



# Positive association of low-level environmental phthalate exposure with sperm motility was mediated by DNA methylation: A pilot study

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## HIGHLIGHTS

- DEHP exposure associated with decreased sperm LINE-1 DNA methylation levels.
- Sperm LINE-1 DNA methylation levels negatively associated with sperm quality.
- LINE-1 DNA methylation mediate environmental level DEHP exposure and sperm motility.

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## ABSTRACT

Accumulating evidence indicates that phthalate exposures may affect human semen quality. Epigenetic modifications such as DNA methylation might be linked chemical exposure and spermatogenesis epigenetic reprogramming. In the present study, we investigated associations between phthalate exposures, DNA methylation and sperm quality in undergoing fertility assessment male population. Urine was used for phthalate exposures monitoring, six selected metabolites (i.e., monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono-n-butyl phthalate (MBP), monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP) and mono (2-ethyl-5-oxohexyl) phthalate (MEOHP)) were measured by using HPLC–MS/MS. Sperm quality parameters were determined by computer-assisted semen analysis (CASA). Sperm DNA methylation patterns (long interspersed nuclear element-1(LINE-1), H19 and LIT1) were analysed employing high-melting resolution (HRM) PCR. Urinary MMP, MEHP, MEOHP, sum of DEHP metabolites ( $\sum$ DEHP) and sum of selected phthalates metabolites ( $\sum$ PAEs) were significantly positively associated with sperm motility. Sperm LINE-1 DNA methylation were found to be negatively associated with  $\sum$ DEHP exposure and sperm quality (ejaculate volume, total sperm number and motility). Epigenetic modification LINE-1 DNA methylation demonstrated mediating effects in association between DEHP exposure and sperm motility, and 20.7% of the association was mediated by serum LIEN-1 DNA methylation. These results extend the previous studies in association between phthalate exposures and classical semen parameters, mainly of inverse association, and sperm DNA methylation may be linked phthalate exposures and male reproductive health outcome.

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## 1. Introduction

The noncovalent binding of phthalate diesters in many matrices result in they can easily leach out of these products (Blount et al., 2000), which makes that phthalates are the pervasive environmental pollution and they are present in food and various other

commodities (ATSDR, 2002). Humans are exposed to these ubiquitous chemicals via multiple routes (Hauser and Calafat, 2005) and the phthalate diesters are rapidly metabolized into their corresponding monoesters when they enter the human body. Some of phthalate monoesters can be further oxidized and conjugated by glucuronide before excretion into urine or feces (Lorber et al., 2009). Because the vast majority of phthalate metabolites can be excreted in urine within 48 h, their metabolites forms have been extensively used as the exposure biomarkers in human bio-monitoring (Guo et al., 2011).

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Some phthalates, such as di-(2-ethylhexyl) phthalate (DEHP) and di-n-butylphthalate (DBP), are endocrine disruptors and classified as the reproductive toxicants in the EU (Gray et al., 2000). Toxicological studies have demonstrated that these chemicals can directly impact testicular Sertoli and Leydig cells (Sobarzo et al., 2015; Ge et al., 2007), then the subsequent disruption of steroidogenesis may result in the altered spermatogenesis in testis (Aly et al., 2016). However, the mode of disruption for phthalate is still inconsistent both in epidemiological and toxicological studies (Bloom et al., 2015; Cai et al., 2015; Ge et al., 2007; Savchuk et al., 2015; Wang et al., 2018). Several epidemiological studies have showed that phthalate exposures may associate with the decreased sexual steroid secretion, shortened anogenital distance (AGD), and lowered semen quality (Pan et al., 2015; Swan et al., 2015; Wang et al., 2017, 2018). However, recent *in vitro* or *in vivo* laboratory studies also indicated that phthalates have distinct effects between the low- and high exposure doses; at the low-level dose the secretion of sex hormones was induced *via* changing the steroidogenesis-related proteins and genes (Ge et al., 2007; Li et al., 2016; Savchuk et al., 2015). Some epidemiological results also implied that environmental relevant phthalate exposures may associate with the increased steroid sexual hormone (urinary MBzP exposure concentration mean was 1.7 and 47 ng/mL) (Mouritsen et al., 2013; Tian et al., 2018) and the improved semen quality parameters (urinary MEHP exposure concentration median was 1.2 ng/mL and MEP was 152 ng/mL) (Bloom et al., 2015; Duty et al., 2004). Although the observed effects are inconsistent, phthalate exposures dose have action on the spermatogenesis *via* steroidogenesis pathway.

In addition to change the steroidogenesis, increasing toxicological studies have revealed that the sperm epigenetic modification can be modified by some environmental factors including phthalates (Stenz et al., 2017; Prados et al., 2015; Manikkam et al., 2013). DNA methylation play the important role in sperm maturation and male mammalian germ line will undergo an extensive epigenetic reprogramming in spermatogenesis. The widespread erasure of DNA methylation followed by the *de novo* DNA methylation is a normal procedure (Houshdaran et al., 2007; Rousseaux et al., 2005); generally, the reducing DNA methylation is a trend in spermatogenesis (Houshdaran et al., 2007). Abnormal epigenetic modification may result in an impaired spermatogenesis and the compromised sperm function. The global DNA methylation surrogate (LINE-1) hypermethylation has been associated with the reduced sperm motility (Tian et al., 2014), lowered sperm quality (Houshdaran et al., 2007) and decreased sperm morphology (Cassuto et al., 2016). The abnormally hypomethylated paternal imprinted gene H19 or/and abnormally hypermethylated maternal imprinted gene LIT1 may also associate with the poor sperm quality and the increased risk of imprinting syndrome in offspring (Li, 2002; Klaver et al., 2013; Rotondo et al., 2013). Very limited epidemiological evidence also indicated that phthalate exposures may influence the sperm DNA methylation of men undergoing IVF (*in-vitro* fertilization) treatment (Wu et al., 2017a).

