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Associations of urinary 5-methyl-2′-deoxycytidine and 5-hydroxymethyl-2′-deoxycytidine with phthalate exposure and semen quality in 562 Chinese adult men

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ABSTRACT

5-methyl-2′-deoxycytidine (5mdC) and 5-hydroxymethyl-2′-deoxycytidine (5hmdC), products of DNA methylation and hydroxymethylation processes, have been detected previously in human urine, but their associations with environmental chemicals or healthy outcomes are unclear. The present investigation explored the associations between urinary 5mdC and 5hmdC with phthalate exposure and semen quality. We assessed semen parameters including sperm concentration, motility, and morphology, before measuring urinary 5mdC, 5hmdC and 13 phthalate metabolites among 562 subfertile men from Nanjing, China. Urinary 5mdC and 5hmdC were positively associated with the levels of low molecular weight phthalate metabolites (Low-MWP), high molecular weight phthalate metabolites (High-MWP), and the sum of all phthalate metabolites (ΣPAEs), respectively. Urinary 5mdC was associated with below-reference sperm concentration (odds ratios for increasing quartiles = 1.0, 2.2, 3.0, 2.0; p for trend = 0.02), sperm motility (1.0, 1.1, 1.9, 1.3; p for trend = 0.05), and sperm morphology (1.0, 1.4, 2.3, 1.5; p for trend = 0.05). Sperm concentration was associated with the highest quartile of urinary 5hmdC [odds ratio = 1.9 (95% CI: 1.1, 3.6)]. Our findings showed significant associations between urinary 5mdC and 5hmdC with phthalate metabolites and semen parameters, which suggested urinary 5mdC and 5hmdC may be promising biomarkers in future epidemiological studies.

1. Introduction

Phthalates are a group of global high-production-volume industrial chemicals that have been widely used as plasticizer in consumer products (Schettler, 2006). Since they are not covalently bound to plastics, phthalates can be released into the environment and have been widely detected in China [e.g., air (Wang et al., 2016), soils (Ma et al., 2015), drinking water (Li et al., 2010) and edible food commodities (Ji et al., 2014)]. Humans may be exposed to these chemicals via ingestion, inhalation, and dermal absorption (Wittassek et al., 2011). Urinary metabolites of phthalates have been widely detected in different human populations (Barr et al., 2003; Guo et al., 2011; Joensen et al., 2012). Human epidemiologic studies have reported associations between exposure to some phthalates and adverse male reproductive outcomes, including reduced sperm quality, increased sperm DNA damage, and altered serum hormone levels, although these associations are not entirely consistent (Bloom et al., 2015; Duty et al., 2003; Hauser et al., 2006; Hauser et al., 2007; Lenters et al., 2015; Joensen et al., 2012; Mendiola et al., 2012; Specht et al., 2014; Thurston et al., 2015; Wang et al., 2015a, 2015b). In one animal exposure study, anti-androgenic effects on male reproductive development, decreased testes and epididymis weight, and increased incidences of external reproductive tract malformation in male rats were observed (Saillenfait et al., 2008). Other animal studies concluded that phthalates exhibit marked differences in toxicity depending on their chemical structure and duration of exposure (Corton and Lapinskas, 2005; Hannas et al., 2011; Johnson et al., 2012).

In addition, phthalates are believed to have epigenetic effects (Singh and Li, 2012). A recent study indicated that exposure to di(2-ethylhexyl) phthalate (DEHP) significantly increased the methylation level in mice testes and disrupted mRNA levels of DNA methyltransferase 1 (Dnmt1, the main cellular enzyme responsible for the maintenance of DNA methylation patterns in somatic mammalian cells) (Wu et al., 2010). DNA methylation, one form of epigenetic effect, is the process of adding a methyl group to the cytosine nucleotide (5-methylcytosine, 5mC) within a cytosine-phosphate-guanine DNA sequence (Bird and Wolffe, 1999). It is one of the well-characterized epigenetic modifications and plays a critical role in many biological processes, which include regulation of gene expression, genomic

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imprinting, cellular differentiation, spermatogenesis, embryogenesis, and X-chromosome inactivation (Bird, 2002; Gehring et al., 2009; Li et al., 1993; Panning and Jaenisch, 1996). The increasing relevance of epigenetic modifications induced by phthalates to human health and disease has expanded our understanding towards these chemicals (Singh and Li, 2012).

In the mammalian model, 5mC is sequentially oxidized into 5-hydroxymethylcytosine (5hmC), further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by ten-eleven translocation (Tet) (Ito et al., 2010; Tahiliani et al., 2009). These three oxidized methylcytosines may indicate the occurrence of both passive and active DNA demethylation and also serve as stable epigenetic marks (Ko et al., 2015). 5hmC is mainly involved in the up-regulation of gene expression, affecting cellular processes such as differentiation, somatic cell reprogramming, and embryonic development (Gao et al., 2013; Ito et al., 2010; Koh et al., 2011). Although the details of the biological mechanism remains elusive, aberrant (either hypo- or hyper-) methylation and/or hydroxymethylation of certain genes and tissues, is associated with various diseases, e.g., cancers, Huntington's disease, and Alzheimer's disease (Chen et al., 2013; Coppieters et al., 2014; Jin et al., 2011; Wang et al., 2013).

