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DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites

R.Hauser[1,2,8], J.D.Meeker[3], N.P.Singh[4], M.J.Silva[5], L.Ryan[6], S.Duty[1,7] and A.M.Calafat[5]

[1]Department of Environmental Health, Harvard School of Public Health, [2]Vincent Memorial Obstetrics & Gynecology Service, Andrology Laboratory and In Vitro Fertilization Unit, Massachusetts General Hospital, Boston, MA, [3]Department of Environmental Health Sciences, University of Michigan, Ann Arbor, MI, [4]Department of Bioengineering, University of Washington, Seattle, WA, [5]National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, [6]Department of Biostatistics, Harvard School of Public Health and [7]Department of Nursing, School for Health Studies, Simmons College, Boston, MA, USA

[8]To whom correspondence should be addressed at: Department of Environmental Health, Harvard School of Public Health, Occupational Health Program, Building 1, Room 1405, 665 Huntington Avenue, Boston, MA 02115, USA. E-mail: rhauser@hohp.harvard.edu

BACKGROUND: The ubiquitous use of phthalate esters in plastics, personal care products and food packaging materials results in widespread general population exposure. In this report, we extend our preliminary study on the relationship between urinary concentrations of phthalate metabolites and sperm DNA damage among a larger sample of men and include measurements of mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), two oxidative metabolites of di-(2-ethylhexyl) phthalate (DEHP). METHODS: Among 379 men from an infertility clinic, urinary concentrations of phthalate metabolites were measured using isotope-dilution high-performance liquid chromatography–tandem mass spectrometry. Sperm DNA damage measurements, assessed with the neutral comet assay, included comet extent (CE), percentage of DNA in tail (Tail%) and tail distributed moment (TDM). RESULTS: Monoethyl phthalate (MEP), a metabolite of diethyl phthalate, was associated with increased DNA damage, confirming our previous findings. Mono-(2-ethylhexyl) phthalate (MEHP), a metabolite of DEHP, was associated with DNA damage after adjustment for the oxidative DEHP metabolites. After adjustment for MEHHP, for an interquartile range increase in urinary MEHP, CE increased 17.3% [95% confidence interval (CI) = 8.7–25.7%], TDM increased 14.3% (95% CI = 6.8–21.7%) and Tail% increased 17.5% (95% CI = 3.5–31.5%). CONCLUSIONS: Sperm DNA damage was associated with MEP and with MEHP after adjusting for DEHP oxidative metabolites, which may serve as phenotypic markers of DEHP metabolism to 'less toxic' metabolites. The urinary levels of phthalate metabolites among these men were similar to those reported for the US general population, suggesting that exposure to some phthalates may affect the population distribution of sperm DNA damage.

Key words: phthalates/urinary metabolites/DNA damage/comet assay/human sperm

Introduction

Phthalates, diesters of phthalic acid, are multifunctional chemicals widely used in personal care products, in food packaging and processing materials, and to soften a wide range of plastics, including medical products such as polyvinyl chloride blood and intravenous bags (Nässberger *et al*., 1987; ATSDR, 1995, 2001, 2002; Bradbury, 1996; Koo *et al*., 2002). General population exposure to phthalates occurs via dietary ingestion, dermal absorption, inhalation and parenteral exposure from medical devices containing phthalates (ATSDR, 1995, 2001). In the 2001–02 National Health and Nutrition Examination Survey (CDC, 2005), four phthalate monoesters—monoethyl phthalate (MEP), mono-(2-ethylhexyl) phthalate (MEHP), monobutyl phthalate (MBP) and monobenzyl phthalate (MBzP)—were present in most participants, thus confirming widespread general population exposure to phthalates (CDC, 2005). The oxidative metabolites of di-(2-ethylhexyl) phthalate (DEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) were present in nearly all subjects at urinary concentrations higher than those of MEHP, the hydrolytic metabolite of DEHP (CDC, 2005).

Despite widespread general population exposure to phthalate esters, very limited epidemiologic data exist on the potential general population effects of phthalate exposure on male reproductive function. Toxicological studies have consistently shown that some phthalates are reproductive and developmental toxicants. Most of the toxicological studies focused on gestational exposure windows, but there is also evidence that pubertal and adult exposure to dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP) and DEHP results in testicular toxicity, including impaired spermatogenesis (Agarwal *et al*., 1985; Parmar *et al*., 1986; Srivastava *et al*., 1990; Park *et al*., 2002).

