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ORIGINAL ARTICLE

Serum Diethyl Phthalate Levels in Infertile Females with Endometriosis

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ABSTRACT

Background: Endometriosis has a complex multifactorial pathophysiology and is a leading cause of female infertility. Emerging evidence suggests the role of endocrine disrupting chemicals and environmental factors such as Diethyl phthalate (DEP) in the pathophysiology of the disease.
Aim: To investigate the serum DEP levels in females with infertility having endometriosis and normal healthy counter parts.
Methods: Married females (n=50) age 20-40 years, diagnosed with endometriosis and having history of >1 year of infertility, were selected as cases. Age matched women (n=50) with proven fertility and screened negative for endometriosis were included as controls. Females on any medicine, having co morbid conditions were also excluded from the study. DEP concentration in serum was determined by using High performance liquid chromatography (HPLC).
Results: Significantly higher levels (p=0.003) of DEP were seen in endometriosis females (3.76± 1.28 ng/ml) as compared to controls (2.61± 1.72 ng/ml). The comparison of DEP levels between different stages of endometriosis revealed an increasing, but no significant trend with advancement of the disease.
Conclusion: High serum DEP levels in patients substantiate their role in disease pathophysiology. Therefore, it may be advisable to pay attention while using such compounds. There is an increased need to regulate the levels of such industrial compounds manufactured for daily use of human beings by efficient and judicious quality assurance plastics and by using the standards set by WHO and/or FDA.
Keywords: Phthalate, Endometriosis, Diethyl phthalate, plasticizers, Infertility

INTRODUCTION

Endometriosis, is disease in which the presence of endometrial tissue in ectopic sites, other than uterine cavity and is one of the commonest causes of female infertility[1]. About 15% of peoples in industrialized world is consults for infertility[2]. The frequency of the endometriosis in Pakistan reported to 20.2% in cases of primary infertility[3] which is consistence with results of other studies of different populations[4,5].

New evidence propose that possible role of environmental contamination of endocrine disrupting chemicals (EDC's) in the pathophysiology of endometriosis[6]. An endocrine disrupting chemical is an exogenous substance that hinder with production, binding, and removal of natural sex hormones[7]. Some chemicals stimulate the growth of endometrial cells that play an important role in endometrial angiogenesis[8,9]. Among such EDC's phthalates have attained special focus due to their existence in everyday life products[10].

Phthalates are esters of Phthalic acid. A class of organic chemicals that are used as plasticizer to increase the pliability of polyvinylchloride (PVC). They are used in immense range of products for example food packaging materials, medical devices, medicines, kids' toys as well as beauty and personal care brands[11]. Out of the primary phthalate compounds, diethyl phthalate (DEP) is most frequently used plasticizer globally. Its most common use is in cosmetics, pharmaceutical and food packaging materials[12].

Once inside the body these phthalates are promptly metabolized to their monoesters after hydrolysis and conjugation, and then excreted in urine, feces and other body secretions. Primary and secondary phthalate metabolites are bio-active[13,14]. Public and scientific concern is being focused to inquire the role of phthalate exposure in estrogen related disease such as endometriosis. Studies have shown accumulation of these substances in blood, follicular fluid, urine, saliva and sweat of infertile females[15,16,17]. So far, fewer reports have been published on the subject and nothing exists from Pakistan. A Few authors reported that there is positive association between phthalate exposure and endometriosis[18,19], while others reported contradictions[20,21]. Pakistan is a country where plastic and plastic related products are very heavily used, particularly by the lower middle class and modest communities. Plastic containers are even used for carrying very hot food items and apart from their industrial use, small factories or cottage industries making plastic utensils and shopping bags use recycled plastic materials, which make these items quite hazardous to the general health of the public. Also there are women of the reproductive age who work in these home-based factories where there is least concept of quality control, quality assurance and health hazards. Keeping in view of these facts we conducted the present study to understand whether exposure to diethyl phthalate is associated with risk of endometriosis.

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MATERIAL AND METHODS

The case control study was approved by Ethical review board of University. Sample size was calculated by using the following formula:

$$n = \frac{(z_{1-\alpha/2} + z_{1-\beta})(\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)2}$$

Study population comprised of 50 young (20-40 years) females laparoscopically diagnosed of endometriosis and had a history of infertility (>1 years). Staging of the disease was done according to ASRM criteria for endometriosis staging[22]. A comparable number (n=50) of same age group healthy females who gave birth a child in last two years, also negative for endometriosis screened by ultrasonography followed by detailed clinical evaluation and examination by specialized doctors served as controls. Females on any medication especially hormone treatment, history of infertility other than endometriosis or having co-morbid conditions, were excluded from the study**.** After taking written well informed consent from all study participants, a detailed medical, socioeconomic and occupational history with detailed general physical and systemic clinical evaluation was done by a qualified doctor.

