Environmental Science & Technology

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Urinary Concentrations of Phthalates in Couples Planning Pregnancy and Its Association with 8-Hydroxy-2′-deoxyguanosine, a Biomarker of Oxidative Stress: Longitudinal Investigation of Fertility and the Environment Study

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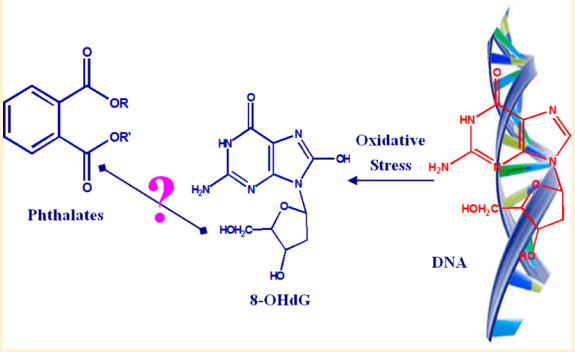
Supporting Information

ABSTRACT: Oxidative stress has been recognized as one of the most important contributors to infertility in both males and females. Exposure to many environmental chemicals, such as phthalates, has been shown to induce oxidative stress. In a longitudinal study designed to assess exposure to environmental chemicals and fecundity in couples who were planning pregnancy, 894 urine samples were collected from 469 couples from Michigan and Texas during 2005−2009. The concentrations of 14 phthalate metabolites and a marker of oxidative stress, 8-hydroxy-2′-deoxyguanosine (8-OHdG), were determined in these samples. Concentrations, profiles, and estimated daily intakes (DIs) of phthalates were positively associated with 8-OHdG. The median concentrations of monomethyl phthalate (mMP), monoethyl phthalate (mEP), mono(3-carboxypropyl) phthalate (mCPP), mono-*n*-butyl phthalate (mBP), mono(2-isobutyl) phthalate (miBP), monobenzyl phthalate (mBzP), Σ_5mEHP (sum of five metabolites of di(2-ethylhexyl) phthalate (DEHP)) and Σ_{14}phthalates (sum of 14 urinary phthalate metabolites) were 0.48, 85.2, 4.50, 7.66, 4.36, 3.80, 54.8, and 249 μg/g creatinine, respectively. The estimated DI values for DEHP in 39 individuals were above the U.S. Environmental Protection Agency's (EPA) reference dose (RfD) of 20 μg/kg-bw/day. The mean and median concentrations of 8-OHdG were 6.02 and 3.13 μg/g creatinine, respectively, which were significantly higher in females than in males. Statistically significant associations were found between 8-OHdG and urinary concentrations of mEP, and Σ_5mEHP for females. Similarly, a significant association was found between 8-OHdG and DIs estimated for select phthalates. Our results suggested that phthalate exposure increases oxidative stress, which can be a mechanism for the diminished fertility observed in couples who were highly exposed to select phthalates.



INTRODUCTION

The diesters of phthalic acid (also known as phthalates) were introduced in the 1920s as plasticizers to soften plastics. Because phthalates make products flexible and durable, they have been widely used in a variety of commercial and personal care products, including electronics, medical devices, toys, nail polishes, and perfumes.[1] Because phthalates can leach from these products, they are now ubiquitous in the environment. Phthalates have been found in indoor dust, foodstuffs, indoor and outdoor air, and personal care products in the U.S.[2−4] Following exposure, phthalate diesters are metabolized to the corresponding monoesters in human bodies and excreted in urine. According to the National Health and Nutrition Examination Survey (NHANES), 100% of the U.S. population has been exposed to at least one of the several phthalates.[5]

Several studies have reported that phthalates are potential reproductive toxicants and endocrine disruptors, with a more

Received: May 21, 2014
Revised: July 23, 2014
Accepted: July 28, 2014
Published: July 28, 2014

pronounced effect in males.[6−8] A negative association between semen volume and urinary concentrations of dibutyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) and a positive association between sperm malformation and concentrations of DEHP have been reported in males from China.[9] Similarly, a positive association was observed between sperm DNA damage and urinary concentrations of phthalate metabolites in males from the U.S.[10] Studies also have shown that phthalate exposure can alter reproductive and thyroid hormone levels in males.[7,11] Laboratory animal studies have shown that DEHP exposure can impair fertility in female mice.[12] An epidemiological study in Denmark reported an association between pregnancy loss and high exposure to DEHP in females.[13] Several earlier studies have investigated the association of urine phthalate levels in pregnant women with outcomes for fertility and reproduction.[14−17] These population-based studies provide evidence for widespread human exposure to phthalates and associate such exposures to impaired fertility and fecundity. Although several mechanisms for reproductive toxicity of phthalates have been proposed, the health effects from low-level exposures are still not clearly understood. Analysis of the effect biomarkers in human biospecimens, as well as exposure levels of phthalates, would provide further insights into the mode of toxicity of phthalates.

