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The risk of endometriosis and exposure to dioxins and polychlorinated biphenyls: a case–control study of infertile women

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BACKGROUND: A case–control study was designed to determine the possible association between chronic exposure to dioxins and polychlorinated biphenyls (PCBs), and the occurrence of endometriosis. The study group consisted of 42 infertile endometriosis cases and 27 mechanical infertile controls, both groups attending one of the collaborating Centres for Reproductive Medicine, enrolled between 1996–1998. METHODS: Exposure assessment to dioxin-like compounds was determined through CALUX (chemical-activated luciferase gene expression)-bioassay to measure dioxin-like total toxic equivalents (dioxins and co-planar PCBs), whereas non-co-planar PCBs were determined through chemical analysis. RESULTS: No association was found between median dioxin-like total toxic equivalents (TEQ) and the occurrence of endometriosis in infertile women [cases (n = 34): 29; controls (n = 27): 24; NS]. When patients were subdivided based on an arbitrary cut-off value of 100 pg TEQ/g serum lipids, no statistically significant association between very high exposure to dioxin-like compounds and endometriosis was found [crude odds ratio (OR) = 4.33; confidence interval (CI) 0.49–38.19; NS]. After adjusting for body mass index, and alcohol consumption, the risk increased slightly to OR = 4.6 (CI 0.48–43.62; NS). There was no confounding by age, ovulatory dysfunction, caffeine intake, smoking or exposure to non-co-planar PCBs. CONCLUSIONS: The study results showed no statistically significant association between exposure to dioxin-like compounds and the occurrence of endometriosis in infertile women.

Key words: CALUX-bioassay/dioxin-like compounds/endometriosis/infertility/polychlorinated biphenyls

Introduction

Endometriosis is an enigmatic gynaecological disorder that is characterized by the ectopic presence of both endometrial glands and stroma (Olive and Schwartz, 1993). The disease is thought to occur through the transtubal spreading and implantation of menstrual endometrium, and is associated with pelvic pain, dyspareunia and infertility. Among the environmental pollutants that have been suggested as being linked to endometriosis are the polyhalogenated aromatic hydrocarbons (PHAH), a class of widespread environmental contaminants which includes polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) (Rier *et al.*, 1993; Lebel *et al.*, 1998). Because the aetiology of endometriosis seems to be multifactorial, it has been suggested that dioxin exposure may contribute to an imbalance of sex hormones or alter growth factors and the immune response (Mayani *et al.*, 1997; Osteen and Sierra-Rivera, 1997). Dioxins alter tissue-specific responses to hormones via modulation of steroid receptor expression (Safe *et al.*, 1991). Along with inhibition of T-lymphocyte function (Neubert *et al.*, 1991) and decreasing natural killer cell activity in plasma and peritoneal fluid, dioxins may stimulate peritoneal fluid macrophages and thus affect angiogenesis and local concentrations of cytokines (e.g. interleukin-1) and growth factors (Clark *et al.*, 1991; Koninckx, 1999). Alternatively, cellular changes or genetic background may predispose an individual to the immunological modulation caused by dioxin exposure, leading to infiltration and adhesion of endometrial cells in the peritoneum (Koninckx, 1999).

Extensive experimental studies have pointed out that most toxic actions induced by 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD) are mediated via the arylhydrocarbon receptor (AhR) (Safe, 1990). Six other PCDDs, 10 PCDFs, and 12 PCB congeners (non-*ortho*, and to a lesser extent some

mono-*ortho* substituted congeners) can also assume a co-planar configuration. Hence, they also interact with the AhR, and they produce the same spectrum of responses in animal and cell models as TCDD, depending on their binding affinity to the AhR (Van den Berg *et al.*, 1998).

PCB congeners possessing two or more chlorine substituents at the *ortho* positions of the biphenyl rings are non-co-planar, and do not bind with high affinity to the AhR (Battershill, 1994). Exposure to these non-co-planar PCB congeners reportedly results in a variety of toxic effects in experimental animals, including neurochemical, neurotoxic, carcinogenic, and endocrine changes (Safe, 1990; Brouwer *et al.*, 1995). However, the spectrum of activity produced by the latter congeners has not been fully explored and the mechanisms of action remain to be fully elucidated (Fischer *et al.*, 1998).

