

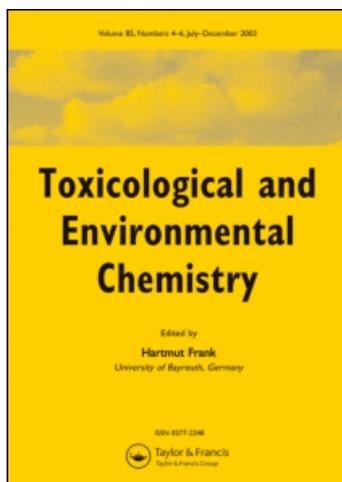
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### Salivary cortisol and cortisone levels, and breast milk dioxin concentrations in Vietnamese primiparas

Dang Duc Nhu<sup>ab</sup>; Teruhiko Kido<sup>a</sup>; Rie Naganuma<sup>a</sup>; Hiroyuki Suzuki<sup>a</sup>; Naoko Kuroda<sup>a</sup>; Seiji Honma<sup>c</sup>; Pham The Tai<sup>d</sup>; Shoko Maruzeni<sup>d</sup>; Muneko Nishijo<sup>d</sup>; Hideaki Nakagawa<sup>d</sup>; Nguyen Ngoc Hung<sup>b</sup>; Le Thi Hong Thom<sup>b</sup>; Le Ke Son<sup>e</sup>

<sup>a</sup> School of Health Sciences, College of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Ishikawa, Japan <sup>b</sup> Division for Mitigation of the Consequences of the Chemicals Used During the War on Human Health (10-80 Division), Hanoi Medical University, Hanoi, Vietnam <sup>c</sup> ASKA Pharma Medical Co. Ltd., Kawasaki, Japan <sup>d</sup> Department of Epidemiology and Public Health, Kanazawa Medical University, Ishikawa, Japan <sup>e</sup> Vietnam Environment Administration, Ministry of Natural Resources and Environment, Vietnam

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## Salivary cortisol and cortisone levels, and breast milk dioxin concentrations in Vietnamese primiparas

Dang Duc Nhu<sup>ad</sup>, Teruhiko Kido<sup>a\*</sup>, Rie Naganuma<sup>a</sup>, Hiroyuki Suzuki<sup>a</sup>, Naoko Kuroda<sup>a</sup>, Seiji Honma<sup>b</sup>, Pham The Tai<sup>c</sup>, Shoko Maruzeni<sup>c</sup>, Muneko Nishijo<sup>c</sup>, Hideaki Nakagawa<sup>c</sup>, Nguyen Ngoc Hung<sup>d</sup>, Le Thi Hong Thom<sup>d</sup> and Le Ke Son<sup>e</sup>

<sup>a</sup>School of Health Sciences, College of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Ishikawa, Japan; <sup>b</sup>ASKA Pharma Medical Co. Ltd., Kawasaki, Japan; <sup>c</sup>Department of Epidemiology and Public Health, Kanazawa Medical University, Ishikawa, Japan; <sup>d</sup>Division for Mitigation of the Consequences of the Chemicals Used During the War on Human Health (10-80 Division), Hanoi Medical University, Hanoi, Vietnam; <sup>e</sup>Vietnam Environment Administration, Ministry of Natural Resources and Environment, Vietnam

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There is a great deal of concern regarding the adverse effects of polychlorinated dibenzodioxins (PCDD) and polychlorinated dibenzofurans (PCDF) present in Agent Orange and other herbicides on Vietnam's population and ecosystems. The purpose of this study was to determine the effect of dioxin exposure on adrenal steroids in saliva, and dioxin levels in breast milk, of primiparas in an Agent Orange/dioxin hot-spot and a non-exposed area in Vietnam on the basis of epidemiological research. The subjects were 35 lactating women who had recently given birth to their first or second child. A further sub-study involved eight primiparas from each area. All subjects were aged between 20 and 30 years with infants aged between 4 and 16 weeks. The mean concentration of PCDD, PCDF, and PCDD + PCDF toxic equivalents (TEQ) in breast milk in the hot-spot area was significantly higher than in the non-exposed area. Cortisol and cortisone levels in the saliva of primiparas in the hot-spot area were also significantly higher than those in the non-exposed area. There was a significant negative correlation between cortisol and the cortisol/cortisone ratio and PCDD + PCDF and PCDF TEQ levels in the hot-spot area. Furthermore, the correlation between cortisol and cortisone and the PCDD + PCDF, PCDD, and PCDF TEQ in the combination of hot spot + non-exposed area was significant according to the curve (bell style). Our results suggest that Agent Orange/dioxin exposure still exerts a major influence on the salivary hormones of the Vietnamese population.