The linkage of phthalate exposures to adverse outcome of male reproduction remains inconclusive, also a little is known about the effect of phthalate exposures on the imprinted genes and global DNA methylation in sperms. Currently we investigated the potential spermatogenesis effect of phthalate exposures with the viewpoints of sperm quality (concentration, morphology and motility) and its DNA methylation changes (for both the global DNA and some selected imprinting genes) in a cross-sectional male population. The major goal is to verify the male reproductive effect of the low-dose environment relevant phthalate exposure.

## 2. Materials and method

### 2.1. Study subjects

The men included in this study were a subset of participants who came to Reproduction Department of fertility assessment (Liu et al., 2012; Tian et al., 2014). The 118 volunteers were recruited from the Reproduction Department of the Chongqing Institute of Science and Technology for Population and Family (CISTPF), and the local ethics committee approved the study. The purposes of the study were explained to the prospective participants, who each agreed to participate by signing a consent form. A face to face interview was arranged for each participant to collect the individual information of age, personal background, medical history, life-style factors, occupational and environmental exposures, sexual, and physical activity. The exclusion criteria for participation in the study were as follows: metabolic disease, urogenital diseases, abnormal mass index and occupationally pollutants exposure. Finally, 86 eligible participants were involved in this study, who have sufficient semen samples for semen quality and DNA methylation analysis and urine sample for phthalate metabolites measurement.

### 2.2. Sperm collection and analysis

Semen analyses were performed in accordance with the World Health Organization (WHO, 2010) guidelines. Sperm specimens were obtained *via* masturbation after a required abstinence period of at least two days. After liquefaction at 37 °C for no more than 30 min, the semen volume was measured with a serologic pipette. Sperm motion and conventional semen parameters were determined according to the World Health Organization (WHO) guideline (World Health Organization, 2010). As have been previously described (Kruger et al., 1986; Tian et al., 2014), the semen concentration, motility and morphology parameters were analysed using a Micro-cell slide and computer-aided semen analysis (CASA, WLJY 9000, Weili New Century Science and Tech Den., Beijing, China). Sperm morphology was determined using air-dried smears stained with a modified Wright-Giemsa stain, total sperm count was calculated as the product between ejaculate volume and sperm concentration, total sperm motility [progressive motility (PR) + non-progressive motility (NP)] were calculated (World Health Organization, 2010).

### 2.3. Sperm DNA methylation analysis

The measurement method for sperm global DNA surrogate (LINE-1) and imprinted gene (H19 and LIT1) has been described previously (Tian et al., 2014). Briefly, sperm DNA were extracted using the QIAamp DNA minikit (Qiagen, Hilden, Germany) following the manufacturer's instruction. Isolated DNA (200 ng) was subjected to sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) in accordance with manufacturer's instructions. High-resolution melting (HRM) analysis was applied to quantitatively measure the methylation status of selected imprinted (H19 and LIT1) and global DNA methylation surrogate (LINE-1) genes. Primers for HRM analysis are shown in supplementary data Table S1. Methylation standards were constructed by diluting 100% methylated control plasmid in a pool of unmethylated control plasmid, selected standards were confirmed with bisulfite sequence PCR (BSP) method (supplementary data, Figs. S1–4). The PCR cycling conditions and HRM data analysis were presented in previous publication (Tian et al., 2014). The standard curve was then used to calculate the methylation levels for each sample.

#### 2.4. Urine collection and phthalate metabolites analysis

Participants provided a spot urine sample (morning spot urine before eating or drinking) at the same day as semen sample procurement. The collected urinary samples were stored at  $-80^{\circ}\text{C}$  until analysis. Six urinary phthalate metabolites MMP, MEP, MBP, MBzP, MEHP and MEOHP were analysed. The contents of both free monoester phthalates and their conjugated forms were analysed using our previous method (Liu et al., 2012; Tian et al., 2018). Briefly, urine samples were initially spiked with the  $^{13}\text{C}$ -labeled internal standard of monoester phthalates and deconjugated using *E. coli*  $\beta$ -glucuronidase, then purified by a SPE cartridge (Oasis HLB column, Waters), and finally analysed by an isotope dilution method using LC-ESI-MS/MS. Two method blanks, two quality control samples (human urine spiked with phthalates) and two sets of standards were also run together with the unknown samples in each analytical batch. The spiked recoveries for the phthalate metabolites were 89%–104%, relative standard deviation (RSD) were 0.8%–14.6%. The limit of detection were 0.21–0.51 ng/mL. Phthalate metabolite concentrations were adjusted by urinary creatinine to adjust the influence of urine dilution.

#### 2.5. Statistical analysis

Descriptive statistics were calculated for the demographic characteristics of participants, along with the distributions of urinary phthalate metabolites and gene DNA methylation levels of sperm. The total concentrations of DEHP metabolites  $\Sigma\text{DEHPm}$  and  $\Sigma\text{DEHPM}$  were the mass and molar sum of MEHP and MEOHP, respectively. The  $\Sigma\text{PAEm}$  and  $\Sigma\text{PAEM}$  were the mass and molar sum of MMP, MEP, MBP, MBzP, MEHP and MEOHP (Mouritsen et al., 2013). Given the low detection frequency (44.2%) of MBzP, it was not used in the further statistics. As urinary phthalate metabolite concentrations were not normally distributed, we transformed the metabolites using natural logarithm (ln) and adjusted by creatinine for following data analysis. Multiple linear regression was used to assess the DNA methylation association with the natural ln-transformed phthalate metabolites concentrations and semen quality parameters, while the relevant confounders of age, body mass index (BMI), smoking, alcohol drinking, and abstinence time were adjusted. Potential mediating effects of DNA methylation in the association of phthalate exposure with semen quality were evaluated by causal step regression (Baron and Kenny, 1986). The regression was tested in three-step multiple linear regressions. Data analyses were conducted using the SPSS 19.0 statistical package (SPSS Inc., USA).  $P$  value  $< 0.05$  was defined as a statistical significance.

