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Hormone levels in the saliva of lactating Vietnamese mothers in a dioxin hot-spot area

Dang Duc Nhu, Teruhiko Kido, Rie Naganuma, Kae Saito*, Hiroyuki Suzuki, Naoko Kuroda, Seijiro Honma**, Pham The Tai***, Shoko Maruzeni***, Muneko Nishijo***, Hideaki Nakagawa***, Nguyen Ngoc Hung****, Le Thi Hong Thom****

Abstract

Objective Between 1961 and 1971 the US military used over 80 million litres of herbicides in southern Vietnam. This study aims to assess hormone levels in the saliva of lactating Vietnamese mothers and human health effects in a dioxin hot spot (Phu Cat district, Binh Dinh province) and a non-exposed (Kim Bang district, Ha Nam province) area in Vietnam. *Materials and Methods* An epidemiological study was carried out in both areas. The subjects were 58 lactating females in the hot spot area and 53 lactating females in the non-exposed area. All were aged between 20 and 30 years with infants aged between 4 and 16 weeks. Information about disease risk factors was obtained through interviews with mothers. Breast-milk samples were taken from all subjects, whereas saliva samples were obtained from 41 mothers in the hot spot area and 19 in the non-exposed area. Body measurements for both mothers and their infants were compared between the two areas. *Result and Discussion* The weight and chest circumference of infants in the hot spot area were significantly lower than those in the non-exposed area, whereas age (weeks) is also significantly younger in hot spot area than non-exposed area. Present maternal and family diseases in the hot spot area were significantly higher than those in the non-exposed area. Maternal eyesight in both eyes did not differ significantly between the two areas. The cortisone levels in saliva have been found to be closely related to those in breast milk samples of both mothers in hot spot and non-exposed areas. No significant difference was found between salivary hormone levels of mothers in the hot spot and non-exposed areas.

Key words

TCDD, Saliva, Hormone, Hot spot, Vietnam

Introduction

During the Vietnam War, between 1961 and 1971 the US military initiated the use of herbicides in Vietnam for general defoliation and crop destruction through a program codenamed Operation Ranch Hand[1]. Initial figures showed that over 72 million litres of herbicide were applied over approximately 10－12% of southern Vietnam[2,3], although this quantity was recently revised upwards since spray records from the war revealed that over 80 million litres of herbicide were used in Vietnam[4]. Sixty-one percent of the chemical herbicide 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

Division of Health Sciences, Graduate School of Medical Science, Kanazawa University
* International Medical Center of Japan
** ASKA Pharma Medical Co. Ltd
*** Department of Epidemiology and Public Health, Kanazawa Medical University
**** 10-80 Division, Hanoi Medical University

fraction of the Agent Orange mixture contained the highly toxic chemical 2,3,7,8-tetrachlorodibenzo -*p*-dioxin (TCDD)[1]. It has been estimated that Southern Vietnam is contaminated with somewhere in the region of 600 kg of dioxin[4].

TCDD is one of 75 congeners of dioxin that can be created by changing the position and number of chlorine atoms around the dioxin structure. Several TCDD contamination episodes have allowed identification of the health effects associated with high-dose exposure to TCDD, including skin disorders and liver damage. The adverse reproductive effects observed in these populations include increases in spontaneous abortions, low birth weight, growth retardation, and hypoplastic deformed nails[5]. TCDD is also a known risk factor for cancer[6,7] and can produce birth defects at microgram levels. Hormone levels can be altered by low-dose exposure to TCDD, and fetal death can occur after high-dose exposure. An association has been established between maternal TCDD exposure in animal studies and adverse birth outcomes, including increased fetal mortality[8,9], immune deficiency[10], decreased estradiol and progesterone levels[11], altered serum testosterone level[12] and reduced fertility and fecundity[8,11,13,14].

