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Association between phthalate metabolites and biomarkers of reproductive function in 1066 Chinese men of reproductive age

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HIGHLIGHTS

- Urinary MBP and MiBP in Chinese men were higher than the values in other countries.
- Serum levels of INSL3 was negatively associated with MEHP and %MEHP in urine.
- Acrosin activity was negatively associated with MBP, MiBP, MEHP and %MEHP.
- Serum levels of T and LH were negatively associated with MBP and MiBP simultaneously.

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ABSTRACT

Phthalates are suspected endocrine disrupting chemicals that impair male reproductive function in animal and epidemiological studies. We investigated associations between urinary phthalate metabolites and acrosin activity, along with that between insulin like-factor 3 (INSL3), a Leydig cell function marker, in Chinese adult men and assessed the association between the metabolites and male reproductive function. Serum levels of INSL3 and other hormones, semen parameters, and urinary concentrations of 14 phthalate metabolites in 1066 men were measured. The unadjusted concentrations of phthalates were included as independent variables and urinary creatinine as a separate covariate. INSL3 was negatively associated with mono-2-ethylhexyl phthalate (MEHP) and %MEHP [percentage of MEHP to all di(2-ethylhexyl) phthalate (DEHP) metabolites]. Acrosin activity was negatively associated with mono-*n*-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP), MEHP and %MEHP. MBP and MiBP were also negatively associated with total testosterone (T), free androgen index (FAI), free testosterone (FT), luteinizing hormone (LH) and sperm morphology and positively associated with DNA fragmentation index (DFI). A negative association between %MEHP and sperm motility was observed. Several other metabolites were also associated with reproductive function. This is the first report on the inverse associations of phthalate metabolites with acrosin activity and INSL3. Phthalates may cause multiple adverse results on reproductive function at environmental levels.

1. Introduction

Phthalates are phthalic acid esters mainly used as plasticizers in textiles, toys, coatings, pharmaceuticals and personal care products [1,2]. Since phthalates are not covalently bonded to plastics, they can be released into the environment. Due to their widespread occurrence and suspected endocrine disrupting properties [3], there is growing concern that environmental exposure to phthalates may impair male reproductive function.

Humans are primarily exposed to diester phthalates through ingestion, inhalation or dermal contact, after which they are metabolized into monoesters and excreted in urine [4]. Phthalate metabolites in urine have been used extensively as biomarkers of human exposure [5], with biomonitoring studies reporting on human exposure to several phthalates [6,7]. Phthalates have shown adverse health effects in rodents, particularly anti-androgenic

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effects on male reproductive development after prenatal exposure [8]. Epidemiological studies have suggested a negative association between phthalate exposure and semen quality and sex hormone levels [9–12]. Evidence of these associations is not entirely consistent, however, suggesting that additional parameters and markers of testicular function need to be explored to evaluate potential health risks on male reproduction.

Insulin like-factor 3 (INSL3), which is a major secretory product of Leydig cells, plays a crucial role in testicular descent [13] and germ cell survival [14], and is associated with spermatogenesis [15,16] and sperm morphology [17] in adult men. Compared with testosterone, INSL3 production is increasingly used as an excellent and more sensitive marker of Leydig cell function [18]. Research reported inhibited INSL3 expression in fetal rat testes after exposure to di-*n*-butyl phthalate (DBP) and DEHP [19]. However, evidence regarding the association of INSL3 with phthalate exposure is still lacking in epidemiological studies.

Acrosin, a sperm-specific trypsin-like acrosomal proteinase, is capable of hydrolyzing the zona pellucida in the oocyte and plays an important role in fertilization [20], such that fertilization is prevented if acrosin activity is inhibited. In clinical studies, acrosin activity is regarded as a potential marker for semen quality [21]. Research found that acrosin activity levels were correlated with sperm morphology and in vitro fertilization rate [22], and insufficient levels were associated with unexplained male infertility [23].

We recruited 1066 Chinese men of reproductive age in the present study. Six hormones, including INSL3, T, LH, follicle-stimulating hormone (FSH), estradiol (E_2), and sex hormone-binding globulin (SHBG), were measured in sera samples. Seven semen quality parameters, including sperm acrosin activity, were also evaluated. Levels of 14 phthalate metabolites were measured in urine samples. Associations between levels of urinary phthalates and reproductive hormones and semen quality were assessed.

