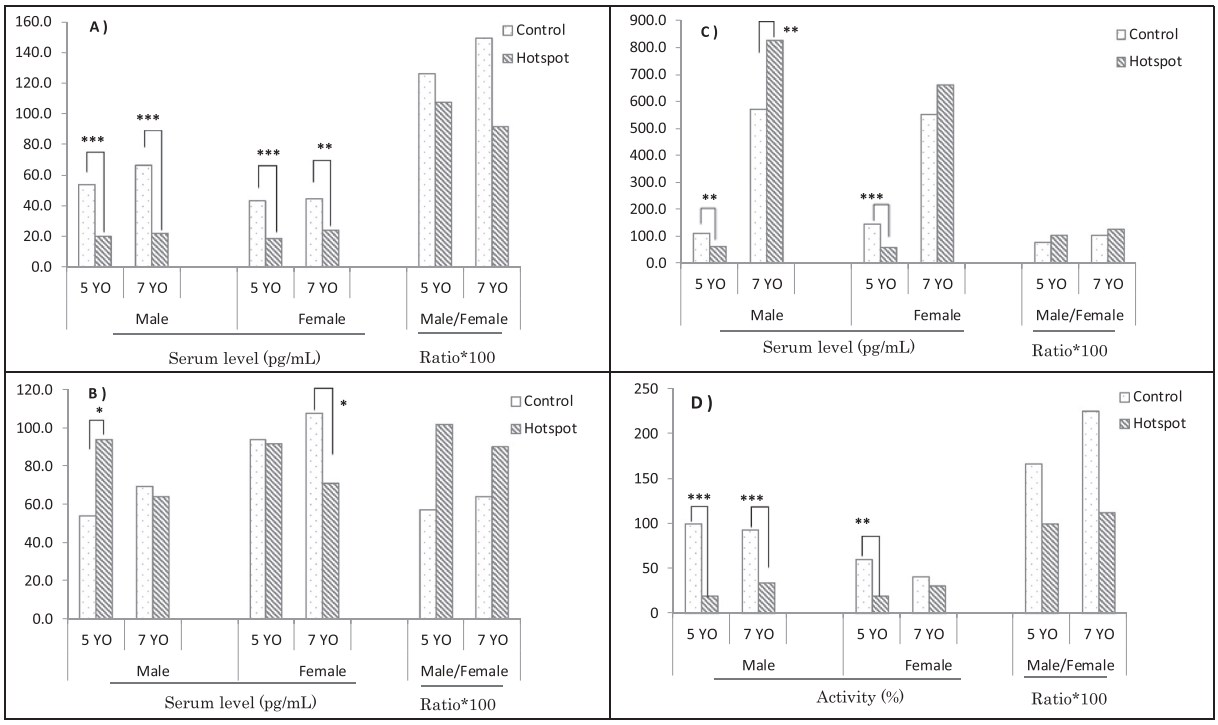

Fig. 2. Comparison of hormone levels and enzyme activity between the two areas in 5- and 7-year-old, by sex and by sex ratio. A): Testosterone, B): A-dione, C): DHEA, D): 17β-HSD. ***:$p < 0.001$, **:$p < 0.01$, *:$p < 0.05$.

3.4. Relationship between TEQ total PCDD/Fs in maternal breast milk and serum steroid hormone or enzyme activity in children

The correlations between the hormone level or enzyme activity in the children and TEQ total PCDD/Fs level in the breast milk are separately shown in Figs. 3 and 4 for the boys and girls. In the boys, the serum testosterone level and 17β-HSD activity had a strong inverse correlation with the TEQ total PCDD/Fs level in the breast milk ($r = -0.47$, $p = 0.001$, and $r = -0.62$, $p < 0.001$, respectively). However, this was not seen in the girls ($r = -0.21$, $p = 0.139$, and $r = -0.18$,

