IMPACT OF HIGH PLASMA CONCENTRATIONS OF DIOXIN AND POLYCHLORINATED BIPHENYLS (PCBS) IN SOUTH INDIAN WOMEN WITH ENDOMETRIOSIS

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SUMMARY: To estimate the levels of Dioxin and PCBs present in the plasma of women with different stages of endometriosis and relation between Dioxin, PCBs and their possible impact on the pathogenesis of endometriosis.

Design: A prospective case control study.

Setting: Department of Reproductive Medicine, Bhagawan Mahavir Medical Research centre, Maternal Health and Research Trust, and Owaisi Hospital AND Research Center Hyderabad, Andhra Pradesh, India.

Patient(s): 97 women with endometriosis undergoing laparoscopy and 102 controls

Intervention(s): Heparinised blood samples were collected for dioxins, PCBs estimation.

Main outcome Measure(s): The levels of dioxins and PCBs were measured via gas chromatography.

Women with endometriosis showed significantly higher concentrations of dioxin and PCBs when compared with the control group. The correlation between the concentrations of dioxin -TCDD, PCBs, and difference in the severity of endometriosis was strong and statistically significant at $p<0.05$ for all the four compounds PCB-1: $r=+0.53$; $P<0.0001$, PCB-5: $r=+0.67$; $P<0.0001$, PCB-29: $r=+0.64$; $p<0.0001$, PCB-98: $r=+0.43$; $p<0.0014$ and concentration of dioxin-TCDD: $r=+0.36$, $p<0.0001$.

These results suggest that women having higher concentration of Dioxin and PCBs might have an increased susceptibility to endometriosis.

Key Words: Dioxin, Endometriosis, PDBs, Pathogenesis

INTRODUCTION

Endometriosis is a common gynecological disorder, affecting at least 10% of reproductive -aged women and is characterized by the growth of endometrial tissue outside the uterine cavity. Retrograde menstruation is one of the proposed mechanisms for the presence of endometrial cells in entopic sites; however this phenomenon occurs in approximately 90% of women while the prevalence of endometriosis is much lower. Hence, other factors are thought to contribute to the disease, including exposure to environmental pollutants (1). Among the environmental pollutants suggested to be linked to

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endometriosis are the polychlorinated aromatic hydrocarbons (PHAH), a class of widespread environmental contaminants which include polychlorinated di benzoic-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) (2). As the etiology 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 immune response (3). Dioxin alters tissue specific responses to hormones via modulation of steroid receptor expression (4).Alternatively, cellular changes or genetic background may predispose an exposure, leading to infiltration and adhesion of endometrial cells in the peritoneum (5). Extensive experimental studies have pointed out that most toxic actions induced by 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD) are mediated via the aryl hydrocarbon receptors (AhR) (6). Six other PCDDs, 10 PCDFs, and 12 PDB congeners (non-ortho, and to a lesser extent some mono-ortho substituted congeners) can also be assumed a coplanar configuration. Hence, they also interact with the AhR and produce the same spectrum of response in animal and cell models as TCDD, depending on their binding affinity to the AhR (7). 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 (8). In view of the accumulating data, we carried out a case -control study aimed to estimate the levels of dioxin and PCBs present in the plasma of south Indian women with different stages of endometriosis.

MATERIAL AND METHODS

Reagents

The following reagents were used: Anti -coagulant Heparin (GREINER, Germany), all the solvent used for GC - analysis were of analytical grade purity (High Pressure Liquid chromatography (HPLC) grade; Qualigens Ltd, Mumbai, India).

Source and Collection of Samples

The blood samples employed in this study were obtained with written informed consent from endometriotic patients (n=97), between 28.5± 6.5 years of age, recruited for infertility treatment at three collaborating centers Bhagawan Mahavir Hospital and Research center (BMHRC), Maternal Health and Research Trust (MHRT) and Owaisi Hospital and Research Center, Department of Reproductive medicine Hyderabad, which receives cases from all over the region of Andhra Pradesh, India. The endometriosis was staged as second and third during the operation, according to the revised American fertility Society Classification systems (9). The control group comprised of (n=102) women, between the (28.4 ± 4.8) years of age without endometriosis, proven fertile women, recruited in the same gynaec clinics with some other problems like symptoms of pain, dysmenohrea, dyspareunia underwent laparoscopic surgery. The protocol was approved by the ethics committee on human research of Bhagawan Mahavir Hospital and Research center.

Table 1: Demographic details of case and control groups.

