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Abstract

Dioxin (PCDDs+PCDFs), is one of the most toxic chemical substances known. Although it is suspected to cause endocrine disruption, very few epidemiological studies into its effects on human steroid hormones have been performed. The aim of this study is to elucidate the association of dioxin exposure with steroid hormone levels in the saliva and serum of Vietnamese women. Two areas, namely Phu Cat (hot-spot) and Kim Bang (non-exposed area), were selected for study. The study subjects consisted of 51 and 58 women, respectively. Saliva, blood and breast milk samples were collected from subjects in both areas. Cortisol, cortisone, dehydroepiandrosterone, androstenedione, estrone and estradiol levels in serum and saliva were determined by liquid-chromatography/tandem mass spectrometry (LC-MS/MS); dioxin levels in breast milk were measured by gas-chromatography-mass spectrometry (GC-MS). The dioxin concentration in breast milk from women in the hot-spot area was 3-4 times higher than in women from the non-exposed area. Good correlations were found between the levels of six steroid hormones in saliva and those in serum, respectively. Salivary and serum cortisol and cortisone levels in women from the hot spot were significantly higher than in those from the non-exposed area ($P<0.001$) and those in all subjects were positively associated with dioxin levels in Vietnamese women ($P<0.01$). These results suggest-that dioxin influences steroidogenesis in humans. Saliva sample can be used for hormone analysis and are therefore excellent specimens in epidemiological studies.

Introduction

Dioxin is one of the most toxic chemical substances known and is a persistent environmental contaminant. During the Vietnam War (1961–1971), the US Air Force sprayed over 80 million litres of chemical herbicides on southern battlefields for general defoliation and crop destruction [1]. This chemical herbicide was contaminated with highly toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin-TCDD) [1]. Kreuzer et al. [2] have estimated that the half-life of dioxin in human adults is 7–11 years. Although the war in Vietnam ended more than 40 years ago, dioxin hot spots have been found in and around three former US airbases [3-5]. TCDD accumulates in the fatty tissues of the body as a result of dioxin's lipophilic nature [6,7]. As such, studies on dioxin levels in lactating mothers are mainly carried out using breast milk. The dioxin levels in breast milk in sprayed areas in Vietnam are still higher than in non-exposed areas [8].

Some of the adverse effects associated with dioxin exposure may be considerably mediated by alterations in endocrine function [9-12]. There have been a few scientific studies concerning the effect of dioxin exposure on human sex steroid hormones in Seveso residents and chemical industry workers [13,14]. Recently, in an epidemiological study, we demonstrated that salivary cortisol, cortisone, estradiol and androstenedione (A-dione) levels in primiparae were related to dioxin levels in their breast milk [15-17].

The purpose of this study was to further clarify the relationship between dioxin exposure and steroid hormone levels in the serum and saliva of Vietnamese mothers by using a larger number of subjects residing in a dioxin hot spot and a non-exposed area. A further aim was to compare salivary steroid hormone levels with serum steroid hormone levels determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Materials and methods

Study area

Agent Orange/Dioxin hot-spot: The hot-spot area selected was Phu Cat Airbase, where chemical herbicides were stored and the aircraft used to spray Agent Orange/Dioxin during the Vietnam war washed. This site is located in Phu Cat district, Binh Dinh province, and is one of the three dioxin hot spots in south Vietnam. The study subjects were chosen from the population that had been living in and around Phu Cat airbase after the war. Records show that 17,000 drums of Agent Orange, 9000 drums of Agent White and 2900 drums of Agent Blue were stored there [18].

Control area: The non-exposed area selected was Kim Bang district, Ha Nam province, in the north of Vietnam, which was not exposed to chemical defoliants during the war and is a rural area that has not been affected by industrial pollution..

Subjects and methods

The study subjects consisted of 51 lactating women from the hot-spot area and 58 from the non-exposed area. All mothers were aged between 20 and 30 years with children aged between 13 and 16 months at the time of the study. A sample of breast milk was collected from lactating women in September 2008 and serum and saliva samples were collected from the same subjects one year later (August). All blood, breast milk and salivary samples were collected in the morning (between 8.00 and 10.00 AM). The temperature in both areas was around 30-34^{0}C at this time. After local government officials and medical staff had explained the purpose of this study to 109 lactating females (51 from the hot-spot area and 58 from the non-exposed area), all agreed to participate in the study. A sample of about 20 mL of breast milk was collected from all participants. Saliva samples (2–3 mL) were self-collected with support from medical staff. After 5 min rinsing the mouth with water, the saliva collected directly into a 15-mL bakelite test tube. Medical staff used a 10-mL syringe to extract about 10 mL of venous blood from lactating women and the serum was separated from this blood sample by centrifugation (7000rpm x 10 minutes). The saliva and serum samples obtained were conserved in chemically cleaned cooling containers and frozen in dry ice over several days. All samples were then transported to Japan and were stored at -70^{0}C until analysis.

Mothers were asked to provide information concerning their family, age, family income and residence period. The lactating mother's body mass index was determined and compared between the two areas.

The Medical Ethics Committee of Kanazawa University approved this study (Permission number: Health 89).

Reagents

Cortisol, cortisone, dehydroepiandrosterone (DHEA), androstenedione (A-dione), estrone and estradiol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Estrone-$^{13}C_4$, Estradiol-$^{13}C_4$ and Progesterone-$^{13}C_3$ were purchased from Hayashi Chemical Co Ltd. (Oosaka, Japan). Cortisol-$^{2}H_4$ was obtained from C/D/N Isotopes (Pointe-Claire, Canada) and DHEA-$^{2}H_4$ was provided by Aska Pharma Medical Co. Ltd. (Kawasaki, Japan). $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,7,8-TCDF were obtained from Wellington Laboratories (Canada). Picolinic acid, 2-methyl-6-nitrobenzoic acid anhydride, 4-dimethylaminopyridine, pentafluorobenzyl bromide and 2-fluoro-1-methylpyridinum *p*-toluenesulfonate were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Triethylamine was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Oasis MAX (60mg, 3ml), Bond Elut C18 and InterSept pharm cartridges were purchased from Waters Co. (Milford, MA, USA), Valian (CA, USA), and GL Science (Tokyo, Japan), respectively.