10-80 Division, Hanoi Medical University, Vietnam, and Hatfield Consultants Ltd., a Canadian environmental consulting company, have demonstrated that areas of the Aluoi Valley sprayed with herbicide do not retain high levels of TCDD due to tropical rains, erosion, chemical breakdown, and other environmental factors over the past 30 years[15]. However, the main areas of concern include those where Agent Orange and other defoliants were spilled, loaded onto aircraft, applied by truck-mounted sprayers, or transported. The resultant dioxin load in soils near former military installations was significantly higher than that resulting from aerial applications, and dioxin hot spots or reservoirs continue to exist to this day[16]. Dioxin hot spots are areas where soil/sediment still has very high dioxin levels due to higher levels of dioxin loading during the Vietnam War.

A project to locate dioxin hot spots was carried out in several provinces in southern Vietnam between October 2002 and December 2005. The results showed that dioxin contamination in soil and sediment was higher than normal and identified the US bases at Bien Hoa, Da Nang and Phu Cat as significant hot spots whose soil/sediment dioxin levels exceeded the maximum levels permitted in many western countries. Bien Hoa had the highest dioxin level (833 pg/g toxic equivalents-TEQ), with a 2,3,7,8-TCDD reading of 797 pg/g (sediment)[17]. In this project, Hatfield Consultants and 10-80 Division analysed 18 samples from Phu Cat. The highest dioxin levels in sediment and soil were 201 and 169 pg/g, respectively. The highest sediment TCDD level was recorded at a location (194 pg/g) downstream of a dioxin mitigation site located by Vietnamese authorities.

Between 2002 and 2007, a Japanese medical research team researched the long-term effects of Agent Orange/dioxin on human health of the people living in a sprayed area in southern Vietnam. This study consistently found significantly higher dioxins levels in serum, breast milk, and adipose tissue from the inhabitants of sprayed areas (Quang Tri province) than those in non-sprayed areas (Ha Tinh province), although no significant difference was found as regards early indicators of adverse health effects, such as liver or thyroid function and immunological activity[18]. Kido et al.[19] showed that the visual acuity of both eyes for people in sprayed areas was significantly lower than those in non-sprayed areas under all conditions, with a change of contrast from 100 to 2.5%, except for a 2.5% contrast in the left eye. The simple relationships between dioxin levels and visual acuity were shown to be tenuous for both eyes. Furthermore, this study demonstrated a statistically significant difference in sister chromatid exchange (SCE) values between individuals in sprayed areas and non-sprayed areas and that log SCE values are statistically correlated with log TEQ values in individuals[20]. The number of past pregnancies and deliveries and the rate of abortion/stillbirth were also significantly higher in the sprayed areas than in the non-sprayed areas[21].

A previous report found that TCDD has no

effect on progesterone levels or on the conversion of added Dehydroepiandrosterone into estradiol[22]. These authors demonstrated that the effect of TCDD on estradiol production was not due to a direct effect on cytochrome P450 aromatase (P450arom) but to altered estrogen production by luteal cells. A related study has shown that the effect of TCDD in rodents and other species leads to changes in steroid hormone levels[23]. The adverse effects of TCDD also decrease estrogen secretion from porcine granulose cells and progesterone from porcine luteal cells in vitro[24], and this compound also inhibits androgen secretion upon interaction with cytochrome P450 17 alpha-hydroxylase (P450c17)[25] and obstructs ovulation, with a concomitant reduction in steroid hormone secretion[26,27]. Another study demonstrated that TCDD decreases basal progesterone production by placental cells and increases conversion of Dehydroepiandrosterone into estradiol and testosterone into estradiol[28]. A recent study showed that biosynthesis of androgens, cortisol, and aldosterone is altered by dioxin-like PCB126 in human adrenocortical H295R cells[29].

Most studies of dioxin contamination in humans or the environment were undertaken in herbicide sprayed and dioxin hot spot areas. In the present study, we analyzed hormone levels in saliva and breast milk of Vietnamese people in areas affected by TCDD. This is one of the first and new studies to investigate effect on the human body and hormone in dioxin hot spot and herbicide sprayed areas in southern Vietnam.