Characteristic	No. of cases/ controls	Case group	Control group
Age in years [mean± SD] *	97/102	28.5 ± 6.5	28.4 ± 4.8
Body mass index [kg/m2] *	97/102	23.7± 2.0	23.6 ± 1.7
Age at menarche [years] *	97/102	12.6 ± 1.3	12.5 ± 1.1
Marital status: married/Unmarried	97/102	97	102
Menstrual cycle: regular/irregular	97/102	89/8	93/9

*Not significant between the case and control groups

Table 2: Demographic details of case and control groups.

Characteristic	No. of cases/ controls	Case group	Control group
Symptoms: pelvic pain	97/102	89 (91.7%)	46 (45%)
Dyspareunia		33 (34%)	17 (16.6%)
Dysmenorrhea			
Mild		29 (29.8%)	26 (25.4%)
Moderate		4 (4.1%)	5 (4.9%)
Severe		4 (4.1%)	1 (0.9%)
No dyspareunia and Dysmenorrhea		27 (27.8%)	53 (51.9%)
Ectopic pregnancy	97/102	1 (1.0%)	3 (2.9%)
Infertility:	97/102	74 (76.2%)	---
Primary Infertility		23 (23.7%	---
Secondary Infertility			
Duration of infertility in years (mean ± SD)	97	3.9 4.2	---

Table 3: Dioxin and PCBs concentrations in case and control groups.

Congener	Case group (n=86) μg/ml	Control group (n=91) μg/ml	95% CI	P value
PCB-1 (co-planar)*	0.42 ± 0.37	0.05 ± 0.14	0.45 to 0.28	<0.0001
PCB-5 (co-planar)*	0.30 ± 0.35	0.01 ± 0.06	0.36 to 0.21	<0.0001
PCB-29 (co-planar)*	0.34 ± 0.40	0.02 ± 0.08	0.40 to 0.23	<0.0001
PCB-98 (Non-coplanar)*	0.14 ± 0.23	0.00 ± 0.03	0.18 to 0.08	<0.0001
Dioxin-TCDD*	0.003 ± 0.011	0.000 ± 0.000	0.005 to 0.001	0.005

Data are represented as mean ± SD. P<0.05 is considered statistically significant.

*Significant between the groups; μgml-1(micrograms per milliliter).

Collection of Sample

6-8 ml of heparinised peripheral blood was collected during the first visit and 6-8ml non-heparinised peripheral blood was collected during the patients follow up , about week later into sterile syringes from all the 97cases and 102 controls (total=199) to estimate the PCBs, Dioxin. Plasma and serum were isolated from 86/97 cases and 91/102 control case because of insufficient blood samples for GC analysis.

Estimation of Dioxins by Gas Chromatography and Mass spectrometry

Blood specimens for dioxin analysis were frozen at -20°C and transported to the residue control laboratory. Dioxin was extracted and quantitated by Gas Chromatography (GC) and Mass Spectrometry (MS), as previously described (10) with certain modification: 6-10ml of blood was extracted three times with 15ml n-hexane: actone (9:1 v/v). The combined extracts were concentrated by evaporation and applied to solid phase extraction colums. Dioxin was eluted with methylene chloride which was evaporated to dryness; the residue was dissolved in 0.05ml of toluene immediately prior to GC analysis which was carried out as per the instructions of the suppliers from (Supelco, Germany) and in house modification done at Center of Cellular and Molecular biology (CCMB) on GC-2010 series gas chromatograph (Shimadzu, Japan), Equipped with capillary column injection port. The concentration of the dioxins was detected by the Gas Chromatography. The operating condition was as follows: the source of temperature was maintained at 280°C and aquadropole at 100, injector temperature was 250°C and detector at 320°C. A PTE Tm-5 fused capillary column, 30x0.25cm of internal diameter and 0.25μm of film thickness (Supelco, Germany), at programmed temperature of 100°C/min, then raised to 280°C at rate of 1°C/min and then maintained at 280°C for 8 minutes. Mode of injection was spilt less and total flow was maintained at 10ml/min and the purge flow was 3ml/min. All the solvent used were of analytical grade purity (High Pressure Liquid chromatography (HPLC) grade; Qualigens Ltd, Mumbai, India). The LOD of TCDD was 0.0003μg/ml (S/N ration: 5) and the quantification limit of the TCCD is 0.001μg/ml (S/N ration: 8) with a recovery of 90%.

