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Full Length Research Paper

Determination of serum levels of three phthalate esters in patients with polycystic ovary syndrome

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The present study aimed to detect serum levels of three phthalate esters in polycystic ovary syndrome (PCOS) patients and explore the association between these phthalate esters and PCOS. Eighteen PCOS patients and 16 age-matched women without PCOS or laparoscopy-proven endometriosis but with infertility related to tubal defects or pelvic adhesions were recruited into the present study. Serum levels of three phthalate esters (diethyl phthalate [DEP], dibutyl phthalate [DBP] and diisooctyl phthalate [DEHP]) were measured using high performance liquid chromatography. Results showed PCOS patients had significantly higher levels of DEP and DBP than those in control group (DEP: 0.45±0.24 vs 0.26±0.10 mg/ml; DBP: 0.53±0.15 vs 0.41±0.14 mg/ml). Meanwhile, the levels of DBP, DEP, and DEHP declined in sequence in PCOS women. But no statistically significant difference in DEHP level was noted between these two groups; in the PCOS patients, there was no significant correlation between the serum levels of three phthalate esters and estradiol (E2), testosterone (T), follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), body mass index (BMI), and age. Our results suggest that phthalate esters may have an etiological association with PCOS.

Key words: Polycystic ovarian syndrome, phthalate esters, endocrine disrupting chemicals.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disease in women. It was first reported in 1935 by Stein and Leventhal, and is also known as Stein-Leventhal syndrome. PCOS has been a hot topic in the field of gynecology and reproductive endocrinology and proposes a challenge to clinical physicians (Chen, 2007). Despite extensive studies of PCOS have been conducted, the exact pathogenesis of PCOS remains unclear. Phthalate esters are a type of fat-soluble compounds and have been regarded as the major environmental estrogens, belonging to the endocrine disrupting chemicals (EDCs). A large number of studies have demonstrated that phthalate esters have reproductive and developmental toxicity and teratogenicity.

But most of these studies are confined to animal experiments and *in vitro* researches, while epidemiological surveys of the affected population, particularly those examining the effects of phthalate esters on women are limited. In this study, we determined the serum levels of three phthalate esters in women to explore the association between serum phthalate esters and PCOS and provide evidence for studies on the pathogenesis of PCOS.

MATERIALS AND METHODS

Subjects

A total of 18 patients with PCOS (PCOS group) and 16 patients without PCOS or laparoscopy-proven endometriosis but with infertility associated with tubal defects or pelvic adhesions (control group) were recruited into the present. The diagnosis of PCOS was based on the criteria from 2003 Rotterdam conference: (1) oligo- or anovulation, (2) clinical signs of hyperandrogenism and/or hyperandrogenism, (3) polycystic ovaries: the number of follicles with a diameter of 2-9 mm in one or both ovaries ≥ 12, and/or ovarian volume≥ 10 ml; (4) inclusion of 2 of 3 above criteria and

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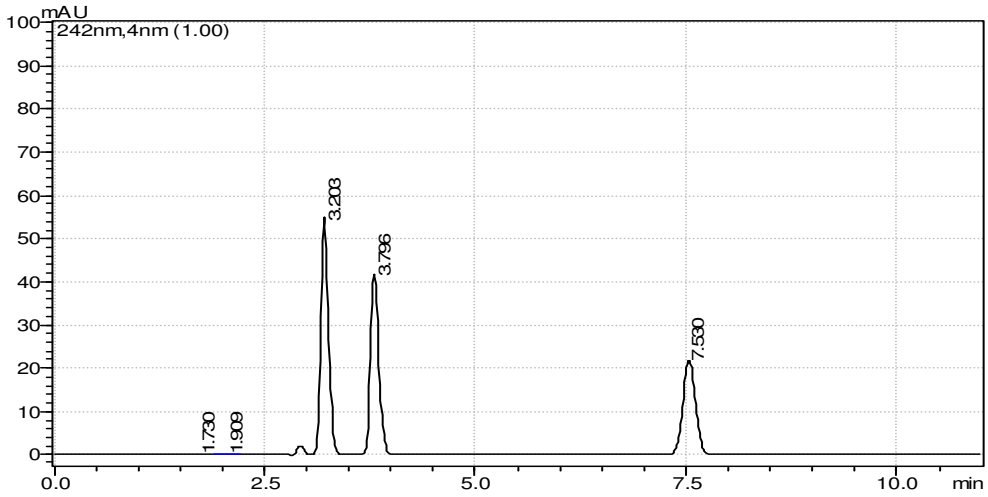

Figure 1. Chromatographic separation effect and retention time of the three mixes DEP, DBP, and DEHP.

exclusion of other causes of hyperandrogenism: congenital adrenocortical hyperplasia, Cushing syndrome, androgen-secreting tumors, as well as other diseases leading to ovulation disorders such as hyperprolactinemia, premature ovarian failure and pituitary or hypothalamic amenorrhea, and abnormal thyroid function. All patients in this study did not take hormones in latest three months, and signed the consent before study.