Ten milliliters of blood was drawn under aseptic conditions. Serum was separated after centrifugation; and stored in glass vials at -80 ◦C for later analysis. High performance liquid

chromatography (Perkin Elmer series 200 UV pump) was used to determine diethyl phthalate concentrations.

Reagents and solutions preparation: Stock solution of HPLC grade (Merck chemicals, Germany) 99.99% diethyl phthalate (DEP) and its internal standards were prepared in HPLC grade (Fisher scientific, Germany) acetonitrile, and stored at -20˚C to test the linearity. With a DEP concentration of 10, 50, 100, 250, 500, and 1000 ng/ml, Calibrators were prepared in serum. A total of 200 µL of serum was added with 50 µL of internal standard, 400 µL of 1 mol/L NaOH (HPLC grade; Sigma Aldrich, USA), 100 µL of 50 % H_3PO_4 (HPLC grade; Sigma Aldrich USA) and 600 µL of 50 % acetonitrile (Fisher scientific, Germany).

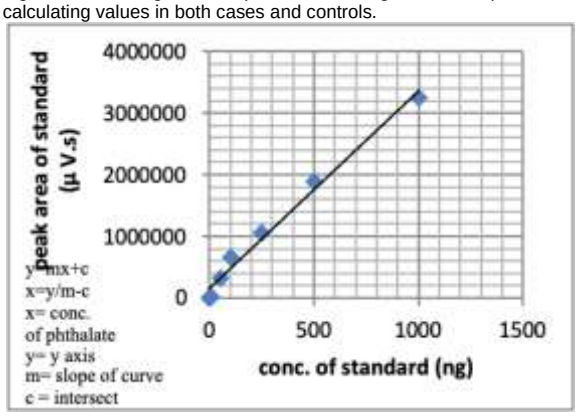
High Performance Liquid Chromatography (HPLC): After 10 minutes at 1500 rpm centrifugation, the supernatant was separated, and 10 µL of the supernatant was injected into the HPLC glass vials via syringe filter (25mm/ 0.22 micrometer), and put in auto- sampler (Perkin Elmer series 200). To avoid potential contamination, all glass vials were clean with methanol, acetone and acetonitril. HPLC was carried out using Perkin Elmer series 200 UV pump, attached to a Diode Array UV/ visible Perkin Elmer series 200 detector. A Perkin Elmer 250 x 4.6 mm (internal diameter) Brownlee analytical C18 (5-µm particle size) column (USA) was used. Results were analyzed on Total chrome Navigator 3.1.5 software. The mobile phase consisted of a mixture of 1ml/L acetic acid (Sigma Aldrich, USA) in water and 1ml/L acetic acid in acetonitrile (10/90, vol/vol). Elution was performed with the use of an isocratic mode at 0.5 ml/min. Total chromatographic run time was 10 minutes. Peak for DEP was obtained at 6.43 minutes (retention time). Concentration of DEP was calculated by using straight line equation.

Statistical analysis: By using IBM-SPSS version 20, data was analyzed. The variables were analyzed and expressed as Mean(±SD). Two sample Student's't' test, was applied to compare the phthalate esters concentrations in both cases and controls. A *p* value of ≤ 0.05 was considered as statistically significant. To compare the levels of DEP between different stages of endometriosis one-way ANOVA was applied.

RESULTS

Individual values for DEP in endometriosis females and controls were calculated by using straight line equation among peak area of standard and concentration of standard, and putting individual peak areas for cases and control in straight line equation, as expressed in fig. 1.

Fig. 1: DEP straight line equation according to least square method for calculating values in both cases and controls.



Description of chromatographs: Fig. 2a, 2b, 3a and 3b describe DEP chromatographs, obtained through High performance liquid chromatography in cases and controls respectively. The X-axis shows the retention time (minutes), and Y-axis shows milli-Absorbance Units (mAU).

Figures 2a and 3a are real time chromatographs, showing the peak of DEP at its respective retention times in cases and controls respectively. Fig. 2b and 3b, are reprocess results collected from total chrome navigator 3.1.5 software, showing DEP peaks in cases and controls at their respective retention times.

Fig. 2a: HPLC Real time chromatograph of cases showing the DEP peak. Generated from: Total chrome navigator; 3.1.5 software.