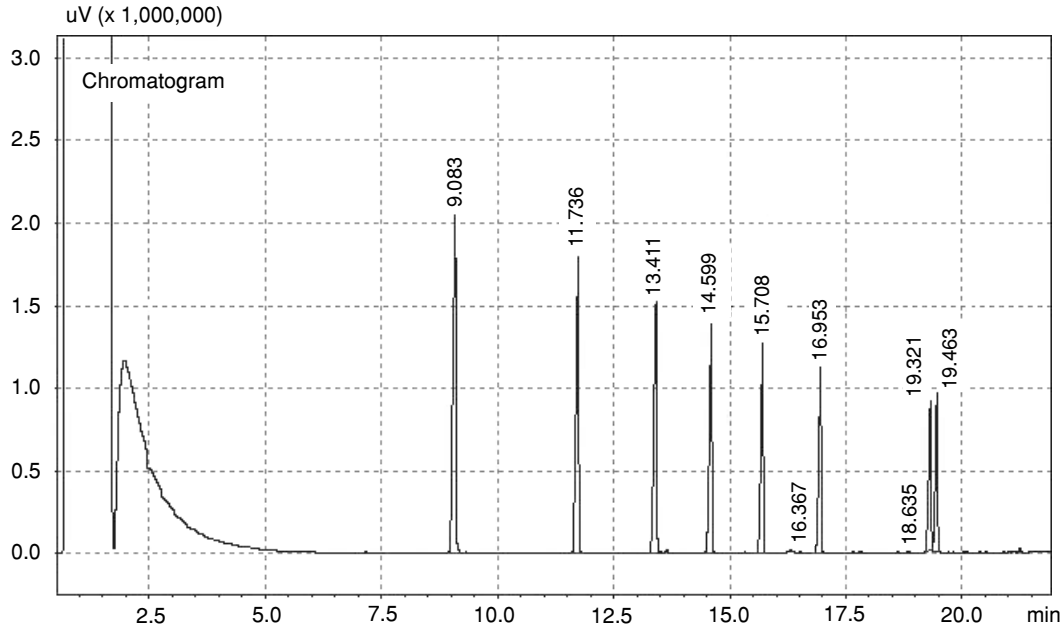
Table 4: Concentration of Dioxin and PCBs in control and different stages of endometriosis.

Congeners	Control group (n=91) μg/ml	Case group stages (n=86); μg/ml				Correlation coefficient (r)
		I (n=35)	II (n=27)	III (n=14)	IV (n=10)	
PCB-1	0.05 ± 0.14	0.23 ± 0.26	0.42 ± 0.28	0.60 ± 0.27	0.84 ± 0.56	+0.53*
PCB-5	0.01 ± 0.06	0.10 ± 0.12	0.24 ± 0.21	0.62 ± 0.39	0.75 ± 0.43	+0.67*
PCB-29	0.02 ± 0.08	0.13 ± 0.15	0.29 ± 0.31	0.50 ± 0.34	0.99 ± 0.54	+0.64*
PCB-98	0.00 ± 0.03	0.03 ± 0.10	0.11 ± 0.18	0.34 ± 0.32	0.26 ± 0.31	+0.43*
TCDD	0.000 ± 0.000	0.000 ± 0.001	0.002 ± 0.009	0.005 ± 0.013	0.013 ± 0.022	+0.36*

The correlations between the concentrations of Dioxin-TCDD and PCBs in different stages of endometriosis were strong and statistically significant at P < 0.05 for all compounds.

Figure 1: Map of South India (Squares indicate cities of the sampling locations).

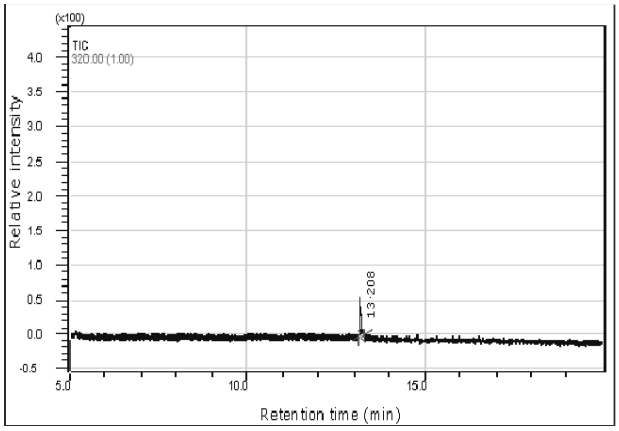
Figure 2: PCBs standard graph.