Collection of serum

From all subjects, 5 ml of fasting venous blood were collected and centrifuged at 2000 r/min for 10 min, followed by collection of the supernatant.

Experimental equipment

Main instruments

Shimadzu LC-2010A high performance liquid chromatograph, LD4-2 centrifuge, silicon-controlled thermostatic water bath, electronic balance, QT-1 vortex oscillator.

Main reagents

DEP (analytical pure) was purchased from Union Pharmaceutical Co., Ltd (batch number 8254A), and DBP (chromatographically pure), DEHP (analytical pure), methanol (HPLC grade, purity ≥ 99.9%) and n-hexane (analytical grade) were purchased from Guangzhou Chemical Reagent Factory (batch number: 20090301-2). Anhydrous sodium sulfate was from Tianjin Chemical Reagent Factory (batch number: 080620).

Methods

Sample preparation

First, 2.5 ml of n-hexane were added to 1 ml of serum and the resultant mixture was vibrated on a vortex oscillator for 5~10 min followed by centrifugation at 2000 r/min for 5 min (centrifuge radius 15 cm). The upper n-hexane was transferred into a test tube, and dehydrated with anhydrous Na_2SO_4 twice. The samples were evaporated to dryness in water bath (70°C), and the resultant products were dissolved in 300 µl of methanol for determination.

Chromatography

For qualitative analysis, methanol was mixed with water at a ratio of 95:5, which was used as the mobile phase and the separation effect of DEP, DBP, and DEHP was determined. The chromatographic separation effect and retention time of the mix are presented in Figure 1.

Preparation of single standard series

Then, 100 mg of each standard of DEP, DBP, and DEHP were dissolved in methanol at a final concentration of 1 mg/ml (100.0 ml) as stock solution. The single-standard stock solutions were sealed and preserved in a refrigerator.

Delineation of standard curves

Standard solutions of various concentrations of each standard (1.0, 3.0, 5. 0, 7.0 and 10.0 µg/ml) were prepared. Then 10 µl of each working solution was loaded and each working concentration was assayed in triplicates to construct the concentration-peak height standard curve of each standard (Figures 2 - 4). The regression equations of three standard curves were present below. The linear range of the method was 1.0~10 µg/ml. The lower detection limits were all:

DEP: Y = 49713.8X + 3190.3; r = 0.9998
DBP: Y = 39911.2X + 691.7; r = 0.9999
DEHP Y =30402.7 X + 1155.4; r = 0.9999

Sample determination

The processed samples were analyzed by HPLC. Data were qualified using retention time and quantified using peak height. The