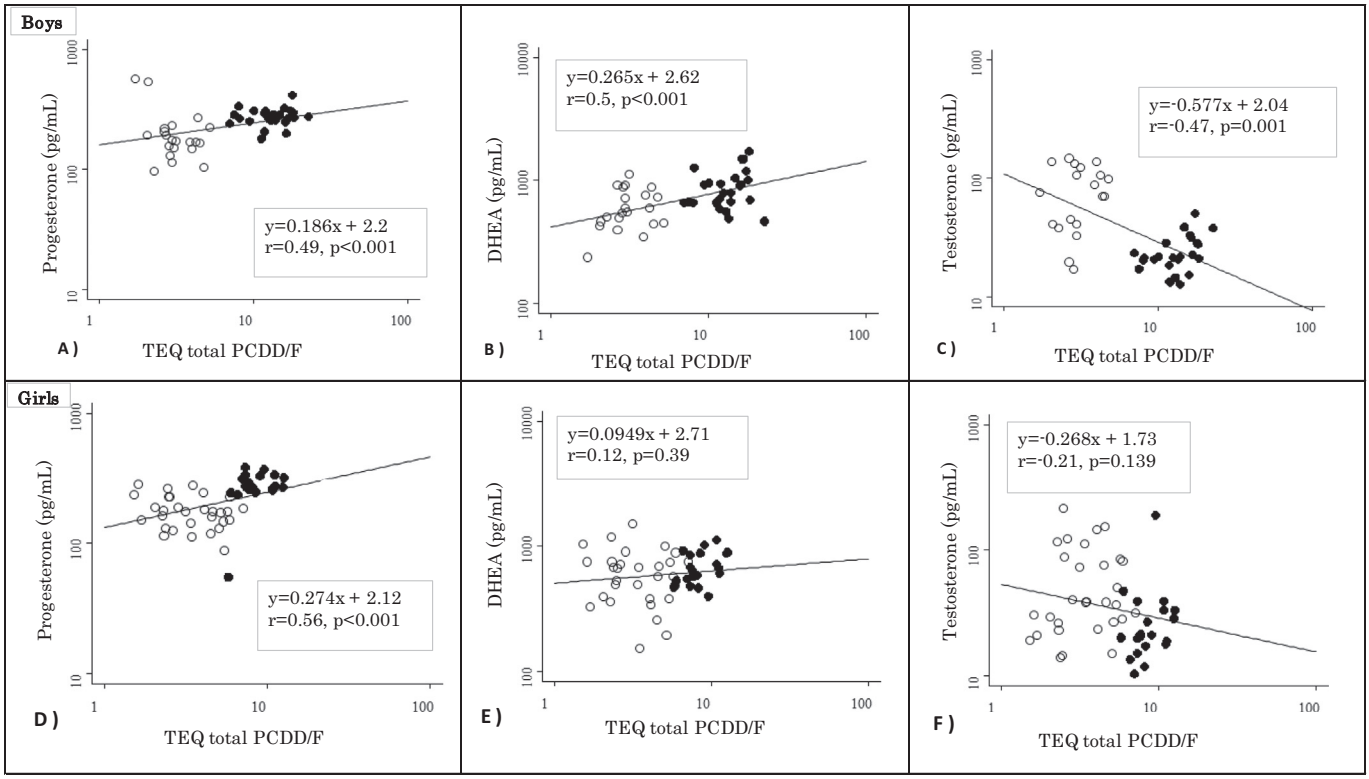

Fig. 3. Correlations of TEQ total PCDD/F TEQ in maternal breast milk and steroid hormones in 7-year-old children from hotspot and non-sprayed area. A), D): Progesterone; B), E): DHEA; C), F): Testosterone ●: Hotspot ○: Non-sprayed.