Reagent A: Picolinic acid (40mg), 2-methyl-6-nitrobenzoic acid anhydride (40mg), dimethylaminopyridine (20mg)/mL of tetrahydrofuran. Reagent B: 2% 2-fluoro-1-methylpyridinum *p*-toluenesulfonate/ ml of dichloromethane.

Instrurment

The LC-MS/MS system was as following. An API 5000 triple stage quadrupole mass spectrometer (Applied Biosystems Inc., Foster city CA, USA) connected to an LC-20AD pump and SIL HTC autosampler (Shimadzu, Kyoto, Japan). An ESI ion source device was employed for estrone and estradiol. Column: xterra-C-18 (Waters ,USA)

An API 4000 triple stage quadrupole mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) with electrospray ionization ion source, Agilent 1100 HPLC system (Agilent Techologies, Waldbronn, Germany), and a PTC pal auto-sampler (CTC Analytics Zwingen, Switzerland) were

employed for analysis of neutral steroids. Column: Cadenza CD-C18 (250mm x 3 mm, internal diameter 3 um, Imtact, Kyoto, Japan)

The gas chromatography-mass spectrometry (GC-MS) system was as following. A high resolution mass spectrometer (HRMS: JEOL MS station-JMS700) equipped with gas chromatograph (GC: HP-6980, Hewlett-Packard, Palo Alto, CA). Column: ENV-5MS with 30m x 0.25 mm ID of 0.25 µm film thickness (Kanto Chemical Co., Inc.)

Analysis of serum steroids by LC-MS/MS

The analytical procedure of serum steroids was modified with method of Yamashita et al [19] . Human serum (200 µL) was mixed with purified water to a volume of 1.0 mL and then mixed with cortisol-$^{2}H_4$ 1 ng, DHEA-$^{2}H_4$ 100 pg, progesterone-$^{13}C_3$ 100 pg, estrone-$^{13}C_4$ 100 pg and estradiol-$^{13}C_4$ 100 pg /100 µL as Internal standard (IS). After samples were extracted with 3 mL ethyl acetate and the extract applied to the Bond Elut C18 cartridge to remove impurity. After elution of steroids fraction with 80% acetonitrile solution, the residue was reacted with reagent A [19,20]. The reaction mixture was then applied onto an InterSept pharm cartridge to remove excess reagents. A part of residue involved in the picolinoyl derivatives and non- derivatives steroids, were directly assayed by LC-MS/MS as described by Yamashita [19] .

The estimation ions are as follows: cortisol and cortisol-$^{2}H_4$: 468.2/309.2; 472.2/454.3; cortisone and cortisol-$^{2}H_4$: 468.2/309.2; 472.2/454.3; DHEA and DHEA-$^{2}H_4$:394.3/175.1; 398.1/179.4; A-dione and Progesterone-$^{13}C_3$: 287.4/109.0; 318.3/100.1; estrone and estrone-$^{13}C_4$: 376.1/156.9; 380/160.8; estradiol and estradiol-$^{13}C_4$: 483.3/264.0; 487.2/268.2. The lowest analytical limits for cortisol, cortisone, DHEA, A-dione , estrone and estradiol were 50, 50, 5, 10, 1.0 and 0.5 pg/assay, respectively. The accuracy and precision were both within ±20% of the lowest level in intra-day and inter-day assays and both were within ±15% for concentrations other than the lowest concentration.

Analysis of salivary steroids by LC-MS/MS

Human saliva (1.0 - 1.5 mL) was mixed with the same IS in analysis of serum steroid. The extracts were then applied to a Bond Elut C18 cartridge to separate the polar (cortisol and cortisone) with 20% acetonitrile solution (2 mL) and non-polar steroid fractions using 80% acetonitrile solution (3 mL). The non-polar fraction was applied to an ion cartridge column prewashed with methanol (3 mL), 0.1 M

NaOH (1 mL) and water (3 mL), successively. The non-polar fraction was separated into a neutral fraction with methanol and an estrogen fraction (estrone and estradiol) with 1% formic acid-methanol. The organic phase for both fractions was evaporated to dryness. After deriving of the estrogen fraction with 2% pentafluorobenzyl bromide-acetonitrile (100 µL) and 5% KOH-ethanol solution (50 µL) at 53^0C, the derivatives obtained were furthermore separated into the estrone and estradiol derivatives on an Intersept SI cartridge column using 15-50% ethyl acetate–hexane. The estrone-3-pentafluorobenzyl fraction was converted to the estrone-3-pentafluorobenzyl-17-hydorazino-2-methylpyridinium derivative by method of Higashi [21], whereas the estradiol-3-pentafluorobenzyl fraction was converted into estradiol-3-pentafluorobenzyl-17-O-2-pyridinium ether using reagent B [22]. Both derivatives were purified on a Bond Elut C18 cartridge column to remove excess reagent and purified estrogen derivatives were mixed with 100 µL of 1% formic acid/methanol/acetonitrile (20:1:1) and a 20 µL aliquot of this solution was estimated by LC-MS/MS. The neutral fraction was treated according to the picolinic acid method described above for serum samples. The fraction obtained was estimated by LC-MS/MS (API 5000). The estimation ions are as follows: cortisol and cortisol-$^2H_4$: 363.3/121.2; 367.3/121.2; cortisone and cortisol-$^2H_4$: 361.2/162.8; 367.3/121.2; DHEA and DHEA-$^2H_4$: 394.3/175.1; 398.1/179.4; A-dione and Progesterone-$^{13}C_3$: 287.4/109.0; 318.3/100.1; estrone and estrone-$^{13}C_4$: 556.3/313.1; 560.3/379.3; estradiol and estradiol-$^{13}C_4$: 544.2/339.0; 548.2/343.2.