Two recent human studies have explored associations between conventional measures of semen quality and urinary concentrations of phthalates (Duty *et al*., 2003a; Jonsson *et al*., 2005). In the US study (Duty *et al*., 2003a), associations were found between MBP and MBzP and lower sperm concentration and motility. However, the Swedish study did not find similar relationships (Jonsson *et al*., 2005). Interestingly, neither study reported associations between semen quality and MEHP.

In the Swedish and US studies, the relationships between urinary concentrations of phthalate metabolites and sperm DNA damage were also investigated. In the US study, sperm DNA integrity was assessed using the neutral single-cell microgel electrophoresis assay (comet assay). An association between increased sperm DNA damage and MEP was found, but there were no associations with the other phthalate monoesters (Duty *et al*., 2003b). By contrast, the Swedish study did not find associations between any of the phthalate monoesters and sperm DNA damage measured with the sperm chromatin structure assay (Jonsson *et al*., 2005).

In the present report, we extend our previous study on sperm DNA damage (Duty *et al*., 2003b) by including a larger sample of men and measurements of two oxidative metabolites of DEHP. Consistent evidence shows that sperm DNA damage adversely affects male fertility, contributing to poorer embryo development and lower pregnancy rates among partners of men undergoing assisted reproductive treatments (Duran *et al*., 2002; Morris *et al*., 2002; Agarwal and Allamaneni, 2004; Borini *et al*., 2006).

Materials and methods

Subjects

The study was approved by the Harvard School of Public Health and Massachusetts General Hospital (MGH) Human Subjects Committees, and all subjects signed an informed consent. Subjects were recruited from an ongoing study on the relationship between environmental agents and male reproductive health. They were male partners of couples who presented to the MGH Andrology Laboratory, Boston, MA, USA, between April 2000 and May 2004 for semen analysis as part of an infertility investigation. Eligible men were those between 20 and 54 years of age. Men presenting for post-vasectomy semen analysis were excluded.

Semen sample collection

Semen was produced on site at MGH by masturbation into a sterile plastic specimen cup after a recommended period of abstinence of 48 h. After liquefaction at 37°C for 30 min, semen parameters and characteristics were measured (Duty *et al*., 2003a). The remaining unprocessed semen was frozen in 0.25-ml cryogenic straws (CryoBiosystem, IMV Division, San Diego, CA, USA) by immersing the straws directly into liquid nitrogen (–196°C). Our previous research showed that this freezing method produced comet assay results that were highly correlated with results from fresh, unfrozen samples (Duty *et al*., 2002). Semen samples were later analysed in batches. Straws were thawed by gently shaking in a 37°C water bath for 10 s, and the semen was immediately processed for comet assay.

Neutral comet assay

The comet assay procedure used has been previously described (Singh and Stephens, 1998; Duty *et al*., 2003b). Briefly, 50 µl of a semen/agarose mixture was embedded between two additional layers of agarose on microgel electrophoresis glass slides. Slides were then immersed in a cold lysing solution to dissolve the cell membrane and make chromatin accessible for the enzyme digestion steps. After 1 h cold lyses, slides were transferred to a solution for enzyme treatment with 10 µg/ml of RNase (Amresco, Solon, OH, USA) and incubated at 37°C for 4 h. Slides were then transferred to a second enzyme treatment with 1 mg/ml of proteinase K (Amresco) and incubated at 37°C for 18 h. The slides were placed on a horizontal slab in an electrophoretic unit, equilibrated for 20 min and underwent electrophoresis for 1 h. DNA in the gel was then precipitated, fixed in ethanol and dried. Slides were stained and observed with fluorescence microscope. Comet extent (CE), tail distributed moment (TDM) and percentage of DNA located in the tail (Tail%) were measured on 100 sperm cells in each semen sample using VisComet software (Impuls Computergestutzte Bildanalyse GmbH, Gilching, Germany). CE is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. Tail% is a measurement of the proportion of total DNA that is present in the tail. TDM is an integrated value that takes into account both the distance and intensity of comet fragments:

$$\mathrm{TDM} = \Sigma(I * X) / \Sigma I$$

where ΣI is the sum of all intensity values that belong to the head, the body or the tail, and X is the x-position of the intensity value. Comets with high damage (CHD), which are cells too long to measure with VisComet (>300 µm), were counted for each subject and used as an additional measure of DNA damage.