**Keywords:** saliva; cortisol; cortisone; dioxin; hot spot; Vietnam

### Introduction

Between 1961 and 1971, the US military used over 80 million liters of chemical herbicides in Vietnam for general defoliation and crop destruction in a program code named

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\*Corresponding author. Email: kido@mhs.mp.kanazawa-u.ac.jp

Operation Ranch Hand (Stellman et al. 2003). Herbicides were applied to approximately 10–12% of southern Vietnam (Westing 1984; IOM 1994), and 65% of the chemical herbicides used between 1965 and 1970 was Agent Orange, a 50/50 mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The 2,4,5-T fraction of the Agent Orange mixture was contaminated with the highly toxic chemical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin-TCDD) (Stellman et al. 2003), one of the most toxic man-made substance known (Schechter et al. 2006).

Many Vietnamese people were heavily exposed to Agent Orange/dioxin and that exposure is now manifesting as health problems including skin disorders and liver damage (Kimbrough et al. 1977; Selevan, Sweeney, and Sweeney 2003) and is responsible for the adverse reproductive effects, including an increased rate of spontaneous abortions, low birth weight, birth defects, and fetal mortality (Allen et al. 1979; Roman et al. 1995; Selevan, Sweeney, and Sweeney 2003), and reduced fertility and fecundity (Allen et al. 1979; Barsotti, Abrahamson, and Allen 1979; Umbreit, Hesse, and Gallo 1987; Gray and Ostby 1995). TCDD is also a known risk factor for cancer (Fingerhut et al. 1991; Steenland et al. 1999) and has elevated the risk of developing diabetes mellitus and cardiovascular diseases (Steenland et al. 1999).

Hormone levels are also known to be altered by low-dose exposure of TCDD, and fetal death occurred after high-dose exposure. Similarly, animal studies established an association between maternal TCDD exposure and immune deficiencies (Weisglas-Kuperus et al. 2000), decreased estradiol and progesterone levels (Barsotti, Abrahamson, and Allen 1979), and altered serum testosterone levels (Egeland et al. 1994). Peterson, Theobald, and Kimmel (1993) showed that the exposure of rodents and other species to TCDD resulted in the changes in steroid hormone levels. Li and Wang (2005) demonstrated that the biosynthesis of androgens, cortisol, and aldosterone is altered by dioxin-like PCB126 in human adrenocortical H295R cells.

Several studies showed that dioxin contamination in the soil and sediments from some regions in Vietnam was high, and the regions were identified as the US bases at Bien Hoa, Da Nang, and Phu Cat as significant hot spots whose soil/sediment dioxin levels exceeded the maximal levels permitted in many western countries (Dwernychuk 2005; Dwernychuk et al. 2006). Other investigators reported the elevated levels of TCDD in food, wildlife, and even the inhabitants of contaminated areas (Schechter et al. 2001, 2002, 2003; Dwernychuk et al. 2002).

Recently, Nhu et al. (2009) and Saito et al. (2010) found that the mean dioxin levels in soil and breast milk in sprayed areas were significantly higher than those in non-sprayed areas. The purpose of this study was to determine the effect of dioxin exposure on adrenal steroids in saliva and dioxin levels in breast milk of primiparas in an Agent Orange/dioxin hot spot compared to a non-exposed area in Vietnam.

## **Materials and methods**

### ***Study area***

The study was conducted in Phu Cat district (Binh Dinh province) and Kim Bang district (Ha Nam province). Phu Cat airbase is one of three main dioxin hot spots in southern Vietnam. Subjects are known to have been living in and around the airbase prior to the war. Kim Bang district is located in northern Vietnam and did not experience herbicide operations during the war, which is why it was selected as the control site.

## ***Subjects and methods***

### *Breast milk and salivary samples*

Breast milk and saliva samples were obtained from lactating women, aged between 20 and 30 years, from both districts in the morning (between 8:00 and 10:00 AM) in September 2008. The local authorities and medical staff explained the purpose of the study to 35 lactating women, 18 from Phu Cat district and 17 from Kim Bang district, who had recently given birth to their first or second child. Eight primiparas were chosen from each district. All lactating women consented to donate milk samples (10–20 mL) and were breast-feeding infants aged 4–16 weeks. Samples were collected by the mothers themselves and the medical staff at each local clinic and were frozen immediately after collection. Saliva was collected by rinsing their mouths with water and then transferring the resulting mixture directly into a Bakelite test tube (15 mL). All samples were stored at  $-70^{\circ}\text{C}$  until analysis. Mothers were asked to provide the information regarding age, family income, and residence period. The body measurements for mothers (body height and body weight) were compared between the two areas. The Medical Ethics Committee of Kanazawa University approved this study (Permission No; Health-89), and informed consent was obtained from each participant.

## ***Analytical methods***

### *Saliva hormone analysis*

Cortisol- $^2\text{H}_3$  (1 ng) was added to 1–2 mL of the saliva solution as internal standard and the hormones were extracted with ethyl acetate. The extract was applied to a Bond Elut C18 cartridge column to separate the polar (cortisol and cortisone) steroid fractions, which were directly estimated by LC–MS/MS (LC–MS, liquid chromatography–mass spectrometry) following a previously reported method (Matsui et al. 2009). The lowest analytical limits for cortisol and cortisone were 50 and 50 pg per assay, respectively.