The lowest analytical limits for cortisol, cortisone, DHEA, A-dione , estrone and estradiol were 50, 50, 2, 10, 0.5 and 0.1 pg/assay, respectively. The accuracy and precision were both within ±20% of the lowest level in intra-day and inter-day assays and both were within ±15% for concentrations other than the lowest concentration.

GC-MS analysis of dioxin in breast milk

Breast milk samples were analyzed following a previously reported method [8,23]. After extraction of the fat from 10 g of breast milk, 40-80 pg of 17-$^{13}C_{12}$ labeled PCDD/PCDF congeners was added as an internal standard.

A series of purification operations involving alkali digestion and chromatography on a multi-layer silica gel column and an active carbon-dispersed silica gel column were carried out to separate and collect the PCDDs/PSDFs. The final sample extract was evaporated to dryness under a nitrogen steam,

then re-dissolved by addition of 20 μL of nonane containing 40 pg of $^{13}C_{12}$-1,2,3,4-TCDD and $^{13}C_{12}$-1,2,7,8-TCDF as external standards. Finally, determination was performed using a gas chromatograph equipped with a high resolution mass spectrometer .

Dioxin analyses were performed in the selected ion-monitoring mode (SIM) at a resolution of 10,000 and converted to toxic equivalents (TEQ) using the World Health Organization (WHO) toxicity equivalency factors (TEFs) [7,24].

Quality control and quality assurance were implemented following guidelines described in the Japanese Industrial Standard (JIS). Eligibilities for dioxin analysis were certified using natural reference powder milk CRM607 provided by the European Commission. The recovery rate was typically in the range 60-95% and the detection limits determined at a signal-to-noise ratio of 3 (S/N = 3) on a lipid basis. Values for congener concentrations below the detection limits were set to half the detection limits.

Statistical analysis

Data are indicated as mean ± standard deviation in the case of a normal distribution and as median (inter-quartile range) for a non-normal distribution, as determined using the Shapiro-Wilk test. Pearson's correlation coefficients were calculated. Statistical comparisons of the mean differences between two areas were performed using Student's t-test in the case of a normal distribution or the Wilcoxon signed-ranks test for a non-normal distribution. The significance level was set to $p < 0.05$. All statistical analyses were performed using the SPSS 12.0 software, JMP@9 software package (SAS Institute) and Microsoft Excel 2010.

Results

Comparison of study subject characteristics between the dioxin hot-spot and the non-exposed area

Table 1, which presents the characteristics of lactating women from the two study areas (n=109), shows the age, weight, height and BMI. These values do not differ significantly between the two areas. Likewise, the family income and residence period are similar for both areas.

Comparison of hormone levels for mothers from the dioxin hot-spot and non-exposed area

Table 2 shows the salivary concentrations of six steroid hormones (cortisol, cortisone, DHEA, A-dione, estrone, and estradiol) for all study subjects (n=109) from the dioxin hot-spot and non-exposed areas, as determined by LC-/MS/MS analysis. Salivary cortisol and cortisone levels were found to be significantly higher in women from the dioxin hot-spot area than those from the non-exposed area ($p<0.001$). There were no significant differences between the two areas for any of the other salivary hormones.

Table 3 shows the serum levels of the six steroid hormones studied for the two areas. Cortisol and cortisone levels can be seen to be significantly higher in women from the hot-spot area than in those from the non-exposed area ($p<0.001$ and $p<0.01$, respectively). There were no significant differences in serum DHEA, A-dione, estrone, and estradiol levels.

Comparison of TEQ dioxin levels in lactating women's breast milk for the dioxin hot-spot and non-exposed area

The median total toxic equivalence (TEQ) of polychlorinated dibenzodioxins (PCDDs, 6.29 pg/g lipid), polychlorinated dibenzofurans (PCDFs, 4.44 pg/g lipid) and total PCDDs+PCDFs (11.04 pg/g lipid) for the dioxin hot-spot area (n=51) was significantly higher than for the non-exposed area (PCDDs: 1.87 pg/g lipid; PCDFs: 1.41 pg/g lipid; total PCDDs+PCDFs: 3.15 pg/g lipid). Specifically, dioxin levels in the hot-spot area were more than three-times higher than those in the non-exposed area ($p < 0.001$).

Correlation between steroid hormones in saliva or serum and dioxin in breast milk

Fig. 1 shows the correlation between salivary cortisol or cortisone and dioxin levels in the combination of dioxin hot-spot area and non-exposed area. Significantly positive correlations between salivary cortisol or cortisone and breast milk dioxin levels (total PCDDs+PCDFs) can be seen, ($p<0.001$).

Fig. 2 also shows the significant correlation between serum cortisol or cortisone and breast milk dioxin levels (total PCDDs + PCDFs), ($p<0.01$ and $p<0.001$).

Correlation between salivary steroid hormones and serum steroid hormones

We determined six kinds of steroid hormones in saliva and serum by LC-MS/MS. Fig. 3 shows a significantly positive correlation between cortisol, cortisone, DHEA, estrone, A-dione and estradiol in serum and saliva for the combination of dioxin hot-spot area and non-exposed area, respectively ($p< 0.001$).

Discussion

The study region in this investigation (Phu Cat) is one of three major dioxin hot-spot areas in Vietnam. Indeed, according to Dwernychuk [3,25], the concentration of TCDD recorded at Phu Cat is 194 pg/g in sediment, and the Hatfield Consultancy [26] has reported that the maximum TCDD level of 236,000 pg/g TCDD in soil taken from the vicinity of Phu Cat is much higher than the internationally recognized standard of 1000 pg/g TCDD in soil.