Urinary phthalate metabolites

Phthalate metabolites were measured in urine because of potential sample contamination from the parent diester and because the metabolites, as opposed to the parent diesters, are the active toxicants (Peck and Albro, 1982; Gray and Gangolli, 1986; Li *et al*., 1998). One spot urine sample was collected in a sterile specimen cup, prescreened for phthalates, on the same day as the semen sample. The analytical approach for measuring MEP, MBP, MBzP, monomethyl phthalate (MMP), MEHP, MEHHP and MEOHP in urine involved enzymatic deconjugation of the metabolites from their glucuronidated form, solid-phase extraction, separation with high-performance liquid chromatography and detection by isotope dilution tandem mass spectrometry (Silva *et al*., 2003, 2004b). Detection limits were in the low nanogram per millilitre range. Isotopically labelled internal standards and conjugated internal standards were used to increase precision. Along with the unknown samples, each analytical run included calibration standards, reagent blanks and quality control materials of high and low concentration to monitor for accuracy and precision. Analysts at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, were blind to all information concerning subjects. Urinary phthalate metabolite concentrations were normalized for dilution by specific gravity (SG).

Using the urinary concentrations of MEHP, MEHHP and MEOHP, we calculated the percentage of these DEHP metabolites excreted as MEHP (%MEHP). To calculate %MEHP, we converted the urinary concentrations of MEHP, MEHHP and MEOHP to nanomoles per millilitre, divided the MEHP concentration by the sum of MEOHP, MEHHP and MEHP concentrations and multiplied by 100. Concentrations below the limit of detection (LOD) were assigned a value of ½ LOD for the calculations. To our knowledge, the use of %MEHP as a phenotypic marker of DEHP metabolism and excretion is novel and has not been used in human health studies.

Statistical analysis

Values for CE, TDM and Tail% represent the arithmetic mean of 100 sperm cells scored per slide for each ejaculate. Because the SG-adjusted

phthalate metabolite concentrations were distributed log-normally, they were logarithmically transformed for the analyses, and Spearman's correlation coefficients were used to explore correlations among phthalate metabolites and comet assay parameters. The CE and TDM were normally distributed, whereas Tail% followed a log-normal distribution. However, because statistical results were similar using either untransformed or ln-transformed Tail%, for consistency and interpretability, we presented only the untransformed Tail% results. The number of CHD in each subject's semen sample was not normally distributed, and an arcsine transformation was used (Zar, 1984).

Multiple linear regression analyses were used to explore relationships between individual comet assay parameters and ln(SG-adjusted urinary metabolite concentrations). Inclusion of covariates was based on statistical and biologic considerations (Hosmer and Lemeshow, 1989). Age was modelled as a continuous variable, and abstinence time was modelled as an ordinal five-category variable (≤2 days, 3, 4, 5 or >6 days). Smoking status was dichotomized by current smoker versus never smoked or former smoker, and race was categorized into four groups: white, African American, Hispanic and other. Regression coefficients were back transformed to represent a change in comet assay parameter for an interquartile range (IQR) increase in SG-adjusted metabolite concentrations to improve interpretability of results.

Results

The demographic distribution of the 379 men with both comet assay results and urinary concentrations of phthalate metabolites is summarized in Table I. Men were primarily Caucasian (85%) with a mean (SD) age of 36.3 years (5.4). Seventy-four percent were never smokers, and only 9% were current smokers.

The distribution of SG-adjusted urinary phthalate metabolite concentrations is summarized in Table II. MEP was detected in 100% of subjects, MBP and MBzP in at least 90% of subjects and MEHP and MMP in at least 75% of men. MEHHP and MEOHP were detected in >95% of subjects. The sample size for MEOHP and MEHHP was approximately half that for MEHP and the other phthalate metabolites, because analytical methods for the quantification of these analytes were only recently implemented in our ongoing study.

Table I. Subject demographics (n = 379)

Characteristic	Mean (SD)	n (%)
Age	36.3 (5.4)	
BMI	27.9 (4.6)	
Race		
White		321 (85)
Black/African American		16 (4)
Hispanic		17 (4)
Other		25 (7)
Abstinence time		
≤2 days		91 (24)
3 days		115 (31)
4 days		66 (18)
5 days		42 (11)
≥6 days		61 (16)
Smoking[a]		
Never smoker		280 (74)
Ever smoker		97 (26)
Current smoker		33 (9)
Former smoker		64 (17)

n, number of subjects; SD, standard deviation; BMI, body mass index.
[a]Information on smoking status missing for 2 subjects, abstinence time missing for 4 subjects.

The distribution of comet assay parameters is summarized in Table III. The parameters CE and TDM were highly correlated (Spearman's R = 0.87, P < 0.0001). By contrast, Tail% was weakly correlated with TDM and CE (R = –0.11, P = 0.04, and R = 0.22, P < 0.0001, respectively), indicating that they are partially independent measures of sperm DNA integrity.