### *Breast milk dioxin analysis*

Breast milk samples were analyzed following previously reported procedures (Tawara et al. 2003; Nishijo et al. 2008), as was their fat content (Patterson et al. 1987). The dioxin concentrations obtained were converted into 2,3,7,8-TCDD toxic equivalents (TEQ) using the international World Health Organization (WHO) toxicity equivalency factors (TEFs) from 1997 (Van den Berg et al. 1998) to 2005 (Van den Berg et al. 2006). Non-detectable (ND) and non-destructive readout (NDR; chromatographic peaks detected but did not meet quantification criteria) data were not rejected.

### *Statistical analysis*

Data are shown as median (interquartile range), and Spearman's rank correlation coefficients were calculated. Statistical comparisons were made using the Wilcoxon signed rank tests and *t*-tests for categorical variables. The significance level was set at  $p < 0.05$ . All statistical analyses were performed using the JMP<sup>®</sup>8 software package (SAS Institute, Japan).

## Results

The results are shown in a series of five tables and three figures, including the correlations obtained by comparing the cortisol and cortisone levels, and the cortisol/cortisone ratio in saliva, and dioxin concentrations in breast milk.

Table 1, which presents the characteristics of all study subjects, shows that the age and residence period were significantly higher for lactating women in the hot-spot than in the non-exposed area, whereas height, weight, body mass index (BMI) and family income did not differ significantly. The salivary cortisone levels were significantly higher in the hot-spot than in the non-exposed area (Table 2), whereas cortisol levels and cortisol/cortisone ratio did not vary markedly between the two. The mean dioxin levels for the hot-spot area in terms of polychlorinated dibenzodioxins (PCDD) TEQ [6.70 (4.87–9.54)], polychlorinated dibenzofurans (PCDF) TEQ [5.73 (4.06–7.28)], and PCDD + PCDF TEQ [12.32 (9.06–16.64)] were significantly greater than those for the

Table 1. Comparison of characteristics of lactating women from hot-spot and non-exposed regions with herbicide.

	Hot-spot area ( <i>n</i> = 18)	Non-exposed area ( <i>n</i> = 17)	<i>p</i> -value
Age (years)	24.5 (22.8–27.3)	23.0 (20.5–25.0)	* <sup>b</sup>
Height (cm)	153.4 (149.1–155.4)	150.4 (146.8–153.8)	n.s. <sup>a</sup>
Weight (kg)	48.8 (45.0–51.6)	49.0 (46.0–53.3)	n.s. <sup>a</sup>
BMI (kg m <sup>-2</sup> )	20.6 (19.8–22.4)	21.1 (20.7–23.1)	n.s. <sup>a</sup>
Family income (× 10 <sup>4</sup> VND/month)	300 (175–325)	200 (110–300)	n.s. <sup>a</sup>
Residence period (years)	20.0 (19.0–25.0)	18.0 (17.0–23.0)	* <sup>a</sup>

Notes: Data are shown as median (inter-quartile range). n.s., not significant.

<sup>a</sup>Wilcoxon signed rank test.

<sup>b</sup>*t*-test.

\**p* < 0.05.

Table 2. Mean dioxin in breast milk and salivary hormone levels of lactating women in both areas.

	Hot-spot area ( <i>n</i> = 18)	Non-exposed area ( <i>n</i> = 17)	<i>p</i> -value
	Median	Median	
Cortisol (pg mL <sup>-1</sup> )	3292.3 (1264.90–4251.30)	1391.70 (1025.00–2280.00)	n.s. <sup>a</sup>
Cortisone (pg mL <sup>-1</sup> )	13,300.00 (9426.00–19,568.00)	8941.00 (6450.00–14,321.00)	* <sup>a</sup>
Cortisol/cortisone	0.19 (0.16–0.22)	0.16 (0.12–0.25)	n.s. <sup>b</sup>
TEQ PCDDs (pg g <sup>-1</sup> )	6.70 (4.87–9.54)	2.74 (0.99–3.30)	*** <sup>a</sup>
TEQ PCDFs (pg g <sup>-1</sup> )	5.73 (4.06–7.28)	2.34 (1.55–2.73)	*** <sup>a</sup>
TEQ PCDDs + PCDFs (pg g <sup>-1</sup> )	12.32 (9.06–16.64)	5.08 (2.28–6.03)	*** <sup>a</sup>

Notes: Data are shown as median (inter-quartile range).

n.s., not significant.

<sup>a</sup>Wilcoxon signed rank test.

<sup>b</sup>*t*-test.

\**p* < 0.05; \*\*\**p* < 0.01.