We have demonstrated in this study that dioxin levels in maternal breast milk are three- to fivefold higher in the hot-spot area than in the non-exposed area despite the fact that more than 40 years have passed since the end of war. This result is consistent with our previous study [15]. As similar study in Seveso estimated that the TCDD concentration in females was fivefold higher in the exposed area than in a control area after 30 years [27]. These studies suggest that the dioxin burden in humans continues in the long term after environmental exposure.

Salivary cortisol and cortisone levels were found to be significantly higher in the dioxin hot-spot than in the non-exposed area ($p < 0.001$). Furthermore, serum cortisol and cortisone levels were also significantly higher in the dioxin hot-spot ($p<0.001$ and $p<0.01$, respectively). We have recently reported a similar study of salivary steroid hormone levels in primiparae in a dioxin hot-spot area [15-17]. Our present study of all lactating women (first, second or third child), which included a larger number of subjects (N=109), revealed significantly higher serum and salivary cortisol and cortisone levels in lactating women from the dioxin hot-spot area (Table 2 and 3, $p< 0.001$).

The relationship between dioxin and hormone levels show linear in the dioxin level range 2-25 pg/g lipid (Fig. 1~2, $p<0.01$). The adrenal gland is a major accumulation site for lipophilic dioxins and PCBs in the body. Three types of steroid hormones (cortisol, aldosterone and DHEA) are synthesized in the adrenal gland and their levels, and therefore ratios, are regulated by ACTH. In our study, the concentrations of cortisol and cortisone in both serum and saliva were found to be significantly higher in the hot-spot than those in the non-exposed area, whereas the DHEA level varied less between the two areas. These results clarified one of the aims of the present study.

Our previous study on 18 primiparae in a dioxin hot-spot area, which also showed increased salivary cortisol and cortisone level, also gave an inverted U-shape for the TEQ dioxin levels [15]. The current

study in 109 lactating mothers also showed increased cortisol and cortisone levels in serum and saliva. However, the model between steroid hormone levels and dioxin could be fit by a straight line rather than a non-linear inverted U-shaped curve. The current findings, which differ somewhat with respect to the previous study in primiparae, may be due to the fact that this study included lactating mothers who had given birth to their first, second or third child.

The levels of the other hormones studied herein (DHEA, androstenedione, estrone, and estradiol) did not change significantly between the hot-spot and the non-exposed area. Our previous study showed that the mean salivary estradiol or androstenedione level did not differ significantly between the dioxin hot-spot and non-exposed areas, although the curve between salivary estradiol or androstenedione levels and dioxin levels was U-shaped in primiparae [16]. In the Seveso study, men aged 22-31 years showed reduced sperm concentrations and serum estradiol, but increased FSH levels, 22 years post-exposure. In contrast, men aged 32-39 years showed an increased total sperm concentration and serum FSH, and a reduced estradiol concentration, compared with those from the control area. Serum testosterone levels did not vary [13]. These results suggest that exposure at certain times of life may affect the subsequent impact of this exposure. Steroid hormone levels in serum are distributed into three phases: the free type (1-3%), the bioavailable type (30-40%) and the inactive type (50%). Salivary cortisol has been reported to correlation well with serum free-cortisol concentration [28]. Salivary steroid hormones are known to be excreted from serum as the free type. The total serum cortisol to cortisone ratio is approximately 3:1, whereas the salivary ratio is approximately 1:6. This marked difference in the cortisol/cortisone ratio between serum and saliva is due to two main effects. First, salivary gland membranes contain the enzyme 11β-HSD 2, which irreversibly converts cortisol into cortisone as it passes through this membrane. Second, more than 90% of circulating hormone in human serum is bound to proteins such as corticoid binding globulin (CBG) and albumin. Cortisone binds CBG in serum with a 10-fold lower association constant than cortisol. Consequently, the proportion of free cortisone in serum is much higher than that for cortisol. These findings are reflected in the salivary steroid concentrations. We also determined the ratios of free to protein-bound hormones from salivary and serum hormone concentrations. The ratio of each salivary to serum hormone concentration was significantly higher in the dioxin hot-spot than in the non-exposed area

except for DHEA (data were not shown). These higher ratios may be related to any one of the numerous effects of dioxin exposure on endocrine disruption. In other words, dioxin may have an effect on steroid binding proteins, and may influence the natural balance of hormone circulation.

It should be noted that low doses of this chemical also affect immune function [29]. Thus, in the study that examined the effects of TCDD on Vietnam War air force veterans, Pavuk et al. [30] found an increase in TSH levels but no change in T3 or free T3 levels. However, studies on endocrine disruption by chemicals can be difficult to interpret and are readily misinterpreted, thus meaning that the same factor could stimulate or inhibit [31].

We found a strongly positive correlation between salivary hormones and serum hormones ($p<0.001$) (see Fig.3). We chose saliva as the matrix for steroid hormone analysis in this study as it is non-invasive and easy to collect from subjects, even from children, and is feasible for use in epidemiological studies.

The LC-MS/MS method is an excellent technique with a higher sensitivity and accuracy than standard immunoassay methods. Furthermore, this technique can also be used to simultaneously analyze six steroids, including those, such as cortisol and cortisone, with a similar molecular structure using only 0.1 mL of serum. We have also established an analytical method for estradiol involving chemical derivatization that allows even trace amounts to be detected. This method has a limit of quantification of 0.1 pg/mL by LC-MS/MS.

There are some limitations to our study. The subjects in our study were women (n = 109) who had given birth up to one year previously and therefore may have returned to a normal menstrual cycle with the physiological activity inherent to normal hormone regulation [32]. It is therefore probably difficult to elucidate sex hormone levels, such as progesterone or estrogen, in women with varying menstrual cycles.

In summary, the relationship between dioxin exposure and endocrine disruption requires further clarification in order to be able to evaluate adverse human health effects caused by dioxin and/or other environmental chemicals. It is especially important to continuously observe maternal health throughout life and to investigate any possible influence on the development of their children at dioxin hot-spot in Vietnam.