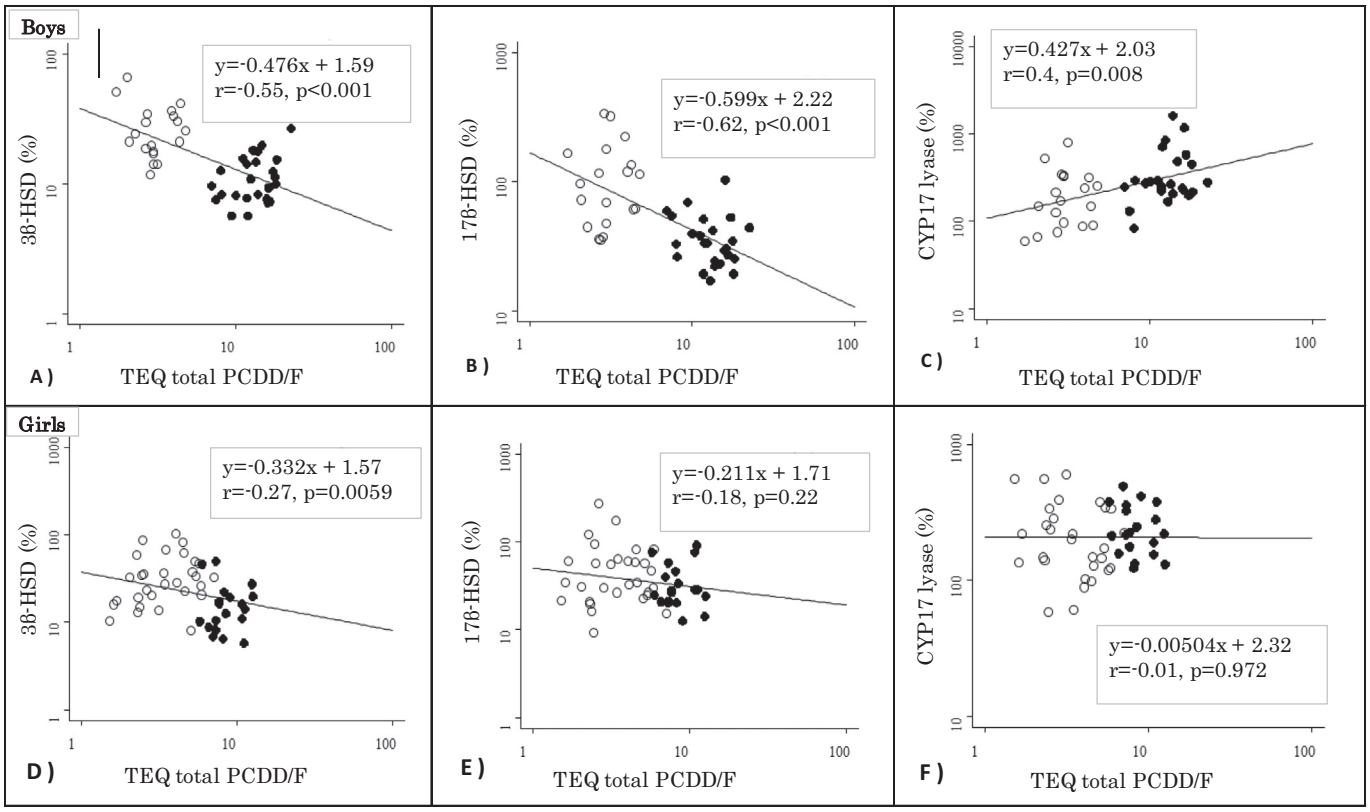

Fig. 4. Correlations of TEQ total PCDD/F TEQ in maternal breast milk and enzyme activity in 7-year-old children from hotspot and non-sprayed area A), D): 3β-HSD, B), E): 17β-HSD, C), F): CYP17 lyase ●: Hotspot ○: Non-sprayed.

$p = 0.22$, respectively). On the other hand, a positive correlation ($r = 0.49$, $p < 0.001$, and $r = 0.56$, $p < 0.001$) was observed between the progesterone and TEQ total PCDD/Fs in the boys and girls (Fig. 3-A). In the boys, DHEA and CYP17 lyase were specifically observed to have positive correlations ($r = 0.5$, $p < 0.001$, and $r = 0.4$, $p < 0.01$) with the TEQ total PCDD/Fs in the breast milk (Figs. 3 and 4). However, this was not observed in the girls ($r = 0.12$, $p = 0.39$, and $r = -0.01$, $p = 0.972$). The cortisol, cortisone and 17-OH progesterone concentrations were not significantly correlated with the dioxin congeners in either the boys or girls (data not shown).

Table 4
Correlation of body parameters in 7-year-old and dioxin congeners in breast milk using multiple regression analysis.

Sex	Dioxin congeners	Height (cm)			Weight (kg)			Head (cm)			Chest (cm)		
		β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2
Boys	2,3,7,8-TeCDD	0.107	0.497	0.055	0.041	0.790	0.099	0.098	0.537	0.043	−0.089	0.554	0.143
	1,2,3,7,8-PeCDD	0.131	0.407	0.060	0.164	0.286	0.124	0.147	0.355	0.054	0.044	0.770	0.137
	1,2,3,4,7,8-HxCDD	0.101	0.518	0.054	0.122	0.424	0.112	0.085	0.588	0.041	−0.001	0.997	0.136
	1,2,3,6,7,8-HxCDD	0.088	0.574	0.051	0.073	0.633	0.103	0.066	0.679	0.038	−0.044	0.769	0.137
	1,2,3,7,8,9-HxCDD	0.181	0.247	0.076	0.153	0.315	0.121	0.081	0.609	0.040	0.027	0.857	0.136
	1,2,3,4,6,7,8-HpCDD	0.111	0.477	0.056	0.060	0.693	0.101	0.029	0.852	0.035	−0.052	0.726	0.138
	OCDD	−0.048	0.761	0.046	0.014	0.926	0.098	0.058	0.712	0.037	−0.096	0.519	0.145
	TEQs total PCDD	0.125	0.430	0.059	0.134	0.386	0.115	0.130	0.414	0.050	0.008	0.958	0.136
	TEQs total PCDF	0.133	0.396	0.061	0.101	0.509	0.108	0.091	0.567	0.042	−0.021	0.889	0.136
	TEQs total PCDD/F	0.130	0.409	0.060	0.119	0.436	0.112	0.112	0.481	0.046	−0.006	0.967	0.136
Girls	2,3,7,8-TeCDD	−0.303	0.039*	0.135	−0.307	0.034*	0.160	−0.177	0.203	0.211	−0.283	0.053	0.143
	1,2,3,7,8-PeCDD	−0.204	0.174	0.087	−0.245	0.097	0.127	−0.163	0.244	0.206	−0.230	0.122	0.117
	1,2,3,4,7,8-HxCDD	−0.305	0.046*	0.130	−0.289	0.057	0.144	−0.319	0.024*	0.271	−0.234	0.125	0.116
	1,2,3,6,7,8-HxCDD	−0.211	0.169	0.088	−0.213	0.160	0.112	−0.254	0.071	0.239	−0.168	0.269	0.093
	1,2,3,7,8,9-HxCDD	−0.204	0.180	0.086	−0.197	0.189	0.107	−0.244	0.081	0.236	−0.148	0.329	0.088
	1,2,3,4,6,7,8-HpCDD	−0.065	0.667	0.052	−0.099	0.511	0.080	−0.246	0.077	0.237	−0.086	0.568	0.075
	OCDD	−0.138	0.362	0.066	−0.155	0.301	0.094	−0.266	0.054	0.247	−0.127	0.398	0.083
	TEQs total PCDD	−0.242	0.106	0.103	−0.258	0.079	0.134	−0.187	0.178	0.214	−0.238	0.107	0.121
	TEQs total PCDF	−0.153	0.313	0.070	−0.154	0.303	0.093	−0.119	0.397	0.195	−0.167	0.267	0.093
	TEQs total PCDD/F	−0.220	0.143	0.093	−0.225	0.130	0.118	−0.171	0.223	0.209	−0.215	0.149	0.111