Table 3. Correlation between dioxin in breast milk and salivary hormone levels of the lactating women in both areas ( $n = 35$ ).

	TEQ PCDDs		TEQ PCDFs		TEQ PCDDs + TEQ PCDFs	
	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value
Cortisol	0.208	0.230	0.171	0.326	0.208	0.231
Cortisone	0.298	0.082	0.229	0.186	0.288	0.093
Cortisol/cortisone	0.018	0.919	0.059	0.737	0.038	0.827

Note: *r*, Spearman's rank correlation coefficient.

Table 4. Comparison of characteristics of primiparas from hot-spot and non-exposed regions with herbicide.

	Hot-spot area ( $n = 8$ )	Non-exposed area ( $n = 8$ )	<i>p</i> -value
Age (years)	25.0 (20.8–27.0)	21.5 (19.3–24.5)	n.s.
Height (cm)	152.3 (148.2–157.9)	152.0 (150.3–154.9)	n.s.
Weight (kg)	49.8 (45.6–51.9)	48.8 (45.8–52.8)	n.s.
BMI ( $\text{kg m}^{-2}$ )	20.8 (20.1–22.1)	21.0 (20.2–21.4)	n.s.
Family income ( $\times 10^4$ VND/month)	200 (163–375)	150 (63–300)	n.s.
Residence period (years)	22.5 (20.0–26.0)	20.5 (18.0–26.0)	n.s.

Notes: Data are shown as median (inter-quartile range). Wilcoxon signed rank test. n.s., not significant.

non-exposed area {PCDD TEQ [2.74 (0.99–3.30)], PCDF TEQ [2.34 (1.55–2.73)], and PCDD + PCDF TEQ [5.08 (2.28–6.03)]}. The Spearman rank correlation coefficient showed no significant association between dioxin levels in breast milk and salivary hormone levels for mothers from both areas (Table 3).

The general characteristics of the sub-group of primiparas are presented in Table 4, which shows no marked differences between the two regions in terms of age, height, weight, BMI, family income, or residence period. Table 5 presents the mean dioxin and salivary hormone levels of primiparas. Cortisol and cortisone levels in the hot-spot area were found to be significantly higher than those in the non-exposed area, although the difference in cortisol/cortisone ratio was not significant. Table 5 also shows that the mean PCDD TEQ levels in breast milk in the hot-spot area ( $9.4 \pm 3.9$ ) were significantly higher than that for the non-exposed area ( $3.2 \pm 0.7$ ). Furthermore, the mean PCDF TEQ and PCDD + PCDF TEQ concentrations in breast milk in the hot-spot area ( $6.8 \pm 2.4$  and  $16.2 \pm 5.9$ , respectively) were significantly higher than those in the non-exposed area ( $3.0 \pm 0.8$  and  $6.1 \pm 1.3$ , respectively).

A plot of the cortisol in saliva and dioxin in breast milk of primiparas (Figure 1) shows a significant negative correlation between cortisol and PCDF TEQ and PCDD + PCDF TEQ levels in the hot-spot area. There was also a significant correlation between the cortisol and PCDD TEQ, PCDF TEQ, and PCDD + PCDF TEQ levels in the combination hot-spot and non-exposed area. Figure 2 shows the significant correlation between the cortisone and PCDD TEQ, PCDF TEQ, and PCDD + PCDF TEQ levels in the combination of hot-spot and non-exposed area. A plot of the cortisol/cortisone ratio

Table 5. Mean dioxin and salivary hormone level of primiparas in both areas.

	Hot-spot area ( $n = 8$ )	Non-exposed area ( $n = 8$ )	$p$ -value
Cortisol ( $\text{pg mL}^{-1}$ )	3993.30 (2816.30–4542.50)	1140.00 (241.70–1861.50)	*
Cortisone ( $\text{pg mL}^{-1}$ )	18,292.00 (12,038.00–21,255.00)	6450.00 (1212.00–8706.00)	**
Cortisol/cortisone	0.21 (0.17–0.27)	0.19 (0.09–0.26)	n.s.
TEQ PCDDs ( $\text{pg g}^{-1}$ )	9.11 (6.12–13.04)	3.29 (2.55–3.80)	***
TEQ PCDFs ( $\text{pg g}^{-1}$ )	6.55 (4.78–7.72)	2.73 (2.40–3.39)	**
TEQ PCDDs + PCDFs ( $\text{pg g}^{-1}$ )	15.65 (10.99–22.13)	6.03 (4.95–6.94)	**

Notes: Data are shown as median (inter-quartile range). Wilcoxon signed rank test.

n.s., not significant.

\* $p < 0.05$ ; \*\* $p < 0.01$ ; and \*\*\* $p < 0.01$ .

in saliva and dioxin in breast milk of primiparas (Figure 3) shows a significant negative correlation between the cortisol/cortisone ratio and PCDF TEQ and PCDD + PCDF TEQ levels in the hot-spot area.

