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Original Article

Phthalate ester concentrations in blood serum, urine and endometrial tissues of Chinese endometriosis patients

Jing Sun[1], Bo Chen[2], Liqian Zhang[2], Dong Zhao[1], Shu Guang Li[2]

[1]Department of Gynecology, Shanghai First Maternity and Infant Hospital of Tongji University, 536 Changlel Road, Shanghai 200040, China; [2]Key Laboratory of Public Health Safety of Ministry of Education, School of Public Health, Fudan University, 130 Dongan Road, Shanghai 200032, China

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Abstract: Objective: To explore differences of phthalic acid ester (PAE) concentrations in serum and urine as well as endometrial tissues in patients diagnosed with endometriosis (EM) compared to healthy controls in Shanghai. Patients and methods: We designed a single-center case-control study based on the measurement of PAE concentrations in serum and urine of patients and controls as well as in pathological tissues from EM patients which were measured using either high performance liquid and/or gas chromatography. A total of 289 female subjects were included in the study (115 cases diagnosed with EM and 174 healthy women as controls). Results: Positive detection rates of Dibutyl phthalate (DBP) and Di (2-ethylhexyl) phthalate (DEHP) were > 90% in both measured groups for all measurements, but for diethyl phthalate (DEP) the range was from 0-16.4%. The serum DBP and DEHP concentrations in patients with EM were significantly higher than in healthy women ($P < 0.05$). The urine concentration of primary DEHP metabolites (∑DEHP) was also higher in EM patients ($P < 0.05$). In patients diagnosed with EM, DBP and DEHP concentrations in pathological tissues were 4 and 14.4 times higher respectively, as those in serum. Conclusion: Significantly enhanced blood serum DBP and DEHP concentrations and significant increases of their primary metabolites in urine of EM patients compared to the controls indicated that PAE affected EM. In addition, the high concentration of DBP and DEHP in resected endometrial tissues of EM patients supported this finding.

Keywords: Endometriosis, phthalic acid ester, DEHP, DBP, DEHP metabolite

Introduction

Phthalate acid esters (PAE) or phthalate esters (PE) are used as plasticizers, which are additives that increase the flexibility, workability and dispensability of plastics or elastomers. The most common use of PE is to increase the flexibility of polyvinyl chloride (PVC) [1]. Because PAE are not chemically bonded to the surface structure, they can be easily released from the surface, particularly in PVC containing materials [2].

In recent years, studies have been conducted to evaluate the amount of PAE migration from food and beverages into the foodstuff packaging [3-5], but other potential sources of PAE uptake in humans, ranging from cosmetics, personal care products, home furnishings, toys, pharmaceuticals, nutritional supplements, insecticides to medical instruments have also been reported [6, 7]. Although the lethal dose (LD) of phthalic acid is relatively high (7,900 mg/kg in rats [8], PAE are categorized as endocrine disrupters including estrogenic, anti-androgenic and anti-thyroid effects [9-11], working in μM dose ranges. Due to the extensive use of PAE and their ability to diffuse through the placenta and breast milk, the majority of modern humans are exposed to PAE from as early as the prenatal period [12, 13]. Since children experience significantly higher daily exposure to phthalates in relation to their body weight than adults, the presence of PAE in toys has been raised as a potential health risk [14, 15].

It has been shown by epidemiological studies that PAE affect female reproduction to various extents, including correlations with precocious puberty, EM, infertility, menoxenia, and a short-

Table 1. Basic information about the patients and healthy controls

Basic information	Total		Control		Case		*P*-value
	n	%	*n*	%	*n*	%	
Age (years)							0.116
≤ 35	223	77.2	140	80.5	83	72.2	
> 35	66	22.8	34	19.5	32	27.8	
BMI (kg/m^2)							1.000
≤ 22.9	249	86.2	150	86.2	99	86.1	
> 22.9	40	13.8	24	13.8	16	13.9	
Education							0.001
College and above	199	68.9	96	55.2	103	89.6	
High school and below	90	31.1	78	44.8	12	10.4	
Smoker							0.161
No	284	98.3	169	97.1	115	100	
Yes	5	1.7	5	2.9	0	0	
Alcohol consumption							0.070
No	277	95.8	170	97.7	107	93.0	
Yes	12	4.2	4	2.3	8	7.0	
Feeling pressure							0.329
No	119	41.2	76	43.7	43	37.4	
Yes	170	58.8	98	56.3	72	62.6	
Daily schedule							0.762
Regular	233	80.6	139	79.9	94	81.7	
Irregular	56	19.4	35	20.1	21	18.3	
Menarche (year)							0.128
≤ 14	217	75.1	125	71.8	92	80.0	
> 14	72	24.9	49	28.2	23	20.0	
Menstrual status							1.000
Regular	243	84.1	146	83.9	97	84.3	
Irregular	46	15.9	28	26.1	18	15.7	
Reproductive history							0.037
No	174	60.2	96	55.2	78	67.8	
Yes	115	39.8	78	44.8	37	32.2	
Abortion history							1.000
No	249	84.1	146	83.9	97	84.3	
Yes	46	15.9	28	16.1	18	15.7	
Uses contraception							0.007
No	251	86.9	159	91.4	92	80.0	
Yes	38	13.1	15	8.6	23	20.0	
Family history of gynecological diseases							0.001
No	246	85.1	158	90.8	88	76.5	
Yes	43	14.9	16	9.2	27	23.5	

ening pregnancy. However, disagreements still exist between the findings of several published studies [16-21].