The deoxynucleoside of 5mC and 5hmC, namely 5-methyl-2′-deoxycytidine (5mdC) and 5-hydroxymethyl-2′-deoxycytidine (5hmdC) were successfully identified and quantified in urine in recent studies (Hu et al., 2012; Yin et al., 2015). Several biological processes, such as apoptosis, can cause DNA to degrade into single deoxynucleosides (Kawane et al., 2014; Nagata et al., 2003). It is hypothesized that urinary 5mdC and 5hmdC molecules may result from metabolism of methylated DNA (e.g., DNA degradation or DNA excision repair) (Hu et al., 2012; Yin et al., 2015). It is likely that urinary 5mdC and 5hmdC contents may, to some extent, reflect metabolism status and function of tissues or organs in human body.

Proper DNA methylation and demethylation processes are maintained throughout spermatogenesis to ensure a highly specific DNA methylation pattern in sperm cells, further guaranteeing appropriate sperm function, and any alteration of these processes may contribute to altering sperm function and fertilization efficiency (Boissonnas et al., 2013; Rajender et al., 2011). Several processes such as DNA damage and apoptosis may affect the metabolism of DNA and finally affect semen quality (Agarwal et al., 2003). In addition, phthalates have been proved to increase the production of reactive oxygen species (ROS), and consequently induce DNA lesions and apoptosis (Rusyn et al., 2001; Tetz et al., 2013). In conclusion, we hypothesized that phthalate-induced oxidative stress caused apoptosis or DNA lesions resulting in the release of excessive degradation products, 5mdC and 5hmdC, into urine. The levels of urinary 5mdC and 5hmdC might be associated with semen quality, considering that 5mdC and 5hmdC might be involved in the process of methylation or demethylation during spermatogenesis, and their levels might reflect the status of reproductive function, to some extent. Therefore, to test our hypothesis, (1) we determined the levels of urinary phthalate metabolites and urinary 5mdC and 5hmdC content in 562 adult men; (2) investigated the possible association between urinary phthalate metabolites and 5mdC and 5hmdC content; (3) and explored a link between urinary 5mdC and 5hmdC and semen quality in the present investigation.

2. Materials and methods

Details of standards and reagents, sample preparation, instrumental analyses and quality control and assurance data including blank, matrix recoveries, limit of quantifications (LOQs) are provided in the Supporting Information (SI).

2.1. Study population and sample collection

The study population was recruited from the Reproductive Medical Center, Nanjing Jinling Hospital, Nanjing, Jiangsu, China. Participants were male partners of subfertile couples recruited at their first visit to the clinic, before knowledge of the diagnosis. A total of 963 eligible men visited the clinic between April 2014 and March 2015, and 593 of them (61.6%) agreed to participate in the current investigation. They were asked to fill out a detailed questionnaire, which included demographics (e.g., age, race, body weight, and height), health condition and lifestyle factors (e.g., smoking status, alcohol consumption status, abstinence time, occupation, education level and reproductive and medical history). The criteria for sample selection were as follows: 1) between 18 and 50 years; 2) capable of communication in Chinese and completing the questionnaire. Out of the 593 participants, 31 were excluded: because 4 of them reported sexually transmitted diseases; 12 of them were suffering severe varicoceles that might alter semen quality; and 1 of them was undertaking Chinese medicine to improve semen quality. 14 samples were not included because of missing either semen or urine samples. No participants reported occupational exposure to phthalates. The final sample size for this investigation was 562 adult men. The research activity was approved by the Human Subject Committee of Nanjing Jinling Hospital, and the informed written consent was collected from all participants.

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 the laboratory in Beijing. Samples were shipped on dry ice and stored at −20 °C until analysis. The urine cups and cryovials were tested to ensure no contamination from the container.

Semen specimens were collected on the same day as urine samples. Semen was produced on site by masturbation into a sterile polypropylene specimen container after a recommended abstinence period of 2 days. The samples were immediately sent to the laboratory and analyzed.

2.2. Measurements of urinary 5mdC and 5hmdC

Urinary 5mdC and 5hmdC were measured following a previously published method, with modification (Yin et al., 2015). In brief, urine samples were thawed, vortexed, and then heated up to 37 °C for 20 min to dissolve any precipitates that developed during storage. After that, the samples were centrifuged at 5000 rpm for 5 min; 200 μL of urine was then transferred to a new tube and spiked with mass-labelled internal standards (final concentration 50 nM for each analyte) and 200 μL of 1% (v/v) formic acid; they were then vortex mixed and extracted using Bond Elut Plexa PCX SPE cartridges (3 mL, 60 mg, Agilent Technologies, Santa Clara, CA).

Levels of 5mdC and 5hmdC were analyzed by an Acquity ultra-performance liquid chromatograph (UPLC) coupled to a Xevo TQ-S triple quadrupole mass spectrometer in positive electrospray ionization (ESI+) mode (Waters, Milford, MA, USA). Chromatographic separation was achieved using an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 μm, Waters, MA, USA) with mobile phases: 2.0 mM NH_4HCO_3 in water (A) and methanol (B) at a flow rate of 0.3 mL/min. Calibration curves (range: 0.5–128 nM) exhibited excellent linearity ($R^2 > 0.999$). Matrix recoveries were all >94%. The inter-day precisions presented by relative standard deviations (RSD) were all <8%. The limits of quantitation (LOQs) were defined as the lowest standard having a signal-to-noise ratio >10, and were 0.2 nM for 5mdC and 0.1 nM for 5hmdC.

Urinary creatinine was measured spectrophotometrically to correct for the urinary dilution effect. Adjusted concentrations of 5mdC and 5hmdC were expressed as μg/g creatinine.

