Original Article

Evaluation of the Phthalate Esters in South Indian Women with Endometriosis

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Abstract

Background: To evaluate the possible association between phthalate esters (PEs) and the occurrence of endometriosis. Blood samples were collected from 99 infertile women with endometriosis (study group); 135 age-matched women without endometriosis (control group) but with infertility related to tubal defects, fibroids, polycystic ovaries, idiopathic infertility and pelvic inflammatory diseases diagnosed by laparoscopy with no evidence of endometriosis or other gynecological disorders during laparoscopic sterilization.

Materials and Methods: This is a prospective case-control study, which recruited women undergoing infertility treatment at three collaborating centers (BMMHRC: Bhagwan Mahavir Medical Hospital and Research Centre, MHRT: Maternal Health and Research Trust, and Owaisi Hospital and Research Center) of Reproductive Medicine Hyderabad, which receives cases from all over the region of Andhra Pradesh, India. The concentrations of Phthalate Esters were measured by using the High Performance Liquid Chromatography (HPLC).
Evaluation of Phthalate Esters concentrations in women with endometriosis compared with women who are free from the disease.

Results: Women with endometriosis showed significantly higher concentrations of Phthalate esters (Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Di-n-butyl phthalate (DnBP), Butyl benzyl phthalate (BBP) and Bis (2-ethylhexyl) phthalate (BEHP)) compared with control group. We found that (38%) of the cases with endometriosis and (21%) of the control group. The correlation between the concentrations of Phthalate esters and different severity of endometriosis was strong and statistically significant at $p<0.05$ for all five compounds (DMP): $r=+0.57$, $p<0.0001$; DnBP $r=+0.39$, $p<0.0001$; BBP: $r=+0.89$, $p<0.0001$; DnOP: $r=+0.66$, $p<0.0001$ and BEHP: $r=+0.33$, $p<0.0014$.

Conclusion: This study for the first time from Indian subcontinent demonstrates that possibly Phthalate Esters might have a role in etiology of endometriosis.

Keywords: Endometriosis, Phthalate Esters, Female Infertility, Environmental Hazards

Introduction

Endometriosis is a gynecological disorder characterized by the presence of ectopic endometrial glands and stroma. It affects approximately 15% of women of childbearing age and is consistent with the estrogen dependent nature of the disease (1-6). It is frequently associated with chronic pelvic pain, dysmenorrhea, menorrhagia, and dyspareunia (7, 8). Despite the widespread occurrence of this disorder, the etiology of the disease is still to be elucidated.

Environmental pollutants that have previously shown to be linked to endometriosis are polyhalogenated aromatic hydrocarbons (PHAH), a class of widespread environmental contaminants that includes polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs)

and biphenyls (PCBs) (9).
Phthalates are chemicals used in the manufacturing of medical supplies, plastic wraps, automobile parts, beverage containers and the linings of metal cans and can act as powerful endocrine disruptors. Several studies indicate that phthalates can negatively affect reproductive function in laboratory animals (10, 11). DEHP (Diethyl hexyl phthalate), DINP (Di-iso-nonyl phthalate) and DIDP (Di-iso-decyl phthalate) have been tested and found not to have any oestrogenic activity (12).
We, therefore, carried out a preliminary study in order to evaluate the concentrations of phthalate esters in the serum of women suffering with endometriosis and compare it with those from women who are free from

the disease.

Materials and Methods

Patients

This is a prospective case-control study, which recruited the women undergoing infertility treatment at three collaborating centers (BMMHRC: Bhagwan Mahavir Medical Hospital and Research Centre, MHRT: Maternal Health and Research Trust, and Owaisi Hospital and Research Center) of Reproductive Medicine Hyderabad, which receives cases from all over the region of Andhra Pradesh, India. A proforma containing information about absent, obstetric and gynecological history including age at menarche, length of menstrual cycle, associated symptoms, duration and amount of blood loss, duration of infertility, and socio-demographic details like age, body mass index (BMI) and limited information on diet was used for this study. The ethical committee of our institute approved the research protocol for this study. Informed consents were obtained from all the participants in this study.