It has been reported recently that in Belgium the incidence and severity of endometriosis in women, as well as the degree of dioxin pollution, is among the highest in the world (Koninckx *et al.*, 1994). In view of the accumulating data, we carried out a case–control study aimed to assess whether dioxin-like toxic equivalents in serum are related to endometriosis within a female infertile population.

Materials and methods

Patients

This prospective case–control study recruited patients undergoing infertility treatment at one of the collaborating Centres for Reproductive Medicine spread over Belgium (University Hospitals of Antwerp, Ghent, and Leuven), enrolled between 1996 and 1998. All women underwent a laparoscopy as part of their infertility work-up. A couple was defined as 'infertile' when pregnancy had not been achieved after one year of unprotected sexual intercourse.

The case group consisted of 42 patients with laparoscopy-confirmed endometriosis. Endometriosis was staged as minimal (AFS I) in 21, mild (AFS II) in 7, moderate (AFS III) in 10, and severe (AFS IV) in 4 patients, according to the revised classification of the American Fertility Society (rAFS) (American Fertility Society, 1985).

The control group comprised 27 women without endometriosis and with infertility related to tubal disease, tuboperitoneal factors, cervical factors, or uterine factors. In these women no evidence of endometriosis was found at laparoscopy. An association between these causes of female infertility and exposure to PHAHs has not been established.

This study had been accepted by all Ethical Committees (Protocol nos. 96/44/107, 97/100, and ML 536, for UZA, UZG, and UZL respectively). All patients acknowledged their participation by signing an Informed Consent Form.

Medical records and interviews

Medical records of the subjects were reviewed to obtain information on diagnosis (tubal, tuboperitoneal, cervical, uterine factor, endometriosis, ovulatory dysfunction) and anthropometric variables (age, body mass index). A 20-minute telephone interview was conducted, eliciting detailed personal data and documenting factors potentially associated with endometriosis and with PHAH exposure. The enrolment questionnaire was designed primarily to obtain information on the patient's eating, drinking and smoking pattern.

During the interview all subjects were asked 'what is your average weekly consumption of glasses of alcohol (including wine, beer, liquors)'. Women consuming $\geqslant$6 glasses/week were considered to be alcohol drinkers.

We estimated each person's total daily caffeine intake by assuming that there was 107 mg/cup in coffee of unknown preparation method, 34 mg/cup in tea (Pastore and Savitz, 1995), and 26 mg/glass (200 ml) in regular or light cola. Furthermore, the patient's current and former smoking pattern was expressed in cumulative dose of exposure, in terms of packages of cigarettes per year.

Peripheral blood analysis

During the preoperative interview, 25 ml of blood per patient was collected in a vacuum system tube, transported in a cooling pail, and centrifuged (15 min, 2000 *g*) within 24 h after collection. All serum samples were stored at –20°C until analysed.

Lipid determination

Serum cholesterol (free and cholesterol esters), triglycerides, and phospholipids were determined in duplicate as a measurement of lipid content, using enzymatic spectrophotometric determination with commercially available reagents from Elitech Diagnostics (Sées, Normandie, France).

Rationale for the analytical methods used to quantify dioxins and polychlorinated biphenyls

Dioxins and co-planar polychlorinated biphenyls

The TCDD toxic equivalency factor (TEF) concept (Safe, 1990), allows conversion of a PHAH chemical data set into the AhR-related toxic potency of a mixture of PHAHs. Concentrations of individual PHAHs are multiplied by their respective TEF-values and added together to give the total TCDD toxic equivalency (TEQ) value. In recent years, bioassays have been developed that can measure the total TEQ-value of complex mixtures directly without the need for extensive clean-up and chemical analysis procedures. One of the novel in-vitro reporter gene assays, the CALUX (chemical-activated luciferase gene expression)-bioassay is based on AhR-mediated firefly (*Photinus pyralis*) luciferase expression in genetically modified cell lines (Aarts *et al.*, 1995).