2.3. Measurements of urinary phthalate metabolites

The 13 phthalate metabolites included monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono(3-carboxypropyl) phthalate (MCPP), mono-n-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP), monobenzyl phthalate (MBzP), mono-2-ethylhexyl phthalate (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), monoisononyl phthalate (MiNP), and mono(carboxy-isooctyl) phthalate (MCiOP). In brief, 1 mL of each urine sample was spiked with mass-labelled standard, 1 mL of sodium acetate solution (0.1 M, pH 6.5), 10 μL of β-glucuronidase (200 units/mL), and 20 μL 4-methylumbelliferyl glucuronide (100 ng/mL). The samples were incubated at 37 °C for 120 min to deconjugate the glucuronidated phthalate metabolites; 0.5 mL of formic acid (0.5 M) was added in order to stop the enzymatic reaction. After that, the sample mixture was extracted using Oasis HLB 3 mL/60 mg SPE cartridge (SI).

Sample extract was analyzed by the Acquity UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) in negative electrospray ionization (ESI-) mode. Chromatographic separation was accomplished using an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 μm, Waters, MA, USA). The column temperature was set to 40 °C. Sample injection volume was 5 μL. The mobile phases were 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B) (Silva et al., 2003).

The LOQs were 0.2 ng/mL for MMP, MBP, and MiBP, and 0.1 ng/mL for the other phthalate metabolites (details on the measurements are provided in the SI).

Hereafter, the concentration of low-molecular weight metabolites (<250 Da, low-MWP) was defined as the sum of MMP, MEP, MBP and MiBP, while the concentration of high-molecular weight metabolites (>250 Da, high-MWP) was the sum of MCPP, MEHP, MEHHP, MEOHP, MECPP, MCMHP, MBzP, MiNP and MCiOP. The concentration of ΣPAEs was the sum of all 13 phthalate metabolites.

2.4. Semen analyses

Specimens were allowed to liquefy for 30 min at 37 °C in a water bath. After that, semen was weighed. Sperm concentration and progressive motility were analyzed by a computer-aided sperm analysis (CASA) system (CFT-9201, Jiangsu Rich Life Science Instrument Co., Ltd., Nanjing, China). The analyses were accomplished within 30 min to reduce the effect of time interval (from ejaculation to analysis) on sperm motility (Elzanaty and Malm, 2007). Sperm morphology was evaluated using Diff-Quik staining in duplicate. For each specimen, at least 200 spermatozoa were analyzed. If the difference between the two replicates was acceptable (within 95% confidence interval), the average number was reported. If the difference was not within the confidence interval, assessment was repeated with two new aliquots of the semen sample. All semen analyses were performed and assessed in accordance with the World Health Organization (WHO) manual (WHO, 2010). For quality control, two known concentrations of Latex beads (Hamilton-Thorne Inc., Beverly, MA, USA) were used to confirm the accuracy of the CASA counts. A daily measurement of a well-stored semen sample was also performed manually for sperm concentration. For sperm morphology, a number of slides of good and poor quality semen samples was evaluated each day. All the measurements for semen quality were performed by two well-trained technicians without knowledge of the subject's information. One technician measured sperm concentration and motility and the other measured sperm morphology. The technicians had joined a continuous quality control system under the supervision of the Quality Control Committee of Nanjing Jinling Hospital.

2.5. Statistical analyses

Descriptive statistics are provided for subject demographics, urinary 5mdC and 5hmdC, phthalate metabolites and semen parameters. Correlations between 5mdC, 5hmdC and potential covariates were evaluated by Spearman Rank correlations. When the concentrations of the phthalate metabolites were below the LOQ, a value of LOQ/√2 was employed.

Linear regression analyses were performed between urinary 5mdC, 5hmdC and phthalate metabolites. To achieve normal distribution of the residuals, the concentrations of 5mdC, 5hmdC and phthalate metabolites were natural log (ln) transformed. We first conducted the crude analyses and then added potential variables to examine the associations adjusted by covariates. Alcohol consumption status was negatively correlated with 5mdC and 5hmdC. No significant correlations were found between age, BMI, smoking and 5mdC and 5hmdC. However, these parameters might affect the excretion of 5mdC and 5hmdC in urine through biological mechanisms. Based on statistical and biological considerations, age (continuous), body mass index [BMI, (weight in kg divided by the square of height in m); continuous], smoking and alcohol consumption status [never, occasionally (less than once a day) or often (more than once a day), categorical] were included in adjusted model 1. Adjusted model 2 included these covariates plus urinary creatinine as a separate covariate (continuous). Furthermore, we divided the concentrations of phthalate metabolites into quartiles (as categorical variables) to examine the nonlinear associations across phthalate quartiles.

Multivariate logistic regression models were used to explore the relationships among 5mdC, 5hmdC and semen parameters. Semen parameters were dichotomized based on WHO reference values (WHO, 2010) for sperm concentration (<15 million/mL), total sperm count (<39 million), sperm motility (<32% motile) and Tygerberg Kruger strict criteria for sperm morphology (<4% normal). The comparison group was defined as men with all four semen parameters at or above the reference values. In addition, the concentrations of creatinine adjusted 5mdC and 5hmdC were categorized into quartiles. The regression models were adjusted by covariates including age, BMI, smoking status, drinking status and abstinence time (continuous).

Linear regression analyses were conducted to explore the associations between ln-transformed phthalate metabolites and semen parameters. All the semen parameters were not transformed. Covariates included age, BMI, smoking status, drinking status, urinary creatinine, and abstinence time. Further, mediation analysis was conducted to evaluate the size of the effect of phthalate exposure on semen parameters that was mediated by urinary 5mdC or 5hmdC (Valeri and Vanderweele, 2013). The indirect effect (Beta coefficient) was estimated using a linear mediation model, and its *p*-value was obtained in bootstrap analysis.