Materials and methods

1. Study area

Phu Cat district is located in Binh Dinh province and Kim Bang district is in Ha Nam province, where the study was carried out. Phu Cat airbase was a Ranch Hand site during the war and is one of three dioxin hot spots in Vietnam. Herbicide storage, loading and plane washing at Phu Cat has been confirmed. Run-off from the wash area was directed into a small lake used by local inhabitants for raising fish and waterfowl. The population has been living in and around the Phu Cat airbase since before the war. The control site was Kim Bang district in Northern Vietnam, which did not experience herbicide operations during the war.

2. Subjects and methods

The subjects consisted of 58 lactating females in the hot spot area and 53 lactating females in the non-exposed area. All were aged between 20 and 30 years and had infants aged 4–16 weeks in September 2008. The mothers were interviewed to determine parity, reproductive and residential history and other specific risk factors for pollution exposure. Saliva and breast-milk samples were collected from volunteer donors in the morning (between 8:00 and 10:00 AM) in both areas. Saliva was collected from some of the mothers (41 from Phu Cat district and 19 from Kim Bang district) 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℃ until analysis. Mothers were asked to provide information regarding age, number of infants and number of infants they have breast-fed (including children of relatives, etc.). The body measurements for mothers (body height, body weight and eye sights) and those for their infants (body height, body weight and head, chest and abdomen circumferences) were compared between the two areas. The medical ethical committee of Kanazawa University approved this study (Permission No; Health-89), and informed consent was obtained from each participant.

1) Analytical method

Steroid hormone levels in the saliva of normally lactating Vietnamese mothers, including cortisol (F), dehydroepiandrosterone (DHEA), androstenedione (4AD), estradiol (E2), progesterone (P4) and testosterone (T), were measured. The sample treatment and assay of hormone in saliva and breast milk with internal standards (IS) (500 pg: cortisol F-d_4, 200 pg: DHEA-d_4, 4AD-d_7, and 100 pg: P4-$^{13}C_3$, T-d_3, 100pg: E2-^{13}C4) was based on the methods previously reported[30,31]. Saliva and breast-milk samples were analyzed using an API 4000 electrospray ionization (ESI) mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). High-performance liquid chromatography

(HPLC) was performed on an Agilent 1100 95 device (Agilent Technology, CA, USA) fitted with an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a Cadenza CD-C18 column (3 μm, 3 x 150 mm) (Imatake, Kyoto, Japan). Mass spectrometry (MS) measurements were performed in the positive-ion mode and the infusion volume was 10 μL. The ion spray voltage and ion source temperature were set to 5000 V and 500℃, respectively.

2) Statistical analysis

Data are indicated as mean ± standard deviation (SD). Pearson correlation coefficients were calculated. Statistical comparisons were made using the chi-square and Wilcoxon signed rank tests for categorical variables. All statistical analyses were performed using the JMP®6 software package (SAS Institute, Japan).

Results

The 58 infants in the hot spot area and the 53 infants in the non-exposed area had a mean age of 10.7 ± 3.1 and 12.0 ± 2.7 weeks, respectively. The results presented in Table 1 show that the weight and chest circumference of infants in the hot spot area were significantly lower than those in the non-exposed area ($p < 0.05$). However, age (weeks) is also significantly younger in hot spot area than non-exposed area. There was no significant difference between infant height, head circumference,

Table 1 Comparison of infant characteristics between the hot spot and non-exposed areas