The left-hand side of the curve (bell style) in Figures 1 and 2 demonstrates that for the combination hot-spot and non-exposed area, both the dioxin as well as cortisol and cortisone levels are increasing, whereas the right-hand side of the curve shows a negative relationship, where the dioxin levels are rising but the cortisol and cortisone levels are decreasing.

## Discussion

The study region in this investigation is one of the known dioxin hot spots in Vietnam. The high TCDD levels in this area are due to the spillages that occurred during the loading of the herbicide into the crop-spraying planes during the Vietnam War (Dwernychuk 2005). Despite the fact that a significant amount of time has passed since the end of the war, herbicide residues still exert adverse effects on those inhabitants living in the sprayed/hot-spot areas and on the ecosystem (Schechter et al. 2001; Mai et al. 2007). Dioxin is a persistent environmental toxin (soil and sediment) that bio-accumulates in fish and animals which are then consumed by humans. Several studies reported that the half-life of dioxin in the human body is 7–11 years (Pirkle et al. 1989; Kreuzer et al. 1997).

Dwernychuk et al. (2002) demonstrated the apparent food-chain transfer of TCDD from contaminated soil to cultured fish pond sediments and then onto fish and ducks, and finally to humans, where it was detected in whole blood and breast milk. Our study found dioxin levels to be present in the breast milk of primiparas (Table 5). Indeed, all lactating women who had just given birth to their first or second child (Table 2), possessed higher levels in the hot-spot than in other previously studied herbicide-sprayed areas, such as Quang Tri (PCDD + PCDDF TEQ of  $11.3 \pm 7$ ; Nhu et al. 2009), and the non-sprayed area studied here. It was not possible to find a correlation between dioxin levels in breast milk and salivary hormone levels of lactating women in either area. Previous studies demonstrated that the mean decrease in TEQ levels in breast milk was 22% for the second child and 42% for the third child with respect to milk from primiparas (Beck, Dross, and Mathar 1994), with a 72% decrease in dioxin levels for mothers who breast-fed