Although inconsistent relationships of comet assay parameters with smoking status and age existed, these were considered potential confounders, because several studies have reported increased sperm DNA damage in smokers and men with increased age (Fraga *et al.*, 1996; Sun *et al.*, 1997; Ündeğer *et al.*, 1999; Møller *et al.*, 2000; Singh *et al.*, 2003). However, the crude and adjusted coefficients in the multiple regression models were similar, indicating minimal confounding by age and smoking status.

The multiple regression models are summarized in Table IV. After adjusting for age and smoking status, for an IQR increase in SG-adjusted MEP concentrations, CE increased 6.6 μm [95% confidence interval (CI) = 0.94–12.3; P-value = 0.02] and TDM increased 2.72 μm (95% CI = –0.46–5.00; P-value = 0.02). This represents a 5.2 and 4.8% increase, respectively, in CE and TDM relative to the study population median. Tail% and CHD were not associated with MEP. MBP was associated with an increase in the percentage of DNA in the comet tail (1.63%, 95% CI = 0.20–3.08). By contrast, MBzP was associated with an increase in CE (5.12 μm, 95% CI = 0.98–9.25) and TDM (2.49 μm, 95% CI = 0.82–4.13). MMP was inversely associated with CE and Tail%.

MEHP was positively associated with Tail% (3.06 μm, 95% CI = 1.33–4.79; P-value = 0.0006). This represents a 12% increase in Tail% relative to the study population median. Given that MEHHP and MEOHP were strongly correlated (r = 0.98), their relationships with comet assay parameters were nearly identical (Table V). For instance, after adjusting for age and smoking status, for an IQR increase in SG-adjusted MEHHP and MEOHP concentrations, CE decreased 8.5 and 8.6 μm, respectively, and TDM decreased 4.53 and 4.42 μm, respectively. For MEHHP, these results represent a 6.6 and 8.0% decrease in the study population median CE and TDM, respectively. Interestingly, although MEHP was positively correlated with the oxidative metabolites MEHHP (R = 0.76, P < 0.0001) and MEOHP (R = 0.73, P < 0.0001), MEHP was not inversely associated with CE and TDM.

Given the inverse relationships of MEHHP and MEOHP with the comet assay parameters and what is known about the metabolism of DEHP, we hypothesized that oxidative metabolism of DEHP may have a protective effect for sperm DNA damage. To explore this hypothesis, we performed analyses in which MEHP and either MEHHP or MEOHP were in the same regression model (both MEHHP and MEOHP were not included because of their strong correlation). The regression coefficients for models with MEHP and either MEHHP or MEOHP were nearly identical, and we chose to present only the MEHHP results.

Table II. Distribution of specific gravity-adjusted phthalate metabolites in urine (ng/ml)

Phthalate metabolite	*n*	Mean	Geometric mean	Selected percentiles								
				Minimum[a]	5th	10th	25th	50th	75th	90th	95th	Maximum
MEP	379	472	171	8.7	19.7	28.2	57.2	154	513	1201	2030	5396
MBP	379	74.5	17.9	<LOD	3.2	5.0	11.1	18.4	32.3	51.2	72.8	14 459
MBzP	379	13.4	7.2	<LOD	<LOD	1.9	4.0	7.9	15.0	23.8	46.2	229
MMP	379	9.17	3.6	<LOD	<LOD	<LOD	1.3	4.0	9.7	20.2	29.1	278
MEHP	379	25.8	7.6	<LOD	<LOD	<LOD	2.9	7.7	19.7	57.9	112	876
MEHHP	204	142	55.9	<LOD	6.8	13.4	23.0	48.6	111	306	601	2886
MEOHP	204	92.4	21.4	<LOD	7.3	8.8	15.3	32.0	71.3	198	444	1780

MEP, monoethyl phthalate; MBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MMP, monomethyl phthalate; MEHP, mono-(2-ethylhexyl) phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate.
Half the limit of detection (LOD) was imputed for phthalates below the LOD (ng/ml) (when no quantification was given) as follows: MEP, 0.605; MBzP, 0.235; MBP, 0.47; MEHP, 0.435 and MMP, 0.355.
[a]Limit of detection (in ng/ml): MEHP, 1.2; MEP, 1.0; MBP, 0.6; MBZP, 0.8; MMP, 0.71; MEHHP, 0.95; MEOHP, 1.07. The percentage of sample above the LOD were as follows: MEHP, 89.9%; MEP, 100%; MBP, 97.6%; MBzP, 94.1%; MMP, 76.2%; MEHHP, 99.5%; MEOHP, 99.2%.