Conflict of interest

The authors declare no conflict of interest.

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References

1. Stellman JM, Stellman SD, Christian R, Weber T & C Tomasallo. The extent and patterns of usage of agent orange and other herbicides in Vietnam. *Nature* 2003 **422** 681–687.
2. Kreuzer PE, Csanady GA, Baur C, Kessler W, Papke O, Greim H & Filser JG. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and congeners in infants. A toxicokinetic model of human lifetime body burden by TCDD with special emphasis on its uptake by nutrition. *Archives of Toxicology* 1997 **71** 383–400.
3. Dwernychuk LW. Dioxin hot spots in Vietnam. *Chemosphere* 2005 **60** 998–999.
4. Schecter A, Dai LD, Papke O, Prange J, Constable JD, Matsuda M, Thao VD & Piskac AL. Recent dioxin contamination from Agent Orange in residents of a Southern Vietnam city. *Journal of Occupational and Environmental Medicine* 2001 **43** 435–443.
5. Mai TA, Doan TV, Tarradellas J, de Alencastro LF & Grandjean D. Dioxin contamination in soils of southern Vietnam. *Chemosphere* 2007 **67** 1802–1807.
6. Fingerhut MA, Sweeney MH, Patterson DG, Piacitelli LA, Morris JA, Marlow DA, Hornung RW, Cameron LW, Connally LB, Needham LL & Halperin WE. Levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the serum of US chemical workers exposed to dioxin contaminated products: interim results. *Chemosphere* 1989 **19** 835–840.
7. Van den Berg M, Birnbaum L, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N & Peterson RE. The (2005) World Health Organization re-evaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicological Sciences* 2006 **93** 223–241.
8. Pham TT, Nishijo M, Kido T, Nakagawa H, Maruzeni S, Naganuma R, Anh NT, Morikawa Y, Luong HV, Anh TH, Hung NN, Son le K, Tawara K & Nishijo H. Dioxin concentrations in breast milk of Vietnamese nursing mothers: A survey four decades after the herbicide spraying. *Environmental Science and Technology* 2011 **45** 6625–6632.

9. Bestervelt LL, Pitt JA, Nolan CJ, Cai Y, Piper DW, Dybowski JA, Dayharsh GA & Piper WN. In vitro 2,3,7,8-tetrachlorodibenzo-p-dioxin interference with the anterior pituitary hormone adrenocorticortropin. *Toxicol Sci* 1998 **44** 107-115.

10. Peterson RE, Theobald HM & Kimmel GL. Developmental and reproductive toxicity of dioxins and related compounds: Cross-species comparisons. *Critical Reviews in Toxicology* 1993 **23** 283–335.

11. Bookstaff RC, Moore RW & Peterson RE. 2,3,7,8 -Tetrachlorodibenzo-p-dioxin increases the potency of androgens and estrogens as feedback inhibitors of luteinizing hormone secretion in male rats. *Toxicology and Applied Pharmacology* 1990 **104** 212- 224.

12. Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC & Hauser R. Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. *Endocrine Reviews* 2009 **30** 293-342.

13. Mocarelli P, Gerthoux P M, Patterson DG, Milani S, Limonta G, Bertona M, Signorini S, Tramacere P, Colombo L, Crespi C, Brambilla P, Sarto C, Carreri V, Sampson EJ, Turner WE & Needham L. Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. *Environ Health Perspectives* 2008 **116** 70-77.

14. Egeland GM, Sweeney MH, Fingerhut MA, Wille KK, Schnorr TM & Halperin WE. Total serum testosterone and gonadotropins in workers exposed to dioxin. *Am J Epidemiology* 1994 **139** 272-281.

15. Nhu DD, Kido T, Naganuma R. Suzuki H, Kuroda N, Honma S, Tai PT, Maruzeni S, Nishijo M, Nakagawa H, Hung NN, Thom LTH & Son LK. Salivary cortisol and cortisone levels, and breast milk dioxin concentrations in Vietnamese primiparas. *Toxicological & Environmental Chemistry* 2010 **92** 1939-1952.

16. Nhu DD, Kido T, Hung NN, Thom LT, Naganuma R, Son LK, Honma S, Maruzeni S, Nishijo M & Nakagawa H. Dioxin levels in the breast milk and estradiol and androgen levels in the saliva of Vietnamese primiparae. *Toxicological & Environmental Chemistry* 2011 **93** 824-838.

17. Manh HD, Kido T, Okamoto R, XianLiang S, Hoang N, Nakano M, Tai PT, Maruzeni S, Nishijo M, Nakagawa H, Suzuki H, Honma S, Tung DV, Nhu DD, Hung NN & Son LK.

The relationship between dioxins and salivary steroid hormones in Vietnamese primiparae. *Environ Health Prev Med* 2013 **18** 221-229.

18. Young AL. The history, use, disposition and environmental fate of Agent Orange. *Environmental Science and Pollution Research (ESPR)* 2010 **17** 1016-1019
19. Yamashita K, Miyashiro Y, Maekubo H, Okuyama M, Honma S, Takahashi M & Numazawa M. Development of highly sensitive quantification method for testosterone and dihydrotestosterone in human serum and prostate tissue by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids* 2009 **74** 920–926.
20. Yamashita K, Okuyama M, Watanabe Y, Honma S, Kobayashi S & Numazawa M. Highly sensitive determination estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry . *Steroids* 2007 **72** 819-827.
21. Higashi T. Salivary hormone measurement using LC/MS/MS: specific and patient-friendly tool for assessment of endocrine function. *Biol Pharma Bull* 2012 **35** 1401-1408.
22. Arai S, Miyashiro Y, Shibata Y, Kashiwagi B, Tomaru Y, Kobayashi M, Watanabe Y, Honma S & Suzuki K. New quantification method for estradiol in the prostatic tissues of benign prostatic hyperplasia using liquid chromatography–tandem mass spectrometry. *Steroids* 2010 **75** 13–19.
23. Tawara K, Honda R, Nishijo M & Nakagawa H. Pretreatment procedure of dioxin analysis for a small volume of human breast milk [in Japanese]. *Journal of Kanazawa Medical University* 2003 **28** 17–25.
24. Van den Berg M, Birnbaum L, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F & Zacharewski T. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environmental Health Perspectives* 1998 **106** 775–792.
25. Dwernychuk LW, Hung TM, Boivin TC, Bruce GS, Dung PT, Son LK, Hatfield CT, BruceTG, Dung GS, Son PT, Hatfield LK, Dung CT & Allan NT. The Agent Orange dioxin issue in Vietnam: A manageable problem. *Organohalogen Compounds* 2006 **68** 312–315.