### 3. Results

#### 3.1. Study participant demographics

The demographic characterization for the current investigated men who were seeking fertility assessment (Table 1). The mean age and body mass index (BMI) were  $31.6 \pm 4.8$  years old and  $23.9 \pm 4.0 \text{ kg/m}^2$ , respectively. Abstinence time and lifestyle habits (i.e., smoking and alcohol consumption) were also listed in Table 1. Most of the participants have abstinence time longer than three days (90.7%). About half of the participants were smokers (47.7%) or alcohol drinker (58.1%). Among the 86 specimens, semen volumes ranged from 0.6 to 4.7 mL, concentration 2.11 to  $216.09 \times 10^6/\text{mL}$ , morphology 3.0–35.0% and motility 2.82–81.97%. In the study population, 6 (7.0%) participants had a semen volume  $< 1.5 \text{ mL}$ , 4 (4.7%) had a sperm concentration  $< 15 \times 10^6/\text{mL}$ , 7 (8.1%) had total sperm number per ejaculum  $< 20 \times 10^6$ , and 14 (16.3%) had  $< 40\%$

**Table 1**  
Subject demographics of the participants (n = 86).

Characteristic	Mean (SD)	Number(%)
Age (years)	31.6 (4.8)	
BMI ( $\text{kg/m}^2$ )	23.9 (4.0)	
Abstinence time (days)		
≤2		8 (9.3)
3–4		36 (41.9)
5–6		31 (36.1)
≥7		11 (12.7)
Smoking status		
No smoking		45 (52.2)
Smoking		41 (47.7)
Alcohol drinking		
None		36 (41.9)
Drinker		50 (58.1)
Semen parameters		
Ejaculate volume (ml)	2.67 (0.89)	
Concentration ( $10^6/\text{mL}$ )	76.80 (47.56)	
Total sperm number ( $10^6$ )	202.65 (143.74)	
Morphology (%)	15.27 (6.97)	
Motility (%)	55.52 (16.33)	

BMI: body mass index.

motile sperm and 3 (3.5%) had  $< 4\%$  sperm morphology according to WHO reference standards (World Health Organization, 2010).

#### 3.2. Urinary phthalate metabolites, sperm DNA methylation

Six urinary metabolites of phthalate diesters, i.e., MMP, MEP, MBP, MBzP, MEHP and MEOHP, were measured as indicators of the participants' internal exposure to phthalates (Table 2). Except for MBzP, the selected phthalate metabolites were detectable in almost all samples with media concentrations in the range from 0.50 to 15.02 ng/mL (not adjusted for creatinine) and 0.85–20.53  $\mu\text{g/g}$  (creatinine-adjusted). The short straight alkyl chain MMP, MEP, or MBP concentration was higher than the branched alkyl chain MEHP and aromatic alkyl chain MBzP concentration.

Differentially-methylated regions (DMRs) in promoters of the paternally imprinted *H19* gene, maternally imprinted *LIT1* gene and the genome-wide repetitive element (*LINE-1*) were analysed by HRM. Table 2 shows the distribution of DNA methylation of each gene. Mean DNA methylation levels varied widely between different genes, ranging from 0.65% to 74.72%. The median methylation levels of *LINE-1*, *H19* and *LIT1* were 50.36%, 85.06% and 0.12%, respectively.

#### 3.3. Phthalate exposures and semen quality parameters

Multiple linear regression models were used to model the associations between phthalate exposures and semen quality parameters. Semen quality parameters were modelled as the function of ln-transformed phthalates concentration with the adjustments for age, BMI, smoking, drinking and abstinence time. The associations between urinary phthalate metabolites and semen quality parameters are shown in Table 3. The urinary MMP, MEHP, MEOHP,  $\Sigma\text{DEHPm}$ ,  $\Sigma\text{DEHPM}$ ,  $\Sigma\text{PAEm}$  and  $\Sigma\text{PAEM}$  were positively associated with sperm motility (all  $P < 0.05$ ) (Table 3). Especially, an ln-transformed unit increase in urine concentration of  $\Sigma\text{DEHPM}$  was associated with a 4.01% (95% CI: 0.27%–7.75%) increase in sperm motility. However, no significant associations were observed between the phthalate exposure and other semen quality parameters (ejaculate volume, concentration total sperm number and morphology). In addition, we found significantly inverse associations between *LINE-1* DNA methylation level and motility, volume and total sperm number (all  $P < 0.05$ ) (Table 3).

**Table 2**  
Distribution of the phthalate metabolite concentrations in urine samples and DNA methylation in sperm (n = 86).