EM is a clinical condition characterized by endometrial tissues appearing in other parts of the body than the corpus uteri. Although EM is usually referred to as a benign lesion, it has been categorized as a neoplastic process [22]. The most common locations for EM to occur are visceral organs and the peritoneum, but implantation in the ovary is also a common finding. In this case, the endometrial cyst of the ovary is termed a “chocolate cyst” [23]. In

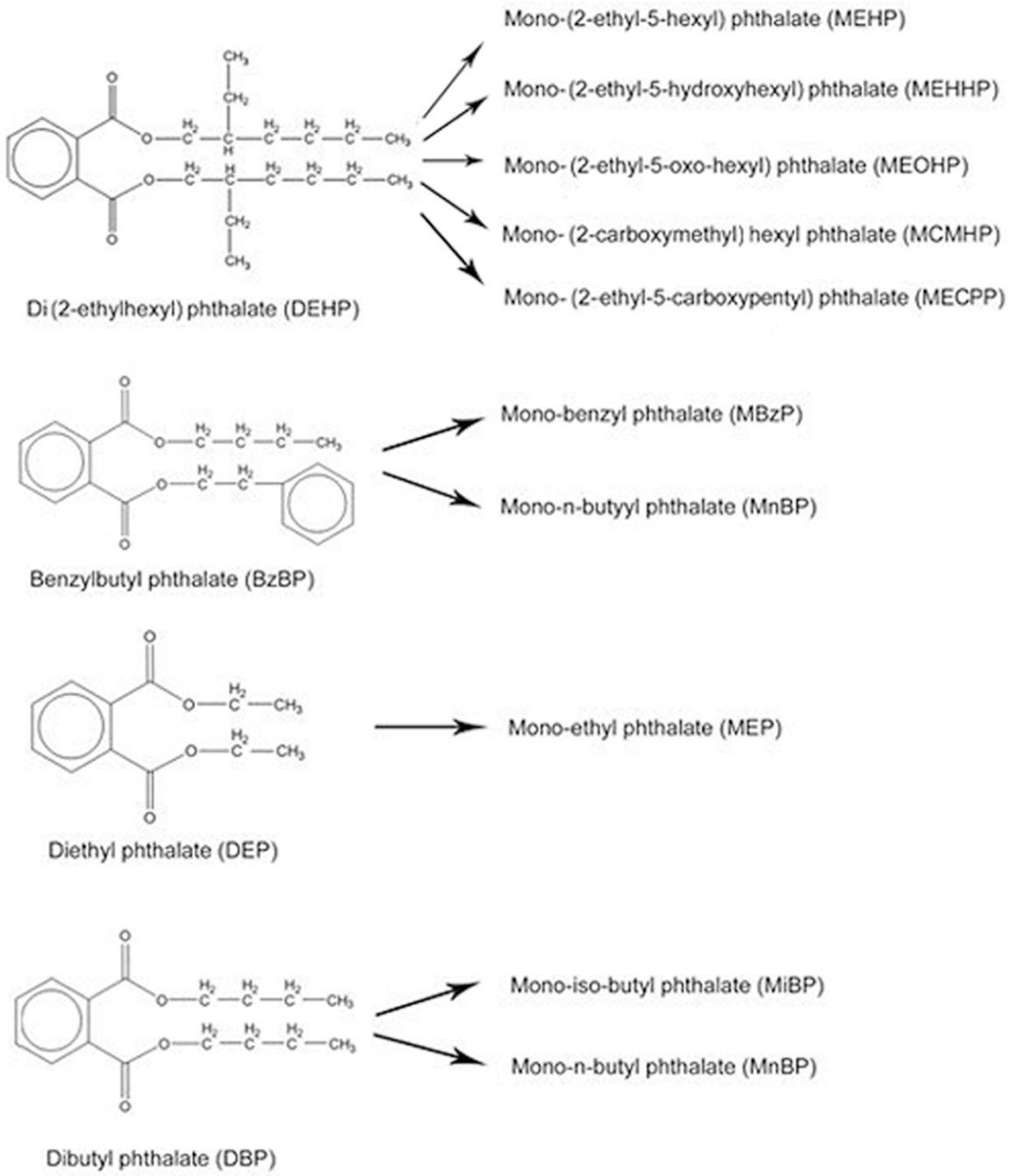

Figure 1. PAE analyzed in blood, urine and endometrial tissues of EM patients and controls. The compounds are shown on the left side of the figure and on the right their primary metabolites.

recent years, more and more females of childbearing age have been diagnosed with EM, with an incidence rate between 5 and 15% being most prevalent in females aged between 25 to 45 years of age [24].

Several studies suggested correlations between EM and multiple environmental factors [25, 26] and PAE serum concentrations have been widely used as exposure markers [27-29]. Cobellis et al. analyzed the serum and peritoneal fluid from 35 Italian women diagnosed with EM by High Performance Liquid Chromatography (HPLC) and found that the DEHP concentration in the sera of EM patients was significantly higher than that in the control

group. The authors suggested a potential role of PAE in the pathogenesis of EM [17]. Reddy at al. validated the risk of PAE in promoting the pathogenesis of EM based on their research in India. They found similar results when comparing PAE concentrations in the serum of patients and healthy controls. Additionally, they further demonstrated that the serum level of PAE was positively correlated with the severity of EM [30]. In contrast, a Japanese study of 166 infertile women diagnosed with EM failed to produce the same results when they analyzed urine samples [31]. Despite the extensive use of PAE-related materials in China, limited research has been conducted about correlations between PAE and EM and it remains a concern whether urine or serum PAE metabolite concentrations are directly correlated with the pathogenesis of EM.

In the present study, serum and urine samples as well as endometrial tissues were collected from outpatients diagnosed with EM and the PAE concentrations analyzed. Combined with multiple clinical indices, the effects of exposure to PAE on the pathogenesis of EM were evaluated.