2. Materials and methods

2.1. Study population

The study population was recruited from the Reproductive Medical Center, Nanjing Jinling Hospital in Nanjing, China. Participants were male partners of infertile couples who presented to the medical center between November 2012 and July 2014 for reproductive health analysis. Participants included men with male factor infertility as well as men who were partners of women with female factor infertility. In brief, participants were asked to fill in a detailed questionnaire including demographics, health and lifestyle factors (e.g. age, race, body weight, height, smoking status, alcohol drinking status, abstinence time, occupation, education level and reproductive and medical history). Samples of urine, semen and non-fasting blood were collected. Participants who had azoospermia, suffered from sexually transmitted diseases or severe varicoceles, or took Chinese medicine to improve semen quality were excluded from the present investigation. The final sample size for this investigation was 1066 adult men. The research was approved by the Human Subject Committees of Nanjing Jinling Hospital, and informed written consent was collected from all participants.

2.2. Measurements of urinary phthalate metabolites

Spot urine samples were first collected in sterile polypropylene cups, and then transferred to 5 mL polypropylene cryovials and stored at −80 °C until delivery to our laboratory in Beijing. Urinary concentrations of 14 phthalate metabolites: monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono(3-carboxypropyl) phthalate (MCPP), MBP, MiBP, monobenzyl phthalate (MBzP), MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-[(2-carboxymethyl) hexyl] phthalate (MCMHP), mono-*n*-octyl phthalate (MOP), monoisononyl phthalate (MiNP), and mono(carboxy-isooctyl) phthalate (MCiOP) were measured following a previously published method [24], with modification. Briefly, urine samples were spiked with mass-labelled internal standards, enzymatic deconjugated, purified by solid-phase extraction and analyzed by LC–MS/MS. Details of standards and reagents, sample preparation, instrumental analysis and quality control data are given in the supporting materials (Tables S1 and S2).

The proportion of DEHP excreted as the primary metabolite MEHP was calculated and expressed as %MEHP [%MEHP = MEHP (nanomoles per milliliter)/MEHP + MEHHP + MEOHP + MECPP + MCMHP (nanomoles per milliliter) × 100%]. Similarly to %MEHP, the proportions of DEHP excreted as each secondary metabolite (e.g. %MEHHP, %MEOHP, %MECPP, and %MCMHP) were also calculated. %MiNP was calculated as MiNP (nanomoles per milliliter)/MiNP + MCiOP (nanomoles per milliliter) × 100%. Urinary creatinine was measured spectrophotometrically to correct for urinary dilution effect.

2.3. Reproductive hormone analyses

Venous blood samples were centrifuged and sera collected and stored at −80 °C until analysis. Sera were analyzed for T, LH, FSH, E_2, SHBG and INSL3 levels. Levels of T, LH, FSH, E_2 and SHBG were determined by chemiluminescence assay using an automated Unicel Dxi 800 Access Immunoassay System (Beckman Coulter, Inc., USA). The assay sensitivities were 0.35 nmol/L for T, 0.2 IU/L for FSH and LH, 20 pmol/L for E_2 and 0.33 nmol/L for SHBG. The intra-assay coefficients of variation (CVs) for T, LH, FSH, E_2 and SHBG were less than 5%, and the interassay CVs were less than 8%. The free androgen index (FAI) was calculated as the ratio of T to SHBG, and free testosterone (FT) was calculated from concentrations of T and SHBG using a constant albumin level of 43.8 g/L [25]. Serum INSL3 was detected by radioimmunoassay (RIA) (Phoenix Pharmaceuticals Inc, CA, USA) in 500 randomly selected subjects. No demographical difference was found between the selected and the whole subjects. Sensitivity determined as the lower detection limit was 10 pg/mL and the intra- and inter-assay CVs were 4% and 10%, respectively.

2.4. Semen analyses

Semen specimens were collected on the same day as blood and urine samples. Specimens were allowed to liquefy for 30 min at 37 °C. After liquefaction, semen volume was assessed by weight. Sperm concentration and progressive motility were analyzed by a computer-aided sperm analysis (CASA) system. Sperm morphology was evaluated using Diff-Quik staining. All analyses were performed and assessed in accordance the with World Health Organization (WHO) manual [26]. The DNA Fragmentation Index (DFI) was assessed using a sperm chromatin structure assay (SCSA) by flow cytometry [27]. For DFI, 634 subjects were randomly selected and did not differ regarding the demographical characteristics. Sperm acrosin activities were analyzed as the method established by Kennedy et al. [28]. Briefly, for each liquefied semen sample, a pre-calculated volume that contained 7.5 million spermatozoa was added to a buffered Ficoll solution. After centrifugation, the seminal plasma and Ficoll supernatant were removed. The washed sperm pellet was suspended in a substrate (*N*-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride, BAPNA)-detergent (Triton X-100) buffer (pH = 8.0). The mixture was incubated at 23 °C for 3 h. Using BAPNA as a specific substrate, acrosin activity was determined

spectrophotometrically and presented as μIU/10^6 sperm. The CVs for acrosin activity were less than 15%.