Based on the CALUX-derived TEQ-levels, the impact of co-planar PCBs on endometriosis can be interpreted, since it has been shown recently that the contribution of dioxin-like PCBs to the total TEQ-value is almost equal in human matrices to the contribution of dioxins and furans (Brouwer *et al.*, 1995). The most recently updated TEF-values of dioxin-like PCBs are compiled in Table I (Van den Berg *et al.*, 1998).

Non-co-planar polychorinated biphenyls

Combining the *a priori* environmental exposure to a mixture of (non)-dioxin like PCBs, and the correlation between CALUX-based TEQ-values and the sum of non-co-planar PCBs (Pauwels *et al.*, 2000), the association between TEQ-levels and endometriosis may be feasibly confounded by the effects of non-co-planar PCB congeners. Therefore, four non-co-planar PCB congeners (IUPAC Nos. 118, 138, 153, 180) were determined by chemical analysis through gas chromatography using electron-capture detection (GC-ECD).

CALUX-bioassay

The CALUX-bioassay analysis was performed as described in detail (Murk *et al.*, 1997). The extraction and clean-up method involves essentially *n*-hexane extraction of blood serum aliquots (1–1.5 ml)

Table I. World Health Organization toxic equivalency factors (TEFs) for dioxin-like PCBs for humans (Van den Berg *et al.*, 1998; with permission)

Congener IUPAC no.	Structure	TEF[1]
Non-ortho substituted		
PCB-81	3,4,4′,5-TetraCB	0.0001
PCB-77	3,3′,4,4′-TetraCB	0.0001
PCB-126	3,3′,4,4′,5-PentaCB	0.1
PCB-169	3,3′,4,4′,5,5′-HexaCB	0.01
Mono-ortho substituted		
PCB-105	2,3,3′,4,4′-PentaCB	0.0001
PCB-114	2,3,4,4′,5-PentaCB	0.0005
PCB-118	2,3′,4,4′,5-PentaCB	0.0001
PCB-123	2′,3,4,4′,5-PentaCB	0.0001
PCB-156	2,3,3′,4,4′,5-HexaCB	0.0005
PCB-157	2,3,3′,4,4′,5′-HexaCB	0.0005
CB-167	2,3′,4,4′,5,5′-HexaCB	0.00001
PCB-189	2,3,3′,4,4′,5,5′-HeptaCB	0.0001

[1]The reference value derives from 2,3,7,8-TCDD with a TEF-value of 1.
Abbreviations: CB = chlorinated biphenyls; PCB = polychlorinated biphenyls; TEF = toxic equivalency factor.

and removal of acid-labile matrix components by passage through a silica column containing 33% (w/w) concentrated H_2SO_4. This extract was diluted in dimethylsulphoxide (DMSO) for CALUX measurement using rat H4IIE hepatoma (H4L1.1c4) cells. These cells stably transfected with an AhR-controlled luciferase reporter gene construct (*pGudluc1.1*) and were grown confluent in 96-well view plates and exposed in triplicate to the PHAH samples and TCDD standards for 24 h, using DMSO (0.5% v/v) as a vehicle.

After removal of the medium, cells were washed twice with phosphate-buffered saline (Oxoid, Hampshire, UK). The cells were harvested in 75 µl cell lysis reagent (Luciferase Assay System; Promega, Leiden, The Netherlands), and centrifuged at 2000 *g* for 1 min. For measurements of luciferase activity, 20-µl aliquots of the supernatants were pipetted into a 96-well microtitre plate, 100 µl luciferin assay mix at room temperature was added. After thorough mixing, the light production was measured in a Luminoskan RS Luminometer (Labsystems, Helsinki, Finland).

The lipid-corrected CALUX-based TEQs were calculated by comparison of the luciferase activity induced by the sample (three replicates) against a dose-response curve generated from TCDD concentration standards simultaneously analysed. The standard curve was fitted (one-site ligand fit) using SlideWrite 6.00 (Advanced Graphics Software Inc., Encinitas, CA, USA), and the CALUX TEQ-value of an unknown sample was interpolated on this curve.