Study population and methods

Study population

The study was approved by the ethical committee of the Hospital and written informed consent was obtained from all participants. A total of 134 EM patients admitted to Shanghai First Maternity and Infant Hospital for surgery were included in the study from September 2011 to September 2012 and their diagnoses were confirmed by postoperative pathological reports. Another 176 healthy random volunteers were recruited as the control group once diagnoses of related gynecological diseases were excluded for all these participants. Two trained physicians from the hospital conducted the survey and collected the required information, including baseline characteristics and each women's personal health history (**Table 1**).

PAE analyses

Measured PAEs are listed in **Figure 1**.

For all PAEs, standard solutions were created: 0.5 μg/L, 1.0 μg/L, 5.0 μg/L, 10.0 μg/L and 20.0 μg/L for blood as well as 5 ng/mL, 10 ng/mL, 20 ng/mL, 100 ng/mL, 200 ng/mL and 500 ng/mL for urine sample analyses (Sigma-Aldrich, Shanghai, China). Sample dilutions were measured 5 times and linear-regression analyses performed to determine peak areas vs the concentration of each standard solution. Correlation coefficients were > 0.996.

Blood serum tests

3 mL blood was collected from 134 EM patients and 176 healthy volunteers and centrifuged at 4,000 rpm for 10 min. Then 3 mL hexyl hydride was added to 1 mL serum and the mixture was again centrifuged at 4,000 rpm for 10 min. 5 mL supernatant from 2 hexyl hydride mixtures were prepared for gas chromatographic analysis and 1 mL serum hexyl hydride mixture aliquots were processed with a microfiltration membrane and a nitrogen blowing concentrator at 40°C (ANPEL DC12). Finally, gas chromatography (SHIMADZU GC2010 chromatograph) was used to analyze the samples with a pure sample acting as the control.

Processing of urine samples

20 mL morning urine samples from 133 EM patients and 158 controls were collected and stored at -40°C. 1 mL of thawed urine sample was transferred into a 10 mL glass tube and mixed with 20 μL β-glucuronidase (*Helix pomatia* > 2,000 Units, Sigma, Shanghai, China) solution and 2 mL ammonium acetate buffer solution (pH = 5). The glass tube was heated in water bath at 37°C for 3 hours after which 2 mL of 5% ammonium hydroxide was added to alkalify the reaction system. After that the samples were processed using a Solid Phase Extraction Column (SPE) (12 tubes-solid phase extraction apparatus, Supelco, USA). The urine samples passed through the SPE columns (previously activated with 5 mL methanol and 5 mL H_2O) at a rate of 2 mL/min and were then washed with 5 mL methanol, 5 mL H_2O and 5 mL H_2O solution containing 2% formic acid and 20% methanol. The extraction apparatus was subjected to a vacuum until the air pressure dropped to 0.05 Mpa, and then blow-dried. Subsequently, 5 mL of methanol containing 4% formic acid was used to wash out the sample remnants. The eluent solution was blow-dried in a 40°C water bath with gentle nitrogen flow (ANPEL DC12 nitrogen blowing concentrator). 0.5 mL solu-

tion, prepared by dissolving the solute in acetonitrile/water (10/90, V/V), was then mixed for 30 s (vortex mixer), centrifuged for 10 min (4°C, 10,000 r/min), and the supernatant liquid was analyzed by UPLC-Q-TOF-MS (ultra-high performance liquid chromatography-Quadrupole tandem time-of-flight mass spectrometry, Waters, USA). According to the chromatographic behaviors and corresponding qualitative analysis, the test conditions were adjusted. The recovery rate and degree of precision (error value) were then calculated based on the standard curve, in order to determine the analysis conditions of the chromatography and mass spectrometry.

Detection of sex hormones in serum

Serum samples from EM patients were collected and analyzed by the endocrine laboratory in the Shanghai First Maternity and Infant Hospital. The levels of testosterone (T), estradiol (E_2), follicle-stimulating hormone (FSH), progesterone (P), luteinizing hormone (LH) and prolactin (PRL) were quantified by direct chemiluminescent image analysis using a commercial kit (Siemens medical diagnostic products co., LTD). According to the kit introductions and the laboratory conditions, the effective measuring ranges for the 6 sex hormones (*vide supra*) were: (T) 0.01-1.5 ng/mL, (E_2) 11.8-3,000 pg/mL, (FSH) 0.3-200 IU/L, (P) 0.21-600 ng/mL, (LH) 0.07-200 IU/L, (PRL) 0.3-200 ng/mL.

Measurement of urine creatinine (deproteinization)

The amounts of PAE metabolites measured directly could have been affected by an individual's urine output, so they were calibrated by the concentration of urinary creatinine in order to improve interpersonal comparability. Urinary creatinine levels were quantified using a creatinine kit (deproteinization) (Nanjing Jiancheng Technology Co., Ltd) as follows: urinary creatinine (g/L) = [(absorbance of the testing tube - absorbance of the blank tube) ÷ (absorbance of the standard tube - absorbance of the blank tube)] × concentration of the standard tube (50 μmol/L) × 201 × 113.12 g/mol ÷ 1000,000 μg/g; post-calibrated concentration of the PAE metabolites = pre-calibrated concentration of the PAE metabolites (ng/mL) ÷ urinary creatinine (g/L)

Collection and preservation of tissue samples

Pathological tissue samples were transferred into clean glass tubes immediately after resection, and normal saline was added to each tube to maintain the tissue osmotic pressure. After surgery, tissues were weighed, wrapped in aluminum foil, labeled and stored at -80°C.