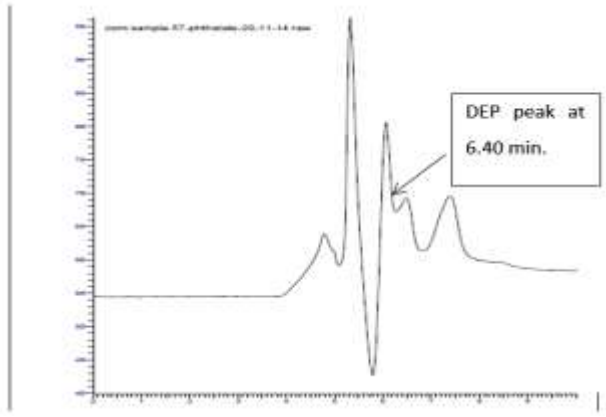


Fig. 2b: HPLC Reprocess Chromatograph of cases showing the DEP peak area. Generated from: Total chrome navigator 3.1.5 software.

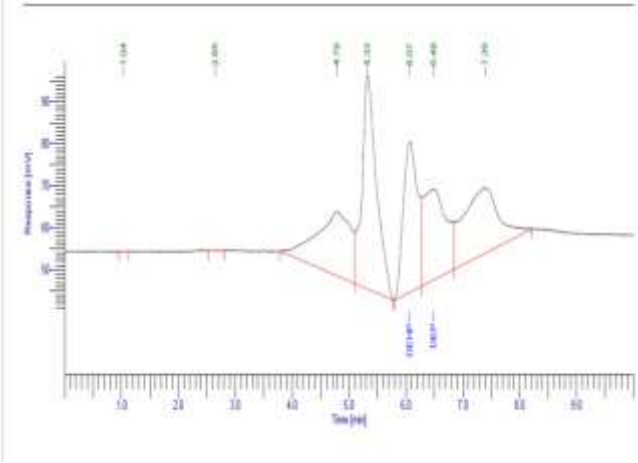


Figure 3a: Real time chromatograph of HPLC of control showing the DEP peak. Generated from Total chrome navigator (3.1.5 software).

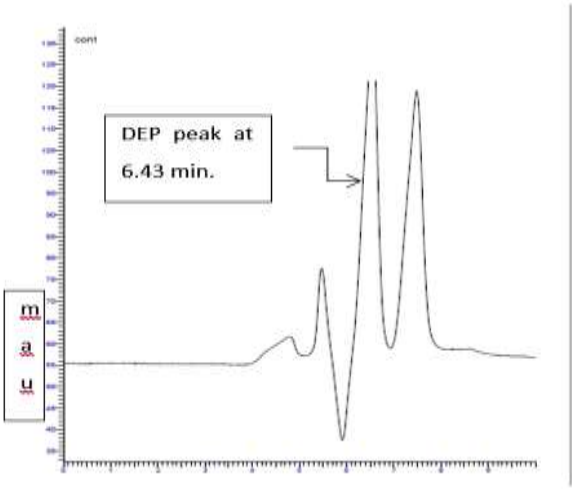
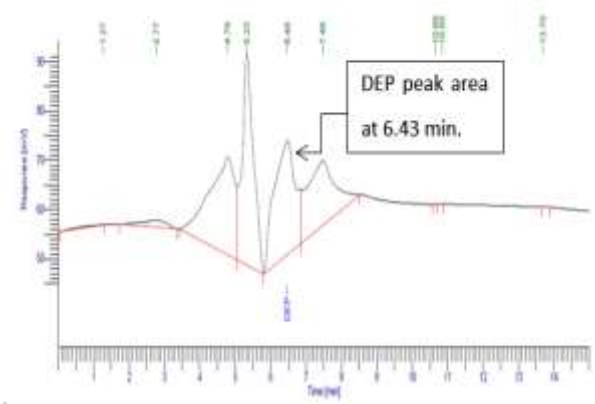
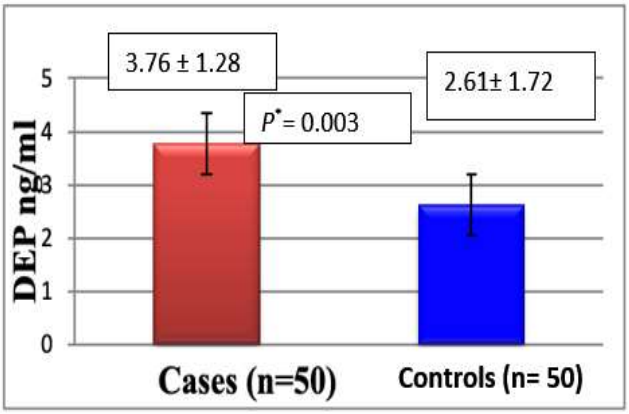


Figure 3b: Reprocess Chromatograph of HPLC of control showing the DEP peak area. Generated from Total chrome navigator (3.1.5 software).



