Analysis of PAEs in semen of infertile men

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Objectives: Phthalates are environmental chemicals with reproductive toxicity and estrogenic effects in animals. They are of increasing concern to human health.

Aim: To determine whether phthalate levels in semen were associated with infertility.

Methods: Using semen samples from 107 infertile and 94 fertile men, the presence and quantity of five phthalate esters were measured using high-performance liquid chromatography (HPLC). Using data collected from questionnaires and clinical examinations, the correlation between phthalate exposure and semen quality was analyzed.

Results: The cumulative levels of the measured phthalate esters were significantly higher in the infertility group compared to the control group ($P < 0.05$). Concentrations of the five phthalate esters in men varied by age with older men showing higher cumulative levels.

Conclusions: The presence of phthalates may contribute to male infertility in our study population.

Keywords: Male infertility, Semen, Phthalates, High-performance liquid chromatography

Introduction

Phthalates are used as plasticizers to improve the flexibility and toughness of products and as raw materials in the production of pesticides, anti-parasitic agents, cosmetics, perfumes, lubricants, and anti-foam agents.¹ The annual global production of phthalates is approximately 11 billion pounds.² Human beings are continuously exposed to phthalates through skin contact through use of products such as cosmetics, perfumes, solvents, and plastics, as well as through the diet and inhalation.³

Phthalates may interfere with the hormones that regulate the human reproductive and developmental processes.⁴ Specifically, phthalates have been implicated in disrupting the human endocrine and reproductive systems. Animal experiments have demonstrated some phthalates to be endocrine disruptors, resulting in abnormalities of the male reproductive system and the inhibition of testicular testosterone production when exposed to it pre- or perinatally.^{5,6} Animal experiments have also confirmed that prenatal phthalates exposure results in Leydig cell dysfunction, leading to the development

of a testicular dysgenesis syndrome.⁷ Moreover, phthalates can directly or indirectly decrease the count and motility of sperm. Ejaculate may be decreased in volume or have an increased number of abnormal sperms. Phthalates are therefore considered to be a threat to human reproduction.^{8–11}

In recent years, epidemiologic studies have investigated the influence of phthalate exposure on male fertility. Most studies have shown that urinary phthalate metabolite levels (of at least one of the metabolites) may affect semen quality. Some researchers have reported that urinary phthalate metabolite levels decrease sperm motility and increase sperm DNA damage.⁴ Although some studies have reported an association between the presence of phthalate metabolites in urine and infertility, reports on the levels of phthalate and infertility are rare. Han *et al.* suggested that phthalate levels in healthy human semen be measured to determine the effect of phthalates on fertility.¹² However, the phthalates levels in the semen of infertility populations have not yet been measured.

In the present study, we investigated the content of five phthalate esters (DEP [di-ethyl phthalate], DBP [di-*n*-butyl phthalate], BBP [butyl benzyl phthalate], DEHP [di-2-ethylhexyl phthalate], and DnOP [di-*n*-octyl phthalate]) in semen from infertile men in a hospital in Harbin, China. High-performance liquid chromatography (HPLC) was used to assess levels of

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phthalic acid esters in semen and findings were analyzed for correlations with infertility and sterility. This study adds important knowledge to the literature because this is the first experimental research to detect and evaluate the presence of phthalic acid esters in semen in an infertile population.

Methods

Study subjects

The Ethical Committee of Harbin Medical University approved this research. A total of 150 infertile men (23–45 years of age) from a hospital in Harbin, China were studied between May 2008 and October 2009. The subjects were evaluated according to the diagnostic criteria for infertility.¹³ All men in the study had lived with their wives for at least 2 years. The reproductive function of their wives was normal and none had become pregnant. Informed consent was obtained from all subjects prior to participation. A total of 107 patients (23–45 years of age and average age, 28.5 years) were formally diagnosed with infertility and recruited for the study (Table 1). Twenty-eight people declined to participate in the study during the recruitment process. The infertility group comprised childless men married at least for 5 years with no known cause of infertility (including reproductive system and reproductive defect diseases), and whose spouses had no known reproductive system diseases. The control group was selected from the same medical center. We matched 94 fertile controls (23–45 years of age) to cases based on socioeconomic status and age. The controls had no known morbidities and had all fathered at least one child.