2.5. Statistical analyses

Descriptive statistics were derived for subject demographics, urinary phthalate metabolites, hormone levels and semen quality parameters. Correlations between phthalate metabolites, reproductive parameters and potential covariates were calculated by Spearman Rank correlations. When the concentrations of the phthalate metabolites were below the Limit of Quantitation (LOQ), a value of LOQ/$\sqrt{2}$ was employed.

Associations between phthalate metabolites and reproductive outcomes were firstly examined in crude models. Multivariate linear regression models were then created to examine the associations adjusted by covariates. To achieve normal distribution and homogeneity of variance of the residuals, concentrations of urinary phthalate metabolite were natural log (ln) transformed. Semen volume, sperm concentration and total sperm number were cubic root transformed. Acrosin activity, DFI and all hormone levels were ln-transformed. Progressive motility and morphology were not transformed because of normal distribution in residuals. Regression coefficients were back-transformed and expressed as percentage change in reproductive outcomes for an interquartile range (IQR) increase in phthalate metabolite concentrations. Further, we divided the concentrations of phthalate metabolites into quartiles (as categorical variables) to examine the associations across phthalate quartiles.

Based on statistical and biological considerations, age (continuous), body mass index [BMI, (weight divided by the square of height); continuous], smoking and alcohol drinking status (never, occasionally or often, categorical), abstinence time (continuous) and time of blood collection [morning (8:00–11:00) or afternoon (13:00–16:00), dichotomous] were included in regression models. To take the urinary dilution effect into account, urinary creatinine was also included as a separate covariate in all models, except for the models of %MEHP or %MiNP (%MEHP and %MiNP represent relationships between urinary concentrations of metabolites that are independent of urinary dilution.). All statistical analyses were conducted using PASW software 18.0 (IBM, USA). Two-tailed p-values of less than 0.05 were considered statistically significant in all models.

3. Results

3.1. Population characteristics

A total of 1066 Chinese adult men, with no reproductive diseases and who had not taken any Chinese medicine for improving semen quality, were included in the present investigation. Participants were primarily young men with a mean age of 29.1 and mean BMI of 24.0 (Table 1). The hormone levels and semen parameters are listed in Table 2. Spearman Rank correlations showed that age, BMI, smoking, drinking, time of blood collection and abstinence time were either correlated with reproductive hormone levels or semen quality parameters (Tables S3–S5); thus, these parameters were included for further regression as covariates.

3.2. Concentrations of urinary phthalate metabolites

Concentrations of unadjusted phthalate metabolites are given in Table 3. Patterns of the creatinine adjusted phthalate metabolites were similar to the unadjusted (Table S6). Phthalate metabolites were detected in most urine samples (>98%), except MBzP and MOP with detection rates of 57.8% and 12.8%, respectively.

Table 1
Subject demographics (n = 1066).

Characteristics	Median (5th, 95th)	n (%)
Age (Year)	29.1 (23.0, 38.00)	
BMI (kg/m^2)	24.0 (19.5, 29.4)	
Abstinence time (day)	4.0 (2.0, 7.0)	
Smoking status		
Never smoke		574 (53.8)
Occasionally[a] smoke		211 (19.8)
Often[b] smoke		281 (26.4)
Alcohol drinking status		
Never drink		457 (42.9)
Occasionally[a] drink		500 (46.9)
Often[b] drink		109 (10.2)
Disease status		
Varicoceles		72 (6.8)
Epididymitis		13 (1.2)
Prostatitis		106 (9.9)
Cryptorchidism		6 (0.5)
Reproductive history		
Previously made partner pregnant	228 (21.4)	

[a] Smoking or drinking less then once a day.
[b] Smoking or drinking more then once a day.

The concentration of MBP was the highest (geometric mean ± SD: 78.7 ± 168.7 ng/mL) among all 14 metabolites, and MOP was the lowest (mean value below LOQ). Because of its low detection rate, MOP was not included for further analysis.

Significant positive correlations were found between all phthalate metabolites. Metabolites of the same diester phthalates were strongly correlated with each other (e.g., r = 0.977 for MEHHP and MEOHP). %MEHP and %MiNP were significantly correlated (r = 0.531).