Chemical analysis of non-co-planar PCBs

All solvents used were purchased pesticide grade from E.Merck (Darmstadt, Germany). The four non-co-planar PCBs were a standard mixture from J.T.Baker at a concentration of 10 ng/µl in iso-octane (J.T.Baker, Deventer, The Netherlands). The $^{13}C_{12}$-labelled CB-149 (internal standard) was obtained from Cambridge Isotope Laboratories (Woburn, MA, USA).

Complete details of the extraction were described and evaluated previously (Pauwels *et al.*, 1999). A brief description of the method is given below. The sample preparation involved addition of $^{13}C_{12}$ CB-149, disruption of protein-binding in an ultrasonic bath using formic acid (1:1, v/v), extraction and concentration of analytes using Empore™ C_{18} SPE disk cartridges (3M, St Paul, MN, USA). A mixture of ethyl acetate and hexane was used for elution. Further clean-up of lipid interferences was accomplished using a sulphuric acid wash of the eluate. Two µl per extract were injected in splitless mode on a GC-µECD system (HP 6890, Palo Alto, CA, USA) by an autosampler (HP 7673). A fused silica DB-XLB capillary column (J&W Scientific, Folsom, CA, USA) of 60 m×0.25 mm i.d.×0.25 µm film thickness was used. Details of the analytical conditions receives ample treatment elsewhere (Pauwels *et al.*, 1999).

Data analysis

A normal distribution could not be assumed for any of the variables (Shapiro-Wilk's *W*-test). Therefore, characteristics of cases and controls were compared using the Mann-Whitney *U*-test for continuous data, and by χ^2 or Fisher's exact test (when appropriate) for categorical data. Crude associations between exposure to dioxins (after dichotomization), PCB congeners (on a continuous scale), and endometriosis were estimated by logistic regression analysis. The initial selection of potential confounders was guided by the literature on known and suspected determinants of endometriosis (Eskenazi and Warner, 1997; Zeyneloglu *et al.*, 1997). Those factors included age, body mass index, ovulatory dysfunction, smoking pattern, alcohol and caffeine consumption (Table II). Multiple logistic regression correcting for selected confounders was performed in order to assess the adjusted association between exposure to dioxins, PCBs and endometriosis [STATISTICA version 5, 1997 (StatSoft, Groningen, The Netherlands); EGRET version 0.03, 1991 (Cytel Software Corp., Cambridge, MA, USA)]. Significant associations are indicated by $P < 0.05$.

Results

Table II represents relevant characteristics of the study population. As can be observed, the cases (n = 42) and the controls (n = 27) were similar with respect to age, ovulatory dysfunction, alcohol and caffeine intake, and smoking pattern. A summary of TEQ-values and non-co-planar PCB congener levels is presented in Table III. Although these figures suggest a trend toward slightly higher exposure for endometriosis patients (median 29 pg TEQ/g lipid) compared with their mechanical infertility counterparts (median 27 pg TEQ/g lipid), none of these differences were statistically significant. For two cases and two controls, no serum was available for PCB-analysis. Consequently, non-co-planar PCB congeners are determined in 40 cases and 25 controls. Endometriosis and mechanical infertile patients are exposed to non-co-planar PCBs in the same range.

The distribution of TEQ-levels in cases and controls is presented in Figure 1. These data show clearly a group in the endometriosis-cases with high TEQ-levels. Consequently, statistical analyses on CALUX-based TEQ-values were executed using only two exposure categories. We thereby chose one cut-off point (100 pg TEQ/g serum lipids) to divide the groups into a non-exposed and an exposed group. After dichotomization, the association between exposure to dioxin-toxic equivalents and occurrence of endometriosis in infertile women was non-significant (OR = 4.33; CI = 0.49–38.19; NS). After adjustment for body mass index, and alcohol consumption, the risk increased slightly (OR = 4.56; CI 0.48–43.62; NS).