All statistical analyses were conducted using IBM PASW statistics 18.0 (SPSS Inc., USA). Two-tailed *p*-values of <0.05 were considered statistically significant in all models.

3. Results

3.1. Population characteristics

A total of 562 semen and urine pair samples were analyzed in the present investigation. The average age and BMI of participants were 28.8 and 24.0, respectively; other details of their demographics and semen parameters are summarized in Table 1. According to the WHO standard (WHO, 2010), the proportions of sperm concentration, total sperm count, sperm motility and sperm morphology below the reference values were 18.9%, 16.7%, 44.8% and 36.1%, respectively; 203 men (36.1%) were found to be above reference value with all the semen parameters.

3.2. Urinary 5mdC, 5hmdC and phthalate metabolites

The concentrations of 5mdC and 5hmdC and unadjusted phthalate metabolites are given in Tables 2 and 3, respectively. Both 5mdC (geometric mean ± SD: 16.3 ± 21.5 ng/mL, range: 0.4–161.0 ng/mL) and 5hmdC (geometric mean ± SD: 4.3 ± 3.2 ng/mL, range: 0.2–33.4 ng/mL) were detected in all urine samples. The percentage of molar concentration ratio of 5hmdC to 5mdC was 5.3–213.6% (mean

Table 1
Subject demographics and semen parameters ($n = 562$).

Characteristic	Mean ± SD	Median (5th, 95th)	n (%)
Maternal age (year)	28.8 (4.6)	28.0 (22.0, 37.7)	
BMI (kg/m^2)	24.0 (3.2)	23.8 (19.3, 25.4)	
Abstinence (days)	4.0 (1.7)	4.0 (2.0, 7.0)	
Smoking status			
Never smoke			296 (52.7)
Occasionally smoke[a]			98 (17.4)
Often smoke[b]			168 (29.9)
Drinking status			
Never drink			235 (41.8)
Occasionally drink[a]			275 (48.9)
Often drink[b]			52 (9.3)
Disease status			
Varicocele			25 (4.4)
Epididymitis			3 (0.5)
Prostatitis			31 (5.5)
Reproductive history			
Reproductive or abortion history			156 (27.8)
No reproductive history			406 (72.2)
Semen volume (mL)	3.5 (1.6)	3.3 (1.3, 6.4)	
Subject < 1.5 mL			48 (8.5)
Sperm concentration (million/mL)	60.4 (60.8)	44.4 (1.7, 189.0)	
Subject < 5 million/mL			106 (18.9)
Total sperm count (million)	202.9 (214.8)	143.7 (2.0, 644.6)	
Subject <39 million			94 (16.7)
Progressive sperm motile (% motile)	32.4 (15.5)	33.8 (4.7, 56.2)	
Subject < 32% motile sperm			252 (44.8)
Sperm morphology (% normal morphology)	4.2 (2.3)	4.5 (0.9, 7.5)	
Subject < 4% normal morphology			203 (36.1)

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

value 29.8%). In 97.8% of samples, the concentration of 5mdC was higher than that of 5hmdC in the same individual. The phthalates were detected in most urine samples (>99%), with the exception of MiNP and MBzP, which had detection rates of 92.5 and 76.0%, respectively.

Spearman correlations showed that 5mdC and 5hmdC were strongly correlated with each other ($r = 0.81$, $p < 0.001$, SI Table S4). Significant positive correlations were also found between all phthalate metabolites (data not shown). To represent similar sources and biological activity, we combined the phthalate metabolites into the following groups (low-MWP, high-MWP and ΣPAEs groups), and their positive linear relationships with urinary 5mdC and 5hmdC are demonstrated (SI Fig. S1, S2).

In multiple linear regression models, significantly positive associations were found between urinary 5mdC and 5hmdC and all phthalate metabolites (SI Table S5). The associations of 5mdC and 5hmdC with low-MWP, high-MWP and ΣPAEs are given in Table 4. Results of the crude model were similar to adjusted model 1. Although the ratios between 5mdC, 5hmdC and phthalate metabolites in the same sample may be independent of urinary dilution, their associations with other parameters were not. Therefore, urine creatinine was included as a separate covariate in our final model (adjusted model 2).

Table 2
Levels of 5mdC, and 5hmdC in urine ($n = 562$).

Compounds	Detection rate (%)	Geometric mean ± SD	Percentile 5th	25th	50th	75th	95th
Unadjusted (ng/mL)							
5mdC	100.0	16.3 ± 21.5	2.7	9.3	18.4	30.8	72.3
5hmdC	100.0	4.3 ± 3.2	1.0	3.2	4.8	6.7	10.9
Unadjusted (nM)							
5mdC	100.0	67.7 ± 89.3	11.0	38.3	76.4	127.8	299.5
5hmdC	100.0	16.7 ± 12.6	4.0	12.4	18.5	25.9	42.4
Creatinine adjusted (μg/g creatinine)							
5mdC	100.0	10.6 ± 57.5	2.3	6.3	11.1	18.1	39.5
5hmdC	100.0	2.8 ± 10.5	1.3	2.1	2.7	3.6	6.2
Urinary creatinine (g/L)	100.0	1.5 ± 0.7	0.5	1.3	1.7	2.2	3.0

Table 3
Unadjusted urinary concentrations (ng/mL) of phthalate metabolites ($n = 562$).