EM tissue samples

1 g pathological specimen was cut into pieces and transferred into a tissue homogenate cup together with 1 mL of normal saline. The mixture was fully homogenized using a high-speed tissue homogenizer at 20,000 r/min. The homogenate was then transferred into a 10 mL glass centrifuge tube and 2 mL of normal saline plus 3 mL of hexane added and then the mixture blended by vortexing for 2 min and by ultrasound for 20 min. The tube was centrifuged for 10 min at 4,000 rpm and the supernatant liquid was removed and the above procedures repeated twice more. The final supernatant was collected and concentrated to 1 mL by nitrogen blowing at 40°C, and chromatographically purified with neutral alumina column chromatography.

The chromatographic column, filled with 20 g neutral alumina and 3 g anhydrous sodium sulfate, was balanced with normal hexane and a total of 1 mL of concentrated supernatant loaded. The eluent solution containing methylene chloride/ethyl acetate (3:2 ratio) was injected slowly. The elution flow rate was set at 2-3 mL/min and 30 mL of eluent solution was collected and concentrated into 5 mL by nitrogen blowing, and then dried in a 10 mL glass centrifuge tube by further nitrogen blowing at 40°C. Finally, the product was dissolved in 200 μL normal hexane and the contents measured using gas chromatography. The peak areas of DEP, DBP and DEHP in different standard solutions were recorded with the injection volume set at 1 μL. The mean peak areas for the plasticizers were calculated in order to plot a working curve. The area of the blank control was subtracted from the peak area of each plasticizer, for which the regression equation was generated against the concentrations of the standard solutions in each sample. According to the condensation ratio of the eluent solution, the lowest detection threshold was then deduced

Table 2. Concentrations of PAE and PAE metabolites in serum (mg/L), urine (ng/mL) and resected pathological endometrium tissues (μg/gr)

	Control				Patients			
Serum	N	Detection rate	GM	Median	N	Detection rate	GM	Median
DEP	176	26 (14.8%)	0.060	N/A	134	22 (16.4%)	0.064	N/A
DBP	176	163 (92.6%)	0.171	0.19	134	132 (98.5%)*	0.304##	0.35
DEHP	176	163 (92.6%)	0.163	0.16	134	127 (94.8%)	0.200##	0.21
T (ng/mL)					106		0.45	0.51
E2 (pg/mL)					106		107.2	51.80
FSH (IU/L)					98		5.67	6.17
P (ng/mL)					106		0.795	0.74
LH (IU/L)					106		6.26	6.43
PRL (ng/mL)					105		14.025	16.16
Urine								
MMP	158		19.08	17.17	133		19.71	17.51
MEP	158		11.79	11.11	133		6.89**	6.96
MiBP	158		20.98	21.99	133		9.53**	10.37
MnBP	158		19.18	20.64	133		9.08**	9.67
MEHP	158		4.45	4.32	133		36.26**	36.61
MEOHP	158		2.29	4.63	133		0.96**	2.26
MEHHP	158		7.09	7.19	133		5.32*	4.43
MECPP	158		9.40	9.20	133		7.56	6.43
MCMHP	158		7.35	16.01	133		10.23	17.36
∑DEHP	158		30.58	41.35	133		60.33**	67.09
Pathological tissue								
DEP	N/A	N/A	N/A	N/A	130	0 (0%)	0.050	-
DBP	N/A	N/A	N/A	N/A	130	117 (90.0%)	0.686	0.72
DEHP	N/A	N/A	N/A	N/A	130	129 (99.2%)	2.351	2.57

∑DEHP=MEHP+MEHHP+MEOHP+MECPP+MCMHP; Mono-(2-ethyl-5-hexyl) phthalate (MEHP); Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); Mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP); Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP); Mono-(2-carboxymethyl)hexyl phthalate (MCMHP); Mono-benzyl phthalate(MBzP); Mono-ethyl phthalate (MEP); Mono-iso-butyl phthalate (MiBP); Mono-n-butyl phthalate (MnBP); Dibutyl phthalate (DBP); Diethyl phthalate (DEP); Diheptyl phthalate (DHEP); testosterone (T); estradiol (E2); follicle-stimulating hormone (FSH); progesterone (P); luteinizing hormone (LH); prolactin (PRL); geometrical mean (GM); $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control with Pearson chi-square test; $^{\#\#}P < 0.01$ compared to control with t-test.

(0.1 μg/g). It was confirmed that the linear correlations were satisfactory for DEP, DBP and DEHP when the concentrations fell in the range between 0.5 μg/L and 100.0 μg/L, and the correlation coefficient for each was > 0.996. Contamination from extra amino acids, polypeptides and proteins in the pathological tissues could be removed using this approach, in order to ensure a lower background noise and higher column efficiency in the separation of chromatographic peaks.

Statistical analysis

All analyses were performed with SPSS for Windows (Version 16.0. Chicago, SPSS Inc.).