26. Hatfield Agent Orange Reports. Evaluation of contamination at the agent orange dioxin hot spot in Bien Hoa, Phu Cat and vicinity, Viet Nam, Hatfield Consultants June 2009 13-15. (http://www.hatfieldgroup.com/wp-content/uploads/AgentOrangeReports/UNDP1391/UNDP1391_Final_VRT_%20Report_and_Appendices.pdf)

27. Warner M, Mocarelli P, Samuels S, Needharm L, Brambilla P & Eskenazi B. Dioxin exposure and cancer risk in the seveso women's health study. *Env Health Perspectives* 2011 **119** 1700-1705.

28. Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M, Taylor J & Young E. Strategies and method for research on sex differences in brain and behavior, *Endocrinolgy* 2005 **146** 1650-1673.

29. Ishimaru N, Takagi A, Kohashi M, Yamada A, Arakaki R, Kanno J & Hayashi Y. Neonatal exposure to low-dose 2,3,7,8-tetrachlorodibenzo-p-dioxin causes autoimmunity due to the disruption of T cell tolerance. *J Immunol* 2009 **182** 6576–6586.

30. Pavuk M, Schecter AJ, Akhtar FZ & Michalek JE. Serum 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) levels and thyroid function in air force veterans of the Vietnam War. *Ann Epidemiol* 2003 **13** 335-343.

31. Gore AC, Heindel JJ & Zoeller RT. Endocrine disruption for endocrinologists (and others). *Endocrinology* 2006 **147** S1–3.

32. Gandara BK, Leresche L & Mancl L. Patterns of salivary estradiol and progesterone across the menstrual cycle. *Annals of the New York Academy of Sciences* 2007 **1098** 446–450.

Figure legends

Fig 1. The correlation between cortisol (A), cortisone (B) concentration in saliva and dioxin level in breast milk in dioxin hot spot and non-exposed areas.

Fig. 2. The correlation between cortisol (A), cortisone (B) concentration in serum and dioxin level in breast milk in dioxin hot-spot and non-exposed areas.

Fig. 3. The correlation between serum hormone and salivary hormone in dioxin hot spot and non-exposed areas. A) Cortisol, B) Cortisone, C) DHEA, D) A-dione, E) Estrone, F) Estradiol

Table 1. Characteristics of lactating mothers in dioxin hot–spot and non–exposed areas

	Hot-spot area (n = 51)	Non-exposed area (n = 58)	p-value
Age (years)	27.3 ± 3.68	26.1 ± 2.82	0.063[a]
Weight (kg)	48.5 ± 6.63	48.8 ± 5.04	0.786[a]
Hight (cm)	152.3 ± 5.47	152.7 ± 5.10	0.721[a]
BMI (kg/m^2)	20.9 ± 2.22	20.9 ± 1.90	0.866[a]
Residence period (years)	21.0 (21.0 – 26.0)	22.5 (18.8 – 25.0)	0.803[b]
Family income ($\times 10^4$VND/month)	200 (100 – 300)	200 (110 – 300)	0.924[b]

Notes: Data are shown as mean ± sd (standard deviation) in normal distribution and as median (inter-quartile range) in non-normal distribution

[a]Student t-test

[b]Wilcoxon Signed Ranks Test

Table 2. Steroid hormone levels in saliva of lactating mothers in dioxin hot– spot and non– exposed areas

	Hot-spot area (n = 51)	Non-exposed area (n = 58)	p-value
Cortisol (ng/ml)	1.89 (1.30–3.16)	1.10 (0.70–1.90)	0.0001[b]
Cortisone (ng/ml)	10.8 (8.41–13.7)	7.74 (5.46–10.6)	0.0001[b]
DHEA (pg/ml)	154.7 (105.8–232.3)	133.8 (104.1–189.6)	0.223[b]
A-dione (pg/ml)	56.6 (42.5–75.6)	55.5 (45.8–74.6)	0.923[b]
Estrone (pg/ml)	1.20(0.58–2.10)	0.84 (0.62–1.47)	0.146[b]
Estradiol (pg/ml)	0.22 (0.13–0.46)	0.18 (0.10–0.33)	0.27[b]

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

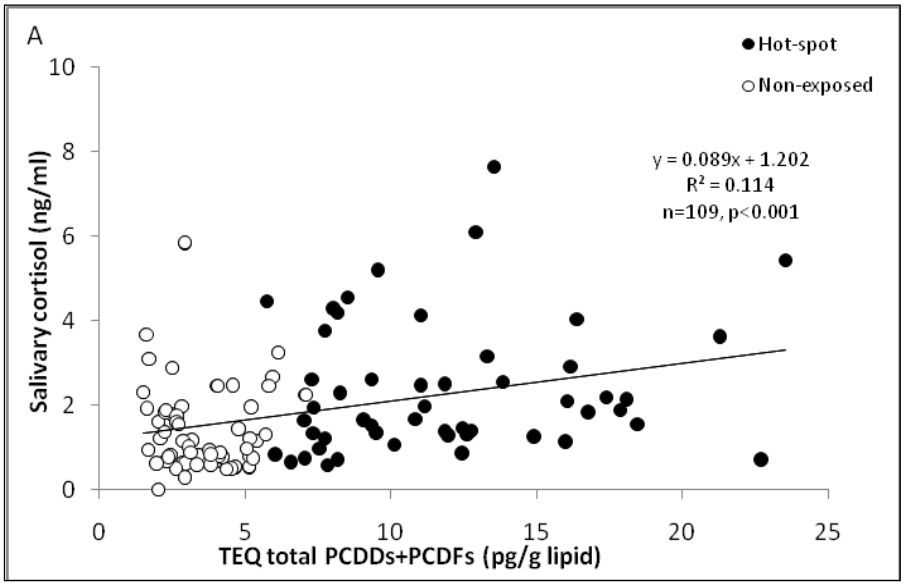
[b]Wilcoxon Signed Ranks Test

Table 3. Hormone levels in serum of lactating mothers in dioxin hot–spot and non–exposed areas