Compounds	Detection rate (%)	Geometric mean ± SD	Percentile 5th	25th	50th	75th	95th
Phthalate metabolites (ng/mL)							
MMP	100.0	16.9 ± 112.3	4.2	8.7	14.7	31.1	106.1
MEP	100.0	14.1 ± 86.9	2.2	5.7	12.4	29.9	130.2
MBP	100.0	89.5 ± 183.9	16.2	49.5	88.6	169.4	448.0
MiBP	100.0	47.6 ± 75.9	9.4	30.6	47.7	77.0	182.0
MCPP	99.3	1.0 ± 2.1	0.2	0.6	1.0	1.7	4.4
MEHP	99.3	4.2 ± 6.7	0.7	2.2	4.0	7.2	18.8
MEHHP	100.0	12.4 ± 19.0	2.7	7.5	13.0	21.0	52.4
MEOHP	100.0	7.7 ± 11.2	1.7	4.7	8.1	13.2	32.5
MECPP	100.0	17.0 ± 26.7	3.8	10.3	17.7	27.1	68.4
MCMHP	100.0	4.6 ± 7.1	1.1	2.9	4.8	7.7	15.2
MBzP	76.0	0.1 ± 4.0	<LOQ	0.1	0.1	0.3	1.2
MiNP	92.5	0.2 ± 1.9	<LOQ	0.1	0.2	0.3	0.7
MCiOP	100.0	1.6 ± 12.2	0.4	1.0	1.5	2.5	6.6
Sums of metabolites[a] (ng/mL)							
Low-MWP		194.5 ± 331.3	43.7	117.0	192.4	341.6	828.1
High-MWP		51.4 ± 70.5	11.8	32.2	52.9	82.6	205.1
ΣPAEs		258.8 ± 355.6	65.6	164.2	263.3	448.1	951.9

[a] Low-MWP, low molecular weight metabolites, sum of MMP, MEP, MBP and MiBP; High-MWP, high molecular weight metabolites, sum of MCPP, MEHP, MEHHP, MEOHP, MECPP, MCMHP, MBzP, MiNP and MCiOP; ΣPAEs, sum of all 13 phthalate metabolites.

To improve the interpretability of the results, the changes in 5mdC and 5hmdC by interquartile range (IQR) increase in phthalate concentrations were computed, and they were adjusted for age, BMI, alcohol consumption, smoking status and urinary creatinine (SI Table S6). 5mdC increased 1.49–1.65 ng/mL (8.1–8.9% of the 18.44 ng/mL median) for an IQR increase in phthalate groups: low-MWP [1.63 ng/mL; 95% confidence interval (CI): 1.49, 1.78], high-MWP (1.49 ng/mL; 95% CI: 1.36, 1.63) and ΣPAEs (1.65 ng/mL; 95% CI: 1.50, 1.80). Similarly, 5hmdC (at the 4.76 ng/mL median) increased 1.56–1.69 ng/mL (32.8–35.4% of median) in association with phthalates: low-MWP (1.64 ng/mL; 95% CI: 1.54, 1.74), high-MWP (1.56 ng/mL; 95% CI: 1.47, 1.65) and ΣPAEs (1.68 ng/mL; 95% CI: 1.59, 1.78).

Fig. 1 presents the associations between changes in 5mdC, 5hmdC and phthalate quartiles. In comparison with the lowest quartile, men in higher quartiles of low-MWP (Fig. 1D), high-MWP (Fig. 1E) and ΣPAEs (Fig. 1F) had a significant increase in 5hmdC. Patterns for 5mdC (Fig. 1A–C) were similar to those of 5hmdC, although the curves appeared to decline slightly at higher phthalate concentrations.

3.3. Urinary 5mdC, 5hmdC, and semen parameters

Compared to men in the lowest 5mdC quartile, men in higher quartiles were more likely to have below-reference sperm concentration [adjusted odds ratios (ORs) for increasing quartiles = 1.0, 2.2, 3.0, 2.0; p for trend = 0.02; Table 5]. There were elevated ORs for the associations between 5mdC levels and total sperm count, but only the OR in the third quartile approached statistical significance. Sperm motility (1.0, 1.1, 1.9, 1.3; p for trend = 0.05; Table 5) and sperm morphology (1.0, 1.4, 2.3, 1.5; p for trend = 0.05; Table 5) were associated with 5mdC levels.

Significant association was found between below-reference sperm concentration and the highest quartile of 5hmdC. The adjusted OR was

Table 4
Association between ln-transformed urinary 5mdC, 5hmdC, and ln-transformed phthalates ($n = 562$).

	Crude model		Adjusted model 1[a]		Adjusted model 2[b]	
Compounds	β (SE)	*p*-Value	β (SE)	*p*-Value	β (SE)	*p*-Value
5mdC						
Low-MWP	0.462 (0.043)	<0.001	0.456 (0.043)	<0.001	0.239 (0.050)	<0.001
High-MWP	0.422 (0.048)	<0.001	0.422 (0.048)	<0.001	0.229 (0.049)	<0.001
ΣPAEs	0.502 (0.046)	<0.001	0.497 (0.046)	<0.001	0.268 (0.053)	<0.001
5hmdC						
Low-MWP	0.463 (0.028)	<0.001	0.460 (0.028)	<0.001	0.256 (0.031)	<0.001
High-MWP	0.471 (0.031)	<0.001	0.471 (0.031)	<0.001	0.297 (0.029)	<0.001
ΣPAEs	0.521 (0.030)	<0.001	0.518 (0.030)	<0.001	0.311 (0.032)	<0.001

[a] Adjusted for age, BMI, smoking, drinking status.
[b] Adjusted for model 1 covariates plus urinary creatinine.

1.9 (95% CI: 1.1, 3.6; Table 5) for the highest quartile. The ORs for the associations between 5hmdC quartiles and other semen parameters were >1.0, but none approached statistical significance.