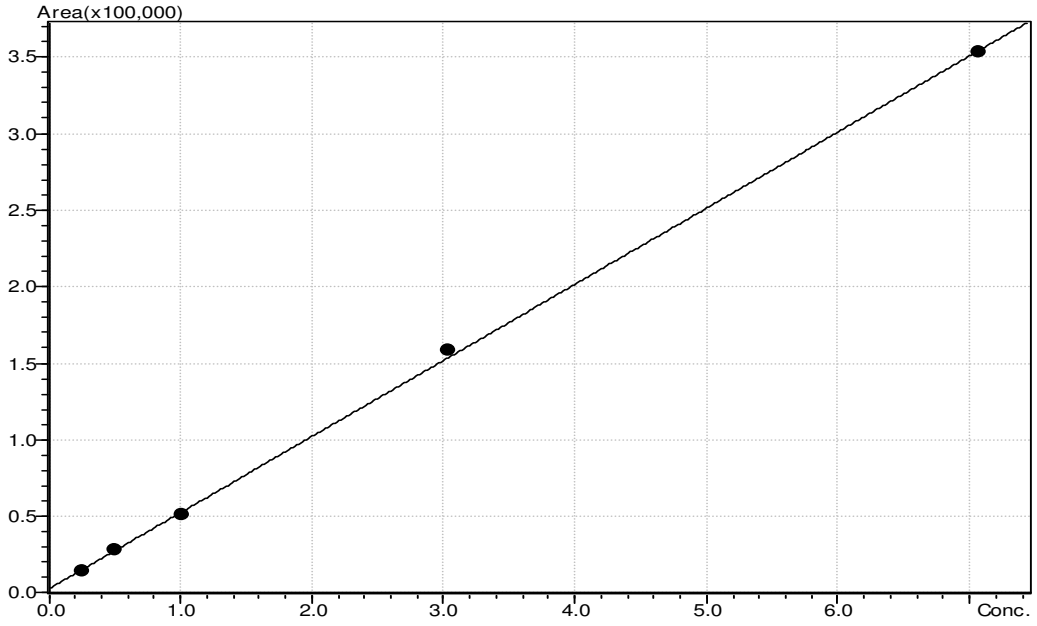

Figure 2. Standard curve of DEP.

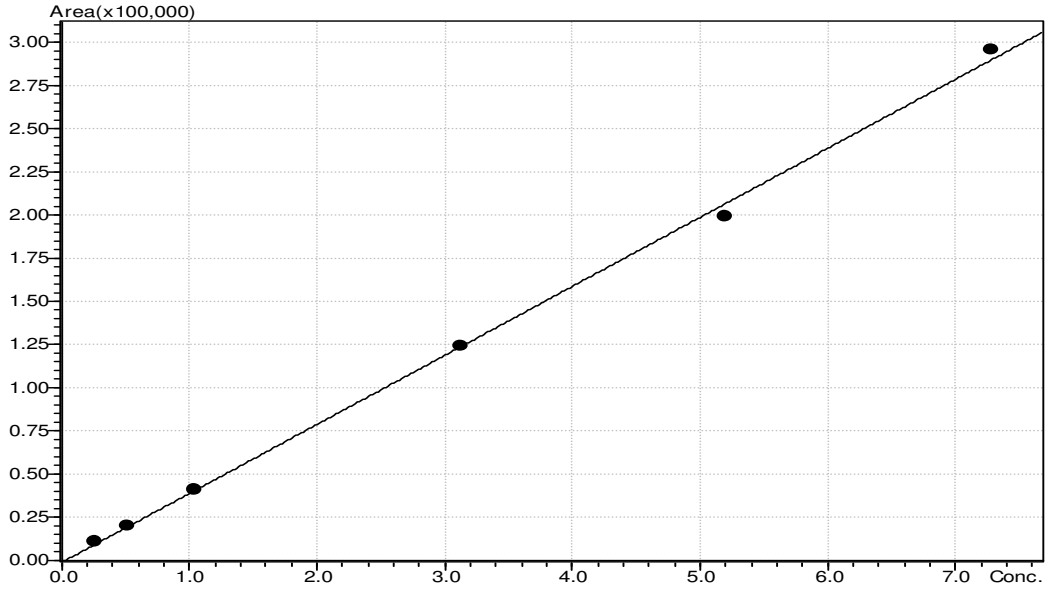

Figure 3. Standard curve of DBP.

levels of three phthalate esters were determined by using the standard curve method.

Statistical analysis

Data are presented as mean ± standard deviation ($\bar{X}$±S). Comparisons of age, BMI, and levels of DEP, DBP, and DEHP between the two groups were performed using t test. Correlations between DEP, DBP or DEHP and E2, T, FSH, LH, PRL, BMI and age were evaluated by using Spearman correlation analysis. All statistical analyses were done with SPSS. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Age and BMI at baseline between two groups

There were no statistical differences in age and BMI between the two groups (Table 1).