Statistical analyses for PCB congeners were executed on a continuous scale. The regression coefficients and corresponding odds ratios did not reveal a significant association between exposure to the four individual congeners and the occurrence of endometriosis. In multiple logistic regression, the association

Table II. Relevant characteristics of the endometriosis cases and mechanical infertile controls, 1996–1998

	Cases (n = 42)	Controls (n = 27)	P-Value
Median age in years (range)	31.0 (25–42)	32 (24–41)	NS
Median body mass index (kg/m^2) (range)	21.2 (17.2–30.7)	22.9 (18.4–36.4)	0.012
No. (%) with ovulatory dysfunction	9 (21.4)	8 (29.6)	NS
No. (%) of caffeine drinkers			NS
no caffeine	3 (7.1)	2 (7.4)	
1–300 mg/day	21 (50.0)	6 (22.2)	
>300 mg/day	18 (42.9)	19 (70.4)	
No. (%) who drank ⩾6 glasses of alcohol/week	3 (7.1)	5 (18.5)	NS
No. (%) who were non-smokers	26 (61.9)	12 (44.4)	NS
Median (range) of pack-years per smoker	7.0 (2–17.0)	6.5 (1.1–23.8)	NS

Table III. CALUX-based TEQ-values and non-co-planar PCB-concentrations in serum of women with endometriosis (cases) and mechanical infertility (controls)

	Cases		Controls		
	n	Median (range)	n	Median (range)	
TEQs[1]	34[2]	29 (0–160)	24[2]	27 (0–135)	NS
PCBs[3]					
PCB-118	40	26 (8–117)	25	21 (9–53)	NS
PCB-138	40	69 (13–137)	24[4]	59 (27–143)	NS
PCB-153	40	89 (21–181)	25	78 (46–152)	NS
PCB-180	39[4]	68 (12–138)	25	55 (30–115)	NS

[1]CALUX-based TEQ-values for 42 cases and 27 controls (pg TEQ/g lipid).
[2]Positive samples: values above detection limit (32 fg TEQ/well).
[3]PCB-levels for 40 cases and 25 controls (ng/g lipid).
[4]Lower numbers indicate missing data due to interferences in the GC-chromatogram.
Abbreviations: PCB = polychlorinated biphenyls; SD = standard deviation; TEQ = toxic equivalence.

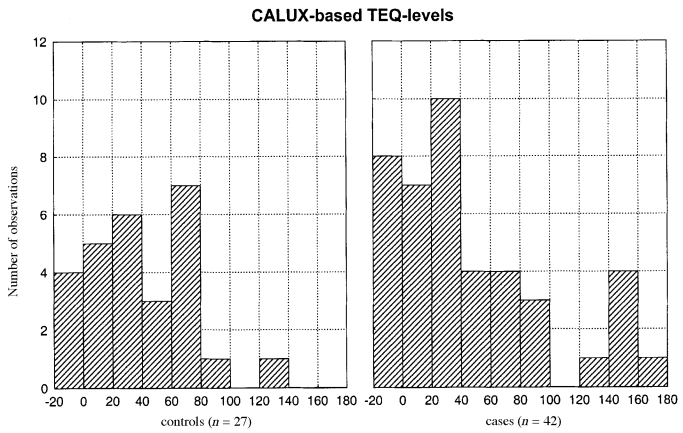

Figure 1. Distribution of TEQ-levels in cases and controls. All data are expressed in pg TEQ/g serum lipids.

between endometriosis and PCB-138, 153 and 180 even declined (data not shown). The relation of the latter congeners with endometriosis was partly explained by PCB-118, the only congener with a dioxin-like toxic capacity (TEF = 0.0001).

For the different associations, confounding was hardly present. For PCB-118 for instance, the (logistic) regression coefficient changed from 0.027 (crude) to 0.029 in the best fitting model, adjusting for body mass index and caffeine intake (P = not significant).

Discussion

The association between endometriosis and exposure to dioxins is a highly controversial issue. In non-human primates, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been reported to induce a dose-dependent increase in severity of endometriosis (Rier *et al.*, 1993), to facilitate the survival of endometrial implants, and to exert a bimodal effect on endometrial implant growth (Yang *et al.*, 2000). A series of human case–control studies have been conducted on the association between endometriosis and PHAH exposure with important differences in study design, patient selection and assay methods, and therefore provide inconsistent results. An association between endometriosis and organochlorine pollutants was confirmed in two reports (Gerhard and Runnebaum, 1992; Mayani *et al.*, 1997), but refuted in one other publication (Lebel *et al.*, 1998).