	Hot-spot area (n = 51)	Non-exposed area (n = 58)	p-value
Cortisol (ng/ml)	94.2 (71.9 – 141.6)	66.3 (52.2 – 103.8)	0.0001[b]
Cortisone (ng/ml)	26.7 (22.1 – 30.8)	22.0 (17.2 – 27.6)	0.003[b]
DHEA (pg/ml)	4566 (3158 – 6493)	4446 (3319 – 6617)	0.753[b]
A-dione (pg/ml)	1484 (1098 – 2070)	1650 (1259 –2172)	0.155[b]
Estrone (pg/ml)	23.7 (13.8 – 38.3)	26.2 (19.0 – 45.3)	0.176[b]
Estradiol (pg/ml)	21.3 (11.2 – 37.0)	21.9 (11.9 – 39.5)	0.888[b]

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

[b]Wilcoxon Signed Ranks Test



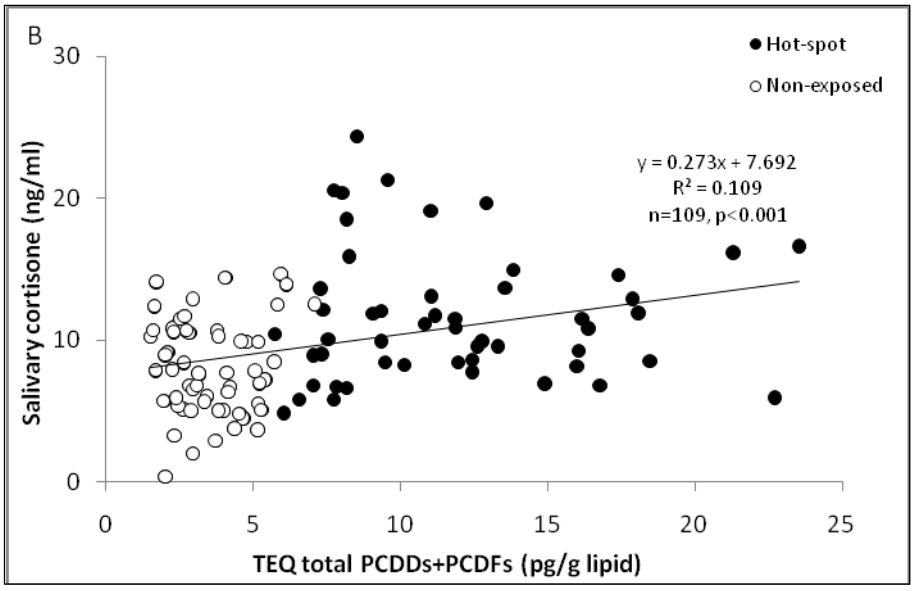


Fig 1. The correlation between cortisol (A), cortisone (B) concentration in saliva and dioxin level in breast milk in dioxin hot-spot and non-exposed areas.