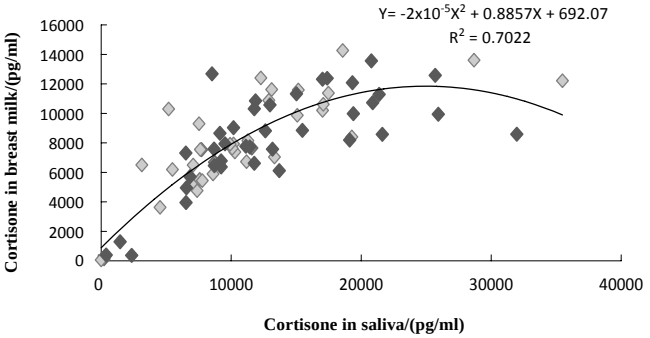
		Hot spot area		Non-exposed area		
		N	mean ± SD number (%)	N	mean ± SD number (%)	p-value
Age	(Weeks)	58	10.7 ± 3.1	53	12.0 ± 2.7	*[1]
Height	(cm)	58	60.7 ± 3.1	53	60.8 ± 2.8	n.s.[1]
Weight	(kg)	58	5.65 ± 0.94	53	5.87 ± 0.89	*[1]
Head circumference	(cm)	58	39.7 ± 1.7	53	40.0 ± 1.9	n.s.[1]
Chest circumference	(cm)	58	39.8 ± 2.8	53	40.7 ± 2.2	*[1]
Abdomen circumference	(cm)	58	38.6 ± 2.7	53	39.3 ± 2.8	n.s.[1]
Birth weight	(kg)	57	3.18 ± 0.46	52	3.25 ± 0.40	n.s.[1]
Birth defect	Yes	58	3 (5.2%)	53	0 (0.0%)	n.s.[2]
Present disease	Yes	58	6 (10.3%)	53	9 (17%)	n.s.[2]
Past disease	Yes	58	2 (3.5%)	53	2 (3.8%)	n.s.[2]

Data are means ± SD or number (%)
[1]Wilcoxon signed rank test, [2]Chi-square, n.s: not significant, *: $p < 0.05$

Table 2 Comparison of maternal characteristics between the hot spot and non-exposed areas

		Hot spot area		Non-exposed area		
		N	mean ± SD number (%)	N	mean ± SD number (%)	p-value
Age	(years)	58	25.8 ± 3.7	53	25.1 ± 2.8	n.s.[1]
Height	(cm)	58	152.0 ± 6.0	53	152.5 ± 5.0	n.s.[1]
Weight	(kg)	58	48.4 ± 6.5	53	48.7 ± 5.0	n.s.[1]
BMI	(kg/m^2)	58	20.9 ± 2.2	53	20.9 ± 1.8	n.s.[1]
Right eye		58	1.2 ± 0.4	53	1.2 ± 0.4	n.s.[1]
Left eye		58	1.2 ± 0.4	53	1.3 ± 0.4	n.s.[1]
Family income	(VND)	55	$(2.4 \pm 2.2) \times 10^6$	51	$(2.1 \pm 1.1) \times 10^6$	n.s.[1]
Alcohol habit	Yes	58	2 (3.45%)	53	0 (0.0%)	n.s.[1]
Smoking habit	Yes	58	0 (0.0%)	53	2 (3.8%)	n.s.[1]
Present disease	Yes	58	21 (36.2%)	53	9 (17%)	*[2]
Past disease	Yes	56	20 (35.7%)	53	13 (24.5%)	n.s.[2]
Reproductive failure	Yes	58	9 (15.5%)	53	10 (18.9%)	n.s.[2]
Present family disease	Yes	58	19 (32.8%)	53	6 (11.3%)	**[2]
Family birth defect	Yes	58	2 (3.5%)	53	0 (0.0%)	n.s.[2]

Data are means ± SD or number (%), BMI = body mass index
[1]Wilcoxon signed rank test, [2]Chi-square, n.s: not significant, *: $p < 0.05$, **: $p < 0.01$



◆: The cortisone levels in hot spot area.
◇: The cortisone levels in non-exposed area.

Fig 1. The correlation between cortisone in saliva and breast milk in Vietnamese mothers

abdomen circumference or birth weight. Three infant birth defects were detected (5.2%) in the hot spot area, although this difference was not statistically significant. Differences in present and past diseases were also not statistically significant.