β: standardized coefficients, *p*: *p*-value, R^2: coefficient of determination.
Dependent variates are body measurements (height, weight, head and chest circumferences).
Independent variates are mother age, residence, full breastfeeding period and dioxin congeners.
* Significant.

3.5. Correlation of body parameters and dioxins in children

Table 4 shows the results of the multiple regression analysis of the children's anthropometric values and dioxin level in the breast milk.

The height and weight ($\beta = -0.303$, $p = 0.039$, and $\beta = -0.307$, $p = 0.034$, respectively) showed weak correlations with the 2,3,7,8-TeCDD levels in the girls, and the height and head circumference ($\beta = -0.305$, $p = 0.046$, and $\beta = -0.319$, $p = 0.024$, respectively) showed weak correlations with the 1,2,3,4,7,8-HxCDD in the girls, but not in the boys.

3.6. Correlation between steroid hormone or enzyme activity in children and dioxin congener levels in breast milk

Table 5 shows the results of a multiple regression analysis of serum progesterone, DHEA, testosterone, 3β-HSD, 17β-HSD, CYP17 lyase, and dioxin. Progesterone was observed to have a weak positive correlation with some of the dioxin congeners in both sexes, but the testosterone, 3β-HSD, and 17β-HSD showed strong reverse correlations with most of the dioxin congeners only in the boys.

4. Discussion

Follow-up studies of the 1–3- and 5-year-old at the hotspot area in Vietnam showed a decrease in the DHEA and testosterone. (Kido et al., 2016; Anh et al., 2017; Oanh et al., 2018; Sun et al., 2020). In this study of the 7-year-old children, we focused on the serum DHEA and testosterone levels, and their biosynthesis key enzyme activities. The study revealed important results, including a decrease in the testosterone levels and a recovery of the DHEA levels comparable to the levels in the non-sprayed area. As seen in Table 3 and Fig. 2, the DHEA levels in the 7-year-old boys and girls in the non-sprayed area were 3.8–5.2 times higher than those of the 5-year-old, showing that the DHEA levels rapidly increased with the age. It is known that DHEA sharply increases starting around school age, peaking in adolescents, and decreased thereafter with age (Rege and Rainey, 2012; Voutilainen and Jaaskelainen, 2015). Based on tracing DHEA in the 1- to 7-year-old in the a non-sprayed area, the critical point of the rapid rise of DHEA was found to be the 7-year-old in both sexes. In the hotspot as well, a sharp increase in the DHEA level was also observed in the 7-year-old. It was reported that in the hotspot area, the DHEA levels were significantly higher in the 1-year-old children than in the non-sprayed area (Anh et al., 2017), and began to decrease starting at 3-year-old, reaching a minimum at 5-year-old (Kido et al., 2016; Oanh et al., 2018; Sun et al., 2020). However, the DHEA levels in the hotspot recovered to levels seen in the non-sprayed area. As a result, the DHEA level due to dioxin exposure in the 3- and 5-year-old children (Kido et al., 2016; Oanh et al., 2018) was not observed in the 7-year-old children.

The production rate of DHEA is based on adrenal tissue differentiation (Parker et al., 1997; Rege and Rainey, 2012; Rennert et al., 2012). It has been reported that the mechanism by which dioxin affects cell

Table 5
Correlation of hormones and dioxin congeners in 7-year-old with breast milk using multiple regression analysis.