3.3. Associations between phthalate metabolites and reproductive hormones

Significant associations with hormone levels were mostly found in MBP, MiBP, MEHP and the phenotypic marker of %MEHP. Their multiple regression models are summarized in Table 4. Associations between hormones and other phthalate metabolites are listed in Table S7. For %MEHHP, %MEOHP, %MECPP, and %MCMHP, no significant associations with any reproductive parameters were found (data not shown). Results of the crude models were similar to the adjusted models (data not shown). There were significant negative associations of INSL3 with MEHP and %MEHP. In adjusted models, INSL3 decreased by 4.3% (95% CI: −7.7, −0.8%) and 5.5% (95% CI: −9.0, −1.9%) with an IQR increase in MEHP and %MEHP, respectively. Levels of T, FAI and FT were inversely associated with MBP, MiBP, MMP and MCiOP. FAI and FT were also negatively associated with MCPP and MiNP. A borderline significant (p = 0.071) negative association between FAI and %MEHP was also observed. MBP and MiBP were associated with a decrease in serum LH [−6.3%, (95% CI: −10.2, −2.4%) for MBP and −6.9% (95% CI: −10.4, −3.2%) for MiBP]. FSH was positively associated with MCMHP. For E_2, a significant negative relationship with MEHP, MBzP, MiNP and MCiOP and statistically suggestive relationship with %MEHP (p = 0.055) were observed. No significant associations were found between SHBG and any phthalate metabolite.

Fig. 1 presents the transformed effect estimates of the phthalate quartile models for the MEHP and %MEHP associations with INSL3. Compared with the lowest %MEHP quartile, INSL3 in the second, third and fourth %MEHP quartiles significantly decreased by 11.6% (−19.2, −3.3%), 15.0% (−22.3, −7.0%) and 10.6% (−18.4, −1.9%), respectively. Patterns for MEHP quartiles were similar to those for %MEHP quartiles, but the decrease in higher MEHP quar-

Table 2
Distribution of reproductive hormones levels and semen quality parameters (n = 1066).

Parameter	Mean ± SD	Percentile				
		5th	25th	50th	75th	95th
Reproductive hormone						
T (nmol/L)	13.1 ± 4.3	6.8	10.4	12.6	15.6	20.8
LH (IU/L)	4.3 ± 3.0	1.8	2.8	3.7	5.0	7.9
FSH (IU/L)	5.4 ± 5.2	2.0	3.3	4.5	6.0	10.7
E_2 (pmol/L)	99.5 ± 47.5	37	67	94	121	188
SHBG (nmol/L)	27.5 ± 11.9	12.1	19.0	25.5	34.2	49.2
FAI	0.53 ± 0.20	0.27	0.40	0.49	0.62	0.91
FT (nmol/L)	0.30 ± 0.10	0.17	0.24	0.29	0.34	0.45
INSL3[a] (ng/mL)	0.94 ± 0.31	0.54	0.73	0.90	1.12	1.48
Semen quality						
Semen volume (mL)	3.2 ± 1.5	1.2	2.0	3.0	4.0	6.0
Sperm concentration (10^6/mL)	65.1 ± 57.6	6.5	24.8	51.3	87.6	171.5
Total sperm number (10^6)	199 ± 240	15	70	149	257	516
Progressive motility (PR, %)	32.2 ± 13.2	11.3	22.6	32.8	41.3	53.1
Morphology (normal forms, %)	4.5 ± 2.0	0.9	3.2	4.6	5.9	7.2
Acrosin activity (μIU/10^6 sperm)	45.0 ± 13.6	36.2	37.6	40.1	49.4	69.1
DFI[b] (%)	17.3 ± 12.6	4.4	8.4	13.7	21.7	42.4

[a] For INSL3, sample size was 500.
[b] For DFI, sample size was 634.

Table 3
Unadjusted urinary concentrations of phthalate metabolites (n = 1066).

Compounds	Detection rate (%)	Geometricmean ± SD	Percentile				
			5th	25th	50th	75th	95th
Phthalate metabolites (ng/mL)							
MMP	100.0	21.0 ± 82.9	4.2	10.2	19.8	40.2	129.8
MEP	100.0	15.2 ± 397.3	2.3	6.6	13.4	32.2	141.7
MBP	100.0	78.7 ± 168.7	13.3	39.2	77.6	162.3	464.7
MiBP	100.0	46.9 ± 78.1	9.6	27.5	47.8	83.9	192.7
MEHP	99.5	4.5 ± 18.0	0.7	2.4	4.6	8.7	27.0
MEHHP	100.0	13.2 ± 58.7	2.9	7.6	13.1	22.5	63.0
MEOHP	100.0	8.4 ± 28.1	1.9	4.8	8.4	14.3	38.6
MECPP	100.0	16.8 ± 48.4	3.8	9.9	17.2	27.1	67.2
MCMHP	100.0	5.1 ± 11.8	1.1	3.1	5.3	8.4	20.1
MBzP	57.8	0.2 ± 9.0	<LOQ	<LOQ	0.1	0.4	2.2
MOP	12.8	<LOQ ± 0.2	<LOQ	<LOQ	<LOQ	<LOQ	0.1
MCPP	99.4	1.0 ± 3.7	0.2	0.5	1.0	1.9	5.2
MiNP	98.3	0.13 ± 9.4	0.03	0.07	0.12	0.22	0.68
MCiOP	100.0	1.3 ± 13.9	0.3	0.7	1.2	2.1	6.3
Percent of metabolites (%)							
%MEHP		9.7 ± 5.7	3.4	7.1	10.3	14.2	20.9
%MiNP		10.0 ± 9.0	3.0	6.5	10.3	16.1	40.0