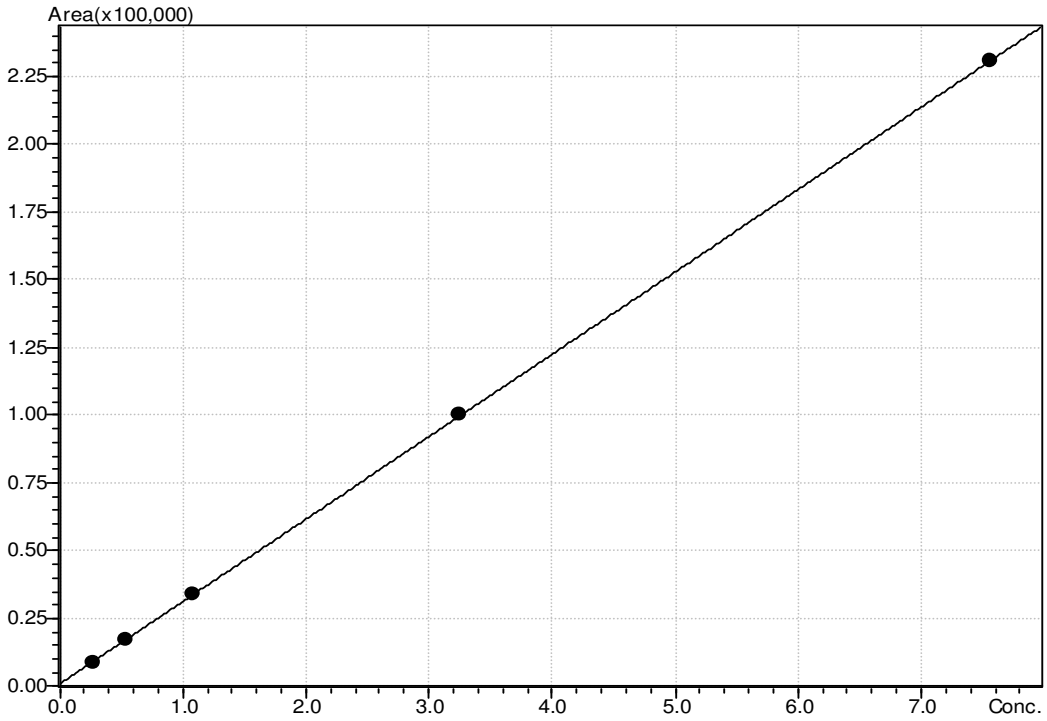

Figure 4. Standard curve of DEHP.

Table 1. Age and BMI of subjects in two groups (X±S).

Group	Age (year)	BMI (kg/m^2)
PCOS	25±3.18	19.79±2.48
Control	27.47±3.78	20.40±2.41
P value	0.052	0.517

* P<0.05.

Table 2. Levels of DEP, DBP, and DEHP in two groups (X±S).

Group	DEP (µg/mL)	DBP (µg/mL)	DEHP (µg/mL)
PCOS	0.45 ±0.24	0.53±0.15	0.41±0.12
Control	0.26±0.10	0.41±0.14	0.40±0.11
P value	0.008*	0.027*	0.853

* P<0.05.

Levels of DEP, DBP, and DEHP in two groups

The data were in a normal distribution and are expressed as mean difference± standard deviation ($\bar{X}$±S) (see Table 2). Our results showed the levels of DEP and DBP in PCOS group were markedly higher than those in control group (P<0.05), but no remarkable difference was noted in DEHP between these two groups. Moreover, DBP, DEP, and DEHP declined in sequence in PCOS group.

Correlations of DEP, DBP and DEHP levels with E2, T, FSH, LH, PRL, BMI and age

Spearman correlation analysis showed that there were no significant correlation between the levels of DEP, DBP and DEHP and E2, T, FSH, LH, PRL, BMI and age. Results are presented in Table 3.

DISCUSSION

PCOS is the most common endocrine disease in women characterized by persistent anovulation, androgen excess and insulin resistance. Its incidence has been estimated at 5-10% (Ehrmann, 2005). The prevalence of PCOS in the infertile Chinese women was reported to be between 30 and 40%. However, there are few reports of PCOS prevalence in affected groups in China. In a study investigating the prevalence of PCOS among women of child-bearing age, Chen and his colleagues from Shandong University revealed that the prevalence of PCOS was 6.46% in Jinan and 7.12% in Yantai city (Chen, 2007). A lot of studies on PCOS have been conducted out of China. Nevertheless, the exact pathogenesis of PCOS remains unclear. It has been postulated that the interaction between some genes and environmental factors may be partly responsible for the onset of PCOS.

Phthalate esters are a class of fat-soluble compounds and have been regarded as the major environmental

Table 3. Correlations between levels of DEP, DBP and DEHP and E2, T, FSH, LH, RPL, BMI and age in PCOS group.