Table III. Distribution of comet assay parameters (*n* = 379)

Parameter	Mean	Geometric mean	Selected percentiles								
			Minimum	5th	10th	25th	50th	75th	90th	95th	Maximum
Comet extent (μm)	130	125	47.6	73.4	82.4	106	128	152	177	191	249
Tail distributed moment (μm)	57.5	55.6	25.7	35.2	39.0	47.5	56.7	66.7	75.8	84.3	107
Percent DNA tail (%)	31.6	28.5	9.90	13.9	16.2	20.1	27.2	43.4	50.5	57.9	79.7
CHD (*n*)	8.9	–	0	0	0	1	4	11	23	34	95

VisComet image analysis software was used to measure Comet extent (microns), a measure of total comet length; Tail Distributed Moment, an integrated measure of length and intensity (microns); percent DNA in tail (%), a measure of the proportion of total DNA present in the comet tail. Number of CHD was a comet with a tail that extended beyond the measuring boundaries in the VisComet image analysis software (>300 microns).

Table IV. Adjusted[a] regression coefficients (95% confidence interval) for comet parameters associated with an interquartile range (IQR) increase in specific gravity-adjusted urinary phthalate metabolite concentrations (*n* = 379)

	Comet extent (μm)	Tail distributed moment (μm)	Percent DNA tail (%)	Long cells-arcsine (*n*)
MEP	6.60 (0.94, 12.3)	2.72 (0.46, 5.00)	–0.26 (–2.52, 2.02)	0.11 (–0.13, 0.33)
MBP	0.17 (–3.46, 3.79)	–0.22 (–1.69, 1.23)	1.63 (0.20, 3.08)	–0.01 (–0.15, 0.14)
MBZP	5.12 (0.98, 9.25)	2.49 (0.82, 4.13)	0.11 (–1.56, 1.77)	0.11 (–0.05, 0.27)
MMP	–6.80 (–12.2, –1.46)	–0.68 (–2.83, 1.50)	–4.95 (–7.02, –2.85)	0.12 (–0.08, 0.34)
MEHP	0.17 (–4.26, 4.58)	–0.74 (–2.51, 1.03)	3.06 (1.33, 4.79)	–0.19 (–0.36, 0.01)
MEHHP[b]	–8.50 (–15.2, –1.79)	–4.53 (–7.11, –1.95)	1.31 (–0.98, 3.60)	–0.16 (–0.42, 0.11)
MEOHP[b]	–8.59 (–15.2, –2.00)	–4.42 (–6.96, –1.89)	1.05 (–1.20, 3.31)	–0.15 (–0.42, 0.11)

MEP, monoethyl phthalate; MBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MMP, monomethyl phthalate; MEHP, mono-(2-ethylhexyl) phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate.
[a]Adjusted for age and smoking status (current and former versus never).
[b]*n* = 204; MEHHP and MEOHP highly correlated (Spearman correlation coefficient = 0.98).

Table V. Adjusted[a] regression coefficients (95% confidence interval) for comet parameters associated with an interquartile range increase in MEHP and MEHHP when both MEHP and MEHHP included in the same model (*n* = 204)

	Comet extent (μm)	Tail distributed moment (μm)	Percent DNA tail (%)
MEHP	22.1 (11.1, 32.9)	8.08 (3.88, 12.3)	4.77 (0.95, 8.58)
MEHHP	–25.5 (–36.2, –14.9)	–10.8 (–14.9, –6.69)	–2.38 (–6.09, 1.34)

MEHP, mono-(2-ethylhexyl) phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate.
[a]Adjusted for age and smoking.

With MEHP and MEHHP in the same model, the association (i.e. change in comet assay parameter for an IQR increase in phthalate metabolite concentration) between MEHP and comet assay parameters became stronger for CE (22.1 μm; 95% CI = 11.1–32.9), TDM (8.08 μm; 95% CI = 3.88–12.3) and Tail% (4.77%; 95% CI = 0.95–8.58). For an IQR increase

in MEHP, this represents increases in the study population median (95% CI) of 17.3% (8.7–25.7%) for CE, 14.3% (6.8–21.7%) for TDM and 17.5% (3.5–31.5%) for Tail%. In addition, the inverse relationship between MEHHP and comet assay parameters became markedly stronger for CE (–25.5 μm; 95% CI = –14.9 to –3.62) and TDM (–10.8 μm; 95% CI =–14.9 to –6.69). This represents decreases in the study population median (95% CI) of 19.9% (–11.6 to –2.8%) for CE and 19% (–26.3 to –11.8%) for TDM. When we included an interaction term for MEHP*MEHHP, it was not statistically significant.