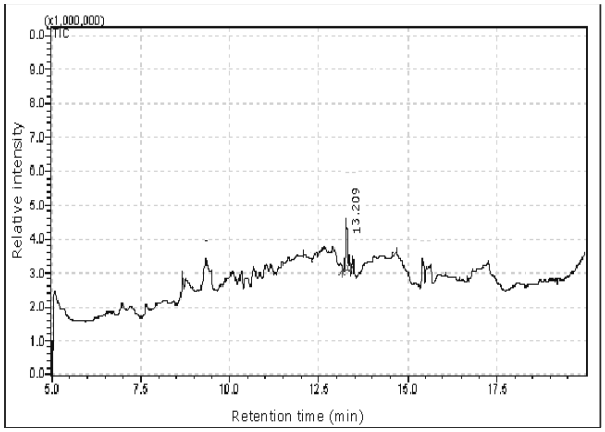
Retention Time	Compound
9.083	2-Chlorobiphenyl (PCB1)
11.736	2, 3-Di chlorobiphenyl (PCB5)
13.411	2, 4, 5-Tri chlorobiphenyl (PCB29)
14.599	2, 2, 4, 4-Tetra chlorobiphenyl (PCB47)
15.708	2, 2, 3, 4, 6-Penta chlorobiphenyl (PCB98)
16.953	2, 2, 4, 4, 5, 6-Hexa chlorobiphenyl (PCB154)
19.321	2, 2, 3, 3, 4, 4, 6-Hepta chlorobiphenyl (PCB171)
19.463	2, 2, 3, 3, 4, 5, 6, 6- Octa chlorobiphenyl (PCB200)

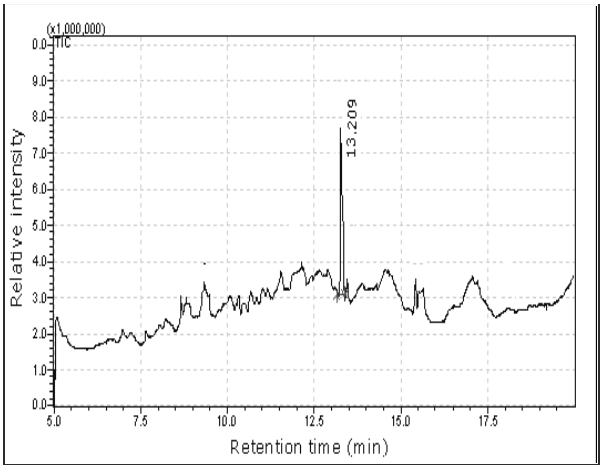
Figure 3: Different stages of endometriosis cases showing Dioxin-TCDD.

*Stage I

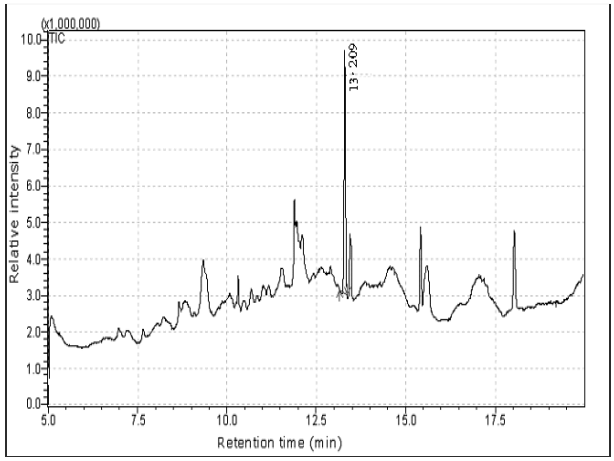


*Stage II





*Stage III



*Stage IV

*Chromatograph of the stage Endometriosis case showing dioxins peak level in Gas Chromatography (GC) analysis.

Estimation of PCBs by GAS Chromatography with Flame Ionization detector (GC-FID)

Plasma was separated from the heparinized blood within 24 hrs after collection by centrifugation (2,500Xg for 15 minutes). The plasma (4-5ml) was pooled and kept frozen at -20°C until PCBs were analyzed. All the solvents used were analytical grade purity (HPLC grade; Qualigens, Ltd, Mumbai India); eight PCB congers mix (PCB Mix525, Supelco. Bellefonte, PA) were selected at a concentration of 500μg/ml in hexane of each PCB. Gas chromatography for the extraction of PCBs were divided into five phases. Extraction of PCBs were performed by previously described method (11) with certain modification (12). The concentrated organic phases in phase four were pooled and dried under nitrogen gas. The sample was resuspended in hexane and then injected for Gas chromatography. Gas Chromatography analysis was carried out as per the instructions (Supelco, Germany), and in house modification on the GC-2010 series Gas Chromatograph (Shimadzu, Kyoto, Japan).