We conducted sensitivity analyses to test the robustness of the results. The associations between the dichotomized semen parameters and creatinine-adjusted 5mdC and 5hmdC quartiles were recalculated after excluding 12 azoospermic men. The ORs for the association between 5mdC level and sperm concentration were slightly attenuated (1.0, 2.2, 2.6, 1.7; p for trend = 0.05). The OR for the third 5mdC quartile with total sperm count was smaller and less statistically significant (1.7; 95% CI: 0.8, 3.5; $p = 0.10$). The other associations between 5mdC, 5hmdC, and semen parameters remained stable. We also analyzed the data excluding 22 men with urine creatinine <0.3 g/L or >3 g/L (WHO, 1996), all associations remained essentially unchanged.

3.4. Phthalate metabolites, semen parameters and mediation analyses

The associations between phthalate groups and semen parameters are displayed in SI Table S7. Significant inverse associations of phthalates with sperm concentration, total sperm count, and sperm morphology were observed. Sperm concentration decreased 8.9–19.0 million/mL for an IQR increase in phthalate groups: low-MWP (−19.0; 95% CI: −26.4, −11.5; $p < 0.001$), high-MWP (−8.9; 95% CI: −15.4, −2.4; $p = 0.007$), and ΣPAEs (−18.9; 95% CI: −26.4, −11.4; $p < 0.001$). Similarly, total sperm count decreased 22.5–53.5 million: low-MWP (−53.5; 95% CI: −79.5, −27.6; $p < 0.001$), high-MWP (−22.5; 95% CI: −44.9, −0.1; $p = 0.049$), and ΣPAEs (−52.3; 95% CI: −78.3, −26.2; $p < 0.001$). Normal sperm morphology decreased −0.37% (95% CI: −0.66, −0.08; $p = 0.012$), −0.31% (95% CI: −0.56, −0.06; $p = 0.014$), and −0.38% (95% CI: −0.67, −0.09; $p = 0.010$) for an IQR increase in low-MWP, high-MWP, and ΣPAEs, respectively. The associations between individual phthalate metabolite and semen parameters are also provided in SI Table S7. Except for MiNP and MCiOP, most of the phthalate metabolites were either significantly ($p < 0.05$) or suggestive significantly ($0.05 < p < 0.1$) associated with decreased sperm concentration, total sperm count and normal sperm morphology. Sperm motility was not associated with any phthalate metabolites or groups.

Since urinary 5mdC and 5hmdC levels were significantly associated with phthalate exposure as well as semen parameters, mediation analysis was performed to examine how the observed relationships between phthalate exposure and semen quality related to 5mdC and 5hmdC (possible mediators). The results are summarized in SI Table S8 and S9. We found that approximately 16.6–16.8% of the effect of phthalate exposure to sperm morphology was mediated by urinary 5mdC. However, the mediation effect was not statistically significant ($p = 0.06$). No mediation effects were found in sperm concentration or total sperm count models.

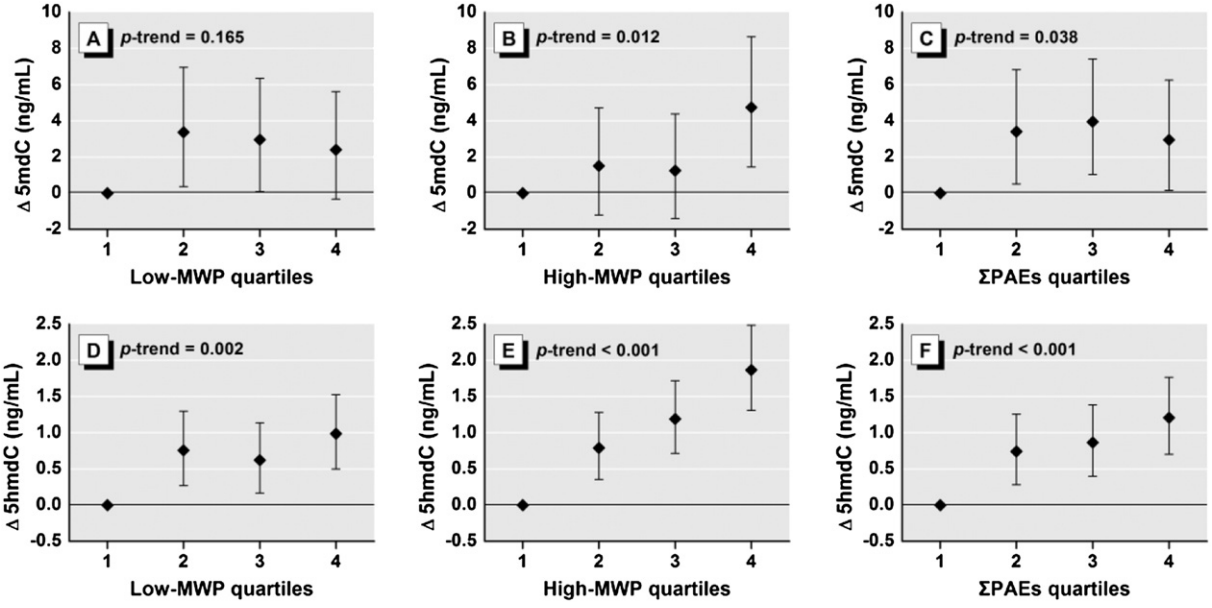

Fig. 1. Associations of changes in urinary 5mdC and 5hmdC with phthalate exposure quartiles (quartile 1 is reference). Models are adjusted by age, BMI, smoking and alcohol consumption status and urinary creatinine. Error bars indicate 95% confidence intervals. *p*-Trend is the *p* value for linear trend across quartiles.