A questionnaire with information on weight, height, smoking and alcohol use, occupational exposure, diet, drinking water sources, duration of

infertility, and frequency of intercourse was administered to all participants.

Measurement of phthalates in human semen

Equipment

The HPLC equipment included a C₁₈ column (250 mm × 4.6 mm × 5 μm; Waters, Bellefonte, PA, USA), AR1140 electronic balance (OHAUS, Pine Brook, NJ, USA), XK96-A rapid mixer (XinKang, Jiangsu, China), TD-4A hydroextractor (YingTai, Changsha, China), KQ-100DB ultrasonic cleaner (Kunshan, Jiangsu, China), and an HGC-12A nitrogen stream instrument (HengAo, Tianjin, China).

Reagents

DEP, BBP, DBP, DEHP, and DnOP at ≥99.0% purity were purchased from the Dr. Ehrenstorfer Co. (Augsburg, Germany). Methanol and acetonitrile (HPLC grade) were purchased from Dikma Technologies (Beijing, China), *n*-hexane (chromatographic grade) was obtained from the Tianjin Guangfu Chemical Reagent Co. (Tianjin, China), and phosphoric acid (analytical grade) at ≥85% purity was purchased from the Tianjin Kermel Chemical Reagent Co (Tianjin, China). Double-distilled water was used in all procedures.

Standard solutions

Stock solutions of the five phthalates (DEP, BBP, DBP, DEHP, and DnOP) were prepared at 1.0 g/L in methanol. A 0.5 mL stock solution was added to a 50 mL volumetric flask, and a constant volume to scale with the mobile phase; the solution was 10.0 mg/L of the standard mixture.

Contamination control

To minimize contamination, all procedures were performed using sterile glass specimen cups. All glass apparatus were soaked in glass cleaner, washed in double-distilled water, rinsed in hexane, and dried at 400°C for 4 hours. Phosphoric acid was analyzed by HPLC to confirm the absence of detectable phthalates. The plastic gloves used to collect and handle semen samples were also analyzed by HPLC, and three new plastic gloves from each batch of samples were handled and analyzed to monitor background phthalate concentrations.

Sample collection

One hundred seven infertile and 94 fertile men were abstinent for 3–5 days before collection of semen samples. Prior to semen sample collection (~3 mL obtained by masturbation), subjects were asked to urinate and wash their hands and penis with water. Condoms were not used in the acquisition process. Samples were collected into pre-cleaned glass centrifuge tubes (10 mL) with glass stoppers. Phosphoric acid was added (200 μL of ≥85% phosphoric acid per 1 mL of semen) after incubation for 1 hour at

Table 1 Demographic characteristics of study participants (*n*=107)

Characteristics	Data	Number (%)
Number of subjects	107	
Age (year)		
Mean	25.51 ± 2.57	
Range	23–45	
Body weight (kg)		
Mean	64.37 ± 4.62	
Range	53–75	
Height (cm)		
Mean	173.12 ± 3.97	
Range	168–182	
BMI (kg/m ²)		
Mean	21.51 ± 1.75	
Range	18.78–22.64	
Smoking status		
No smoking		65 (60.7)
Smoking		42 (39.3)
Drinking status		
No drinking		45 (42.1)
Drinking		62 (57.9)

BMI: body mass index.

room temperature. Samples were stored at -20°C until analysis.

Sample treatment

Following defrosting of frozen acidified semen samples, residual sperm cells were lysed by three to five rounds of freezing and thawing, and samples were then centrifuged at 2500 g for 10 minutes. Aliquots (2 mL) of upper seminal plasma were transferred to another 10 mL glass centrifuge tube using a glass pipette. Extraction solution (6 mL of *n*-hexane/acetonitrile at a 5:1 volume ratio) was added, and following mixing in a vortex oscillator, ultrasound exposure for 10 minutes was used to facilitate extraction. After centrifugation at 3000 g for 10 minutes, the supernatant was transferred to another glass centrifuge tube, and the residue was re-extracted with 6 mL of extraction solution. The two organic phase extracts were combined and evaporated to dryness under N_2 at 50°C . Residues were taken up into 0.2 mL of methanol-saturated *n*-hexane for analysis.