The Pearson chi-squared test was used to compare the detection rates in different groups; neither the amounts of PAE in the serum samples or the concentrations of the post-calibrated PAE metabolites in the urine samples were normally distributed. A *t*-test was performed for each of them based on the geometrical mean values (GM), after the raw results were transformed with natural logarithms; the measurements of PAE in the pathological tissues obeyed the skewed distribution and the GM values were analyzed after the natural logarithm transformation, instead of arithmetic mean (AM) values. A nonparametric Spearman correlation analysis was performed for all the correlation analyses of the continuous data of abnormal

Table 3. Levels of PAE in the serum and correlations with various baseline characteristics

		Serum DEP	Serum DBP	Serum DEHP
	N	Calibrated GM[b]	Calibrated GM[b]	Calibrated GM[b]
Group				
Control	174	0.062	0.167	0.165
Patients	115	0.059	0.327**	0.192*
Age (years)				
≤ 35	223	0.062	0.217	0.178
> 35	66	0.057	0.222	0.168
BMI (kg/m^2)				
≤ 22.9	249	0.062	0.217	0.175
> 22.9	40	0.057	0.226	0.179
Education				
High school and below	90	0.051	0.229	0.159
College and above	199	0.066**	0.214	0.183
Smoker				
No	284	0.061	0.218	0.175
Yes	5	0.053	0.209	0.205
Alcohol consumption				
No	177	0.061	0.220	0.174
Yes	12	0.057	0.194	0.210
Feeling pressure				
No	119	0.065	0.218	0.177
Yes	170	0.059	0.218	0.174
Daily schedules				
Regular	233	0.061	0.221	0.173
Irregular	56	0.062	0.209	0.184
Menarche (year)				
≤ 14	217	0.060	0.217	0.176
> 14	72	0.064	0.224	0.173
Menstrual status				
Regular	243	0.062	0.220	0.179
Irregular	46	0.059	0.210	0.160
Reproductive history				
No	174	0.061	0.220	0.180
Yes	115	0.061	0.217	0.168
Abortion history				
No	249	0.061	0.218	0.179
Yes	40	0.062	0.223	0.158
Uses contraceptives				
No	251	0.061	0.222	0.176
Yes	38	0.063	0.194	0.174
Family history of gynecological diseases				
No	246	0.060	0.219	0.174
Yes	43	0.068	0.214	0.187

Post-calibrated GM[b]: generalized linear models (GLM) were used to generate the post calibrated GM. When analyzing a certain factor, this single factor was set as a fixed variable and the other factors were regarded as concomitant variables (*$P < 0.05$, **$P < 0.01$).

distributions, including the analyses for the amounts of PAE metabolites in the urine, serum PAE, sex hormones or the analysis of PAE levels in pathological tissue. When analyzing correlat-

Table 4. Levels of PAE in urine and correlations with various baseline characteristics

	N	MMP (μg/g Cr)	MEP (μg/g Cr)	MnBP (μg/g Cr)	MiBP (μg/g Cr)	MEHP (μg/g Cr)	MEOHP (μg/g Cr)	MEHHP (μg/g Cr)	MECPP (μg/g Cr)	MCMHP (μg/g Cr)	∑DEHP (μg/g Cr)
Group											
Control	157	38.51	22.22	36.86	32.98	8.41	3.82	13.13	17.41	13.59	87.97
Patients	115	52.40#	19.53	30.42	30.20	110.72##	3.48	16.44	23.03	32.01#	250.64##
Age (years)											
≤ 35	206	41.89	21.33	34.43	31.44	25.43	3.77	14.50	20.05	16.93	139.63
> 35	66	50.70	20.15	32.56	32.82	23.69	3.39	14.25	18.30	30.48	129.15
BMI (kg/m²)											
≤ 22.9	232	42.78	20.29	33.18	31.88	25.20	3.78	14.60	19.89	18.80	137.41
> 22.9	40	50.75	26.02	39.02	31.19	23.88	3.09	13.53	18.03	24.22	134.42
Education											
High school and below	89	52.98	31.25	54.65	55.65	32.49	8.17	22.60	30.39	31.94	201.95
College and above	183	40.04	17.36##	26.98##	24.19##	22.00#	2.49##	11.61##	15.83##	15.36	113.41##
Smoker											
No	267	43.95	21.20	33.92	31.63	24.98	3.63	14.38	19.49	19.34	136.59
Yes	5	40.21	13.83	37.11	39.53	26.10	6.69	18.12	26.23	32.52	155.56
Alcohol consumption											
No	260	43.68	21.01	33.99	31.75	25.28	3.70	14.72	19.97	19.79	139.49
Yes	12	47.89	21.43	33.82	31.79	19.57	3.19	9.59	13.07	14.53	92.85
Feeling pressure											
No	108	45.47	21.87	29.46	32.20	22.83	4.33	15.86	20.49	21.59	142.31
Yes	164	42.86	20.51	37.30	31.50	26.55	3.29	13.57	19.03	18.27	133.62
Daily schedules											
Regular	217	44.30	20.47	32.52	31.25	23.97	3.19	13.41	18.45	19.69	132.29
Irregular	55	42.22	23.48	40.41	33.95	29.46	6.41	19.30#	24.93	18.86	156.80
Menarche (year)											
≤ 14	201	46.06	20.97	33.12	30.97	24.98	3.31	14.44	19.49	22.58	140.61
> 14	71	38.24	21.22	36.53	34.19	25.08	4.94	14.43	19.91	12.94	127.10
Menstrual status											
Regular	229	44.84	20.95	33.45	31.37	25.36	3.43	14.27	19.24	21.03	136.05
Irregular	43	39.06	21.56	36.89	33.95	23.20	5.30	15.39	21.59	13.14	142.17
Reproductive history											
No	157	47.04	21.59	36.23	33.58	23.78	3.70	14.34	19.55	15.82	133.89
Yes	115	39.88	20.31	31.12	29.46	26.79	3.64	14.57	19.67	26.02	141.17
Abortion history											
No	232	43.47	20.76	35.91	33.21	25.61	3.91	14.61	19.77	20.11	138.52
Yes	40	46.25	22.78	24.61	24.61	21.74	2.55	13.46	18.67	16.41	128.25
Uses contraceptives											
No	234	43.25	20.64	35.91	32.85	25.25	3.86	13.96	19.01	17.24	132.29
Yes	38	47.94	23.74	24.19	25.87	23.50	2.69	17.81	23.69	41.93	169.52
Family history of gynecological diseases											
No	229	43.73	20.80	34.78	33.08	24.22	3.14	13.59	18.60	18.47	132.56
Yes	43	44.70	22.31	30.02	25.61	29.58	8.41#	19.95	25.92	26.29	162.88