The case group consisted of 99 women of Indian origin who were diagnosed by laparoscopy and found to have pelvic endometriosis. The severity of the disease was staged according to the revised American Society for Reproductive Medicine (rASRM) classification of endometriosis (13). Endometriosis was staged as minimal (rASRM stage I) in 36, mild (rASRM stage II) in 18, moderate (rASRM stage III) in 30, and severe (rASRM stage IV) in 15 patients. All women were infertile (primary infertility in 76% of cases and secondary infertility in 24% of cases) with infertility duration of 5.4 yr SD (3.8) and their mean age of 26.2 yr SD (4.2) with the following clinical symptoms: dyspareunia (34%) and dysmenorrhoea [mild (28%); moderate (4%); severe (3%)]. In the remaining 31% of cases were asymptomatic. All the patients were menstruating regularly and none of them had received hormonal treatment for at least 3 months prior to laparoscopy.

The control group comprising of 135 women who attended the same hospital for other gynecological pathology (e.g. fibroids, tubal defects, polycystic ovaries, idiopathic infertility and pelvic inflammatory disease) but were laparoscopically confirmed to be without endometriosis. All the patients in the control group were also infertile (primary infertility 79%; secondary infertility 21%) with mean age of 25.6 yr SD (4.3) and mean duration of 5.4 yr SD (2.9) years of infertility. The following symptoms were also present in the women of the control group: dyspareunia (17%) and dysmenorrhoea [minimal (37%); mild (26%); moderate (8%); severe (2%)]. The remaining 27% of women attending the same hospitals for laparoscopic tubal sterilization were no evidence of endometriosis and other gynecological disorders. All women in this group were asymptomatic. The entire case and control group were living in urban areas with no history of any occupational exposure to reproductive toxicants and they also did not smoke or consume alcohol.

Six-eight ml of blood was collected from each patient in a vacuum system tube, transported in a cooling pail, and centrifuged (2500 g for 12 min) within 24 hours after collection. The serum (3-5 ml) was pooled and kept frozen at −80oC until the PEs were analyzed.

The extraction of PEs using High Performance Liquid Chromatography (HPLC) was divided into 5 phases. Extraction of PEs was performed by the method described by Bruce et al (14) with modifications by Rozati et al (15). One milliliter of methanol was added to each sample extraction containing 2-mL aliquots of serum and mixed by vortex; then 3 mL of hexane:ethyl ether (1:1) (HPLC grade; Spectrochem, Ltd., Mumbai, India) was added to extract the mixture. The mixture was agitated on a rotary mixer for 15 minutes and then centrifuged at 2,000X g for 5 min. The organic phase was collected, and the aqueous phase was extracted two more times. The organic phases were pooled and subsequently concentrated to 1 ml by evaporation under nitrogen steam. Figure1 shows the phthalate esters standard graph.

Statistical Analysis

Statistical analysis was performed by using Medcalc 7.6 version software. The body mass index (BMI) was calculated by (quetlet's index) body weight (kg) divided by the square of height in meters. Independent
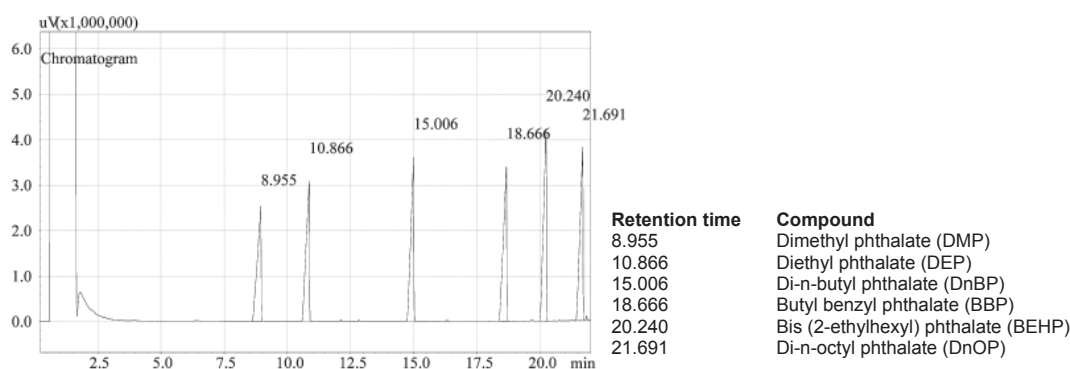