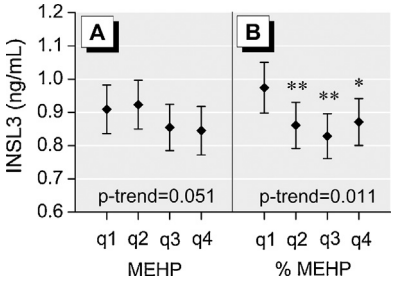

Fig. 1. Estimated insulin like factor 3 (INSL3) from models of (A) mono-2-ethylhexyl phthalate (MEHP) and (B) percent of MEHP to all di(2-ethylhexyl) phthalate metabolites (%MEHP) quartiles. Points correspond to estimated mean values adjusted for age, BMI (body mass index), smoking, drinking, time of blood drawn and urinary creatinine (except for %MEHP), whiskers indicate 95% confidence interval. *p < 0.05; **p < 0.01 compared with lowest quartile. p-trend is the p value for linear trend across quartiles

tiles compared with the lowest quartile did not reach statistical significance.

3.4. Associations between phthalate metabolites and semen parameters

The associations between MBP, MiBP, MEHP, %MEHP and semen quality parameters are displayed in Table 5. Associations between other metabolites are displayed in Table S7. There were significant negative associations between sperm morphology and MBP and MiBP. Following an IQR increase in MBP and MiBP, sperm morphology decreased by 6.5% (95% CI: −10.3, −2.7%) and 5.3% (95% CI: −8.9, −1.8%) relative to the study population median. Similar negative associations were also found in MCPP, MBzP, and MCiOP with less significance (p < 0.05). Sperm concentration and total sperm number were negatively associated with MBzP [3.4% (95% CI: −5.9, −0.8%) and 3.8% (95% CI: −6.4, −1.1%) decrease, respectively, for an IQR increase in MBzP]. Progressive motility was significantly associated with %MEHP (−3.9%, 95% CI: −6.8, −1.0%). There were significant negative associations between acrosin activity and MBP, MiBP, MEHP and%MEHP (p < 0.004). Slightly weaker but similarly negative relationships were observed with MMP, MEP,

Table 4
Percent change[a] (95% CI) in hormone levels associated with an IQR increase in selected phthalate metabolite concentrations ($n = 1066$).

Hormone	MBP		MiBP	
	Percent change (95% CI)	*p*-Value	Percent change (95% CI)	*p*-Value
T (nmol/L)	−3.9% (−6.6, −1.1%)	0.007	−4.1% (−6.7, −1.5%)	0.002
LH (IU/L)	−6.3% (−10.2, −2.4%)	0.002	−6.9% (−10.4, −3.2%)	<0.001
FSH (IU/L)	−2.1% (−6.6, 2.6%)	0.377	−3.0% (−7.2, 1.4%)	0.183
E_2 (pmol/L)	−3.8% (−8.6, 1.3%)	0.138	−1.3% (−5.9, 3.5%)	0.591
SHBG (nmol/L)	1.2% (−2.3, 4.9%)	0.507	−0.5% (−3.7, 2.9%)	0.788
FAI	−5.0% (−7.9, −2.0%)	0.001	−3.7% (−6.5, −0.8%)	0.012
FT (nmol/L)	−4.7% (−7.2, −2.1%)	<0.001	−4.2% (−6.6, −1.8%)	0.001
INSL3[b] (ng/mL)	0.4% (−4.3, 5.3%)	0.875	−0.7% (−5.1, 3.9%)	0.772
Hormone	MEHP		%MEHP	
	Percent change (95% CI)	*p*-Value	Percent change (95% CI)	*p*-Value
T (nmol/L)	−1.2% (−3.5, 1.1%)	0.314	−0.1% (−2.4, 2.3%)	0.925
LH (IU/L)	−0.2% (−3.5, 3.3%)	0.921	1.4% (−2.0, 4.9%)	0.433
FSH (IU/L)	3.1% (−3.4, 4.2%)	0.875	−2.4% (−6.1, 1.5%)	0.222
E_2 (pmol/L)	−4.3% (−8.2, −0.2%)	0.040	−4.1% (−8.0, 0.1%)	0.055
SHBG (nmol/L)	0.9% (−2.0, 3.8%)	0.558	2.3% (−0.7, 5.3%)	0.133
FAI	−2.0% (−4.5, 0.5%)	0.109	−2.3% (−4.8, 0.2%)	0.071
FT (nmol/L)	−1.8% (−3.9 0.3%)	0.097	−1.3% (−3.4, 0.9%)	0.237
INSL3[b] (ng/mL)	−4.3% (−7.7, −0.8%)	0.016	−5.5% (−9.0, −1.9%)	0.003