The general characteristics of the mothers in this study are presented in Table 2. Maternal height, weight, BMI, alcohol habit, smoking habit, reproductive failure, past disease and family birth defects did not differ significantly between the two areas. Present maternal diseases were significantly higher in the hot spot area than in the non-exposed area ($p<0.05$). Furthermore, present family diseases were significantly higher in the hot spot area than in the non-exposed area ($p<0.01$).

Fig. 1 shows the correlation in cortisone levels between saliva and breast milk. The solid parabolic line shows that both cortisone values in saliva and breast milk are positively related until 22,000 pg/ml value of cortisone in saliva although some cortisone values in breast milk are decreasing over 22,000 pg/ml value of cortisone in saliva.

The saliva hormone levels for 41 mothers in the hot spot area and 19 mothers in the non-exposed area are presented in Table 3. The prevalence of low salivary hormone levels in mothers between the hot spot and non-exposed areas was not significant. Table 4 shows that no significant differences were found between the salivary mean hormone levels of mothers in the hot spot and non-exposed areas.

Discussion

The Vietnam War ended over 35 years ago but herbicide residues still have adverse effects on those people who lived in the sprayed/hot spot areas and on the country's ecosystem[32,33,34,35]. Dioxin is persistent in the environment (soil, sediment) and bio-accumulates in fish and animals consumed by humans. The half-life for dioxin elimination by the human body is estimated to be 7 −11 years[36,37] and that for soil is 28.5−274 years[38].

Numerous studies performed over the last decade in humans and other vertebrates have shown that dioxin is a risk factor for reproductive

Table 3 Comparison of the prevalence of low salivary hormone levels for mothers in the hot spot and non-exposed areas

		Hot spot area (n=41)	Non-exposed area (n=19)	p-value
Cortisol (pg/ml)	A (>1000)	34 (82.9%)	18 (94.7%)	n.s.
	B (<1000)	7 (17.1%)	1 (5.3%)	
Cortisone (pg/ml)	A (>8000)	33 (80.5%)	14 (73.7%)	n.s.
	B (<8000)	8 (19.5%)	5 (26.3%)	
Cortisol/Cortisone (pg/ml)	A (>0.12)	34 (82.9%)	17 (89.5%)	n.s.
	B (<0.12)	7 (17.1%)	2 (10.5%)	
Dehydroepiandrosterone (pg/ml)	A (>50)	37 (90.2%)	18 (94.7%)	n.s.
	B (<50)	4 (9.8%)	1 (5.3%)	
Androstenedione (pg/ml)	A (>20)	38 (92.7%)	18 (94.7%)	n.s.
	B (<20)	3 (7.3%)	1 (5.3%)	
Estradiol (pg/ml)	A (>0.05)	32 (78.05%)	18 (94.7%)	n.s.
	B (<0.05)	9 (21.95%)	1 (5.3%)	
Progesterone (pg/ml)	A (>0.01)	41 (100%)	18 (94.7%)	n.s.
	B (<0.01)	0 (0.0%)	1 (5.3%)	
Testosterone (pg/ml)	A (>1.0)	40 (97.6%)	18 (94.7%)	n.s.
	B (<1.0)	1 (2.4%)	1 (5.3%)	

Chi-square, n.s: not significant

Table 4 Comparison of the salivary hormone levels for mothers in the hot spot and non-exposed areas

		Hot spot area n=41	Non-exposed n=19	
		mean ± SD	mean ± SD	p-value
Cortisol	(pg/ml)	$(2.8 \pm 2.1) \times 10^4$	$(3.0 \pm 2.5) \times 10^4$	n.s.
Cortisone	(pg/ml)	$(13.6 \pm 7.0) \times 10^4$	$(14.3 \pm 7.6) \times 10^4$	n.s.
Cortisol/Cortisone	(pg/ml)	0.2 ± 0.1	0.2 ± 0.1	n.s.
Dehydroepiandrosterone	(pg/ml)	$(1.9 \pm 1.2) \times 10^2$	$(1.7 \pm 1.2) \times 10^2$	n.s.
Androstenedione	(pg/ml)	42 ± 19	56 ± 32	n.s.
Estradiol	(pg/ml)	0.2 ± 0.2	0.1 ± 0.1	n.s.
Progesterone	(pg/ml)	3.1 ± 4.1	2.7 ± 4.6	n.s.
Testosterone	(pg/ml)	18 ± 70	5.3 ± 2.8	n.s.