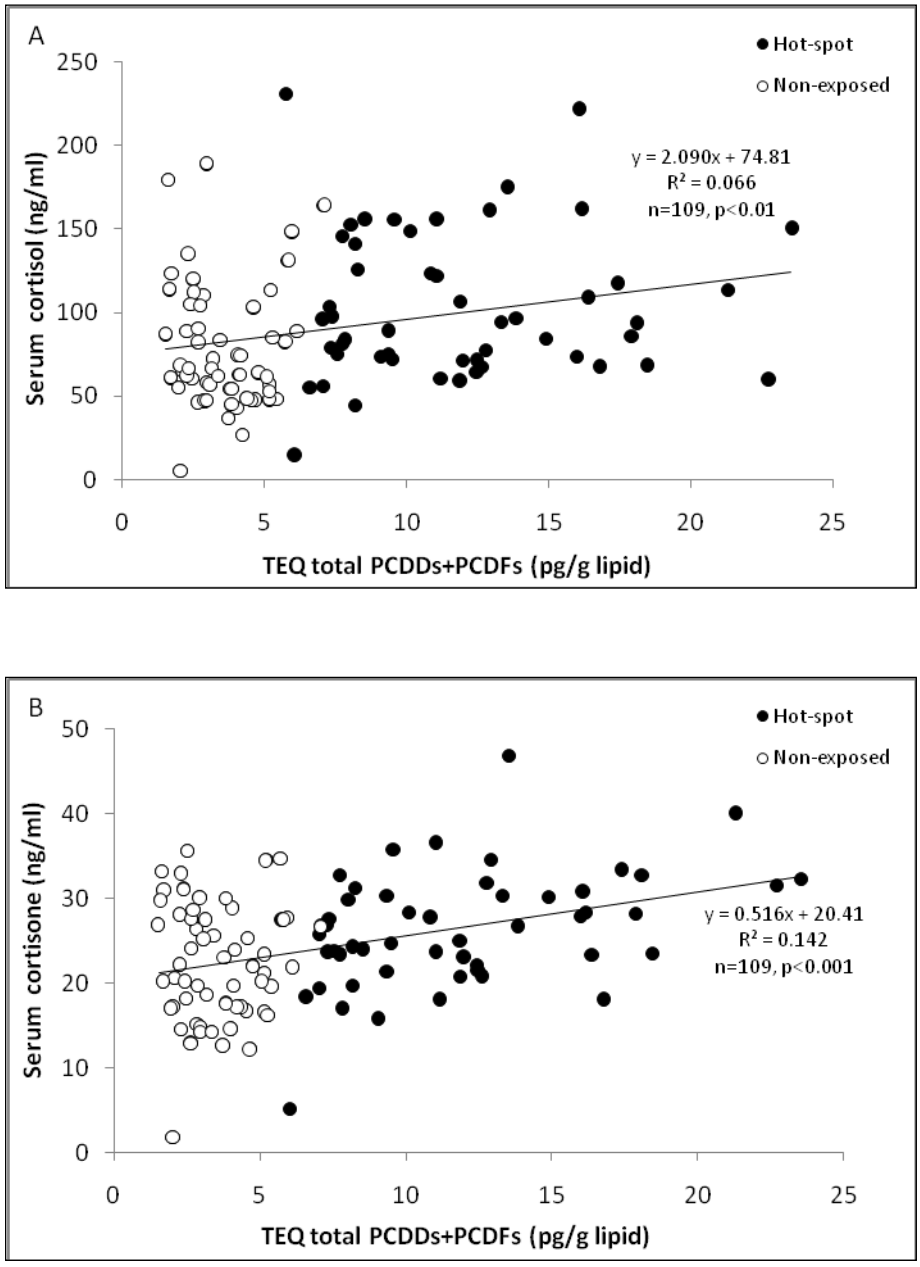


Fig 2. The correlation between cortisol (A), cortisone (B) concentration in serum and dioxin level in breast milk in dioxin hot-spot and non-exposed areas.

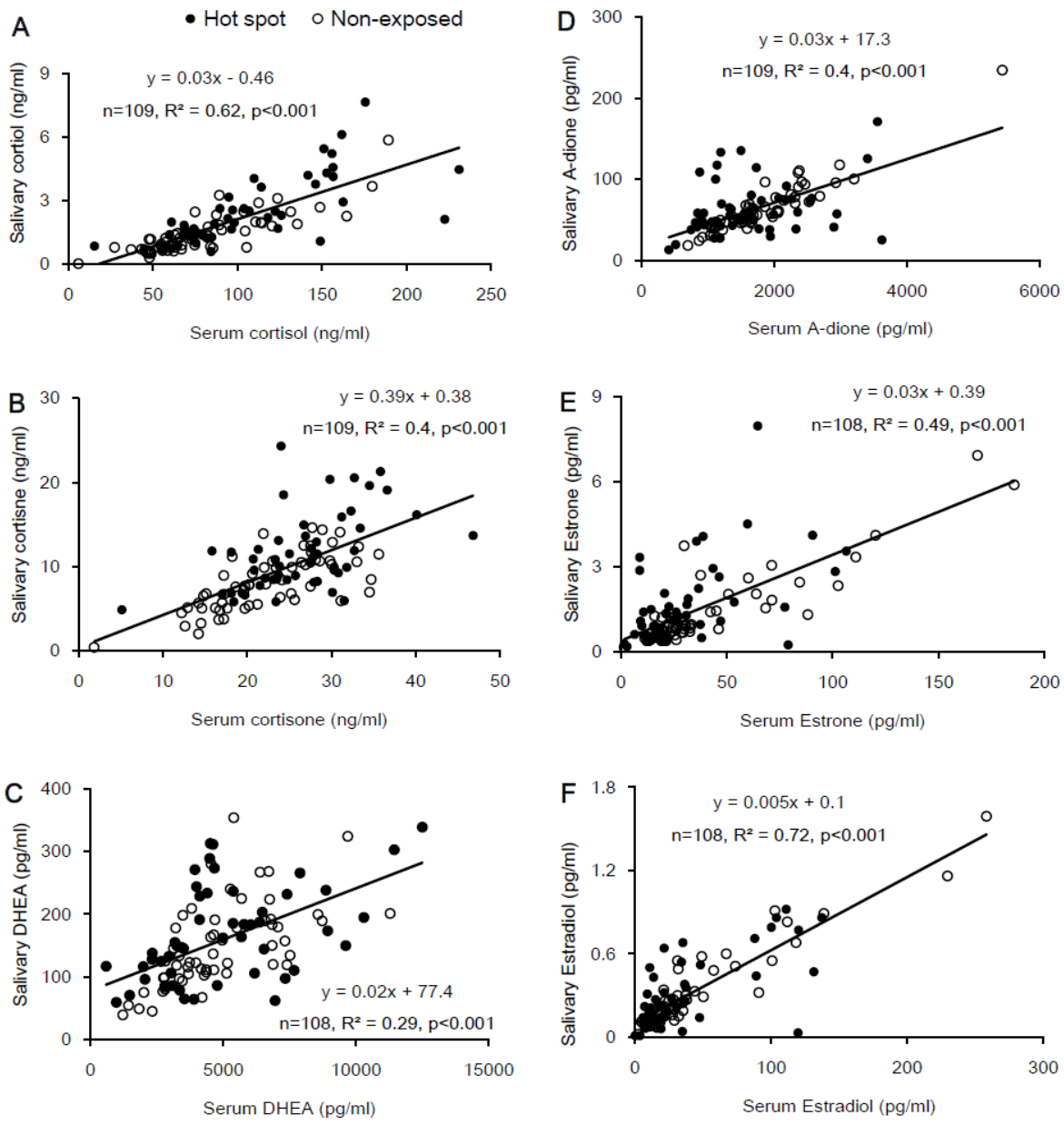


Fig. 3. The correlation between serum hormone and salivary hormone in dioxin hot spot and non-exposed areas. A) Cortisol, B) Cortisone, C) DHEA, D) A-dione, E) Estrone, F) Estradiol