Sex	Dioxin congeners	Progesterone			DHEA			Testosterone			3β-HSD			17β-HSD			CYP17 lyase		
		β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2	β	*p*	R^2
Boys	2,3,7,8-TeCDD	0.335	*	0.128	0.388	*	0.213	−0.622	***	0.426	−0.586	***	0.395	−0.588	***	0.426	0.314	n.s.	0.131
	1,2,3,7,8-PeCDD	0.355	*	0.138	0.529	***	0.329	−0.646	***	0.445	−0.658	***	0.470	−0.649	***	0.488	0.459	**	0.233
	1,2,3,4,7,8-HxCDD	0.411	**	0.184	0.481	**	0.293	−0.605	***	0.411	−0.590	***	0.405	−0.617	***	0.464	0.350	*	0.155
	1,2,3,6,7,8-HxCDD	0.379	*	0.160	0.472	**	0.285	−0.656	***	0.473	−0.627	***	0.448	−0.643	***	0.496	0.425	**	0.211
	1,2,3,7,8,9-HxCDD	0.359	*	0.145	0.503	**	0.312	−0.623	***	0.430	−0.644	***	0.466	−0.599	***	0.441	0.427	**	0.213
	1,2,3,4,6,7,8-HpCDD	0.378	*	0.162	0.478	**	0.296	−0.647	***	0.474	−0.634	***	0.468	−0.605	***	0.461	0.388	*	0.187
	OCDD	0.439	**	0.211	0.446	**	0.266	−0.652	***	0.478	−0.607	***	0.432	−0.621	***	0.478	0.314	n.s.	0.134
	1,2,3,7,8-PeCDF	0.305	n.s.	0.111	0.473	**	0.287	−0.606	***	0.419	−0.628	***	0.456	−0.511	***	0.354	0.464	**	0.248
	2,3,4,7,8-PeCDF	0.342	*	0.129	0.478	**	0.280	−0.579	***	0.368	−0.600	***	0.402	−0.571	***	0.398	0.492	**	0.262
	1,2,3,4,7,8-HxCDF	0.410	**	0.186	0.477	**	0.293	−0.650	***	0.473	−0.655	***	0.490	−0.588	***	0.437	0.440	**	0.228
	1,2,3,6,7,8-HxCDF	0.410	**	0.186	0.494	**	0.309	−0.624	***	0.441	−0.661	***	0.499	−0.558	***	0.403	0.444	**	0.231
	1,2,3,7,8,9-HxCDF	0.297	n.s.	0.103	0.412	**	0.228	−0.491	**	0.284	−0.531	***	0.334	−0.431	**	0.271	0.429	**	0.211
	2,3,4,6,7,8-HxCDF	0.206	n.s.	0.061	0.398	**	0.220	−0.600	***	0.409	−0.558	***	0.372	−0.550	***	0.392	0.435	**	0.222
	1,2,3,4,6,7,8-HpCDF	0.414	**	0.190	0.473	**	0.290	−0.644	***	0.467	−0.654	***	0.492	−0.562	***	0.410	0.417	**	0.209
	1,2,3,4,7,8,9-HpCDF	0.415	**	0.189	0.478	**	0.292	−0.660	***	0.483	−0.672	***	0.510	−0.586	***	0.433	0.420	**	0.210
	OCDF	0.213	n.s.	0.062	0.360	*	0.187	−0.432	**	0.222	−0.462	**	0.257	−0.405	*	0.242	0.458	**	0.222
	TEQs total PCDD	0.357	*	0.141	0.513	**	0.317	−0.662	***	0.469	−0.660	***	0.478	−0.657	***	0.502	0.435	**	0.215
	TEQs total PCDF	0.384	*	0.164	0.493	**	0.305	−0.619	***	0.431	−0.643	***	0.472	−0.570	***	0.413	0.477	**	0.259
	TEQs total PCDD/F	0.375	*	0.155	0.506	**	0.314	−0.653	***	0.465	−0.656	***	0.481	−0.631	***	0.478	0.455	**	0.236
Girls	2,3,7,8-TeCDD	0.163	n.s.	0.052	−0.089	n.s.	0.058	−0.207	n.s.	0.137	−0.189	n.s.	0.176	−0.093	n.s.	0.062	−0.057	n.s.	0.057
	1,2,3,7,8-PeCDD	0.354	*	0.140	0.164	n.s.	0.075	−0.125	n.s.	0.112	−0.271	n.s.	0.209	−0.093	n.s.	0.062	0.102	n.s.	0.064
	1,2,3,4,7,8-HxCDD	0.410	**	0.170	−0.057	n.s.	0.054	−0.208	n.s.	0.135	−0.228	n.s.	0.187	−0.112	n.s.	0.065	−0.044	n.s.	0.056
	1,2,3,6,7,8-HxCDD	0.343	*	0.131	0.043	n.s.	0.053	−0.184	n.s.	0.128	−0.247	n.s.	0.197	−0.133	n.s.	0.069	−0.005	n.s.	0.054
	1,2,3,7,8,9-HxCDD	0.339	*	0.131	0.034	n.s.	0.052	−0.176	n.s.	0.126	−0.220	n.s.	0.187	−0.119	n.s.	0.067	0.050	n.s.	0.056
	1,2,3,4,6,7,8-HpCDD	0.349	*	0.140	0.161	n.s.	0.075	−0.243	n.s.	0.153	−0.378	**	0.273	−0.207	n.s.	0.093	0.147	n.s.	0.074
	OCDD	0.362	*	0.148	0.204	n.s.	0.089	−0.296	*	0.178	−0.426	**	0.307	−0.218	n.s.	0.097	0.241	n.s.	0.106
	1,2,3,7,8-PeCDF	0.329	*	0.133	0.032	n.s.	0.052	−0.152	n.s.	0.121	−0.134	n.s.	0.162	−0.143	n.s.	0.074	−0.053	n.s.	0.057
	2,3,4,7,8-PeCDF	0.270	n.s.	0.097	−0.002	n.s.	0.051	0.008	n.s.	0.099	−0.042	n.s.	0.146	−0.019	n.s.	0.055	−0.096	n.s.	0.063
	1,2,3,4,7,8-HxCDF	0.388	*	0.164	0.143	n.s.	0.070	−0.235	n.s.	0.148	−0.261	n.s.	0.205	−0.258	n.s.	0.114	0.007	n.s.	0.054
	1,2,3,6,7,8-HxCDF	0.337	*	0.128	0.105	n.s.	0.061	−0.141	n.s.	0.116	−0.157	n.s.	0.166	−0.204	n.s.	0.090	−0.047	n.s.	0.056
	1,2,3,7,8,9-HxCDF	0.363	*	0.156	−0.041	n.s.	0.053	−0.211	n.s.	0.141	−0.163	n.s.	0.170	−0.270	n.s.	0.124	−0.005	n.s.	0.054
	2,3,4,6,7,8-HxCDF	0.298	*	0.111	0.190	n.s.	0.085	−0.068	n.s.	0.103	−0.155	n.s.	0.167	−0.199	n.s.	0.091	0.075	n.s.	0.059
	1,2,3,4,6,7,8-HpCDF	0.292	n.s.	0.108	0.211	n.s.	0.093	−0.233	n.s.	0.149	−0.283	*	0.219	−0.252	n.s.	0.113	0.113	n.s.	0.066
	1,2,3,4,7,8,9-HpCDF	0.379	*	0.164	0.119	n.s.	0.065	−0.281	n.s.	0.173	−0.261	n.s.	0.208	−0.322	n.s.	0.151	0.027	n.s.	0.055
	OCDF	0.271	n.s.	0.100	0.134	n.s.	0.069	−0.220	n.s.	0.146	−0.121	n.s.	0.159	−0.258	n.s.	0.119	0.013	n.s.	0.054
	TEQs total PCDD	0.335	*	0.129	0.083	n.s.	0.057	−0.181	n.s.	0.128	−0.277	n.s.	0.212	−0.113	n.s.	0.065	0.049	n.s.	0.056
	TEQs total PCDF	0.345	*	0.136	0.088	n.s.	0.058	−0.132	n.s.	0.114	−0.154	n.s.	0.166	−0.181	n.s.	0.083	−0.051	n.s.	0.056
	TEQs total PCDD/F	0.354	*	0.141	0.084	n.s.	0.058	−0.174	n.s.	0.126	−0.241	n.s.	0.197	−0.145	n.s.	0.073	0.012	n.s.	0.054