Data were subjected Wilcoxon signed rank test, n.s: not significant

and developmental abnormalities[39], spontaneous abortions, low birth weight[5], and increased fetal mortality[9]. However, our study found no significant increase in the prevalence of reproductive failure and family birth defects in the hot spot area. Dioxins are known to affect infant growth and neurological development in both humans and animals. A recent study in Japan, for example, showed that decreased infant head circumference at birth without decreased weight is associated with maternal exposure to 2,3,7,8-TCDD[40]. No statistically significant difference was found between either the body measurements of mothers and some risk factor for childhood disease or the head circumference of infants in the two areas studied.

In this study, we found a correlation between the cortisone levels in saliva and breast milk samples of Vietnamese mothers taken in the morning. This result shows that salivary hormone may be estimated as a good indicator in the epidemiological study at the first time.

The data reported herein show no significant differences between the salivary hormone levels of mothers in the two study areas. We found no difference between the testosterone levels for the two areas in this study, this could be due to insufficient number of saliva samples (41 saliva samples in the hot spot area and 19 in the non-exposed area). Further studies are therefore needed with more saliva samples in order to confirm this finding. Our study is one of the first to investigate hormone levels in the saliva of Vietnamese people in areas affected by TCDD and is likely to lead to an increased interest in the use of salivary hormone levels to measure the effect of this chemical on the human body in dioxin hot spot and herbicide sprayed areas in southern Vietnam. Further research should be carried out to clarify the association between dioxin exposure and human health effects. Hot spot areas may be an especially advantageous location for such studies because of their extreme dioxin levels.

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ベトナムダイオキシン高濃度汚染地域における授乳中の母親の唾液中ホルモン値の検討

Dang Duc Nhu，城戸　照彦，長沼　理恵，齋藤　夏絵*，鈴木　寛之，黒田　尚子，
本間誠次郎**，Pham The Tai***，丸銭　笑子***，　西条　旨子***，
中川　秀昭***，Nguyen Ngoc Hung****，Le Thi Hong Thom****

要　　旨

【目的】アメリカ軍は1961年から1971年の間に8000万リットル以上の枯葉剤を南ベトナムに撒布した。この研究の目的はベトナムの枯葉剤高濃度汚染地域（Binh Dinh省Phu Cat県）と非汚染地域（Ha Nam省Kim Bang県）において枯葉剤（オレンジ剤）の暴露と人体への健康影響との関連を検討することである。**【方法】**2つの地域において疫学的調査を実施した。対象者は授乳中で生後4週から16週の乳児を持つ20－30歳の母親とし、汚染地域では58名、非汚染地域では53名であった。疾患の危険因子等に関する情報は母親への面接調査から得た。母乳をすべての対象者から採取したが、唾液を採取したのは汚染地域41名、非汚染地域19名であった。母親と子どもの身体計測を行い2地域間で比較を行った。**【結果と考察】**汚染地域における乳児の体重と胸囲は有意に非汚染地域よりも小さいが、年齢(週数)も汚染地域の方が有意に低かった。汚染地域における母親とその家族の現病歴は非汚染地域よりも有意に多かった。母親の視力は両眼とも2地域間で有意差は認められなかった。汚染地域と非汚染地域双方の母親の唾液中のコルチゾン値は、母乳中のコルチゾン値と高い相関を示した。汚染地域と非汚染地域間で唾液中のホルモン値には有意差は認められなかった。