%MEHP was log-normally distributed and ranged from 0.16 to 61.4% with a median (25th, 75th percentile) of 10.2% (5.55–16.8%). Interestingly, %MEHP was strongly associated with increased sperm DNA damage. An IQR increase in %MEHP was associated with a 15.0 μm increase in CE (95% CI = 7.9–22.0 μm) which represents a 11.7% increase relative to the study population median CE (95% CI = 6.2–17.2%), a 5.54 μm increase in TDM (95% CI = 2.76–8.32) which represents a 9.8% increase relative to the study population median TDM (95% CI = 4.9–14.7%) and a 3.11% increase in Tail% (95% CI = 0.65–5.57%) which represents a 11.4% increase relative to the study population median Tail% (95% CI = 2.4–20.5%). We recognize that additional DEHP metabolites that we did not measure exist (Koch *et al*., 2005; Silva *et al*., 2006a; Silva *et al*., 2006b); thus, %MEHP as calculated in the present study is not comprehensive.

Discussion

In our preliminary report on the relationships between sperm DNA damage and urinary concentrations of phthalate monoesters in 168 men (Duty *et al*., 2003b), we found a dose–response relationship between sperm DNA damage and urinary concentrations of MEP, but no relationships with other phthalate metabolites (e.g. MEHP, MBP, MBzP and MMP). In the present report on a much larger number of men (n = 379), we reconfirmed the relationship between MEP and comet assay parameters. In addition, when we reanalysed the data after excluding the 168 men from our earlier report, the association of MEP with CE was largely unchanged, although the CIs widened (data not shown). Explanations for our consistent finding of an association between MEP and comet assay parameters are unclear, because in toxicological studies, MEP is not a testicular toxicant (Gray *et al*., 2000). Furthermore, a recent study among young Swedish men did not find an association between MEP and sperm DNA damage (Jonsson *et al*., 2005).

One possible explanation for our observation is that MEP, the main metabolite of DEP, which is widely used in personal care products, is a surrogate for other factors/characteristics that may be associated with sperm DNA damage. For instance, MEP may be associated with unrecognized lifestyle or dietary characteristics that are themselves predictive of sperm DNA damage. It is also possible that the association of MEP with sperm DNA damage may represent a chance finding. Conversely, it may represent a true relationship between MEP and DNA damage in human sperm.

In agreement with our earlier study, we did not find strong evidence of a relationship between MEHP and comet assay parameters when we did not account for the urinary concentrations of the oxidative metabolites. There was a weak association between MEHP and Tail% but no association with the other comet parameters, CE and TDM. Both toxicologic and epidemiologic studies on the relationship between MEHP and DNA damage are very limited. An epidemiologic study on young Swedish men did not find a relationship between MEHP and sperm DNA damage (Jonsson *et al*., 2005). In an experimental study using the neutral comet assay, investigators detected DNA damage in human lymphocytes induced by *in vitro* exposure to DEHP and MEHP (Anderson *et al.*, 1999). However, rat liver microsomes abolished the effect of DEHP, suggesting a lack of a response *in vivo.*

In this study, we hypothesized that the oxidative metabolites of DEHP may serve as a phenotypic marker of DEHP metabolism and may modify risk of sperm DNA damage. Supporting this hypothesis was our novel finding that when we adjusted for the concentrations of the oxidative DEHP metabolites, we found a stronger dose–response relationship between sperm DNA damage and increased MEHP concentrations. In addition, the oxidative metabolites had strong inverse relationships with sperm DNA damage, suggesting that the oxidation of MEHP to MEHHP and MEOHP is ‘protective’ for sperm DNA damage. For instance, among the study population, for a given urinary concentration of MEHP, the greater the urinary concentration of MEHHP or MEOHP, the lower was the sperm DNA damage. Similarly, for a given urinary concentration of MEHHP or MEOHP, the higher the urinary MEHP concentration, the greater was the sperm DNA damage.