Statistically significant difference (p=0.003) in mean DEP serum concentration of cases (3.76±1.28 ng/ml) and controls (2.61±1.72 ng/ml) was observed (figure 4).

Figure 4: Comparison of Means ±SD between cases and controls for DEP statistics according to the "t" test.



Based on the points system as per American Society for Reproductive Medicine classification of endometriosis, patients were assigned to one of the four stages and serum DEP levels were compared among all stages.

Table 1: Mean (±SD DEP) values in all four stages of endometriosis, statistics according to one- way ANOVA.

Stage of endometriosis	I (n=1)	II (n=10)	III (n=21)	IV (n=18)	p-value
Mean ± SD (ng/ml)	1.29 ± 0	3.72 ± 1.32	3.78 ± 1.32	3.89 ± 1.19	0.275

There was an increasing tendency in the Mean values of serum DEP as the stage advances, but the difference was insignificant.
The comparison of mean values of demographic factors i.e. age, age at menarche, weight and height did not differ significantly between cases and control groups (Table 2).

Table 2: Comparison of Anthropometric factors between cases and controls statistics according to the "t" test.

Parameter	Group	Mean (±SD)	p - Value
Age (years)	Cases	29.54 (±5.19)	>0.05
	Controls	29.96 (±3.97)	
Age at menarche (years)	Cases	11.74 (±1.98)	>0.05
	Controls	11.49 (±1.11)	
Weight	Cases	59.24 (±9.95)	>0.05
	Controls	56.50 (±9.25)	
Height (inches)	Cases	61.34 (±3.03)	>0.05
	Controls	61.80 (±2.72)	

DISCUSSION

Endometriosis is a gynecological disorder, which is characterized by the existence of ectopic endometrial tissue. Regardless of the extensive development of endometriosis, the etiology and pathophysiology of the disease is yet to be explicated.

Environmental hazardous toxins have previously shown to be associated to endometriosis. The aim of this study was to identify the levels of phthalates, which are common endocrine disruptors, in age- matched females with diagnosed endometriosis, as well as healthy females. Pakistan lacks a strict quality control over the use of common plastic compounds such as plastic food wraps, medical devices, beverages containers, and the linings of metal cans. A good number of Pakistani females work in the pharmaceutical industry and an equally good number uses daily cosmetics. Hence, the risk of exposure to phthalates in this region is reasonably higher compared to developed countries. Although a variety of phthalate compounds has been studied, we selected DEP which is a parent compound of phthalate, widely distributed and mostly present in plastic, medical devices and daily use products. It enter the body through inhalation, cutaneous contact, and through food and medicine ingestion[23].

The literature for serum DEP levels in infertile females with endometriosis is sparse. This study is the first report from Pakistan demonstrating the relationship between serum DEP levels and infertility with endometriosis. Our study recruited endometriosis patients diagnosed by laparoscopy. Our results showed significantly higher (p=0.003) serum DEP (diethyl phthalate) levels in females with endometriosis, compared to controls. These results are in accord with previous study, which shows high DEP levels but detection rate is low in this study (0-16.4%)[24]. This difference may be due to more judicial use of DEP products in our country especially hot food packaging in plastic bags and containers and poor quality cosmetic products. These results are contrary to previous studies which show undetectable levels of DEP in both cases and controls[24-27]. This difference in results may be due to differences in the techniques, as Reddy et al (2003) used gas chromatography and in Rozati et al (2008) (28) HPLC technique, control and cases criteria was different from ours. DEP used as a solvent and fixative for fragrance, cosmetic ingredients, subsequent contact with skin may lead to high levels of DEP. In order to show correlation between DEP concentrations and severity of the disease, we analyzed the mean DEP values between stages (I, II, III and IV) of endometriosis. This analysis showed no significant difference in mean DEP levels among the stages of endometriosis. However, there was an increasing trend

of mean values of DEP with advancement of stage (Table 1). These results were in contrary to previous study[24] which showed increase phthalate levels with advancement of disease. We compared the anthropometric parameters, i.e. age at menarche, height, weight and age with serum DEP levels, but no statistically significant difference was observed between cases and controls (Table 2). Therefore, no association was ascertained among the anthropometric factors and endometriosis which is in consistency with previous results by Rozati et al[28].

Our results favor the previous studies showing positive association of Diethyl phthalates with endometriosis. Unfortunately, because of our financial limitations, we could not expand our experiments, which may provide more elaborated understanding about the possible role of these phthalate compounds in the pathophysiology and etiology of endometriosis.

CONCLUSION

Further studies are necessary to answer the mechanistic pathway through which these compounds alter the normal physiology and lead to disease development. We proposed that future studies should be conducted on comparison of serum DEP levels in infertile females with endometriosis with infertile females without endometriosis.

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