Retention time	Compound
8.955	Dimethyl phthalate (DMP)
10.866	Diethyl phthalate (DEP)
15.006	Di-n-butyl phthalate (DnBP)
18.666	Butyl benzyl phthalate (BBP)
20.240	Bis (2-ethylhexyl) phthalate (BEHP)
21.691	Di-n-octyl phthalate (DnOP)

Fig 1: Standard Chromatograph showing Phthalate esters of High Performance Liquid Chromatography (HPLC).

two-sample 't'-test was performed for the concentration of PEs between the endometriosis and control groups. Correlation tests were used to determine the association between the concentration of PEs and severity of endometriosis. $p<0.05$ was considered to be statistically significant.

Results

Table I shows the reproductive history of the two study groups. Despite comparable ages at menarche, more women (38%) with endometriosis reported pain during intercourse compared to the control group (21%).
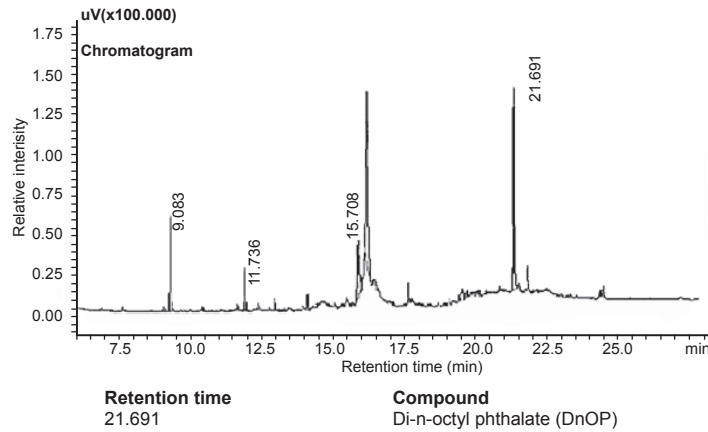

Retention time	Compound
21.691	Di-n-octyl phthalate (DnOP)

Fig 2: Showing the Chromatograph of Control group, Phthalate esters peak levels in High Performance Liquid Chromatography (HPLC) analysis.

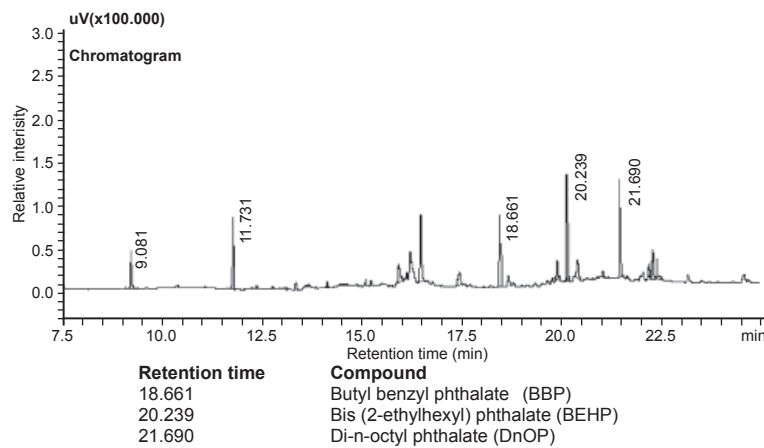| Retention time	Compound
18.661	Butyl benzyl phthalate (BBP)
20.239	Bis (2-ethylhexyl) phthalate (BEHP)
21.690	Di-n-octyl phthalate (DnOP)