Oxidative stress occurs when a normal pro-oxidant to antioxidant ratio is imbalanced. This imbalance can play a key role in the pathogenesis of subfertility in both males and females.[18] The adverse effects of oxidative stress on sperm quality and functions have been well documented.[18] Nevertheless, in females, the impact of oxidative stress on oocytes and reproductive functions remains unclear, given limited study. Both lifestyle factors, such as dietary preferences, tobacco smoke, and alcohol consumption, and exposures to environmental chemicals can trigger oxidative stress. One biomarker for oxidative stress is 8-hydroxy-2′-deoxyguanosine (8-OHdG). Oxidation of DNA occurs normally but increases with exposure to oxidizing agents. Guanosines are susceptible to oxidation, and this reaction can lead to G:C → T:A mutations that could have serious consequences. These oxidized bases are recognized and excised by DNA repair machinery. 8-OHdG is excreted in urine and, as such, provides an assessment of general oxidative stress throughout the body.[19−21]

An association between phthalate exposure and 8-OHdG has been reported in an occupationally exposed population (plastic waste recycling workers) in China.[22] No previous studies have investigated the level of 8-OHdG in a cohort composed of couples who were planning pregnancy and an association of 8-OHdG with phthalate exposure. The human biospecimens collected for the Longitudinal Investigation of Fertility and the Environment (LIFE) study, designed to examine the relationship between environmental chemical exposures, lifestyle, and couples' fecundity and fertility, provided an opportunity to assess the association between phthalate exposure and 8-OHdG levels in urine. In a previous study, we showed that select phthalates were associated with diminished couple fecundity, especially in males, as measured by a longer time-to-pregnancy, in this longitudinally followed cohort recruited prior to conception.[23]

In the present study, 14 phthalate metabolites were quantified in 894 urine samples collected from 469 couples from Michigan and Texas during 2005−2009. Free urinary 8-OHdG, a marker of DNA oxidative damage, also was determined. The objectives of this study were to determine exposure doses, concentrations, and profiles of phthalate metabolites in couples planning pregnancy and to examine the relationship between urinary phthalate levels and 8-OHdG.

MATERIALS AND METHODS

Population and Data Collection. The LIFE study used a prospective cohort design suitable for following couples across sensitive windows of human reproduction and development.[19] In the study, couples who planned to become pregnant were recruited from Michigan and Texas between 2005 and 2009.[24] Further details of the recruitment of the population, study design, and baseline demographic information have been described previously.[19] The selection criteria were as follows: females between 18 and 44 years of age and males ≥18 years of age, partners in a committed relationship, neither partner being medically/surgically sterile, the female's menstrual cycle as between 21 and 42 days, no hormonal birth control injections used in the previous 12 months, and both partners able to communicate in English or Spanish.[24] In this study, baseline urine samples (n = 849) collected from 469 couples at the initial stages of recruitment were analyzed for 14 phthalate metabolites and 8-OHdG. Urine samples were transported to our laboratory on dry ice and stored at −80 °C until analysis. Based on previous stability studies, it was determined that the method used for transportation and storage of urine did not affect the levels of urinary phthalates and 8-OHdG.[25,26]