Semen analysis among study participants

High-performance liquid chromatography analysis

The HPLC system was equipped with an injector, an online solvent degasser, a system controller, a pump, a column oven, a 20 μL injection loop, and an ultraviolet detector. Chromatographic analysis was performed using an analytical Discovery C₁₈ column (id, 4.6×250 mm) with a particle size of 5 μm . Analysis was performed isocratically at a flow rate of 1.0 mL/minute employing a mobile phase of acetonitrile/ultrapure water (95:5 [v/v]) at 30°C . Phthalates were detected at 224 nm using the ultraviolet detector.

Standard curves

Reference standard solutions were diluted with acetonitrile to concentrations of 0.01, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, and 10.0 $\mu\text{g/mL}$; 20 μL of each solution was injected for HPLC analysis under the conditions described above. The abscissa of the standard curve represented the calculated concentration of each phthalate, and the ordinate represented the corresponding A_{224} (detection wavelength) peak areas.

Sample determinations

Samples (20 μL) of semen extracts were analyzed as described above. Analytes were identified based on characteristic retention times and quantified by reference to the standard curves.

Quality control

Five types of phthalates at three different concentrations (0.5, 2.0, and 5.0 $\mu\text{g/mL}$) were added to separate 2 mL aliquots of seminal plasma. All conditions for extraction and chromatography were as described above. Each specimen was measured six times and precision (variance) was evaluated from the relative

standard deviations (RSDs). To evaluate accuracy, we calculated the recovery rates for each of the five phthalates. Prior to extraction, standards were added at different concentrations (0.5, 4.0, and 8.0 $\mu\text{g/mL}$) to samples of seminal plasma (2 mL). For each concentration of each standard, six independent seminal plasma samples were used. All samples were processed, extracted, and analyzed as described above. The coefficient of recovery was calculated separately for each phthalate.

Statistical analysis

Multivariate logistic regression methods were used to analyze the subject-related variables in accordance with the semen quality. The level of significance (α) for introducing a variable was 0.05. The level of significance (β) of excluding the variable was 0.10. A forward stepwise regression method (forward: LR) was used for variable screening to infer risk factors affecting semen quality. The RSD was the ratio of the standard deviation to the mean, expressed as a percentage. The accuracy and general applicability of the quadratic polynomial model was evaluated using the determination coefficient (r^2), and statistical significance of the regression equation was determined by *F*-test. Data are expressed as the mean $\pm$ standard deviation. For concentration levels below the limit of detection (LOD), an imputed value equal to one-half the LOD was used in calculations unless stated otherwise. The Wilcoxon rank sum test method was used for statistical analysis of the case and control groups. A *P* value of 0.05 indicated statistical significance.

Results

Basic analysis

Multivariate logistic regression analysis was performed according to eight factors, including semen quality, body mass index (BMI), sterile time, occupation, cigarette smoking history, alcohol consumption, type of drinking water, and type of subsidiary foods consumed. As shown in Table 2, BMI and infertility were associated with the quality of semen, as follows: $\text{OR}_{\text{BMI}}=1.315$, 95% $\text{CI}_{\text{BMI}}=(1.060-1.632)$; and $\text{OR}_{\text{infertility}}=3.334$, 95% $\text{CI}_{\text{infertility}}=(1.310-8.484)$. Smoking ($P>0.05$, $\text{OR}>1$), drinking ($P>0.05$, $\text{OR}<1$), and the type of drinking water ($P>0.05$, $\text{OR}<1$) were not associated with sperm quality.

Standard chromatogram

To generate a standard chromatogram, a mixed reference standard solution was analyzed by HPLC. As shown in Fig. 1, all five types of phthalates gave clear peaks above baseline and had retention times at 14 minutes under the chromatographic conditions used.

Standard curve and detection limits

The LOD was defined as a signal to noise (S/N) ratio of 3.0. As shown in Table 3, there was a strict linear

Figure 1 The standard high-performance liquid chromatogram of mixed nominal samples. DEP: 3.362 minutes, BBP: 3.914 minutes, DBP: 4.196 minutes, DEHP: 11.131 minutes, DnOP: 12.946 minutes. DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; BP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate.

correlation between sample concentration and peak areas with correlation coefficients ranging from 0.9993 to 0.9999. Because each phthalate had a different structure, charge distribution, and UV absorption characteristics, the LOD varied according to the reference standard. Analyte concentrations at $S/N=3.0$ were in the range of 1.8–5.2 $\mu\text{g/L}$.