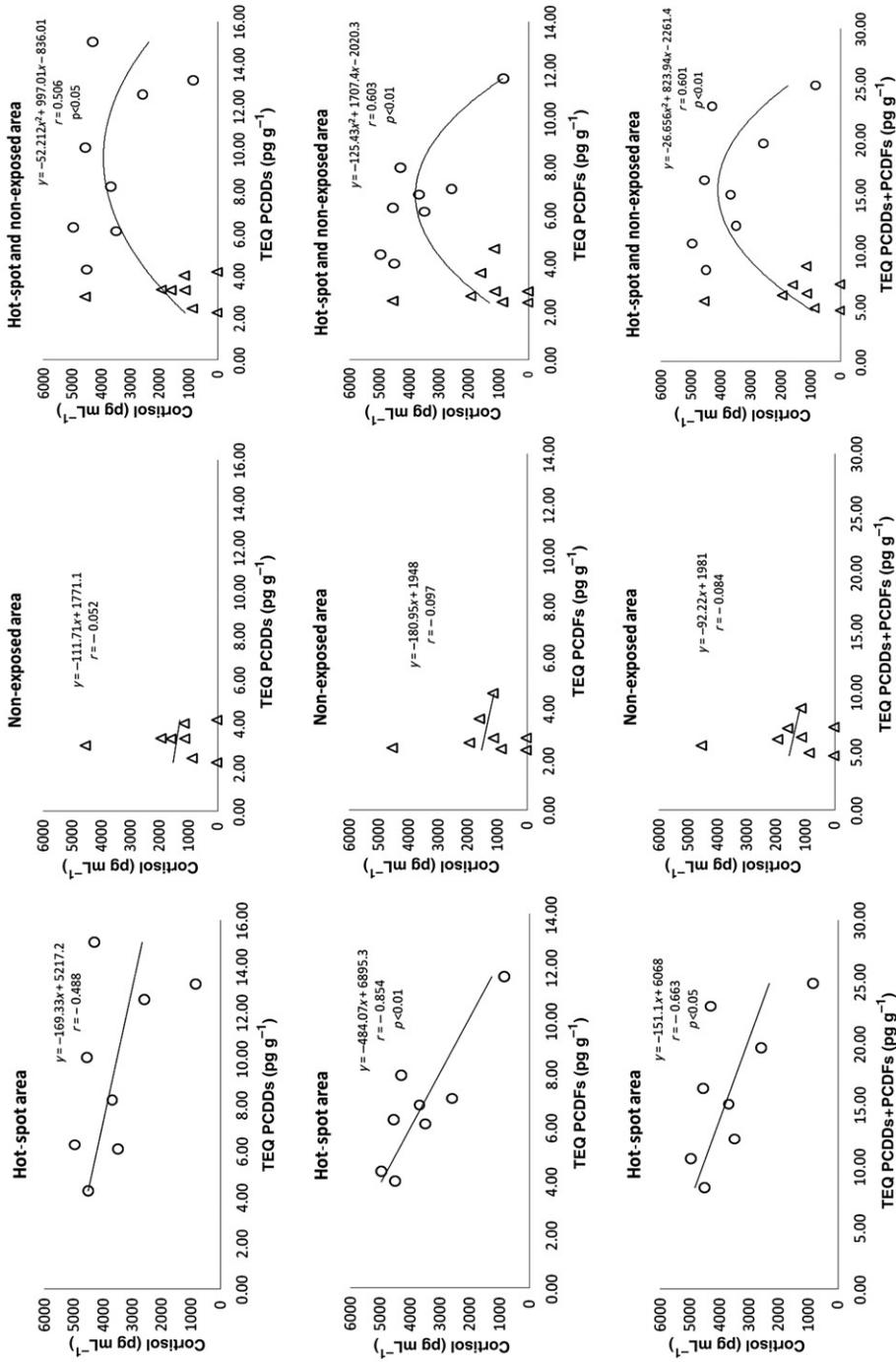


Figure 1. Correlation between free cortisol in saliva and dioxin in breast milk of primiparas.

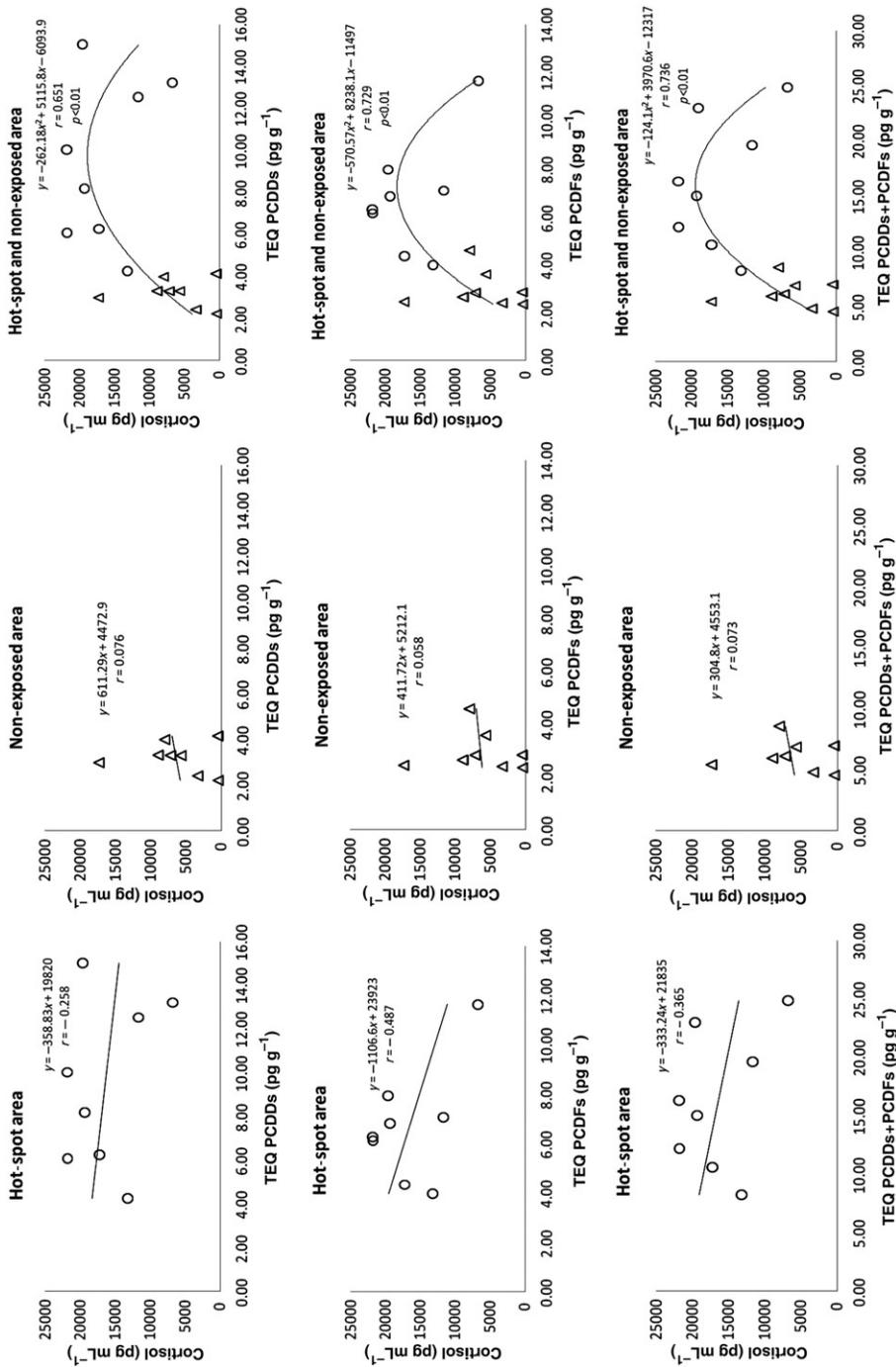


Figure 2. Correlation between cortisone in saliva and dioxin in breast milk of primiparas.