Sample Preparation and Instrumental Analysis. Phthalate metabolites in urine samples were extracted after enzymatic deconjugation, followed by solid-phase extraction (SPE), using an isotope diluted method as described previously.[27] Briefly, 0.5 mL of urine sample was buffered with 200 μL of ammonium acetate (pH = 4.5), and 50 μL of β-glucuronidase (2 μL/mL; from *Helix pomatia*), 25 ng each of ^{13}C-labeled phthalate metabolite standards (as internal standards), and 0.5 mL of Milli-Q water were added. Samples were then incubated at 37 °C for enzymatic deconjugation overnight. A RapidTrace SPE Workstation (Caliper Life Sciences, Hopkinton, MA) was used for the extraction of target analytes. An ABS ELUT-Nexus (Varian, Walnut Creek, CA, USA; 60 mg/3 mL) SPE cartridge was conditioned with 1.5 mL of acetonitrile and 1.2 mL of phosphate buffer (pH = 2). Urine samples were diluted with 1 mL of phosphate buffer and loaded onto the cartridge at a rate of 0.5 mL/min. The cartridge was rinsed with 2.0 mL of formic acid (0.1 M) and 1.2 mL of Milli-Q water and dried under nitrogen for 5 min. Target compounds were eluted from the cartridge with 1.2 mL of acetonitrile, followed by 1.1 mL of ethyl acetate. The eluate was concentrated under a gentle stream of nitrogen to near dryness, and 0.5 mL of acetonitrile:Milli-Q water (1:9) was added. The solution was transferred into a 2 mL glass vial for LC−MS/MS analysis. For the analysis of 8-OHdG, 0.2 mL of urine was diluted 5-fold with Milli-Q water, and 10 ng of labeled internal standard ($^{15}N_5$-8-hydroxy-2′-deoxyguanosine; 99%, Cambridge Isotope Laboratories, Andover, MA, USA) was added and analyzed by multiple reaction monitoring (MRM) in the positive ionization mode by LC−MS/MS.[28]

An API 2000 electrospray triple quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 series high-performance liquid chromatograph (HPLC) (Agilent Technologies Inc., Santa Clara, CA) was used for the measurement of phthalate metabolites. A total of 14 phthalate metabolites, mono(3-carboxypropyl) phthalate (mCPP), monomethyl phthalate (mMP), monoethyl phthalate(mEP), mono(2-isobutyl phthalate (miBP), mBP, mono(2-ethyl-5-carboxypentyl) phthalate (mECPP),

mono-[(2-carboxymethyl)hexyl] phthalate (mCMHP), mono(2-ethyl-5-oxohexyl) phthalate (mEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP), monocyclohexyl phthalate (mCHP), monobenzyl phthalate (mBzP), mEHP, monoisononyl phthalate (mNP), and monooctyl phthalate (mOP), were analyzed. Seven isotopically labeled phthalate metabolites ($^{13}C_4$-mMP, $^{13}C_4$-mEP, $^{13}C_4$-mBP, $^{13}C_4$-mECPP, $^{13}C_4$-mEHP, $^{13}C_4$-mBzP, and D_4-miBP) were used as internal standards. Chromatographic separation was achieved using a Betasil C18 column (Thermo Electron, Bellefonte, PA; 100 mm × 2.1 mm, 5 μm). The mobile phase was 0.1% acetic acid in Milli-Q water (A) and 0.1% acetic acid in acetonitrile (B) at a flow rate of 300 μL/min. Target compounds were determined by MRM in the negative ionization mode. The limits of quantification (LOQ) of phthalate metabolites varied from 0.1 to 0.5 ng/mL. 8-OHdG was analyzed using the same instrumental parameters as presented above, and quantification was achieved by isotope dilution. The LOQ for 8-OHdG was 0.2 ng/mL.

Quality Assurance/Quality Control (QA/QC) and Data Analysis. For each batch of 60 samples, four method blanks, a spiked blank, and a pair of matrix-spiked sample/duplicates were processed. The QA/QC results are provided in Table S1 (Supporting Information). The average recoveries of ^{13}C-labeled internal standards in all samples were between 52% and 68%. The reported concentrations in samples were corrected for the recoveries of the internal standards. The recoveries of target compounds in spiked blanks were between 63% and 69%. The coefficients of variation of duplicate analysis of samples were between 5% and 36%. Trace levels of mEP, mBP, miBP, mECPP, and mEHP were found in procedural blanks, and these levels (average values of the four blanks in each batch) were subtracted from sample values. If the final concentration was a negative value (due to subtraction of background levels), half of the lowest concentration found for each compound was used in data analysis. The sum concentrations of five metabolites of DEHP (mEHP, mECPP, mCMHP, mEHHP, and mEOHP) and the sum of all 14 phthalate metabolites are referred to as Σ_5mEHP and Σ_{14}phthalates, respectively. The reported concentrations were creatinine-adjusted, unless specified otherwise.