[a] Regression coefficients adjusting for age, BMI, smoking, drinking, time of blood drawn and urinary creatinine (except for %MEHP) were back transformed to present the percent change in hormones.
[b] Sample size was 500 for INSL3.

Table 5
Percent change[a] (95% CI) in semen parameters associated with an IQR increase in selected phthalate metabolite concentrations ($n = 1066$).

Semen parameters	MBP		MiBP	
	Percent change (95% CI)	*p*-Value	Percent change (95% CI)	*p*-Value
Semen volume (mL)	0.9% (−0.4, 2.2%)	0.183	0.0% (−1.2, 1.2%)	0.974
Sperm concentration (10^6/mL)	−2.4% (−5.1, 0.3%)	0.078	−2.1% (−4.6, 0.4%)	0.093
Total sperm number (10^6)	−1.6% (−4.4, 1.3%)	0.282	−2.0% (−4.6, 0.6%)	0.139
Progressive motility (%)	−1.3% (−4.8, 2.3%)	0.483	−2.2% (−5.4, 1.1%)	0.193
Morphology (normal forms, %)	−6.5% (−10.3, −2.7%)	0.001	−5.3% (−8.9, −1.8%)	0.003
Acrosin activity (μIU/10^6 sperm)	−5.0% (−8.0, −2.0%)	0.001	−5.8% (−8.5, −3.0%)	<0.001
DFI[b] (%)	5.3% (2.2, 8.3%)	0.001	5.7% (2.8, 8.6%)	<0.001
Semen parameters	MEHP		%MEHP	
	Percent change (95% CI)	*p*-Value	Percent change (95% CI)	*p*-Value
Semen volume (mL)	0.4% (−0.6, 1.5%)	0.431	0.8% (−0.3, 1.9%)	0.135
Sperm concentration (10^6/mL)	−0.8% (−2.9, 1.4%)	0.471	0.3% (−1.9, 2.5%)	0.778
Total sperm number (10^6)	−0.3% (−2.5, 2.0%)	0.825	1.1% (−1.2, 3.4%)	0.358
Progressive motility (%)	−2.1% (−4.9, 0.7%)	0.136	−3.9% (−6.8, −1.0%)	0.007
Morphology (normal forms, %)	−0.7% (−3.7, 2.4%)	0.663	1.3% (−1.8, 4.4%)	0.419
Acrosin activity (μIU/10^6 sperm)	−3.4% (−5.8, −1.1%)	0.004	−4.2% (−6.6, −1.7%)	0.001
DFI[b] (%)	0.6% (−1.8, 3.0%)	0.606	2.0% (−0.3, 4.3%)	0.095

[a] Regression coefficients were adjusted for age, BMI, smoking, drinking, abstinence time and urinary creatinine (except for%MEHP) and were back transformed to present the percent change.
[b] Sample size was 634 for DFI.

MCPP, MBzP, and MiNP ($p < 0.04$). DFI was positively associated with MBP and MiBP, and increased by 5.3% (95% CI: 2.2, 8.3%) and 5.7% (95% CI: 2.8, 8.6%) for an IQR increase in MBP and MiBP, respectively.

Transformed effect estimates from phthalate quartile models of the MBP, MiBP, MEHP and %MEHP associations with acrosin activity are shown in Fig. 2. Compared with the lowest quartile, acrosin activities decreased by 13.3% (95% CI: −20.5, −5.5%), 12.4% (95% CI: −19.7, −4.5%), 8.3% (95% CI: −15.4, −0.6%) and 9.3% (95% CI: −16.3, −1.8%) in the highest MBP, MiBP, MEHP and %MEHP quartiles, respectively.