β: standardized coefficients, *p*, *p*-value, R^2: coefficient of determination.
Dependent variates are progesterone, DHEA, testosterone, 3β-HSD, 17β-HSD and CYP17 lyase.
Independent variates are mother age, residence, full breastfeeding period, BMI and dioxin congeners.
Steroid hormones and dioxin congener values were log-transformed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s; not significant.

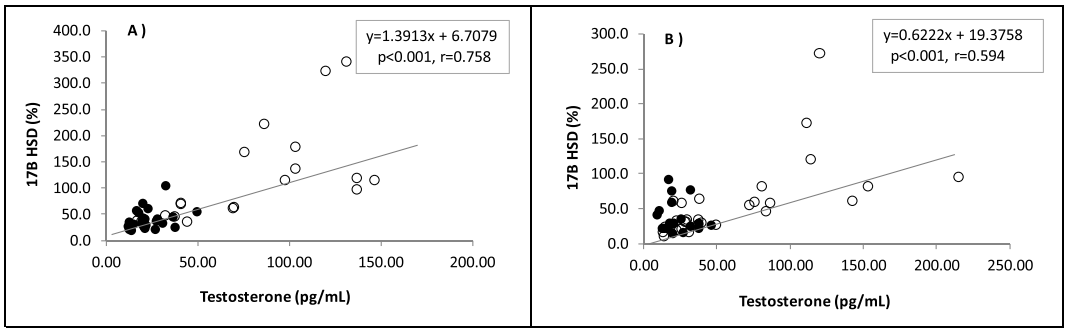

Fig. 5. Correlations of testosterone levels in serum and activity of 17β-HSD in 7-year-old children from hotspot and non-sprayed area A) Boys, B) Girls ●: Hotspot ○: Non-sprayed.