Statistical Analysis

Statistical analysis was performed using Medcalc 7.6 version software (Medcalc software, Mariakerke, Belgium). The BMI was calculated by (Quetlet's Index). Independent two-sample t-test was performed for age, BMI age at menarche and the concentration of PCBs and Dioxin-TCDD between the

cases and the control group. Correlation tests were used to determine the strength of association between concentrations of PCBs and Dioxin-TCDD with severity of endometriosis. $P<0.05$ was considered to be statistically significant.

RESULTS

The demographic and the clinical details of the three study groups (Tables 1 and 2). Despite comparable ages at menarche, more women with endometriosis reported pain during intercourse compared to the control women. No significant difference in age, body mass index (BMI), age at menarche and duration of infertility were observed in between these groups. Figure 1 shows map of sampling locations.

Estimation of Dioxin and PCBs

We observed that cases in the stages III and IV having GSTM1 null mutation had higher concentrations of Dioxin-TCDD when compared to cases in stages I and II and control groups (Tables 3, 4). Gas chromatography with flame ionization detector (GC-FID) analysis for the estimation of PCB standard (Figure 2) and GC MS chromatograph of different stages of endometriosis cases showing Dioxin -TCDD (Figure 3).

DISCUSSION

The relation between endometriosis and exposure to dioxins is highly controversial issue. In continuation with earlier report (13), there is a significant association between PCBs and PEs with endometriosis. Now in the present study, we established an association between dioxins, PCBs and their possible impact on developing endometriosis in the south Indian women, which is the first report from Indian subcontinent. A certain group of women develop endometriosis implies that there is increased susceptibility to development of disease in certain cases. Individual's susceptibility is influenced not only by genetic background but also by the interaction of genes with environmental factors. Dioxin-TCDD, dioxin like PCBs and phthalate esters have been implicated as factors involved in the development of endometriosis 14-15). The lack of detoxification, which is genetically determined, might be a risk factor for endometriosis development (16). Earlier studies attempted to measure the risk of endometriosis for CYP and GST polymorphisms and role of dioxins and dioxin -like compounds (PCBs) in the development of endometriosis. No attempt has been made to measure the level of dioxin-TCDD or dioxin like compounds (PCBs) in the blood of both cases and controls. In view of the controversies surrounding the role of both environmental and detoxification gene polymorphisms, we carried the present study to measure the blood concentration of PCBS and Dioxin-TCDD exposure levels in cases and controls to identify the role and association of these factors in the development of endometriosis in south Indian population. In the present study a significant association ($P=0.037$) between endometriosis patients who were having higher concentration of PCBs and dioxin-TCDD was observed in the south Indian women. The literature is rich in information regarding association of PCBs with endometriosis but PCBs may not be the sole factor for developing endometriosis. The higher concentration of PCBs and Dioxin-TCDD in the plasma of women with endometriosis compared with control possibly suggests an association of PCBs and Dioxin-TCDD with the occurrence of endometriosis. Plasma concentrations of PCBs such as PCB1, PCB5, PCB29 PCB98 and dioxin -TCDD were significantly different from women with endometriosis compared with those free from disease (Table 6). The other compounds of PCBs (PCB47, 54, 171 and 200/201) were not detected in either the study or the control group. The other compounds of PCBs (PCB47, 54, 171 and 200/201) were not detected in either the study or the control group. Our study was well designed and sample size was compared to previous studies (17-18), one of the strong points of our study was the estimation of plasma concentrations of PCBs and Dioxin -TCDD in patients and control cases and their possible impact in the development of endometriosis. Our sample size was smaller than required number for a study of a complex disease to date our knowledge is the first study attempted to resolve the conundrum of conjugation of PCBs and Dioxin-TCDD in the pathogenesis of endometriosis. Our results were similar to the other

reports (19-20) that elevated concentrations of the three PCB congeners found in women with endometriosis in contrast to women free from the disease. However our results conflict with the studies conducted on Italian and Belgian women, no significant difference was found in dioxin -like compound body burdens between cases and controls on a country basis, where as the body burdens of the Italian women were significantly lower than that of Belgian women (21-22). We are unable to compare our study results directly with those of other investigators due to varying methodologies. To conclude within our study patients with increased concentrations of PCBs and dioxin-TCDD, possibly show impact of PCBs and dioxin-TCDD in those genetically predisposed patients with endometriosis. It is noteworthy that cellular proliferation is an important characteristic of both endometriosis and cancer. It seems obvious that genes involved in angiogenesis should also be pursued for their possible role in endometriosis. It might also be expected that women with results of positive to genetic susceptibility would be advised to avoid environmental risk factors and to consider having children sooner than later in life in view of the association between endometriosis and infertility.

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