Statistical Analysis. Data analysis was performed using SPSS, version 17.0. A comparison of concentrations between several subgroups was performed using a nonparametric test (Kruskal–Wallis H or Wilcoxon signed-rank test). The association between 8-OHdG and phthalate metabolite concentrations was tested by Partial Correlations (age, BMI, smoking habit, and race as controlling variables). The relationship of phthalate concentrations between paired males and females was examined by linear regression. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Concentrations and Profiles of Phthalate Metabolites. Phthalate metabolites were found with detection frequencies of >90% for mEP, mCPP, mBP, miBP, and metabolites of DEHP (except for mEHP) and mBzP, ~ 40% for mMP, and ~50% for mEHP. mCHP, mNP, and mOP were found in <5% of the urine samples analyzed; we did not include these compounds in our further discussions.

Concentrations and profiles of phthalate metabolites in urine from the study population are presented in Table 1. mEP and the metabolites of DEHP were the most abundant compounds, accounting for ~90% of the total concentrations in urine, followed by mBzP, mBP, miBP, and mMP. The median concentrations of mMP, mEP, mCPP, mBP, miBP, mBzP, Σ_5mEHP, and Σ_{14}phthalates were 0.48, 85.2, 4.50, 7.66, 4.36, 3.80, 54.8, and 249 μg/g creatinine, respectively (n = 894) (Table 1). The mean concentrations in all samples were 1.3/1.76 (μg/g creatinine/ng/mL unadjusted), 233/259, 10.8/13.8, 14.9/16.9, 6.34/7.33, 6.62/8.59, 164/190, and 437/497 for mMP, mEP, mCPP, mBP, miBP, mBzP, Σ_5mEHP, and Σ_{14}phthalates, respectively. Among the five metabolites of DEHP, mECPP, mCMHP, and mEHHP were the predominant compounds, with median concentrations of 15.8, 13.9, and 12.3 μg/g creatinine, respectively. Our values were similar to the concentrations reported for phthalate metabolites in urine from the general U.S. population in 2003–2004 by NHANES (median concentrations for mEP, mBP, miBP, mBzP, and DEHP metabolites were 177, 17.7, 3.33, 10.5, and 54.2 μg/g creatinine),[29] with the exception of mEP, mBP, and mBzP, which were 2 times lower in our study.

Significantly higher concentrations of phthalate metabolites, except for mMP, were found in females as compared to males (Table 1). Especially, concentrations of mBP were 2 times higher in females than in males (median value). In addition, between paired samples of 424 couples (Wilcoxon signed-rank test), concentrations of phthalate metabolites were significantly higher in females as compared to males, except for Σ_5mEHP. These results were similar to those reported in previous studies; higher levels of phthalates in females were explained by the use of personal care products, such as nail polishes, skin toners, and liquid foundations, which contain high concentrations of phthalates.[30,31] Our previous study showed that phthalate diesters such as DEP were frequently found in leave-on personal care products, by up to 0.4% of the product weight.[4] Lack of differences between males and females for Σ_5mEHP levels can be explained by similar dietary habits within a family, as diet is the major source of human exposure to DEHP.[32] Further, linear relationships were found for mEP (R = 0.11, linear regression model value), miBP (R = 0.21), mBzP (R = 0.22), and Σ_5mEHP (R = 0.11) between paired female and male partners (Figure S1, Supporting Information), which indicated coexposures to phthalates by couples in each family.

Concentrations of mBP and miBP were significantly higher in individuals with lower BMIs (<25.0) (8.77 and 4.66 μg/g creatinine, median) than those with higher BMIs (≥25.0). However, when the urinary concentrations were not normalized for creatinine, no relationship was found between BMI and mBP/miBP concentrations (Figure S2, Supporting Information). This difference can be explained by the excretion of higher levels of creatinine by individuals with higher BMIs (median concentrations of 73, 100, and 135 μg/g for the three BMI groups). In addition, unadjusted concentrations of mEP, mCPP, mBzP, Σ_5mEHP, and Σ_{14}phthalates were significantly higher in individuals with higher BMIs (Table S2, Supporting Information) for both males and females. These results were similar to those reported previously, which showed a positive relationship between the concentrations of mBzP, mEP, mBP, and DEHP metabolites and waist circumference or BMI in males[33,34] (creatinine adjusted) and between the concentrations of mEP and obesity in children[35] (both creatinine adjusted and unadjusted). These results further suggest positive associations between phthalate exposure and overweight or obesity. Among different ethnic groups studied, significantly higher concentrations of Σ_5mEHP were found for Caucasians (59.8 μg/g creatinine) and Asians (54.6 μg/g creatinine) than for other ethnic groups; concentrations of mEP and mBzP were significantly higher for African Americans (183 and 4.62 μg/g creatinine) than for other races, and concentrations of miBP and