An understanding of the metabolism of DEHP (Figure 1) may provide insights into our observations on the relationships between DEHP metabolites and sperm DNA damage. DEHP hydrolyses first to MEHP, which subsequently metabolizes to MEHHP and MEOHP, among other oxidative metabolites (Koch *et al*., 2005; Silva *et al*., 2006b). These DEHP oxidative metabolites are more easily excreted in urine than MEHP. Therefore, oxidation of MEHP could effectively decrease internal body burden of MEHP, which, in turn, may have a

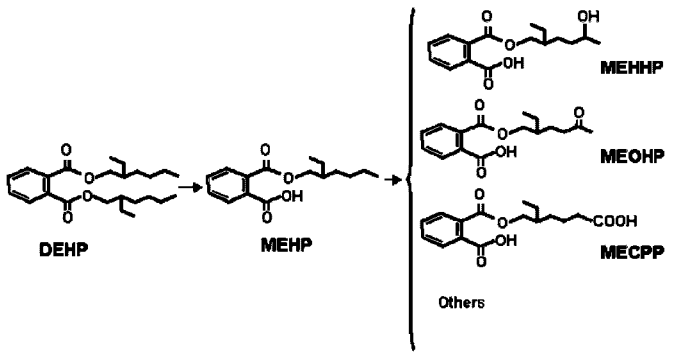

Figure 1. Schematic metabolic diagram of di(2-ethylhexyl) phthalate (DEHP). DEHP, di-(2-ethylhexyl) phthalate; MEHP, mono-(2-ethylhexyl) phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MECPP, mono-(2-ethyl-5-carboxy pentyl) phthalate.

protective effect if MEHP is the bioactive metabolite. DEHP interindividual variability in the percentage of MEHP and of oxidative metabolites that are excreted in the urine has been observed (Becker *et al*., 2004; CDC, 2005; Silva *et al*., 2006a). Therefore, because the proportion of urinary excretion of DEHP as MEHP varies across individuals, urinary concentrations of MEHP alone do not represent total body burden of DEHP exposure. The inclusion of oxidative metabolites, as shown by our study results, may explain the lack of a relationship between MEHP and DNA damage in our earlier study for which we had no available measurements of MEHHP and MEOHP.

Another possible reason for the lack of a strong relationship between MEHP and comet assay parameters, without adjustment for MEHHP and MEOHP, is that MEHP is more susceptible than MEHHP and MEOHP to contamination in the analytical and pre-analytical (e.g. sampling and transportation) phases of the analysis (Koch *et al*., 2003). From the ubiquitously present DEHP, MEHP may be generated during sample collection, transportation and analysis through chemical, microbiological or enzymatic cleavage of the ester bond; unlike MEHP, oxidative metabolites cannot be formed from DEHP contamination. Furthermore, urinary concentrations of MEHHP and MEOHP are at least five times greater than those of MEHP (Barr *et al*., 2003; Koch *et al*., 2003; Becker *et al*., 2004; CDC, 2005; Silva *et al*., 2006a). Therefore, because of the possibility of MEHP contamination, non-differential exposure measurement error at low urinary concentrations of MEHP could exist, thus precluding the detection of associations of MEHP with sperm DNA damage. However, when the regression models included the oxidative metabolites, which are less subject to contamination and more abundant in urine than MEHP, strengthening of the relationship between MEHP and sperm DNA damage occurred. Although our study results support this hypothesis, there may also be other explanations for these observations.

There are limited data on the toxicity of MEOHP and MEHHP. MEOHP and MEHHP antagonized dihydrotestosterone transcriptional activity *in vitro*, indicating anti-androgenic activity (Stroheker *et al*., 2005). In turn, structure-activity studies with MEHP, MEHHP and MEOHP showed a good agreement between the induction of germ cell detachment in culture and testicular toxicity *in vivo*. MEHHP and MEOHP were less toxic in culture than MEHP; thus, MEHP was presumed to be the most bioactive testicular metabolite (Gray and Gangolli, 1986). The relevance of these *in vitro* findings to the observations of associations with sperm DNA damage in men is unclear.

To our knowledge, this is the first study to explore the associations of sperm DNA damage with DEHP oxidative metabolites and %MEHP. Further investigation is warranted on the potential utility of %MEHP as a phenotypic marker of the proportion of DEHP excreted as MEHP and its oxidative metabolites. The timing of collection of the urine sample may partially account for differences in urinary concentrations of MEHP and the oxidative metabolites among individuals, because the oxidative metabolites have a longer half-life than MEHP (Koch *et al*., 2005). For instance, a urine sample collected a few hours after DEHP exposure contains primarily MEHP. By contrast, a urine sample collected 12 h after DEHP exposure may have higher concentrations of MEHHP and MEOHP than of MEHP. The differences in half-lives of DEHP metabolites should be taken into account when interpreting the meaning of %MEHP following a single pulsed exposure to DEHP. However, the interpretation of %MEHP would be more straightforward if the differences in half-lives were not as influential on urinary concentrations as in the case of chronic exposure to DEHP.