Analytes	Detection rate (%)	Mean (SD)	Min	25 <sup>th</sup>	50 <sup>th</sup>	75 <sup>th</sup>	Max
Unadjusted urine phthalate metabolite (ng/mL urine)							
MMP	100	22.37 (23.73)	0.98	8.52	15.02	28.46	170.25
MEP	100	60.64 (231.38)	1.09	5.78	12.39	33.07	2031.27
MBP	98.8	21.83 (35.55)	0	3.89	10.02	19.92	203.39
MBzP	44.2	0.24 (1.34)	0	0	0	0.09	12.24
MEHP	97.7	1.54 (2.23)	0	0.21	0.50	1.93	11.45
MEOHP	100	2.31 (2.00)	0.14	0.92	1.76	3.10	11.60
∑DEHPm		3.85 (3.85)	0.35	1.42	2.50	4.76	20.29
∑DEHPM		0.013 (0.014)	0.001	0.0049	0.0087	0.164	0.07
∑PAEsm		108.93 (237.28)	10.45	34.02	60.64	85.75	2076.77
∑PAEsM		0.55 (1.22)	0.05	0.18	0.30	0.44	10.70
Creatinine-adjusted urine phthalate metabolite (μg/g creatinine)							
MMP	100	39.61 (85.42)	2.50	11.59	20.53	38.50	745.44
MEP	100	93.26 (435.85)	0.46	7.47	16.59	42.96	4015.32
MBP	98.8	30.62 (52.18)	0	5.10	14.19	26.77	283.79
MBzP	44.2	0.27 (1.16)	0	0	0	0.15	10.36
MEHP	97.7	2.88 (6.91)	0	0.25	0.85	2.77	48.77
MEOHP	100	3.38 (4.43)	0.30	1.48	2.17	3.76	29.38
∑DEHPm		6.27 (10.94)	0.46	2.06	3.61	6.35	75.77
∑DEHPM		0.022 (0.039)	0.001	0.007	0.012	0.022	0.27
∑PAEsm		170.03 (449.00)	7.55	46.78	75.02	146.83	4089.06
∑PAEsM		0.85 (2.32)	0.04	0.24	0.36	0.76	21.08
Semen DNA methylation level							
LINE-1 (%)		50.14 (1.93)	44.69	48.68	50.36	51.34	56.05
H19 (%)		74.72 (22.75)	16.40	75.46	85.06	87.37	96.60
LIT1 (%)		0.65 (3.05)	0.01	0.06	0.12	0.38	27.88

∑DEHPm, the mass sum of DEHP metabolites (MEHP and MEOHP), ∑DEHPM, the molar sum of DEHP metabolites (MEHP and MEOHP), ∑PAEsm, the mass sum of selected phthalate metabolites (MMP, MEP, MBP, MBzP, MEHP and MEOHP), ∑PAEsM, the molar sum of selected phthalate metabolites (MMP, MEP, MBP, MBzP, MEHP and MEOHP).

**Table 3**  
Adjusted associations between phthalate metabolites concentrations and DNA methylation with sperm quality parameters. Adjusted for age, smoking, drinking and abstinence time.

Analytes	Ejaculate volume β (95% CI)	Concentration β (95% CI)	Total sperm number β (95% CI)	Morphology β (95% CI)	Motility β (95% CI)
MMP	0.07 (−0.13, 0.26)	2.48 (−8.15, 13.11)	14.30 (−16.54, 45.15)	0.01 (−0.01, 0.04)	4.20 (0.76, 7.64)
MEP	0.08 (−0.13, 0.30)	−1.35 (−8.67, 5.98)	−2.74 (−24.08, 18.60)	0.01 (−0.02, 0.01)	2.18 (−0.22, 4.59)
MBP	0.03 (−0.19, 0.24)	−1.49 (−10.11, 7.14)	1.66 (−23.49, 26.80)	0.01 (−0.01, 0.02)	1.28 (−1.64, 4.21)
MEHP	0.14 (−0.07, 0.36)	3.43 (−3.81, 10.68)	17.59 (−3.44, 38.61)	0.01 (−0.004, 0.02)	1.60 (−0.85, 4.05)
MEOHP	−0.02 (−0.24, 0.20)	8.20 (−5.43, 21.82)	19.29 (−21.40, 59.97)	−0.001 (−0.02, 0.02)	3.56 (−1.17, 8.28)
∑DEHPm	0.11 (−0.10, 0.32)	5.53 (−5.87, 16.94)	26.39 (−6.51, 59.28)	0.01 (−0.01, 0.02)	4.04 (0.29, 7.80)
∑DEHPM	0.11 (−0.10, 0.33)	5.55 (−5.80, 16.90)	−26.40 (−6.34, 59.14)	0.01 (−0.01, 0.02)	4.01 (0.27, 7.75)
∑PAEsm	0.12 (−0.10, 0.33)	−3.01 (−13.47, 7.44)	−1.16 (−31.69, 29.38)	−0.001 (−0.02, 0.02)	4.43 (1.05, 7.80)
∑PAEsM	0.11 (−0.10, 0.33)	−2.82 (−13.25, 7.61)	−0.97 (−31.41, 29.48)	−0.001 (−0.02, 0.02)	4.44 (1.07, 7.80)
LINE-1	−0.28 (−0.49, −0.08)	−2.32 (−7.81, 3.16)	−18.68 (−34.18, −3.18)	0.01 (−0.00, 0.01)	−2.21 (−3.99, −0.43)
H19	−0.06 (−0.27, 0.16)	−0.34 (−0.80, 0.12)	−1.06 (−2.40, 0.29)	0.00 (0.00, 0.001)	−0.01 (−0.16, 0.15)
LIT1	−0.10 (−0.31, 0.12)	−2.60 (−6.03, 0.82)	−7.08 (−17.09, 2.92)	0.01 (−0.00, 0.01)	−0.27 (−1.44, 0.90)

### 3.4. Phthalate exposure and DNA methylation

The ∑DEHP, either as ∑DEHPm or as ∑DEHPM, was significantly associated with the decreased DNA methylation levels of LINE-1 (all  $p < 0.05$ ) (Table 4). The level of LINE-1 methylation declined by −0.46% (95% CI: 0.91, −0.01%) and −0.45% (95% CI: 0.91, 0.00%)

with 1-unit increase in ln-transformed concentration of ∑DEHPm and ∑DEHPM, respectively. However, the correlations between phthalate exposure and the levels of paternally imprinted *H19* gene and maternally imprinted *LIT1* gene were not observed. Nonetheless, the negative tendencies of this association were consistent between H19 and LINE-1.