Note: All PAEs (μg/gCr) are shown as post-calibrated geometrical mean (GM), which was used for generalized linear models (GLM). (When analyzing one certain factor, it was set as a fixed variable and the other factors were regarded as concomitant variables). #$P < 0.05$, ##$P < 0.01$ compare to control or correspondinggroup.

ing factors for PAE serum, urine or pathological tissue concentrations, *t*-tests were performed based on the GM values. Furthermore, generalized linear models (GLM) were adopted in the analysis after correction for other factors

Results

Baseline characteristics of participants

There were no significant differences in baseline characteristics between the two groups,

Table 5. Correlation analysis of the levels of PAE metabolites (μg/g Cr) in urine and PAE (μg/mL) in serum

Metabolites	Serum DEP	Serum DBP	Serum DEHP
MMP			
r	-0.027	0.001	-0.013
P	0.649	0.988	0.823
MEP			
r	-0.035	-0.107	-0.087
P	0.556	0.068	0.137
MiBP			
r	-0.063	-0.173	-0.098
P	0.281	0.003	0.094
MnBP			
r	-0.141	-0.128	-0.130
P	0.016	0.029	0.026
MEHP			
r	-0.034	0.386	0.122
P	0.563	0.000	0.037
MEOHP			
r	-0.068	-0.118	-0.130
P	0.250	0.045	0.026
MEHHP			
r	-0.030	-0.048	-0.105
P	0.616	0.410	0.075
MECPP			
r	-0.056	-0.059	-0.102
P	0.343	0.313	0.084
MCMHP			
r	0.049	0.039	-0.009
P	0.406	0.504	0.872
∑*DEHP*			
r	-0.032	0.145	-0.105
P	0.589	0.014	0.803

∑DEHP = MEHP + MEHHP + MEOHP + MECPP + MCMHP.

including abortion history, menstrual status, menarche, alcohol consumption, feeling pressure and daily schedule. The ages of the research subjects ranged from 17 to 56 years and had a skewed distribution, with 35 years being set as the boundary of the age classification. The degree of obesity was determined based on BMI values, with 22.9 defined as the overweight cut-off according to Asian standards [32]. However, there were significant differences regarding reproductive history, contraceptives consumption and the family history of gynecological diseases between EM patients and the control group (**Table 1**). In addition, educational levels were also obviously different between the two groups ($P < 0.05$).

Concentration of PAE (DEP, DBP, DEHP) and PAE metabolites in serum, urine and pathological tissues

The GM values of serum concentrations of DBP and DEHP in the patient group were obviously higher than in the control group ($P < 0.05$) and the detection rate of serum DBP and DHEP were as high as 92.6-98.5%. In contrast, the detection rate of DEP was very low and ranged from 0-16.4%. The urine concentration of PAE metabolites (MEP, MiBP, MnB, MEOHP, MEHHP and MECPP) was significantly lower in the patient group, while the concentrations of MEHP and ∑DEHP were significantly increased. The detection rates of DBP and DEHP in pathological tissues were similar to serum but the concentrations were tissues were 4 and 14.4 times higher than in the serum respectively (**Table 2**). T, E2, FSH, P, LH and PRL serum concentrations in EM patients did not correlate with PAE concentrations, but there was a positive correlation between PAE levels in pathological tissues and serum levels of E2 and LH ($r = 0.216$, $P = 0.029$; $r = 0.210$, $P = 0.034$).

Comparison of the PAE metabolite concentrations in sera of EM and control subgroups

Concentrations of serum DEP and DEHP in EM patients were much higher than in the control group, and the concentrations of serum DEP were significantly affected by education, being lower in higher educated people (**Table 3**).

Comparison of PAE metabolite concentrations in urine samples of EM and control subgroups

In urine samples taken from patients, the concentrations of MMP, MEHP MCMHP and ∑DEHP were significantly higher than in the controls. Education significantly correlated with the concentrations of PAE metabolites in research subjects. In this study, the subjects in the subgroup "college and above" had much higher urine concentrations of PAE metabolites than the subgroup "high school and below", including MEP, MnBP, MiBP, MEHP, MEOHP, MEHHP, MECPP and ∑DEHP (**Table 4**).

We found that the concentration of serum DBP was negatively correlated with the concentrations of serum MiBP, MnBP and MEOHP ($P <$

0.05), and positively correlated with the concentration of MEHP and ∑DEHP ($P < 0.05$); the concentration of DEHP in serum was negatively correlated with the concentrations of MnBP and MEOHP ($P < 0.05$), and positively correlated with the concentration of MEHP ($P < 0.05$). The data shows that elevated DBP and DEHP concentrations in the serum of patients is related to a significant decrease in urine MnBP and MEOHP concentrations, and a significant increase in MEHP urine concentration (**Table 5**).

Discussion

The detection rate of DEP in EM patient samples was low, while the detection rate of DBP and DEHP were higher, especially for DEHP where the detection rate was close to 100% in endometrial tissue. The concentration of DEHP was also higher compared with DBP. This finding may be explained by the extensive usage of DEHP products in China, which is added into the production of polyvinyl chloride (PVC) materials as plasticizers. PVC is widely utilized in commercial products, including food packaging, floor and decoration materials, as well as in medical equipment, whereas DBP and DEP (lower molecular weights) are used as solvents or as the plasticizer of cellulose acetate. These chemicals are added in the production of paint, personal care supplies, and to pharmaceuticals in order to prolong the characteristics of drug release from formulations *in vivo*.