Precision and accuracy

The RSDs of the mean measured values at three concentration levels (0.5, 2.0, and 5.0 $\mu\text{g/mL}$) were

selected to evaluate precision. As shown in Table 4, the RSDs of the five phthalates varied from 1.3 to 5.7% (inter-assay precision). Reanalysis of the same sample on six sequential days revealed that the intra-day precision was 1.8–4.9% (not shown).

For evaluation of accuracy, recovery was assessed for different phthalates introduced at different concentrations into semen samples prior to extraction. As shown in Table 5, the mean coefficients of recovery for the five types of phthalates in semen were 90.6–99.8%, and RSDs ranged from 1.4 to 5.0%.

Table 2 Logistic regression analyses of factors affecting male infertility

Factor	Estimated value (beta)	SE (beta)	Wald χ^2	<i>P</i> value	OR value	95% of CI
Constant term	−7.7517	2.7356	8.0296	0.004*		
BMI	0.2739	0.1102	6.1776	0.012*	1.315	1.060–1.632
Infertility time	1.2040	0.4766	6.3822	0.011*	3.334	1.310–8.484

* $P < 0.05$.

BMI: body mass index; OR: odds ratio; CI: confidence interval.

Table 3 The equation, the correlation coefficient, and the detection limit of five phthalates

Compounds	Retention time (minutes)	Equation	Correlation coefficient (<i>r</i>)	Detect limit ($\mu\text{g/L}$)
DEP	3.362	$y = 7783.8X + 1414.3$	0.9993	2.8
BBP	3.914	$y = 4271.4X - 31.063$	0.9999	2.4
DBP	4.196	$y = 5054.4X - 53.457$	0.9999	1.8
DEHP	11.131	$y = 5298.4X - 114.54$	0.9997	4.5
DnOP	12.946	$y = 5051.3X - 124.86$	0.9996	5.2

y denotes the corresponding peak areas; *X* denotes the concentration of phthalates.

DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; DBP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate.

Table 4 Results of phthalates precision experiment ($n=6$, $\mu\text{g/mL}$)

Compounds	Background value	Standard sample	Measured value						Mean	RSD (%)
			1	2	3	4	5	6		
DEP	ND	0.5	0.488	0.492	0.497	0.483	0.479	0.495	0.489	1.4
	ND	2.0	1.984	1.957	1.974	1.963	1.995	1.884	1.960	1.9
	ND	5.0	4.865	4.756	4.853	4.773	4.895	4.727	4.812	1.4
BBP	ND	0.5	0.457	0.474	0.451	0.466	0.444	0.459	0.459	2.4
	ND	2.0	1.691	1.729	1.765	1.943	1.921	1.782	1.805	5.7
	ND	5.0	4.513	4.609	4.868	4.783	4.717	4.688	4.697	2.7
DBP	ND	0.5	0.442	0.427	0.447	0.466	0.458	0.415	0.443	4.3
	ND	2.0	1.796	1.677	1.774	1.834	1.817	1.666	1.761	4.1
	ND	5.0	4.641	4.664	4.549	4.695	4.721	4.629	4.650	1.3
DEHP	ND	0.5	0.431	0.444	0.468	0.474	0.484	0.457	0.460	4.3
	ND	2.0	1.703	1.737	1.723	1.694	1.814	1.793	1.744	2.8
	ND	5.0	4.411	4.452	4.515	4.352	4.526	4.319	4.429	1.9
DnOP	ND	0.5	0.437	0.458	0.424	0.419	0.434	0.427	0.433	3.2
	ND	2.0	1.728	1.809	1.867	1.769	1.886	1.704	1.794	4.1
	ND	5.0	4.449	4.515	4.348	4.486	4.619	4.311	4.455	2.5

DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; DBP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate; ND: not detected; RSD: relative standard deviation.

These levels of accuracy were consistent with international standards (WHO/UNEP and AOA) for the analysis of biological samples.

Sample detection and analysis

Five types of phthalates were simultaneously analyzed using the method developed in this study. A total of 201 semen samples from infertile and fertile men were analyzed.

As shown in Table 6, there were significant differences in phthalates in the semen samples in the infertile men and the control group. Phthalate detection rates are shown in Figure 2.

The subjects were then subdivided into five age groups with a 5-year age range. The rates of detection and levels for each phthalate in the semen samples are shown in Table 7 and Figure 3, respectively.