**Table 4**  
Adjusted associations between urinary phthalate metabolite concentrations and DNA methylation.

Phthalate metabolite	LINE-1 β (95% CI)	H19 β (95% CI)	LIT1 β (95% CI)
MMP	−0.04 (−0.47, 0.39)	−0.15 (−5.24, 4.95)	0.36 (−0.33, 1.0)
MEP	−0.06 (−0.36, 0.24)	−2.18 (−5.65, 1.29)	0.10 (−0.37, 0.58)
MBP	−0.12 (−0.47, 0.23)	−0.69 (−4.83, 3.44)	0.29 (−0.26, 0.84)
MEHP	−0.10 (−0.39, 0.20)	−2.33 (−5.71, 1.06)	0.04 (−0.44, 0.52)
MEOHP	−0.53 (−1.09, 0.03)	−3.85 (−10.58, 2.88)	0.13 (−0.79, 1.04)
∑DEHPm	−0.46 (−0.91, −0.01)	−4.45 (−9.85, 0.95)	0.02 (−0.72, 0.76)
∑DEHPM	−0.45 (−0.91, 0.00)	−4.41 (−11.11, 9.16)	0.02 (−0.72, 0.75)
∑PAEsm	−0.10 (−0.53, 0.32)	−3.30 (−8.26, 1.67)	0.19 (−0.49, 0.86)
∑PAEsM	−0.09 (−0.52, 0.33)	−3.17 (−8.13, 1.78)	0.19 (−0.48, 0.86)

Adjusted for age, smoking, drinking and abstinence time.

### 3.5. Mediating effect of DNA methylation

Because DEHP exposure was inversely associated with LINE-1 methylation and positively associated with sperm motility, a causal-step regression mediation analysis was conducted to evaluate the potential mediating role of sperm LINE-1 methylation in the correlation between DEHP exposure and sperm motility. The results showed that LINE-1 mediated the effect of  $\Sigma$ DEHP exposure on sperm motility as all the three regression coefficients ( $\beta_a$ ,  $\beta_b$  and  $\beta_c$ ) were significant ( $p < 0.05$ ). Moreover, in the third step regression  $\beta_b$  (coefficient of LINE-1 with sperm motility) was significant ( $-1.88$ ,  $p = 0.04$ ), whereas  $\beta_c$  (coefficient of  $\Sigma$ DEHP exposure with sperm motility) was not significant ( $3.14$ ,  $p = 0.1$ ) (Fig. 1). Therefore, LINE-1 DNA methylation mediated about 20.7% of the influence of DEHP exposure on sperm motility (indirect effect =  $0.91$ ,  $P = 0.04$ ; total effect =  $4.40$ ,  $P = 0.03$ ; proportion mediated =  $0.207$ ,  $P = 0.04$  in the mediation model). However, the mediation effect of the imprinted genes methylation was not observed (data not shown). Taken together, DEHP exposure may play its action on sperm motility via epigenetic LINE-1 methylation pathway.

## 4. Discussion

The present study showed that some phthalate exposures were positively associated with increased sperm motility, while the sperm global DNA methylation surrogated by LINE-1 negatively associated with some sperm parameters. The LINE-1 methylation may mediate about 20.7% of the positive association between urinary sum of DEHP metabolites and sperm motility. These results extend the previous knowledge of phthalate exposures inversely associated with some semen parameters when they are assumed as the course of anti-androgens, the mediation of LINE-1 methylation to the phthalate exposures associated sperm motility may enhance the observed effects.

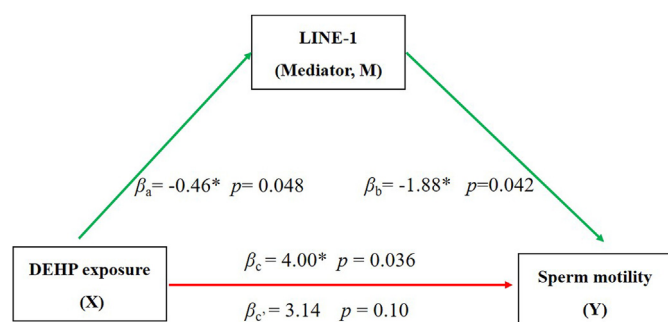
### 4.1. Phthalate exposure level specific associations with male reproduction

Epidemiological results involved phthalates' male reproduction risk (including semen quality) are inconsistent. Although previous human studies had demonstrated the associations between the increased phthalate exposure (i.e., MBP and MEHP) and reduced levels of sperm quality as well as serum androgens in reproductive age males (Wang et al., 2015; Jurewicz et al., 2013; Pan et al., 2015;