In contrast to our earlier report, in this, we found associations between MBzP and MBP with comet assay parameters. It is unclear why MBzP and MBP were associated with increases in different comet assay parameters. Experimental data demonstrating genotoxicity of DBP are limited to two publications by Kleinsasser *et al*. (2000, 2001). In these reports, the alkaline comet assay was used to measure DNA strand breaks in peripheral lymphocytes and human mucosa of the upper aerodigestive tract harvested during surgery of the oropharynx and inferior nasal turbinate.

In previous studies using the neutral comet assay, changes in DNA migration were detected (i.e. CE) at low levels of radiation, 12.5 centigray (rads) of X-rays in human lymphocytes (Singh and Stephens, 1997) and 50 centigray (rads) of X-rays in human sperm (Duty *et al*., 2002). Therefore, we considered CE to represent sensitive quantitative measures of DNA damage. However, Tail% is purported to be a more sensitive measure of DNA damage than TDM and CE. This increased sensitivity results from observations that with increasing DNA damage, the tail length may not continue to increase, but Tail% may increase (McKelvey-Martin *et al*., 1993). In an earlier study, we hypothesized that Tail% may indicate a different form of DNA damage (single-strand breaks) compared to CE or TDM (double-strand breaks) (Meeker *et al*., 2004).

Several mechanisms have been proposed for the presence of DNA damage (strand breaks) in ejaculated sperm. These include aberrant chromatin packing during spermatogenesis (Gorczyca *et al*., 1993; McPherson and Longo, 1993; Manicardi *et al*., 1995), abortive apoptosis in the later stages of germ cell development but before ejaculation (Sakkas *et al*., 1999, 2002) or excessive production of reactive oxygen species (ROS) in the ejaculate (Aitken and Krausz, 2001; Moustafa *et al*., 2004). Some chemicals, including phthalates, have been linked to these mechanisms. Specifically, DEHP decreased cellular levels of glutathione and other antioxidants in rats, leading to increased generation of ROS and increased oxidative stress in the testis (Kasahara *et al*., 2002). Sperm cells are high in unsaturated fatty acids and are particularly susceptible to lipid peroxidation, which results in loss of sperm viability and increased DNA damage (Barroso *et al*., 2000; Aitken and Krausz, 2001; Ollero *et al*., 2001).

In conclusion, among men from an infertility clinic population, increased sperm DNA damage was associated with urinary concentrations of MEP, MBP and MBzP. In addition, MEHP was associated with increased sperm DNA damage only after accounting for the levels of two DEHP oxidative metabolites. One potential explanation for this observation is that the DEHP oxidative metabolites may serve as a phenotypic marker of DEHP metabolism to 'less toxic' metabolites. An alternative explanation is that the relative percentage of DEHP oxidative metabolites in urine may represent a surrogate

for the function of phase 1 enzyme(s). If other genotoxic chemicals requiring phase 1 enzymes for detoxification are associated with sperm DNA damage, men with high %MEHP, which represents low functionality of the phase 1 enzymes, may also be 'poor' metabolizers of the other genotoxic chemicals. Presently, there is no evidence to support this hypothesis.

Finally, and most importantly, the associations found for MEP, MBP, BBzP and MEHP were at urinary concentrations comparable with those found in the general US population (CDC, 2005). Thus, a large proportion of men are exposed to levels of these phthalates that may affect sperm DNA integrity. In this report, the urinary levels of phthalate metabolites were associated with modest percentage increases in study population medians of sperm DNA damage, ranging from 5 to almost 20%. Although the percentage of increase in sperm DNA damage may have a minimal clinical impact on the fertility for a given man, on the population level, the public health significance of a shift in the distribution of sperm DNA damage would be large. The increase in the population median of sperm DNA damage would result in a shift to the right of the distribution curve. This would considerably increase the proportion of men in the far right tail of the distribution with high levels of DNA damage, thus contributing to an increase in the percentage of the population of men with high levels of sperm DNA damage, which, in turn, may adversely affect fertility or success during assisted reproductive treatments (Duran *et al*., 2002; Morris *et al*., 2002; Agarwal and Allamaneni, 2004; Borini *et al*., 2006).

This study was not designed to assess the clinical significance of the effect of phthalates on sperm DNA integrity for an individual, but rather the population effects from exposure to phthalates. This is an important distinction as it defines epidemiologic investigation, the study of the distribution and determinants of health among a population. Clinical relevance generally applies to an individual, whereas in epidemiologic studies, the relevance of the results applies to a population and the potential public health impacts. Both clinical and population relevance are important in their own respects.

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