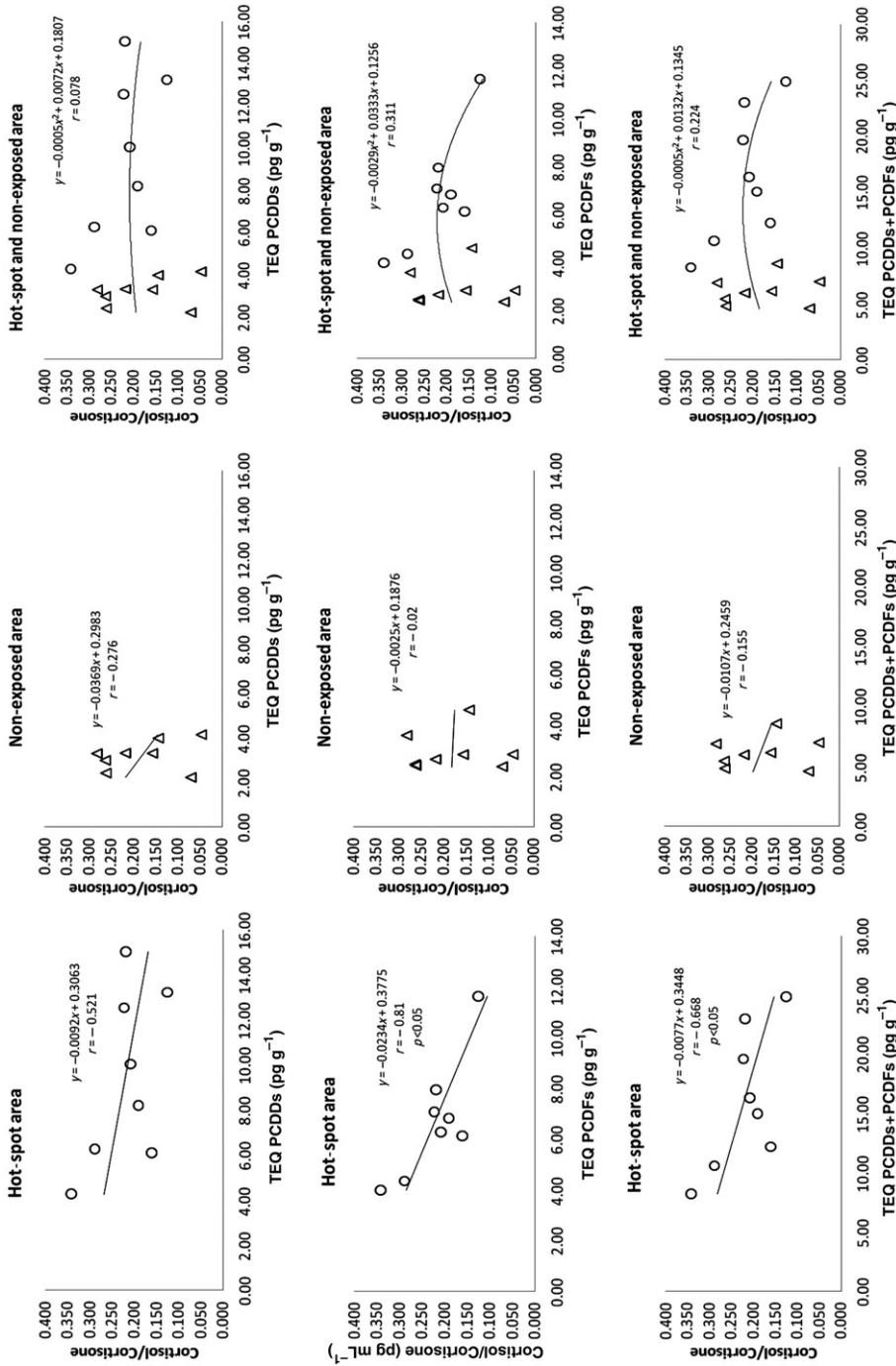


Figure 3. Correlation between cortisol/cortisone ratio in saliva and dioxin in breast milk of primiparas.

their children for 1 year (Furst et al. 1989). The mean TEQ levels in breast milk of primiparas and lactating women in the hot-spot area was 2.5-fold higher than in the non-sprayed area.

Salivary steroid measurements could prove to be a convenient and non-invasive means of determining free steroid concentrations in serum and may have widespread research applications in the field of endocrinology, neuroendocrinology, and reproductive endocrinology. The advantages of LC-MS/MS analysis compared to the radioimmunoassay (RIA) method include higher sensitivity (10- to 100-fold greater than RIA) and accuracy. However, the significant advantage of LC-MS/MS analysis is the ability to determine several steroids simultaneously, which enabled us to use saliva as the matrix for steroid hormone analysis.