4. Discussion

INSL3, a major secretory product of Leydig cells, has been used as a marker of testicular function [18]. In the present study, we observed for the first time, an inverse association between serum INSL3 and MEHP levels in adult men. Considering that INSL3 production is entirely dependent on the state of Leydig cell differentiation and function, its decrease implied that MEHP had an adverse effect on Leydig cells. This result was consistent with previous animal research showing that INSL3 gene expression in rat fetal testes was inhibited after maternal exposure to DEHP [19]. However, a contrary study showed that INSL3 production was not affected when adult human testis explants were cultured with DEHP or MEHP [29]. Since studies focusing on phthalate and INSL3 association remain limited, it is difficult to explain the discord between studies, though disparities in exposure time or subject selection may be responsible. In the future, different aspects of study design should be conducted in various study populations to confirm the association between INSL3 and phthalate exposure.

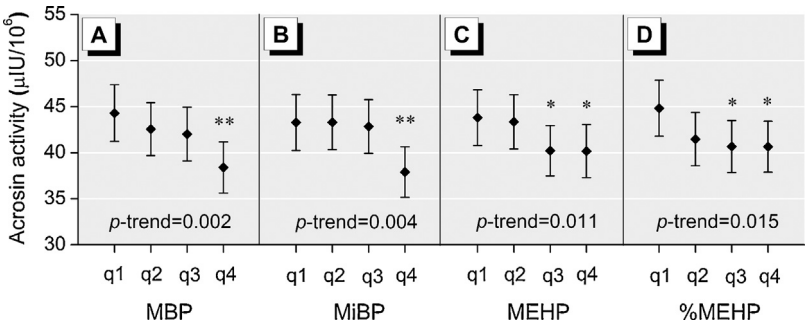

Fig. 2. Estimated acrosin activity from models of (A) mono-*n*-butyl phthalate (MBP), (B) mono-isobutyl phthalate (MiBP), (C) MEHP and (D) %MEHP quartiles. Points correspond to estimated mean values adjusted for age, BMI, smoking, drinking, abstinence time and urinary creatinine (except for %MEHP), whiskers indicate 95% confidence interval. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with lowest quartile. *p*-trend is the *p* value for linear trend across quartiles.

Toxicological studies on experimental animals have shown that some phthalates are testicular toxicants that reduce steroid hormone biosynthesis in Leydig cells [30,31]. In recent years, epidemiological studies have produced consistent trends showing negative associations of MEHP and MBP with serum total testosterone [9,10,32]. In the present study, we found that phthalate metabolites were associated with several hormone levels. The short-chained metabolites MMP and MCPP were negatively associated with FT and FAI and the long-chained metabolites MEHP, MBzP, MiNP and MCiOP were associated with decreased E_2. Interestingly, positive associations of FSH were found with MCMHP but not with other DEHP metabolites. These results indicated that phthalates with different molecular structures might disrupt the synthesis of hormones by different mechanisms. The most striking results were found in MBP and MiBP; not only were levels of T, FT and FAI significantly decreased at higher levels of MBP and MiBP, but so were levels of LH, a gonadotropin secreted by the pituitary. If MBP and MiBP solely impaired testicular steroidogenesis in the Leydig cells, a negative feedback inducing higher LH secretion by the pituitary should be observed. However, in this study, a significant decline in LH was observed. Thus, we speculated that exposure to MBP and MiBP may impair LH production ability in the pituitary, therefore disturbing the hypothalamic-pituitary-testis (HPT) axis. The inhibition of testosterone production may not only be caused by Leydig cell dysfunction, but also, atleast to some degree, be associated with decreased pituitary LH secretion. Our observation is consistent with studies on rats and goldfish [33,34], in which phthalate like DEHP inhibited LH release from the pituitary, resulting in a decrease in testosterone levels and providing evidence for the pituitary effects of phthalates.

In contrast to the present study, a positive association between MBP and LH has been reported in previous research [11]. Other studies found no significant decreases in LH at high PAEs level exposures in various populations [9,10]. These discrepancies might relate to different phthalate exposure levels, or from different study population selection. In this cross-sectional study, participants were male partners of subfertile couples recruited from an infertility clinic. Although this subfertile population might not be representative of the general population, there was no evidence showing that the men who visit infertility clinic were more susceptible to the effects of phthalates on reproductive health. Sample size may be considered another factor affecting epidemiological results. As modest effects of target chemicals can be easily obscured by noise of confounders or intra-individual variation, we collected a relatively large sample size ($n = 1066$) to detect statistically significant associations. In the present study, numerous relationships were tested between phthalate metabolites and reproductive outcomes and the possibility of chance findings existed. The Bonferroni-type correction was not used because the correction may lead to a high rate of false negatives, which will severely reduce our power to detect the important effects of phthalates. Although the possibility that some of our observed associations were chance findings cannot be ruled out, the main results from MBP, MiBP, MEHP, and %MEHP showed a better statistical significance (most of the *p* value <0.01) than the results from other phthalate metabolites (*p* value <0.05) and should not be underestimated. Additionally, the decrease in LH with concurrent T, FAI, and FT decline is a biological pattern that can be explained by HPT axis dysfunction.