Fig 3: Chromatograph of Stage I Endometriosis case Showing Phthalate esters peak levels in High Performance Liquid Chromatography (HPLC) analysis.
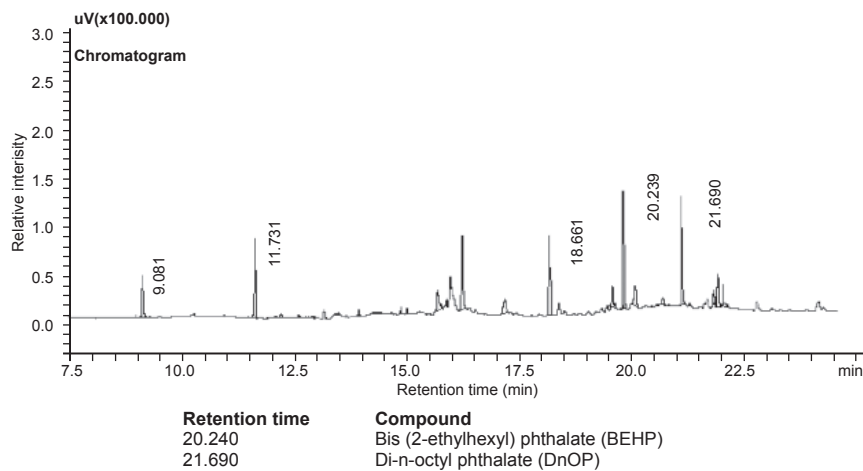

Retention time	Compound
20.240	Bis (2-ethylhexyl) phthalate (BEHP)
21.690	Di-n-octyl phthalate (DnOP)

Fig 4: Chromatograph of Stage II Endometriosis case Showing Phthalate esters peak levels in High Performance Liquid Chromatography (HPLC) analysis.
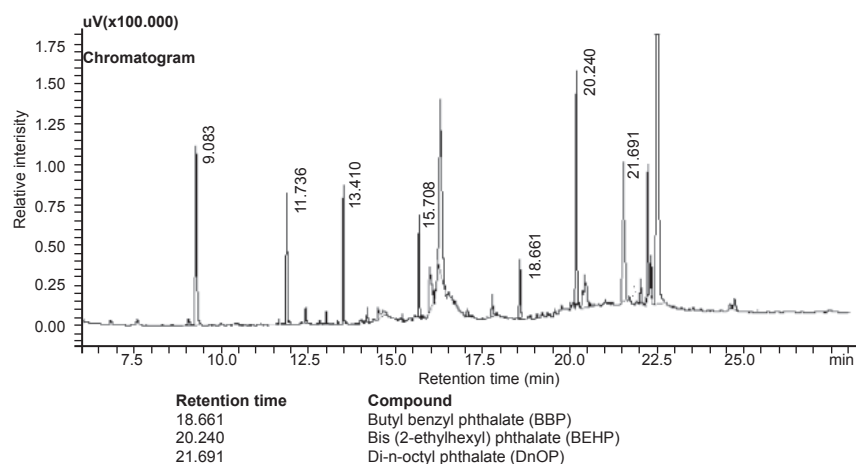
Fig 5: Chromatograph of Stage III Endometriosis case Showing Phthalate esters peak levels in High Performance Liquid Chromatography (HPLC) analysis.