The study of endocrine disruption in humans is extremely complex. Data showed that the dose-response curve between cortisol and dioxin exposure in the combination hot-spot and non-exposed areas showed an inverse U-shape (bell type). This indicates that at less than  $9.6 \text{ pg g}^{-1}$ , PCDD TEQ the cortisol level increases with a rise in PCDD TEQ, whereas above  $9.6 \text{ pg g}^{-1}$ , PCDD TEQ the cortisol level decreases as PCDD TEQ rises. The plots of PCDF TEQ and PCDD + PCDF TEQ versus cortisol in the combination hot-spot and non-exposed area behave similarly, as do those versus cortisone. Previous studies found that endocrine disruption often follows similar dose-response curves (U- or inverted U-shaped dose responses) (Crews and McLachlan 2006; Fenton 2006; Newbold, Padilla-Banks, and Jefferson 2006; Welshons, Nagel, and vom Saal 2006). The combined effect of these multiple actions of endocrine disruptors can, however, be difficult to interpret and is often misinterpreted as variability in the assay system. Indeed, all hormones exert non-linear actions on their targets, which imply that the combined effect of these multiple non-linear dose-responses is not predictable (Gore, Heindel, and Zoeller 2006). Therefore, it is proposed that a moderate increase in dioxin level stimulates the cortisol or cortisone secretion, whereas an excessive rise results in suppression. Several studies demonstrated that TCDD inhibits ovulation and steroid production in rats (Li, Johnson, and Rozman 1995; Ushinohama et al. 2001). In another study, TCDD was shown to inhibit steroidogenesis by interfering with specific steps such as the mobilization of cholesterol to the inner mitochondrial membrane (Moore, Jefcoate, and Peterson 1991). Li and Wang (2005) demonstrated that dioxin-like PCB126 stimulates cortisol biosynthesis by CYP11B1 induction and that it controls CYP21B by suppressing c-AMP induced CYP21B expression. Both these enzymes are essential for cortisol biosynthesis. Also, a high concentration of PCB126 might sensitize adrenocorticotrophic hormone (ACTH) regulation in adrenocortical cells by increasing ACTH receptor levels. Such complex and multiple effects by dioxins on adrenal steroid levels seem to result in non-linear dose-response curves, as also seen in this study. Furthermore, both dioxin and PCB were found to accumulate in the adrenal gland and may therefore alter adrenal steroid synthesis.

Cortisol is a corticosteroid hormone produced by the zona fasciculata of the adrenal cortex, which is part of the adrenal gland. TCDD has been implicated in the decreased bioactivity of ACTH, the primary secretagogue for corticosteroid production (Bestervelt et al. 1993). ACTH increases the concentration of cholesterol in the inner mitochondrial membrane and also stimulates the main rate-limiting step in cortisol synthesis where cholesterol is converted to pregnenolone in a reaction catalyzed by cytochrome P450<sub>scc</sub>, which initiates steroid biosynthesis (Stocco 2000). TCDD was also shown to affect the steroidogenic enzymes in rats by inhibiting cytochrome P450<sub>scc</sub> (DiBartolomeis, Williams, and Jefcoate 1986).

A previous study demonstrated that cortisol levels are increased in TCDD-exposed animals. The rise in corticotrophin-releasing hormone mRNA observed is unlikely to be the result of reduced feedback inhibition since TCDD enhanced cortisol secretion (Shridhar et al. 2001). Balk and Piper (1984) noted stimulation and inhibition of corticosterone in response to TCDD in rats. Our study includes individuals exposed to both high and low levels of dioxins, which may explain contrasting results, with salivary cortisone levels in primiparas from the hot-spot area being approximately 3-fold higher than in those from the non-exposed area and salivary cortisol levels being 3.5-fold greater.

The salivary gland contains 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD), which converts most of the cortisol in blood plasma to cortisone as the hormone passes through this gland (Edwards et al. 1988; Meulenberg and Hofman 1990). Dioxin might therefore stimulate this enzyme in the salivary gland, thereby increasing the rate of conversion of cortisol into cortisone, although further studies are needed with more saliva samples to confirm this hypothesis. It was not possible to find any previous studies involving steroid hormone levels in saliva and their relationship with the effects of dioxin on humans. Therefore, our study is likely to be the first to investigate hormone levels in the saliva of Vietnamese subjects in the areas affected by TCDD and may well lead to an increased interest in the use of salivary hormone levels to measure the effect of this chemical on humans in dioxin hot-spot and herbicide-sprayed areas in southern Vietnam. However, further research is needed to clarify the association between dioxin exposure and human adverse health effects. Hot-spot areas may be an especially advantageous location for such studies because of extremely high dioxin levels.

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