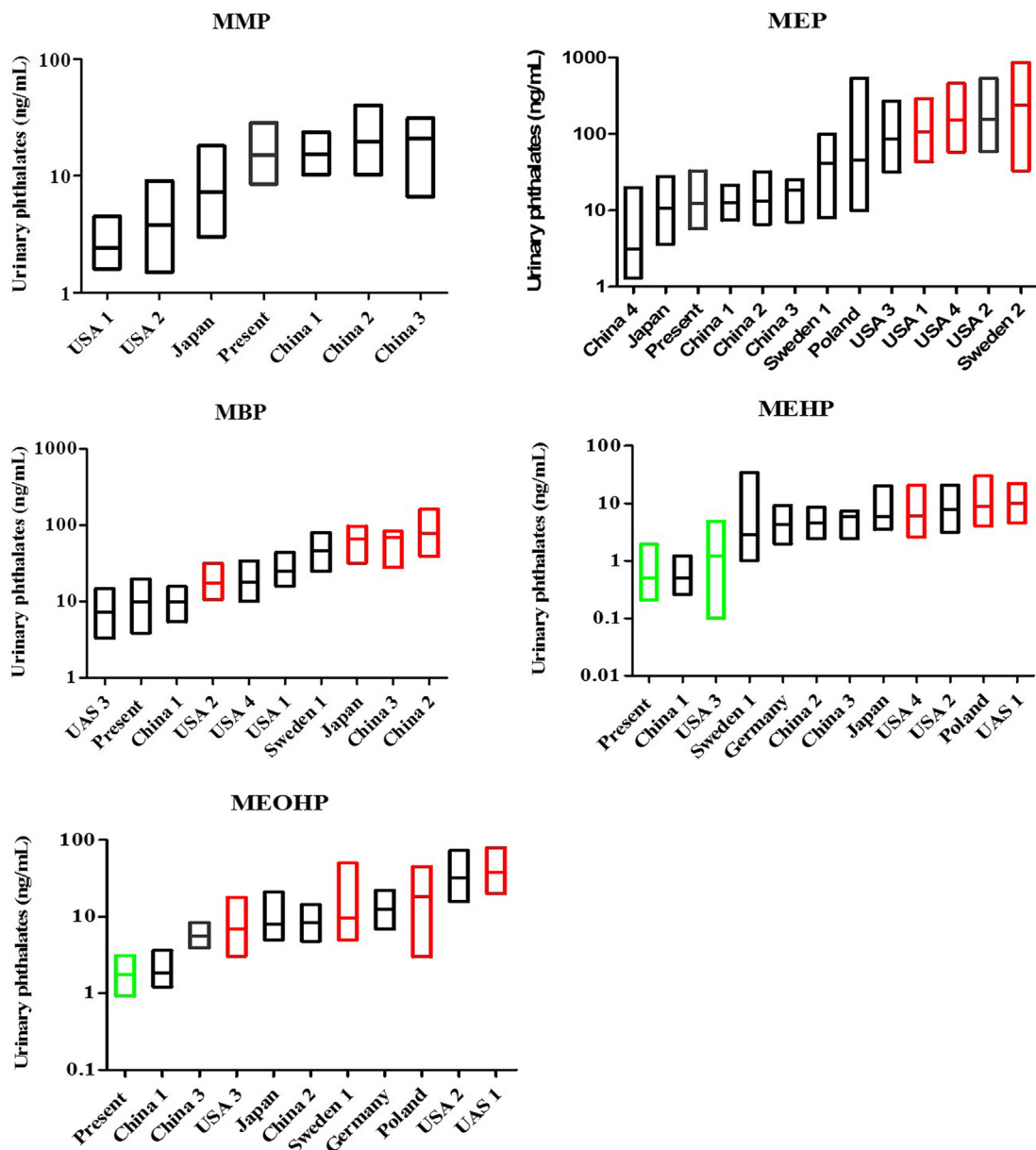
Meeker et al., 2009), other studies show no association between sperm quality or reproductive hormones and urinary phthalate metabolites (i.e., MBP, MEHP, and MBzP) (Jonsson et al., 2005; Han et al., 2014; Lenters et al., 2015). In contrast, significantly positive relationship between DEHP exposure and higher sperm motility were found in a general population study (Bloom et al., 2015). Our present study results are consistent with this positive relationship. Meanwhile, our previous study indicated environmental relevant phthalates induce androgen in general male population (Tian et al., 2018). We also observed that urinary DEHP metabolites was positively associated with preventative metabolomics markers of male infertility, which may contribute to a decreased risk of male infertility (Liu et al., 2017). Therefore, phthalates associated male reproduction effects are exposure level specific.

The low-level phthalates stimulate androgens including testosterone but the higher-level phthalates inhibited their secretion; therefore, the above mentioned inconsistent epidemiological observations may be due to the non-monotonic effects of phthalates in steroidogenesis, which majorly regulated spermatogenesis and male reproduction. Our previous study indicated that phthalates have the biphasic effects on androgen generation in Leydig cells, i.e., the low dose exposure induced androgen secretion while high dose inhibited androgen secretion (Tian et al., 2018). The biphasic effect also was found *in vivo* study that the low dose DEHP and MEHP stimulated mouse testosterone production and advanced puberty onset, whereas they were anti-androgenic at the high doses (Ge et al., 2007). Therefore, the different phthalate exposure doses could make the toxic effects differently.