In several ways, the well designed protocol of our study overcame several shortcomings of the previous publications. Firstly, in our study, the patient population was very well defined: both cases and controls were infertile women undergoing a laparoscopic investigation. In only one other publication (Mayani *et al.*, 1997; 44 cases and 35 controls), the control group consisted strictly of patients with mechanical infertility, whereby the absence of endometriosis was confirmed by laparoscopy. Another report evaluated fertile and infertile women undergoing laparoscopy for pain, infertility or tubal sterilization whereby the controls were indication-matched (Lebel *et al.*, 1998; 68 cases and 70 controls). In one study (Gerhard and Runnebaum, 1992; 24 cases and 484 controls), hormonal disorders were present in both cases and controls, and it was not mentioned whether endometriosis was laparoscopically and histologically confirmed.

Secondly, our study offers a unique combination of measurement of exposure of dioxin-like compounds (dioxins and co-planar PCBs) and non-co-planar PCBs. Dioxin-like compounds were determined using CALUX-bioassay, which has recently been adapted and validated in blood plasma (Murk *et al.*,

1997) and bovine milk (Bovee *et al.*, 1998), whereas non-co-planar PCBs were determined through chemical analysis. In our study, the association between exposure to dioxin-like compounds and endometriosis seemed not to be confounded by the toxic effects of non-co-planar PCBs, since no strong or consistent relationship between the exposure to non-co-planar PCBs and the occurrence of endometriosis could be observed. In the other published studies, dioxin-like compounds were either not measured (Gerhard and Runnebaum, 1992; Lebel *et al.*, 1998) or only TCDD was quantified through chemical analysis (Mayani *et al.*, 1997).

Thirdly, studies that evaluate the association between exposure to PHAHs and endometriosis are likely to be biased by confounding variables, since it is not possible to conduct a randomized controlled trial on this exposure. Adjustment for these variables (age, body mass index, ovulatory dysfunction, smoking pattern, alcohol and caffeine consumption) was performed in our study but not or only partially in the previously published reports (Gerhard and Runnebaum, 1992; Mayani *et al.*, 1997; Lebel *et al.*, 1998).

Fourthly, the validity of our study results should be considered. To reduce the likelihood of selection bias, the inclusion criterion was a laparoscopy. Thereafter, subjects were defined as cases or controls in this survey. Hence, controls derived from the same recruitment population as the case group.

Fifthly, an important problem that might arise when dealing with case–control studies, is (differential) misclassification of exposure. In order to avoid this, the CALUX-bioassay was done blindly. Similarly, the interview was performed by one single staff member, who was unaware of the woman's case or control status. Recall bias can be ruled out because both cases and controls are both infertile patients. Variations in the recording of operative data cannot be excluded entirely. However, all laparoscopies were performed by gynaecologists familiar with the AFS classification for endometriosis (American Fertility Society, 1985).

Because information on alcohol exposure history was obtained by self-report, misclassification of use may have occurred. As we are convinced that these errors are the same for cases and controls, we believe that the misclassification is of the non-differential type, leading to biases towards the 'no association' result. Therefore, we are confident that our finding is not a result of information bias.

This pilot study is focused on the relationship between exposure to dioxins and PCBs and the occurrence of endometriosis in infertile females. We plan to extend it using larger populations, and by the inclusion of fertile patients with and without endometriosis. In this preliminary study, the lack of a statistically significant association between exposure to dioxin-like compounds and endometriosis based on an arbitrary cut-off value, can be explained either by the lack of a real association, or by the lack of power to prove such an association. In order to detect a four-fold increase (OR = 4) with a power of 90% and a significance level of 0.05, 85 cases and 85 controls are required. By increasing those figures by 10% (for multiple regression), a sample size of 100 endometriosis patients and 100 mechanically infertile controls is acquired. Therefore, future epidemiological surveys on PHAHs should enrol larger patient samples and biomonitor a well-defined range of potential culprit PHAHs (dioxins, furans, and selected PCB congeners) by chemical analysis, or by bioassays (toxic biological responses), followed by chemical analysis to identify the culprit compounds.

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