differentiation is through the aryl hydrocarbon receptor (AhR) in the immune T cells, bone cells, kidney cells, etc. (Quintana et al., 2008; Watson et al., 2019; Zhou et al., 2018). Based on these findings, it is speculated that dioxin acts via AhR in the process of inducing the differentiation of the fetal reticular layer in the fetal adrenal gland.

Dioxin delays the process of inducing differentiation from the fetal reticular layer into the adult reticular layer of the adrenal gland, and, according to our previous results, at 1-year-old, a large amount of DHEA was produced from the remaining fetal reticular layer, resulting in high levels (Anh et al., 2017). At 3- to 5-year-old, the DHEA production in the adrenal gland decreases due to delayed differentiation into the adult reticular layer, resulting in a decrease in the serum DHEA (Kido et al., 2016). After birth, the reticular layer of the fetus begins to differentiate, becoming a completely adult reticular layer at 6-year-old. Biochemically, the DHEA was biosynthesized via 17-hydroxypregnenelone from pregnenolone by CYP 17 hydroxylase and 17,20-lyase, (Yoshimoto and Auchus, 2015), and dioxin suppressed the CYP17,20-lyase system (Moran et al., 2003; Li and Wang, 2005).

As shown in Fig. 2(A) and Table 3, the serum testosterone level in the 7-year-old boys from the non-sprayed area was 66.5 pg/mL, which was 18.8% higher than that in the 5-year-old children, while in the 7-year-old girls, the testosterone level was 44.6 pg/mL, which was the same concentration (43 ng/mL) as in the 5-year-old children. The serum testosterone in boys largely increased with age, and by 7-year-old the boys have become masculine from an endocrine perspective. This indicated that the testes of the boys developed during school age, and the superiority of androgens was recognized in the 7-year-old children from the non-sprayed area.

As shown in Table 3, the serum testosterone level in the 7-year-old boys from the hotspot was significantly lower than that in the non-sprayed area ($p < 0.001$). The cause of the decreasing serum testosterone by dioxin is due to the suppression of the 17β-HSD activity. This enzyme is almost exclusively expressed in the testes, and specifically converts the weak androgenic androstenedione to active testosterone (Fig. 5). Furthermore, the serum testosterone level and 17β-HSD activity in the boys showed a strong inverse correlation with the dioxin level in breast milk (Table 5, Figs. 1 and 4). The three mechanisms of the testosterone decline are believed to be as follows:

First, the difference in the testosterone production may be due to the difference in the optical concentration for dioxin action between the adrenal gland and the testes. Dioxin may significantly accumulate in the testes due to lipophilicity, and plays a role in the effects at low doses. Second, testosterone is mainly produced from androstenedione by 17β-HSD in the testis. 17β-HSD is primarily expressed in the testes, and plays the role as a rate-limiting enzyme for the testosterone biosynthesis process. The low doses of dioxin directly affected the expression of the enzyme. Third, the luteinizing hormone (LH) is known to modulate the activity of the testosterone biosynthesis pathway in the testes.

We confirmed the 17β-HSD suppression from the ratio of the testosterone and androstenedione levels in human blood (Oanh et al., 2018; Wojtowicz et al., 2005). However, it is unclear whether the suppression of testosterone by dioxin is an AhR-mediated mechanism of action. Based on the decrease in the sperm and testosterone by the inhibition of 3β-HSD in AhR-knockout mice, Baba proposed a possible mechanism related to the AhR ligands, such as TCDD, on the steroid biosynthesis process (Baba et al., 2008).

In humans, increased hypospadias has been reported in boys when exposed to endocrine disruptors, including dioxins, during the fetal period (Giordano et al., 2010; Ormond et al., 2009; Kishi et al., 2008). It has also been reported that dioxin exposure in children affects androgen production, and reduces the sperm concentration and activity in humans (Goudarzi et al., 2017; Mínguez-Alarcón et al., 2017; Scinicariello and Buser, 2016). On the other hand, Winneke showed a strong positive correlation between the dioxin level in breast milk and feminization behavior in 7-year-old boys through a question-based Pre-School Activity Inventory (PSAI) (Winneke et al., 2014). We confirmed a decrease in the testosterone level and suppression of 17β-HSD activity caused by dioxin in the 5- to 7-year-old boys, indicating delayed male expression in the boys. Based on these findings, one of the factors of feminization found by Winneke may be a decrease in testosterone. Therefore, the feminization phenomenon in boys due to exposure to chemicals can be followed through sex hormones and morphological changes. No significant difference in the androstenedione level was observed between the two areas in the 7-year-old boys, and dioxin had no significant effect on the production or metabolism of androstenedione. In addition, cortisol and cortisone produced only in the adrenal fasciculata layer showed no difference in the amounts between the two areas or between the boys and girls. These hormones were hardly affected by the dioxins (Kido et al., 2016; Oanh et al., 2018; Anh et al., 2017).