The preventive effects of phthalates on sperm quality would be annotated by the low-level exposure, when the higher-level doses were applied, the effect can be inverted (Fig. 2). The endocrine impact of phthalates can show off the antipodal modes in human, i.e., as anti-androgenic agents and to be risk factor or androgenic agents to be prevent factors at outcomes of sperm motility and/or male reproduction. The previous studies have reported the significant associations of phthalates with sperm quality, mainly of DBP and DEHP (Wang et al., 2015; Jurewicz et al., 2013; Bloom et al., 2015; Chang et al., 2017). We compared the present participants' phthalate exposure levels to the previous investigated cohorts (Fig. 2 and Table 5). Our participants simultaneously exposed to multiple phthalates, the median urinary concentrations of MEHP (0.50 ng/mL), MEOHP (1.76 ng/mL) and DBP (10.02 ng/mL) were much lower than the previous studies that the inverse effects were observed (with the median values ranged from 5.0 to 10.1 ng/mL for MEHP, 9.6–32.1 ng/mL for MEOHP and 17.7–83.4 ng/mL for DBP respectively) (Liu et al., 2012; Pan et al., 2015; Wang et al., 2015; Han et al., 2014; Chang et al., 2017; Wirth et al., 2008; Hauser et al., 2006; Bloom et al., 2015; Duty et al., 2004; Axelsson et al., 2005; Jonsson et al., 2005; Jurewicz et al., 2013; Toshima et al., 2012; Herr et al., 2009). Regarding MEP exposure, negative association between sperm quality and MEP was found in the high MEP exposure population (108–240 ng/mL) compared to our present study MEP concentration (12.4 ng/mL) (Wirth et al., 2008; Jonsson et al., 2005). Meanwhile, consistent with our observation, significantly positive association between urinary phthalate metabolites (MEP, MBP and MEHP) and male sperm quality were found in many previous population (Liu et al., 2012; Toshima et al., 2012; Bloom et al., 2015), the ranking of metabolite levels for MEHP (1.2 ng/mL) were similar to our population monitoring results (0.5 ng/mL) (Bloom et al., 2015) (Fig. 2 and Table 5). Apart from the phthalate exposure levels, study design (i.e., participants recruited from the infertility clinical may have more abnormal steroidogenesis and spermatogenesis than from the general population) and genetic polymorphisms were also potential confounders that may introduce uncertainties (Høyer et al., 2018; Yang et al., 2017) in our results.



**Fig. 1.** The association between DEHP exposure and sperm motility was mediated by LINE-1 DNA methylation. Three-step multiple linear regression models are:  $Y = \beta_c X + error_1$ ;  $M = \beta_a X + error_2$ ;  $Y = \beta_c X + \beta_b M + error_3$ ; i.e., sperm motility (Y) with DEHP exposure level (X,  $\beta_c$ ), sperm LINE-1 DNA methylation (M) with DEHP exposure level (X,  $\beta_a$ ), and sperm motility (Y) with DEHP exposure level (X,  $\beta_c$ ) via LINE-1 DNA methylation (M,  $\beta_b$ ). The results were adjusted by factors of age, smoking, drinking and abstinence time. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Fig. 2.** The phthalates metabolites concentrations (ng/mL urine) distribution and association with sperm quality in male of different population. Green color means positive association, black means no significant association and red means negative association. Bottom box represents first quartile, band inside the box represents the median and top box represents third quartile. China 1: Liu et al. (2012), China 2: Pan et al. (2015), China 3: Wang et al. (2015), USA1: Wirth et al. (2008), USA2: Hauser et al. (2006), USA3: Bloom et al. (2015), USA4: Duty et al. (2004), Sweden1: Axelsson et al. (2015), Sweden2: Jönsson et al. (2005), Japan: Toshima et al. (2012), Poland: Jurewicz et al. (2013), German: Herr et al. (2009). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

#### 4.2. Low-level phthalates associated with global hypomethylation of sperm

Male mammalian germ line epigenetic reprogramming is characterized by the erasure of DNA methylation and DNA hypomethylation in spermatogenesis is a general trend (Houshdaran et al., 2007; Rousseaux et al., 2005), which may imply a higher-quality of sperm. Epigenetics has emerged as the potential explanation to elucidate the mechanisms of male compromising fertility, which is a different perspective from steroidogenesis when phthalates' male reproduction effect was assessed. Many studies have indicated that epigenetic modifications, including DNA methylation for both imprinting and developmental genes, play important role in the spermatogenesis (Houshdaran et al., 2007;

Montjean et al., 2015; Rousseaux et al., 2005). In mammalian, widespread erasure of DNA methylation has been found in primordial germ cells when entering the genital ridge during gametogenesis (Hajkova et al., 2002). Demethylation play critical role to correct the errors of methylation and remodel it, which may be a kind of response to the environmental risk induce epigenetic dysfunction (Zhu, 2009). Epigenetic abnormal modification for both imprinting gene and global DNA may result in impaired spermatogenesis and compromised sperm dysfunction. LINE-1 elements count about the 17% of the total human genome and serve as a proxy surrogate of global genomic methylation. Sperm methylation pattern changes may have widespread repercussions on chromatin integrity and then decline the sperm quality. Previous study suggested that global DNA hypermethylation may induce

**Table 5**  
The median values of phthalate monoester metabolites concentrations (ng/mL urine) in different male populations.