When subdivided according to age, as presented in Table 7, there was a higher rate of phthalate detection in the older age groups, and the rate of DEHP detection increased to 81.3% for samples from subjects

in the 35–40-year age group. Overall, the rate of detection order for the five types of phthalates in the semen samples was as follows: DEHP>BBP>DBP>DE>DnOP.

As shown in Figure 3, there was also a difference in the mean concentrations of phthalates by age groups. In general, the concentrations of DEHP, BBP, and DBP were high, but the concentrations of DEP and DnOP were generally lower. The concentration of DEHP in the 35–40-year age group was 504 $\mu\text{g/L}$, and the mean concentration level for the five types of phthalates in the 25–35-year age groups was higher than the other groups.

Discussion

Environmental hormone mimetics are likely to be harmful to human physiology, including the immune system and the brain, but the greatest concern has been expressed in relation to disruption of reproductive function. For example, animal experiments indicate that significant changes in sex ratios observed in fish and

Table 5 The coefficient of recovery of five phthalates in semen ($n=6$, $\mu\text{g/mL}$)

Compound	Background value	Value of standard sample	Measured value (mean)	Mean of recovery rate (%)	RSD (%)
DEP	ND	0.5	0.481	96.2	4.7
	ND	4.0	3.991	99.8	3.9
	ND	8.0	7.556	94.5	2.7
BBP	0.135	0.5	0.594	91.8	1.8
	0.135	4.0	3.861	93.2	4.3
	0.135	8.0	8.024	98.6	3.3
DBP	0.189	0.5	0.678	97.8	2.3
	0.189	4.0	4.012	95.6	3.8
	0.189	8.0	7.621	92.9	5.0
DEHP	0.245	0.5	0.729	96.8	2.9
	0.245	4.0	4.038	94.8	1.4
	0.245	8.0	7.701	93.2	1.7
DnOP	ND	0.5	0.453	90.6	2.5
	ND	4.0	3.807	95.2	4.7
	ND	8.0	7.817	97.7	1.9

DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; DBP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate; ND: not detected; RSD: relative standard deviation.

Figure 2 The detection rate of five types of phthalates in the infertility group and control group. DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; DBP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate.

other animals are ascribed to endocrine disruption, as highlighted by reports from Japan involving female shellfish with male genital organs and from UK involving male rainbow trout positive for female-specific yolk protein *in vivo*.¹⁴

Phthalates are a class of environmental hormone mimetics. Due to low production costs and advantageous chemistry, they are widely used in industry, most commonly as plasticizers to improve the physical qualities of plastic products. However, phthalate additives are not necessarily covalently bound to the product and significant amounts are liberated, particularly when the plastic softens, leading to the release of phthalates into the environment where they accumulate in air, soil, and water.

We measured and analyzed phthalate levels in semen samples from 201 men. Different types and quantities of phthalates were detected in the semen samples from infertile men compared to fertile controls, consistent with previous findings about the types of phthalates found in human semen.^{10,15,16} Results showed significant differences in the five phthalates between the two groups, with higher levels

of accumulated phthalates in the infertility group than in the control group ($P < 0.05$). The type and quantity of phthalates in semen samples are a proxy for the measurement of exposure of phthalates in the reproductive systems of males in our study.

Several studies have indicated that the reproductive system is most sensitive to the adverse effects of endocrine disruptors during development, and early exposure to endocrine disruptors possibly resulting in reproductive tract abnormalities and sexual dysfunction later in life.^{17,18} In addition, the adult reproductive system is sensitive to environmental toxicity, and the potential germ cells of adult males decline with exposure to endocrine disruptors.¹⁹

In this study, phthalate levels were detected in the semen samples. The five types of phthalates in the experimental group were as follows: DEP, 47 cases (43.9%); BBP, 75 cases (70.1%); DBP, 65 cases (60.7%); DEHP, 87 cases (81.3%); and DnOP, 40 cases (37.4%). The detection rates for BBP, DBP, and DEHP exceeded 50%, demonstrating that the majority of cases were exposed to environmental phthalates. Two phthalates, DBP and DEHP, were

Table 6 Comparison of phthalates contents in semen at two groups (mean $\pm$ SD, $\mu\text{g/L}$)

Group	Case	DEP	BBP	DBP	DEHP	DnOP
Infertility group	107	10.61 $\pm$ 14.06 ^a	23.85 $\pm$ 30.86 ^a	21.66 $\pm$ 33.9 ^a	27.66 $\pm$ 37.73 ^a	10.9 $\pm$ 16.56 ^a
Control group	94	5.66 $\pm$ 7.64	8.16 $\pm$ 10.94	4.13 $\pm$ 5.35	13.68 $\pm$ 20.22	5.69 $\pm$ 4.47

^aA statistically significant difference between the infertile group and the control group, $P < 0.05$.

DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; DBP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate.

Figure 3 The concentration of phthalates in semen of infertile men at different ages. DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; DBP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate.

detected at markedly higher levels than the other phthalates analyzed, with concentrations up to 293 and 504 $\mu\text{g/L}$, respectively. Some researchers have reported that 0.61 mg/mL of DEHP and 0.003 mg/mL of DBP exist in the semen of fertile men.¹² We found that the levels of DEHP and DBP in fertile men were higher than expected, and among infertile men, we found higher levels of DEHP and DBP.

Phthalates are known endocrine disruptors with estrogen-like effects and can exert toxic effects on reproductive function and fetal development. Some researchers have indicated that endocrine modulation due to phthalates may be one of the set of subfactors explaining idiopathic infertility, the most common cause of male infertility.²⁰ In addition, recent studies have also reported that prenatal, postnatal, and children's exposure to phthalates may impact early gene expression during embryonic stem cell development and neonates.^{21–25} This has led to increasing

concerns of the possible adverse effects of exposure to environmental phthalates. Some phthalates induce reproductive and developmental toxicity in laboratory animals (DBP, DEHP, and di-isobutyl [DIBP]) and are classified as reproductive toxicants in the EU, while others are not (DEP, di-isononyl [DINP], and di-isodecyl [DIDP]).^{6,26,27}

The reproductive dysfunction effect of DEHP and DBP has been documented in animals and human beings. According to the US REPROTOX database, tests of *in vivo* toxicity have shown that when pregnant female rats are exposed to DEHP, this may lead to fetal rat proptosis, exencephalia, vascular tail and skeletal abnormalities, and other deformities. *In vitro*, DBP has an anti-androgen effect, independent of androgen receptors. DBP does not directly act on the androgen receptor, and does not combine with the rat and human androgen receptor, but indirectly interferes with hormone signaling pathways by acting

Table 7 The detection rate of phthalates in semen of $N=107$ infertile men at different ages

Ages	N	DEP		BBP		DBP		DEHP		DnOP	
		##	Rate (%)	#	Rate (%)	##	Rate (%)	#	Rate (%)	##	Rate (%)
20~	12	4	33.3	8	66.7	5	41.7	9	75.0	4	33.3
25~	38	17	44.7	26	68.4	23	60.5	31	81.6	15	39.5
30~	33	15	45.5	24	72.7	22	66.7	28	84.9	13	39.4
35~	15	8	53.3	11	73.3	10	66.7	13	86.7	5	33.3
≥40	9	3	33.3	6	66.7	5	55.6	6	66.7	3	33.3
Total	107	47	43.9	75	70.1	65	60.7	87	81.3	40	37.4

DEP: di-ethyl phthalate; BBP: butyl benzyl phthalate; DBP: di-*n*-butyl phthalate; DEHP: di-2-ethylhexyl phthalate; DnOP: di-*n*-octyl phthalate.

on the male testis.^{28–30} Some researchers have reported rat testicular atrophy and weight loss after DBP and DEHP exposure.^{31,32} We found that DEHP and DBP levels were much higher than the other phthalates, providing evidence that DEHP and DBP may be associated with male infertility.

Although phthalate concentrations remain relatively low in primary environmental samples, selective accumulation/amplification and bioeffects of these molecules can take place through bioconcentration, bioaccumulation, and biomagnification.³³ These processes can increase the effective concentration in the ecosystem and correspondingly increase the risk of harm to environmental organisms and human beings. We found that the majority of subjects with male infertility were in the 25–35-year age range (Table 7), and the cumulative levels of phthalates in this group were higher than the older age groups. Further studies are required to better understand the adverse effects of human exposure and on possible measures to reduce the environmental release of industrial phthalates.

Disclaimer Statements

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Conflicts of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of.

Ethics approval The study was approved by the Ethical Committee of the Harbin Medical University and informed consent was obtained from all subjects.

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