In the present study, we found that the serum levels of DBP and DEHP in EM patients were significantly higher than in the healthy control group ($P < 0.05$), and that the detection rate was > 90% in both groups. These findings suggest that DBP and DEHP serum concentrations are viable parameters to measure PAE contamination, which indeed was significantly higher in EM patients.

Our findings are in agreement with a previous study which reported enhanced DEHP serum concentrations in EM patients [17]. In the latter study, the peritoneal fluid of EM patients was found to contain higher levels of DEHP and its metabolite MEHP, which is in accord with our data about enhanced MEHP urine concentrations in EM compared to the controls (4.54 vs 36.26 mg/mL, $P < 0.01$) and the high DEHP concentrations in resected endometrial tissue. Our study revealed that various metabolites of PAE in urine can easily be detected by the UPLC-Q-TOF-MS method and that levels of MEHP and ∑DEHP in urine were significantly increased in EM patients. After uptake of PAE, they are converted by hydrolytic enzymes in the blood into monoesters [33], which are secreted into urine within hours of glucuronidation [34], accounting for the high DEHP serum and ∑DEHP urine concentrations in EM patients.

Huang et al. reported that the level of MnBP in 28 patients with EM was significantly higher than in the 29 subjects of the control group in Taiwan ($P < 0.05$), but he attributed the higher levels to a defect of glutathione S-transferase M1 [35]. Kim et al. examined the levels of serum DEHP and MEHP in 97 advanced-stage EM patients and 169 control subjects and found that both DEHP and its metabolite MEHP were significantly increased in patient serum [36], which was also supported by another study in which enhanced DEHP serum levels (beside other PAE) was significantly correlated with the degree of EM severity [30]. The 4 times and 14.4 times higher than serum DBP and DEHP concentrations in endometrial tissue suggest that these chemicals accumulate in this tissue, but as far as we are aware there is no reference for this finding in the published literature. Since both DBP and DEHP exhibit estrogenic effects in MCF7 cells [37], we suggest that the chemicals activated estrogen receptors in endometrial tissue, which led to an accumulation by an as yet unidentified mechanism. Thus, further analyses into this important area of clinical concern will be necessary.

In this study, we showed that the incidence of EM was lower in women with a more advanced education, which was accompanied by significantly enhanced PAE metabolite concentrations particularly in the urine of lower educated women (**Table 4**). This finding may be explained by different food consumption behaviors, since common cheap food particularly for lunch and breakfast in big Chinese cities is usually delivered in plastic containers and bags, which is not the case for restaurant food, the preferred meals of higher educated people. It is also known that microwave heating of food in plastic containers increases the migration of PAEs into the foodstuff [38]. In summary, DBP and DEHP were detected in > 90% of all participants, whereas DEP detection ranged from 0-16.4%. DBP and DEHP serum concentrations were sig-

nificantly higher in EM patients than in control subjects. Accordingly, particularly ∑DEHP urine concentrations were significantly enhanced in EM patients, and DBP and DEHP levels in resected endometrial tissues of EM patients were 2.3 times and 11.8 times higher than their blood serum levels respectively. Our analyses further revealed that PAE uptake was higher in women with lower educational levels compared to higher educated women.

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Disclosure of conflict of interest

None.

Address correspondence to: Shu Guang Li, Key Laboratory of Public Health Safety of Ministry of Education, School of Public Health, Fudan University, 130 Dongan Road, Shanghai 200032, China. Tel: 086-13701910238; Fax: 086-2154237260; E-mail: leeshuguang@fudan.edu.cn

References

[1] Wittassek M, Koch HM, Angerer J and Bruning T. Assessing exposure to phthalates - the human biomonitoring approach. Mol Nutr Food Res 2011; 55: 7-31.

[2] Liu HC, Den W, Chan SF and Kin KT. Analysis of trace contamination of phthalate esters in ultrapure water using a modified solid-phase extraction procedure and automated thermal desorption-gas chromatography/mass spectrometry. J Chromatogr A 2008; 1188: 286-294.

[3] Cirillo T, Fasano E, Esposito F, Del Prete E and Cocchieri RA. Study on the influence of temperature, storage time and packaging type on di-n-butylphthalate and di(2-ethylhexyl)phthalate release into packed meals. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2013; 30: 403-411.

[4] Guo Z, Wei D, Wang M and Wang S. Determination of six phthalic acid esters in orange juice packaged by PVC bottle using SPE and HPLC-UV: application to the migration study. J Chromatogr Sci 2010; 48: 760-765.

[5] Bradley EL, Burden RA, Leon I, Mortimer DN, Speck DR and Castle L. Determination of phthalate diesters in foods. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2013; 30: 722-734.

[6] Schettler T. Human exposure to phthalates via consumer products. Int J Androl 2006; 29: 134-139; discussion 181-135.

[7] Heudorf U, Mersch-Sundermann V and Angerer J. Phthalates: toxicology and exposure. Int J Hyg Environ Health 2007; 210: 623-634.

[8] Com SL. Material Safety Data Sheet, Phthalic acid MSDS. http://www.sciencelab.com/msds.php?msdsId = 9926545.

[9] Lyche JL, Gutleb AC, Bergman A, Eriksen GS, Murk AJ, Ropstad E, Saunders M and Skaare JU. Reproductive and developmental toxicity of phthalates. J Toxicol Environ Health B Crit Rev 2009; 12: 225-249.

[10] Martino-Andrade AJ and Chahoud I. Reproductive toxicity of phthalate esters. Mol Nutr Food Res 2010; 54: 148-157.