Based on these findings, testosterone perturbation by dioxin has a very important influence on not only the reproductive system, but also the development and function of the neuroendocrine system. In addition, a decreased testosterone level and suppression of the 17β-HSD activity may cause symptoms similar to the 17β-HSD deficiency (anogenital distance, AGD) in the reproductive organs (Mendonca et al., 2017). Sata et al. suggested that the activity decrease of 17β-HSD is a potential risk factor for hypospadias (Sata et al., 2010).

The effects of dioxins, such as decreased DHEA and testosterone, and high androstenedione in the hotspot of children up to 5-year-old, were strong, and these effects were strongly correlated with a specific dioxin congener and its levels in maternal breast milk (Anh et al., 2017; Oanh et al., 2018). However, these changes disappeared or were diminished in the 7-year-old children. This may be due to the effects of the timing and amount of exposure to dioxin, or the effects of cell repair in the growth process of children.

It is speculated that the cause of hormonal disruption by dioxin is primarily the result of internal exposure of the fetus to dioxin during pregnancy. If it is assumed that the main causes are exposure to the postnatal food chain and environmental pollution, dioxins would accumulate in the infant's body with growth, and the resulting hormonal

disruption would increase with age. The exposure to dioxin during the fetal or baby period may be associated with the dioxin half-life in the body of approximately 0.5–2 years (Kreuzer et al., 1997; Kerger et al., 2006), and the dioxin-induced perturbation is diminished in school-aged children. As shown in Table 5, the dioxin congeners had strong correlations with hormone levels and enzyme activity in boys, suggesting that the low-dose effects of dioxin may have appeared in the 7-year-old.

As shown in Table 1, only girls were found to have a negative difference in the anthropometric parameters between the hotspot and the non-sprayed area. Among the 5-year-old girls in the hotspot, the head circumference and chest circumference differed from the non-sprayed area (Oanh et al., 2018). Differences in the anthropometric values appeared as the infants grew, and as shown in Table 4, there was a weakly negative relationship with 2,3,7,8-TCDD and 1,2,3,4,7,8-HCDD. It is believed that dioxin may have affected the growth and development of the body. Whether dioxin affects postnatal physical development is nutritionally relevant, and is not conclusive at this time.

The limitations of this study are as follows: it is unclear whether changes in testosterone, androstenedione, and DHEA kinetics are due to postnatal exposure to dioxins or the effects of exposure to dioxin during pregnancy. To evaluate this prenatal exposure, the dioxin levels and some hormone levels, including estradiol and 16-hydroxy DHEA produced only in the fetal adrenal gland in the umbilical cord blood, must be measured to investigate the health issues in children caused by dioxin. After weaning, a child's main sources of dioxins change to diet, airborne inhalation, and skin contact. Even though the dioxins have a long half-life, care should be taken to avoid any additional intake of dioxins in children after weaning.

5. Conclusions

Serum testosterone levels in the 7-year-old decreased, and suppression of the 17β-HSD activity was observed, particularly in boys. The DHEA levels decreased in the 3- and 5-year-old and clearly recovered to the control level in the 7-year-old children. Reduced testosterone and 17β-HSD activity were inversely correlated to the TEQ total PCDD/Fs in maternal breast milk.

CRediT authorship contribution statement

Yuko Oyama: Conceptualization, Methodology, Data curation, Writing - original draft. **Hoang Duc Phuc:** Writing – review & editing. **Seijiro Honma:** Writing – review & editing. **Nguyen Thi Phuong Oanh:** Software, Data curation, Investigation, Validation. **Nguyen Xuan Hung:** Visualization, Investigation. **Le Thai Anh:** Visualization, Investigation. **Ho Dung Manh:** Visualization, Investigation. **Dao Van Tung:** Visualization, Investigation. **Dang Duc Nhu:** Visualization, Investigation. **Ngo Minh Tan:** Visualization, Investigation. **Pham Van Thuc:** Visualization, Investigation. **Nguyen Hung Minh:** Visualization, Investigation. **Ngo Van Toan:** Visualization, Investigation. **Rie Okamoto:** Supervision. **Shizuko Omote:** Supervision. **Hideaki Nakagawa:** Supervision. **Vo Van Chi:** Visualization, Investigation. **Teruhiko Kido:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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