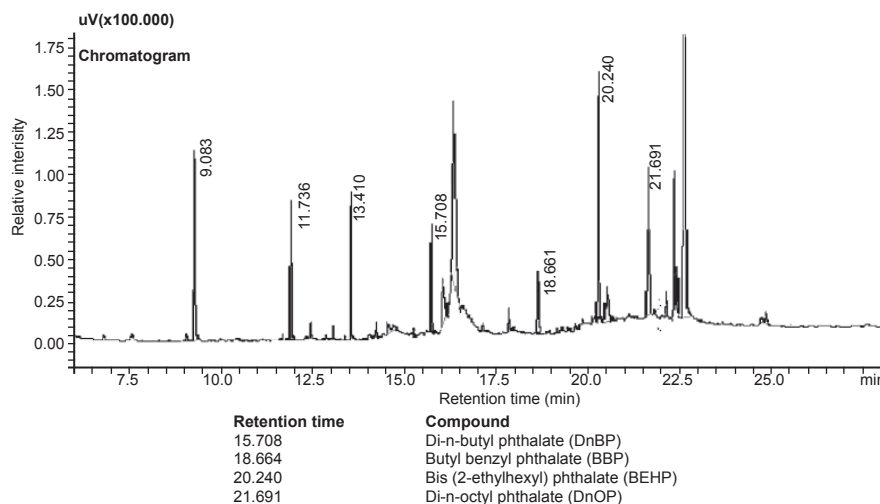| Retention time	Compound
18.661	Butyl benzyl phthalate (BBP)
20.240	Bis (2-ethylhexyl) phthalate (BEHP)
21.691	Di-n-octyl phthalate (DnOP)

Fig 6: Chromatograph of Stage IV Endometriosis case Showing Phthalate esters peak levels in High Performance Liquid Chromatography (HPLC) analysis.

Retention time	Compound
15.708	Di-n-butyl phthalate (DnBP)
18.664	Butyl benzyl phthalate (BBP)
20.240	Bis (2-ethylhexyl) phthalate (BEHP)
21.691	Di-n-octyl phthalate (DnOP)

Table 1: Reproductive history among the cases and control group

Characteristic	Endometriosis (n=99)	Control group (n=135)
Age in years [a]	25.6 (4.2)	26.4 (4.7)
Body mass index (kg m^{-2}) [a]	24.1 (2.2)	23.5 (1.2)
Age at menarche (years) [a]	12.4 (1.1)	12.5 (1.0)
Duration of Infertility (years)	5.4 (3.8)	5.6 (3.7)
Primary infertility [n (%)]	33 (76)	45 (87)
Secondary infertility [n (%)]	12 (24)	4 (13)

Data are presented as mean (SD); $p < 0.05$ considered statistically significant;
a represents not significant in between the group.

Table 2: Phthalate ester concentrations in endometriosis and control group

Groups	DMP µgml^{-1}	DEP µgml^{-1}	DnBP µgml^{-1}	BBP µgml^{-1}	BEHP µgml^{-1}
Control group	0.04 (0.10)	0.9 (0.20)	0.55 (0.89)	0.15 (0.21)	0.11 (0.22)
Study group	0.03 (0.72)	0.89 (0.84)	0.98 (0.96)	3.32 (2.17)	2.15 (1.99)
Control Vs					
Study group; t value	5.13	5.13	-9.52	5.22	0.33
p value [a] <0.0001	<0.0001	<0.0001	<0.0001	<0.0001	< 0.0014

Data are presented as mean (SD); Control group; $p < 0.05$ considered statistically significant; [a] significant in between the group;

No significant difference in age, body mass index (BMI), age at menarche and duration of infertility were observed in between these groups. Table II shows that significant differences in the concentrations of phthalate esters were observed between the women with or without endometriosis [study group: (mean 0.03 [SD 0.72]; 0.89 [SD 0.84]; 0.98 [0.96]; 3.32 [SD 2.17]; 2.15 [SD 1.99] micrograms/ml) ; compared with the control group (mean 0.04 [SD 0.10]; 0.9 [SD 0.18]; 0; 0.55 [SD 0.89]; 0.15 [SD 0.21]; 0.11 [SD 0.22] micrograms/ml). The correlation between the concentrations of PEs as shown in figure 2 (control group) and different severity of endometriosis as shown in figures 3-6 was strong and statistically significant at $p<0.05$ for all five compounds (DMP): r=+0.03, $p<0.0001$; DnBP r=+0.39, $p<0.0001$; BBP: r=+0.89, $p<0.0001$; DnOP: r=+0.66, $p<0.0001$ and BEHP: r=+0.33, $p<0.0014$) as shown in figure 7.
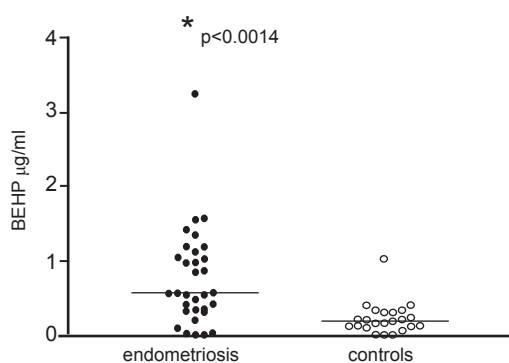