Group		E2 (pg/ml)	T (ng/ml)	FSH (IU/L)	LH (IU/L)	PRL (ng/ml)	BMI (kg/m^2)	Age
DEP	CC	-0.05	-0.359	0.093	0.379	-0.418	-0.157	-0.06
	P	0.859	0.207	0.722	0.133	0.201	0.576	0.734
DBP	CC	-0.019	-0.410	-0.345	0.297	0.114	0.408	-0.239
	P	0.947	0.146	0.176	0.247	0.739	0.132	0.173
DEHP	CC	0.081	-0.057	0.051	0.074	0.373	-0.218	0.151
	P	0.775	0.846	0.844	0.779	0.259	0.435	0.395

Note: *P<0.05; CC, correlation coefficient.

estrogens. They are used primarily as plasticizers to improve the processibility, plasticity, flexibility, and stretchability of plastics. Because of low cost and good performance, phthalate esters have become the most widely used plasticizers in the plastics industry, accounting for 80% of the total amount of plasticizers consumed annually, with millions of tons of phthalate esters consumed each year globally. Even more, this figure is still on the rise (Li et al., 2006). This pervasive use of phthalate esters is illustrated by the fact that they can be found in children' toys, packaging of household chemicals such as soaps, shampoos and perfumes, and medical devices like test tubes, catheters and infusion equipment. Phthalate esters, when used as plasticizers, can bind to plastic molecules via hydrogen bonding or van der Waals force. For this reason, phthalate esters are extremely liable to be released during use when coming into contact with water or organic solvents, thereby causing environmental pollution (Zheng et al., 2006). What is more disturbing is that they can enter the human body by eating, breathing, drinking or direct skin contact before being rapidly metabolized in the body. Since phthalate esters are widely present in cosmetics like nail polish, women seem to be at higher risk for intake of such substances.

DEP, DBP, and DEHP are the three types of phthalate esters most commonly used at present. Phthalate esters are endowed with aromatic groups in the structure and are estrogen analogues, having estrogen-like effects (Sonnenschein and Soto, 1998). Sustained high level of estrogen in the body will sensentize the pituitary to GnRH secrected by the hypothalamus, leading to elevated LH level; meanwhile, presistant estrogen stimulus will have an inhibitory effect on pituitary FSH. Both events will contribute to the development of a pathological endocrine enviroment in the pituitary of PCOS individuals (Li, 2001). In the present study, the levels of DEP and DBP in PCOS group were significantly higher than those in control group (P<0.05). This result suggests the possibility that DEP and DBP may affect humans in a similar manner and thus lead to PCOS. However, more experiments and trials are needed to ascertain it. Previously, animal models of PCOS were successfully established by using estradiol valerate (Brawer et al., 1978, 1986), but no studies have yet reported whether there are insulin resistance or other metabolic disorders in these animal models. Estradiol valerate is a valerate of the natually occurring estradiol and has the pharmocological properties of estradiol.

It has been found that DEHP can lower the serum estrogen levels, prolong the sexual cycle,and suppress the ovulation in Sprague Dawley rats with normal reproductive cycle when animals are fed with DEHP at a dose of 2 g/kg (Davis et al., 1994). This may be partly associated with PCOS. However, DEHP contents in the PCOS women differ insignificantly from those in controls in the current study. The dose of DEHP administered to animals in the above study reached as high as 2 g/kg whereas the amount of DEHP intake by humans in the normal life is by no means anywhere near that level.

Our results also showed that the levels of DEP, DBP, and DEHP in PCOS group had no significant correlation with E2, T, FSH, LH, PRL, BMI, or age. In contrast, Huang et al. (2007) found that serum DBP level was positively correlated with E2 (r=0.410) while DEP negatively with T (r≈0.588) in women. This discrepancy may be due to the small number of subjects in our present study. Phthalate esters are known to be rapidly metabolized in the human body. Within 24 h, about 67.0% (64.6-70.5%) of DEHP can be metabolized into five products, which are excreted along with urine (Koch et al., 2006). The biological half-life of DBP in the body is less than 3 h (Albro, 1986). Therefore, measurement of these three phthalate esters in this study only mirrored the level of short-term exposure whereas measurement of concerning hormones represents the long-term accumulated exposure. It is unlikely to draw an accurate conclusion simply according to a single cross-sectional study. A long-term, well-designed time series study is warranted to validate this.

In addition, our results revealed that DEP, DBP, and DEHP were detectable in both groups. This proves the serous environmental pollution demonstrated by presence of phthalate esters, which is significantly

affecting our society at large and calls for imperative action. In terms of contents, DBP was highest, followed sequencially by DEP and DEHP in the PCOS patients; whereas in controls, DBP, DEHP, and DEP declined in sequence. Although DEHP is the most widely used phthalate ester, its content is lower than that of DBP. Hence, it seems that DBP is well worthy of investigation. Future studies are needed to better understand the mechanism of correlation between environmental factors and PCOS.

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