Country study	Character	Sample size	MMP	MEP	MBP	MBzP	MEHP	MEOHP	Outcomes addressed	Reference
Present (China)	Subfertility	86	15.0	12.4	10.0	<LOD	0.5	1.8	DEHP metabolites positively associated with sperm motility	Present study
China 1	Subfertility	97	15.4	12.6	10.1	<LOD	0.8	1.8	MEP with increased sperm motion parameters	Liu et al. (2012)
China 2	Subfertility	1066	19.8	13.4	77.6	<LOD	2.4	4.8	MBP with low sperm morphology	Pan et al. (2015)
China 3	Subfertility	1040	20.8	18.5	69.9	2.92	5.8	7.9	MBP with low sperm concentration and count	Wang et al. (2015)
China 4	General	232		3.1	18.7		1.1		Not found	Han et al. (2014)
China 5	Subfertility and fertility	290	4.5	16.7	13.5	0.9	5.03	7.7	MEHP and MBzP with low sperm concentration and motility	Chang et al. (2017)
USA 1	Subfertility	45	2.4	108.0	24.7	17.4	10.1	37.6	MEP with low sperm concentration and morphology DEHP metabolites with low sperm concentration,	Wirth et al. (2008)
USA 2	Subfertility	463	3.8	158	17.7	8.0	7.9	32.1	MBP and with low sperm concentration and motility	Hauser et al. (2006)
USA 3	General	501	0.5	86.4	7.3	3.6	1.2	7.0	MMP with low sperm motility, MEHP with increased sperm motility	Bloom et al. (2015)
USA 4	Subfertility	187		152.7	17.8	9.9	6.1		MEP with increased computer-aided sperm analysis motion parameter	Duty et al. (2004)
Sweden 1	General	314		41	47	13	2.8	9.6	MEOHP with low sperm motility	Axelsson et al. (2015)
Sweden 2	Young Soldier	234		240	78	16	<LOD		MEP with fewer sperm motility	Jönsson et al. (2005)
Poland	Subfertility	269		45.2	83.4	5.2	8.8	18.4	DEHP metabolites with low sperm motility, MBP with low sperm motion parameters	Jurewicz et al. (2013)
Japan	Subfertility	42	7.2	10.7	65.7	9.2	5.9	7.9	MBP with increased sperm concentration	Toshima et al. (2012)
Germany	Subfertility	349					4.4	12.7	Not found	Herr et al. (2009)

defective sperm chromatin condensation/DNA integrity which eventually resulting in abnormal spermatozoa (Kumar et al., 2013; Rahiminia et al., 2018). Our results are consistent with the previous studies that the global DNA hypermethylation associated with the reduced sperm quality (Tian et al., 2014; Houshdaran et al., 2007; Rahiminia et al., 2018; Cassuto et al., 2016).

In addition to the global DNA methylation, imprinted gene H19 and LIT1 were investigated. Paternally imprinted gene H19 is only transcribed from the maternally inherited allele, while maternal imprinted LIT1 is only transcribed from the paternally inherited allele (Fedoriw et al., 2012). The abnormally hypomethylated H19 or/and abnormally hypermethylated LIT1 have been found in poor-quality sperms, which may increase the risk of imprinting syndrome in offspring (Gunes et al., 2016; Santi et al., 2017). Although no significant association between the investigated imprinted genes' DNA methylation and sperm quality was observed, we observed that paternal H19 was almost hypermethylated, while maternal LIT1 was hypomethylated in the sperms of our participants, which is consistent with the previous studies (Marques et al., 2008; Hammoud et al., 2010). Especially, the LIT1 methylation showed the unmethylation status with a mean level of 0.65%.

The gamete epigenetic marker of DNA methylation can be modified by environmental endocrine disruptors including phthalate, the effects have been observed both in toxicological and epidemic studies (Chen et al., 2018; Prados et al., 2015; LaRocca et al., 2014; Li et al., 2014; Wu et al., 2017a). Accumulating evidences suggested that phthalate exposures can induce the hypomethylation of global DNA methylation in human blood (Huen et al., 2016), placenta (Zhao et al., 2016) and cord blood (Huang et al., 2018), which were consistent with our findings. The mechanism by which phthalates interfere with DNA methylation remains unclear; one possible explanation for the interference may be related to the oxidative stress. Phthalates are known to increase the production of reactive oxygen species (Kim et al., 2018; Wu et al., 2017b). Cytosine is sensitive to oxidation, leading to formation of 5-hydroxymethylcytosine (5hmc), then may inhibit binding of the methyl CpG-binding domain-proteins (MBDs) to the corresponding CpGs (Valinluck et al., 2004; Rusyn et al., 2001). The weak affinity of DNA for methyltransferase results in global DNA

demethylation. Our *in vitro* study confirmed that phthalate exposure also caused global DNA methylation (LINE-1) DNA hypomethylation in mouse Leydig cell (MLTC-1) (data unpublished, Fig. S5).

This study, to our knowledge, is the first study to report alteration in sperm global LINE-1 DNA methylation in relation to DEHP exposure, and the LINE-1 methylation mediated with phthalate exposures and sperm quality. Our results actually enhanced the evidence that the mode of action of low-level phthalate exposure is positive association with sperm quality (androgenic effect) rather than inverse association (anti-androgenic effect). However, the mechanism behind the mediation of DNA methylation on phthalates' stimulation of sperm motility need to be illustrated in the further study.

There are some limitations in our study. Firstly, the clinical participants may bring few fraction of abnormal semen quality subjects and thus limited the generalization of our findings to the general population. Furthermore, the relatively small sample size may restrict the statistical power of the results. In addition, only one spot urine sample and a single semen samples from each participant, which would be only moderately predictive for long-term exposure and exposure misclassification cannot be fully ruled out (Wan et al., 2019). Finally, only two DEHP metabolites were selected to surrogate DEHP exposure, which would be only moderately reflected the real sum of DEHP exposure situation. Because of sample size limitations, the present observation need to be confirmed with additional larger population studies. Meanwhile, the *in vivo* animal phthalate exposure study will be great help to elucidate the underlying mechanism. Given the sperm DNA methylation link environmental exposure and sperm quality, and sperm DNA methylation abnormalities involvement on potential risk to offspring, sperm DNA methylation could represent a potential approach in the diagnostic flowchart of male infertility and environmental exposure risk assessment.

## 5. Conclusions

In summary, we showed that phthalate exposure was associated with sperm motility for these clinical subfertility males by

suggesting that sperm DNA methylation might be part of the underlying biological pathway linking phthalate exposure with sperm quality.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2018.12.155>.

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