Table 5
Association[a] of below-reference values of semen parameters with creatinine adjusted 5mdC and 5hmdC quartiles ($n = 562$).

	Sperm concentration (<15 million/mL)		Total sperm count (<39 million)		Sperm motility (<32% motile)		Sperm morphology (<4% normal)	
	No.[b]	OR (95% CI)	No.[b]	OR (95% CI)	No.[b]	OR (95% CI)	No.[b]	OR (95% CI)
5mdC								
1st quartile	15	1.0	16	1.0	53	1.0	40	1.0
2nd quartile	29	2.2 (1.1, 4.5)*	24	1.6 (0.8, 3.3)	59	1.1 (0.7, 1.7)	47	1.4 (0.8, 2.3)
3rd quartile	35	3.0 (1.5, 5.8)**	28	2.0 (1.0, 4.0)*	75	1.9 (1.2, 3.1)*	65	2.3 (1.1, 3.7)**
4th quartile	27	2.0 (1.0, 4.0)*	26	1.8 (0.9, 3.5)	65	1.3 (0.8, 2.1)	51	1.5 (0.9, 2.5)
p for trend		0.02		0.22		0.05		0.02
5hmdC								
1st quartile	20	1.0	19	1.0	56	1.0	46	1.0
2nd quartile	26	1.4 (0.7, 2.6)	21	1.1 (0.6, 2.2)	71	1.4 (0.9, 2.3)	48	1.0 (0.6, 1.7)
3rd quartile	28	1.5 (0.8, 2.9)	25	1.4 (0.7, 2.7)	67	1.3 (0.8, 2.2)	55	1.3 (0.8, 2.1)
4th quartile	32	1.9 (1.1, 3.6)*	29	1.8 (0.9, 3.4)	58	1.1 (0.7, 1.8)	54	1.3 (0.8, 2.1)
p for trend		0.22		0.28		0.44		0.63

[a] Adjusted for age, BMI, smoking, drinking status and abstinence time.
[b] Number of subjects in each quartile with below-reference semen parameters. One subject could contribute data to more than one category.
* $p < 0.05$.
** $p < 0.01$.

4. Discussion

Unlike 5hmdC, urinary 5mdC has already been measured in different populations, though with some discrepancies in their levels found among studies. In the present investigation, the level of 5mdC (mean value 17.5 μg/g creatinine; Table 2) in a subfertile male population, was one or two orders of magnitude lower than the values reported in other literatures (Itoh et al., 1995; Lee et al., 2007). Using enzyme linked immunosorbent assay (ELISA), Itoh et al. (1995) reported that the mean levels of urinary 5mdC for healthy subjects and leukemia patients were 1900 and 3600 μg/g creatinine, respectively. Lee et al. (2007) detected 5mdC (mean 559 μg/g creatinine) for Alzheimer's patients using LC-ion trap MS/MS. Contrary to these two findings, subjects in a healthy male cohort from Taiwan (Hu et al., 2012) showed similar levels of 5mdC (mean value 7.04 μg/g creatinine, detected by LC-MS/MS method) to ours.

The discrepancy in 5mdC levels might be related to different analytical methods. Previous methods using ELISA may suffer from poor selectivity and sensitivity. One possible reason for the high levels of urinary 5mdC is likely to be ascribed to a poor specificity of ELISA resulting from its cross-reactivity with non-target analytes (Itoh et al., 1995). Compared to previous methods, LC-MS/MS has advantages in sensitivity, specificity, and structural characterization, and has become a popular method of deoxynucleoside measurement in recent years. Among the studies using LC-MS/MS method, the levels of urinary 5mdC were all detected in the same order of magnitude [mean values were 11.4 ng/mL (Hu et al., 2012), 12.6 ng/mL (Yin et al., 2015), and 16.3 ng/mL (present investigation), respectively].

The discrepancy in 5mdC levels might also be related to the study population, as it has been suggested that levels of urinary 5mdC may depend on subject physical condition (Itoh et al., 1995). In this cross-sectional study, participants were male partners of couples recruited from an infertility clinic, and this subfertile population might have lower semen parameters, and therefore might not be representative of the general male population. Given the results that below-reference semen parameters were associated with elevated urinary 5mdC levels, the elevated 5mdC concentrations (17.5 μg/g creatinine) in our subjects compared to those in healthy males from Taiwan (7.04 μg/g creatinine) might be explained by this reason. It was not known whether demographic or lifestyle factors could affect the levels of urinary 5mdC. In our study, results of the regression model (Table 4) indicated that there was minimal confounding by age, BMI, drinking and smoking status.

In mammalian tissues, the level of 5hmdC is much lower than that of its precursor 5mdC (Jin et al., 2011). Previous investigation reported that 5hmdC was present at the highest abundance in the brain and was lower in the lungs and other tissues (Globisch et al., 2010; Wu and Zhang, 2011). In human brain tissues, 5hmdC levels were relatively high and accounted for 21.1 ± 2.9% of 5mdC, while 5hmdC in human lung tissue was only 2.9 ± 0.8% of 5mdC (Jin et al., 2011). Compared with the ratios in human tissues, the percentage in molar concentration of 5hmdC to 5mdC in urine (mean value 29.8%) was relatively high in the present investigation. It is not known how or why 5hmdC has a higher ratio in urine than in various other tissues. One possible explanation is that urinary 5hmdC and 5mdC are byproducts of genomic DNA metabolism, and 5hmdC in genomic DNA has a more rapid turnover than 5mdC (Yin et al., 2015).