Fig 7: Serum concentrations of BEHP in patients with endometriosis versus healthy control group.

Discussion

This study of infertile women with endometriosis is the first report from the Indian sub-continent demonstrating the relationship between exposure to PEs and the occurrence of endometriosis in infertile women. When the higher concentration of these chemicals in the serum of the study group with endometriosis was compared to the control group, was possibly suggested association of PEs to the occurrence of endometriosis. Phthalate Esters serum concentrations such as DMP, DnBP, BBP, DnOP and BEHP were significantly different from women with endometriosis compared to those who were free from the disease (Fig 1). Diethyl phthalate, (DEP) were not detected in the study as well as in control group.

The chemical found in the highest concentration was the phthalate BBP at 3.32 µgml-1 SD (2.17) fallowed by BEHP 2.15 SD (1.99) µgml-1. DEHP has been shown to cause reproductive and developmental toxicity and are suspected to be endocrine disruptors (16). There is a concern that these compounds may be causing undesirable effects on human reproductive health (17, 18)Furthermore, some of the common phthalates are weakly estrogenic and have more adverse synergistic effects when combined with other chemicals found in the envi-

ronment and food chain.

Our study protocol was well designed compared to the previous studies of Cobellis et al (18). Firstly, our study offered a unique combination of six measurements of phthalate esters mix compared to the previous study in which single compound BEHP and its metabolite MEHP were measured. Secondly, we considered all details about the reproductive history of the patients including BMI, Age at Menarche, duration of infertility and clinical symptoms dyspareunia and dysmenorrhoea, which were other confounding variables but not in cobellis (18) study.

Our results is consistent with the finding of Cobellis (18) who reported the existence of higher concentrations of BEHP in women with endometriosis.

In spite of the high prevalence of endometriosis in all over the world, researchers have been unable to determine its aetiology. It is considered to be due to sex steroids, environmental factors and impaired immune response, which can cause to initiation of endometriosis, although the exact mechanism by which ectopic endometrium attaches to the peritoneum has not yet been identified.

The associated observation demonstrated the possible relationship between endometriosis and environmental contaminants other than DEHP which has been already evidenced (19-21). DEHP has proven detrimental effects on fertility and reproduction in female animal models (22). BEHP exposure of our study subjects with endometriosis shows statistically significant higher concentration compared to the control group as shown in figure 7. The probable association between polychlorinated biphenyls (PCBs) and phthalate esters (PEs), and the occurrence of endometriosis in a prospective case control study found that PCBs and PEs may be instrumental in the etiology of endometriosis (23).

Conclusion

There are enormous uncertainties in assessing what might be a safe level of exposure to hazardous man-made chemicals, especially when they persist in the body for long periods. This is due in part to the lack of toxicity data and exposure data for the vast majority of chemicals which people are exposed. It is difficult to suggest that exposure to a certain chemical at a certain concentration will cause a particular adverse effect. The best way to prevent this ongoing chemical contamination and the threat to future generations is to prevent the manufacture and use of chemicals that are found in elevated concentrations in biological fluids such as blood and breast milk.

Acknowledgment

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