[11] Sugiyama S, Shimada N, Miyoshi H and Yamauchi K. Detection of thyroid system-disrupting chemicals using in vitro and in vivo screening assays in Xenopus laevis. Toxicol Sci 2005; 88: 367-374.

[12] Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen DV, Andersson AM, Toppari J and Skakkebaek NE. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ Health Perspect 2006; 114: 270-276.

[13] Huang PC, Kuo PL, Chou YY, Lin SJ and Lee CC. Association between prenatal exposure to phthalates and the health of newborns. Environ Int 2009; 35: 14-20.

[14] CSTEE. Phthalate migration from soft PVC toys and child-care articles: Opinion expressed ate the CSTEE third plenary meeting Brussels, 24 April 1998 (EU Scientific Committee on Toxicity, Ecotoxicity and the Environment). 1998.

[15] Wormuth M, Scheringer M, Vollenweider M and Hungerbuhler K. What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? Risk Anal 2006; 26: 803-824.

[16] Aldyreva MV, Klimova TS, Iziumova AS and Timofeevskaia LA. [The effect of phthalate plasticizers on the generative function]. Gig Tr Prof Zabol 1975; 25-29.

[17] Cobellis L, Latini G, De Felice C, Razzi S, Paris I, Ruggieri F, Mazzeo P and Petraglia F. High plasma concentrations of di-(2-ethylhexyl)-phthalate in women with endometriosis. Hum Reprod 2003; 18: 1512-1515.

[18] Colon I, Caro D, Bourdony CJ and Rosario O. Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. Environ Health Perspect 2000; 108: 895-900.

[19] Luisi S, Latini G, de Felice C, Sanseverino F, di Pasquale D, Mazzeo P and Petraglia F. Low serum concentrations of di-(2-ethylhexyl)phthalate in women with uterine fibromatosis. Gynecol Endocrinol 2006; 22: 92-95.
[20] Tabacova S, Little R and Balabaeva L. Maternal exposure to phthalates and complications of pregnancy. Epidemiology 1999; 10: 127.
[21] Xianghua L, Zhiming W, Mianzhen W and Desheng W. Changes of Menstrual Function of Female Workers in Plastic Plants. J Environ Health 2003; 20: 98.
[22] Varma R, Rollason T, Gupta JK and Maher ER. Endometriosis and the neoplastic process. Reproduction 2004; 127: 293-304.
[23] Jie L. Obstetrics and gynaecology. 6th edition. People's Medical Publishing House; 2006.
[24] Chunhong P. Endometriosis treatment current situation and prospects. Med Recap 2009; 15: 3282- 3284.
[25] Huang PC, Li WF, Liao PC, Sun CW, Tsai EM and Wang SL. Risk for estrogen-dependent diseases in relation to phthalate exposure and polymorphisms of CYP17A1 and estrogen receptor genes. Environ Sci Pollut Res Int 2014; 21: 13964-13973.
[26] Lifang C, Xianzhi T, Peiquan L, Xiujuan L, Yumei C and Shuling Y. DMPA clinical research for the treatment of endometriosis. Chin Matern Child Health Care 2004; 19: 82-85.
[27] Ke C, Shuguang L and Zhiqiang Z. Serum Phthalate Esters Levels and Their Correlation with Sex Hormones in Human. J Environ Occupat Med 2009; 26: 232-234.
[28] Li S, Dai J, Zhang L, Zhang J, Zhang Z and Chen B. An association of elevated serum prolactin with phthalate exposure in adult men. Biomed Environ Sci 2011; 24: 31-39.
[29] Zhiming C, Xin S, Qianlong Z, Fenghua W, Ying Z, Junyong Z and Shuguang L. GC/MS Determination of Phthalates in Human Serum. PTCA (PartB: Chem Anal 2006; 42: 115-119.
[30] Reddy BS, Rozati R, Reddy BV and Raman NV. Association of phthalate esters with endometriosis in Indian women. BJOG 2006; 113: 515-520.
[31] Itoh H, Iwasaki M, Hanaoka T, Sasaki H, Tanaka T and Tsugane S. Urinary phthalate monoesters and endometriosis in infertile Japanese women. Sci Total Environ 2009; 408: 37-42.
[32] WHO/IASO/IOTF. The Asia-Pacific perspective: redefining obesity and its treatment Health Communication Australia Pty Ltd; 2000.
[33] Calafat AM, Ye X, Silva MJ, Kuklenyik Z and Needham LL. Human exposure assessment to environmental chemicals using biomonitoring. Int J Androl 2006; 29: 166-171; discussion 181-165.
[34] Janjua NR, Frederiksen H, Skakkebaek NE, Wulf HC and Andersson AM. Urinary excretion of phthalates and paraben after repeated whole-body topical application in humans. Int J Androl 2008; 31: 118-130.
[35] Huang PC, Tsai EM, Li WF, Liao PC, Chung MC, Wang YH and Wang SL. Association between phthalate exposure and glutathione S-transferase M1 polymorphism in adenomyosis, leiomyoma and endometriosis. Hum Reprod 2010; 25: 986-994.
[36] Kim SH, Chun S, Jang JY, Chae HD, Kim CH and Kang BM. Increased plasma levels of phthalate esters in women with advanced-stage endometriosis: a prospective case-control study. Fertil Steril 2011; 95: 357-359.
[37] Chen FP and Chien MH. Lower concentrations of phthalates induce proliferation in human breast cancer cells. Climacteric 2014; 17: 377-384.
[38] Moreira MA, Andre LC and Cardeal ZL. Analysis of phthalate migration to food simulants in plastic containers during microwave operations. Int J Environ Res Public Health 2014; 11: 507-526.