Urinary 5mdC and 5hmdC were positively correlated with each other ($r = 0.81$, $p < 0.001$, SI Table S4). This finding is consistent with that of another study, of which levels of 5mC and 5hmC in human buffy coat ($r = 0.32$, $p = 0.03$) and whole blood samples ($r = 0.54$, $p < 0.001$) were positively correlated (Tellez-Plaza et al., 2014). However, it is noteworthy that 5mdC and 5hmdC in urine are free deoxynucleosides which differ from those in genomic DNA. Without the measurement of urinary 2′-deoxycytidine, the ratios of excreted methylated or hydroxymethylated deoxycytides relative to unmethylated deoxycytides could not be obtained. Therefore, in the present investigation, urinary 5mdC and 5hmdC cannot be interpreted as global DNA methylation and hydroxymethylation level.

The effects of phthalates on DNA methylation have been explored in recent years. Animal exposure studies revealed that aberrant methylation of certain genes can be induced by phthalate exposure, and demonstrated that the testicular toxicity of phthalates may be mediated through alterations in DNA methylation (Prados et al., 2015; Wu et al., 2010). Epidemiological study had reported a negative association of phthalate exposure with placental long interspersed nuclear element-1 (LINE-1) methylation (Zhao et al., 2015). Meanwhile, decreased insulin-like growth factor 2 (IGF2) and H19 methylation in human placenta were reported to be associated with high levels of phthalates (LaRocca et al., 2014). In the present investigation, urinary 5mdC and 5hmdC were positively associated with the sum of phthalate metabolites. The biological mechanism for the interference of phthalates with urinary 5mdC and 5hmdC is unclear. Urinary 5mdC and 5hmdC may be the degradation products of genomic DNA metabolism (e.g., DNA degradation, DNA excise repair), which could be affected by several processes such as apoptosis or DNA damage (Kawane et al., 2014; Nagata et al., 2003). Previous research showed that phthalate exposure increased the production of reactive oxygen species (Rusyn et al., 2001). Therefore we speculate that the phthalate-induced oxidative stress caused apoptosis or DNA lesions (Tetz et al., 2013), resulting in high

levels of DNA degradation or excision repair (Nagata et al., 2003; Zhu, 2009), and then the release of excessive products (5mdC and 5hmdC) into the urine.

Previous studies suggest that phthalates are reproductive toxicants that may impair human semen quality (Duty et al., 2003; Hauser et al., 2006). In the present investigation, several phthalate metabolites (e.g. MBP, MiBP, MEP, MMP, MEOHP and MBzP) were negatively associated with semen parameters (e.g. sperm concentration, total sperm count and sperm morphology), which was consistent with the results of previous studies (Bloom et al., 2015; Duty et al., 2003; Hauser et al., 2006; Liu et al., 2012; Specht et al., 2014; Wang et al., 2015a). Although we still do not know if DNA methylation or hydroxymethylation play any specific role in the effect of phthalates on spermatogenesis, the mediation analysis in our study showed a statistically suggestive effect ($p = 0.06$) of phthalate exposure on sperm morphology mediated by urinary 5mdC (SI Table S8). Considering 5mdC might be involved in the methylation or demethylation processes which ensure normal sperm shape and function during spermatogenesis (Rajender et al., 2011), the results of the current investigation may provide new insights. Aside from its inhibition effect on testicular steroidogenesis (Akingbemi et al., 2004), phthalates may affect epigenetic modification, which can lead to impaired sperm quality. However, further investigation is required. In our study men with high levels of urinary 5mdC were more likely to have below-reference sperm concentration, motility and morphology. Sperm concentration was also associated with the highest quartile of 5hmdC. In conclusion, our findings indicate that urinary 5mdC and 5hmdC might be associated with semen quality. 5mdC and 5hmdC may result from the process of methylation or demethylation in testis, and elevated levels of 5mdC and 5hmdC may reflect aberrant methylation or demethylation processes during spermatogenesis which would possibly affect semen quality (Boissonnas et al., 2013). In that case, urinary 5mdC and 5hmdC might be potential biomarkers for male fertility evaluation, to some extent. They may also be a new breakthrough point for epidemiological studies on the impact of phthalates on male fertility.

There are some limitations to this study. First, the level of phthalate metabolites was measured in a single spot urine which may not provide an accurate level of long-term phthalate exposure. Likewise, the temporal variability of urinary 5mdC and 5hmdC is lacking; the variations in levels of 5mdC and 5hmdC need further investigation in the long term. Second, a larger sample size including samples from the general population is needed, as only a subfertile sample was used in this study. A prospective study rather than a cross-sectional study could be more solid and definitive. Third, in addition to conducting the self-reported questionnaires, measuring sera cotinine level may be an objective evaluation of smoking consumption to avoid the issue of under-report. Fourth, semen quality reflects a local condition whereas urinary 5mdC, 5hmdC and phthalate metabolites reflect a systemic condition. To assess the potential epigenetic effect of phthalates on semen quality, measuring the levels of 5mdC, 5hmdC and phthalates in semen rather than urine is more straight-forward and is warranted in future studies. Lastly, as is always the case with epidemiological studies, we are unable to explore the underlying mechanism of the observed association. The biological significance of urinary 5mdC and 5hmdC need to be further explored in future studies.

5. Conclusion

To our best knowledge, this is the first study to measure the level of urinary 5hmdC in a Chinese population, and it is the first epidemiological study to investigate the associations of urinary 5mdC and 5hmdC with phthalate exposure and semen quality. Our results indicated that both 5mdC and 5hmdC in urine were positively associated with phthalate exposure; urinary 5mdC and 5hmdC were associated with below-reference semen parameters. Additional research is needed to elucidate the mechanism between urinary 5mdC and 5hmdC and semen quality.

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Appendix A. Supplementary data

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