In the present study, semen parameters, including sperm motility, morphology, acrosin activity and DFI, were associated with at least one phthalate metabolite. MBP and MiBP seemed to be the primary toxicants with consistent adverse effects on several semen parameters, but not on sperm motility or sperm concentration, which differs from previous studies [12,35]. The biological mechanism linking sperm quality to phthalate exposure remains unclear, though one possible explanation might be that phthalate metabolites such as MBP and MiBP reduced testosterone secretion in the testes, leading to spermatogenesis dysfunction because the maintenance and restoration of spermatogenesis requires a relatively high concentration of testosterone in the adult testis [36].

DFI, representing sperm DNA damage levels, was significantly associated with increasing MBP and MiBP levels in our cohort. Similar findings were found in Polish and Indian cohorts [37,38], in which MBP and DBP were positively correlated with DFI. Associations of sperm DNA damage with MEP and MEHP were also found in a USA population using neutral comet assay [39,40]. Linked with an epidemiological finding that bilirubin, a potent antioxidant, was negatively associated with several phthalate metabolites [41], it is plausible that MBP and MiBP may increase oxidative stress, with excessive production of reactive oxygen species leading to DNA damage in ejaculated sperm.

As a useful indicator of sperm quality, acrosin activity plays an important role in the process of fertilization. Compared with the assessment of acrosome reaction, acrosin amidase measurement is a relatively simple method suitable for large sample size analysis [28]. Using this method, our results pointed toward decreasing fertilizing potential in men with high levels of MBP, MiBP and MEHP. As far as we know, this is the first report on the association between phthalates and acrosin activity. The mechanism of how phthalate affects acrosin activity in sperm requires further research.

%MEHP is a phenotypic marker of less efficient metabolism of DEHP to its oxidized metabolites. A high %MEHP value reflects low detoxification capability and indicates increased susceptibility to DEHP exposure [42]. A few studies have observed the associations of %MEHP with decreased serum T levels [10,11] and DNA damage in sperm [40]. In this study, we compared the regression model results, using MEHP and %MEHP as independent variables, and found that the associations of %MEHP with several reproductive parameters were more significant than the associations of MEHP.

This indicates a need to better understand %MEHP, which might be a more sensitive marker to the effects of DEHP exposure on reproductive health.

In the present study, MBP, MiBP and MEHP were the main toxicants associated with reproductive health parameters. Some of our findings were not observed in existing work or were inconsistent with previous studies, which may have resulted from different phthalate exposure levels. Here, total amount of DBP, di-iso-butyl phthalate (DiBP) and DEHP metabolites accounted for about 74% of all phthalate exposures by weight. The urinary levels of MBP and MiBP (geometric mean 78.7 and 46.9 ng/mL, respectively) were 4–6 times higher than the values in the USA population from the National Health and Nutrition Examination Survey (NHANES 2007–2008), in which the geometric means for MBP and MiBP were 18.9 and 7.15 ng/mL, respectively [6]. Our MBP levels were also higher than studies in Denmark and Poland [11,37]. These regional differences in MBP and MiBP exposure could be related to factors such as dietary exposure. For example, the median of estimated dietary exposures of DBP and DiBP were 243 and 245 ng/kg body weight (bw)/day, respectively, for Chinese adults [43], which were about one order of magnitude higher than those found among American adults (34 and 8 ng/kg bw/day for DBP and DiBP, respectively) [44]. Regional differences in phthalate exposure may help explain why MBP and MiBP showed consistent trends with several reproductive parameters in this study, which were not observed in previous studies.

5. Conclusions

We observed that phthalate metabolites, particularly MBP, MiBP and MEHP, were associated with INSL3, acrosin activity and other reproductive health parameters, indicating their multiple adverse effects on Leydig cell function, pituitary secretion and spermatogenesis. In addition, %MEHP was a potentially sensitive marker to the susceptibility of DEHP exposure. Although causative relationships between exposure to phthalates and adverse health outcomes may never be established in this study, further investigations are still needed to confirm the observed effects of phthalates, especially on testicular marker INSL3 and acrosin activities in different populations and on a larger scale.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.08.011

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