Table 1. Urinary Concentrations (μg/g Creatinine) of 8-Hydroxydeoxyguanosine (8-OHdG) and Phthalate Metabolites by Select Demographic Characteristics of Couples in Texas and Michigan Planning Pregnancy, 2005–2009 (LIFE Study)

	8-OHdG			mMP			mEP			mCPP			mBP			miBP			mBzP			$\sum_5$mEHP			$\sum_{14}$Phthalates		
	5% [a]	50%	95.%	5%	50%	95.%	5%	50%	95.%	5%	50%	95.%	5%	50%	95.%	5%	50%	95.%	5%	50%	95.%	5%	50%	95.%	5%	50%	95.%
Total (n = 894)																											
	0.72	3.13	22.5	ND[b]	0.48	5.73	9.24	85.2	958	0.54	4.50	26.2	1.69	7.66	34.0	0.79	4.36	17.1	0.54	3.80	18.5	13.3	54.8	710	52.6	249	1424
Site																											
Michigan (n = 173)	0.50	3.10	23.7	ND	0.29	4.67 *[c]	8.10	70.6	766	0.01	4.70	43.5	1.20	6.68	27.8	ND	2.78	11.3 **	0.53	4.40	24.0	11.8	60.2	732	48.4	240	1211
Texas (n = 721)	0.83	3.14	22.0	ND	0.56	5.74	9.27	86.5	994	0.63	4.48	25.1	1.76	7.89	35.9	0.96	4.72	18.7	0.60	3.75	17.5	13.2	54.9	719	52.5	251	1559
Gender																											
Females (n = 454)	1.10	3.87	29.7 **	ND	0.49	5.87	8.36	99.5	1023 *	0.64	4.89	22.0 *	2.06	10.2	38.7 **	0.66	5.17	19.8 **	0.71	4.89	21.6 **	13.8	60.4	669 *	54.0	273	1414 *
Males (n = 440)	0.54	2.56	13.2	ND	0.45	4.98	9.42	70.8	887	0.48	4.20	40.9	1.42	5.83	28.3	0.84	3.52	13.8	0.52	2.95	14.9	12.2	49.8	795	51.1	214	1483
Body Mass Index																											
< 25.0 (n = 284)	0.92	4.12	30.7 **	ND	0.48	5.98	8.34	91.4	1021	0.57	4.69	21.1	1.91	8.77	44.2 **	0.62	4.66	19.2 **	0.40	3.88	20.1	13.8	57.0	787	51.6	258	1822
25.0-29.9 (n = 306)	0.85	3.29	17.4	ND	0.45	5.51	9.46	73.0	928	0.70	4.52	47.3	2.01	7.17	27.6	0.85	4.60	16.0	0.55	3.89	17.1	13.5	53.6	792	52.9	245	1565
≥30.0 (n = 305)	0.59	2.47	15.0	ND	0.48	5.45	9.24	87.2	939	0.25	4.30	29.8	1.16	7.00	34.6	0.72	3.68	19.7	0.60	3.77	21.9	12.1	55.8	555	52.9	240	1271
Age (years)																											
≤25 (n = 100)	0.44	2.90	23.8	ND	0.83	8.8	10.2	106	1151	0.95	5.14	21.6	1.61	8.96	36.9	1.02	5.12	21.3	0.52	4.91	25.0	15.8	55.0	322	53.8	244	1396
26-30 (n = 360)	0.80	3.40	18.6	ND	0.49	4.7	9.51	82.0	844	0.61	4.67	32.9	1.77	7.46	33.3	0.66	4.35	16.3	0.75	3.72	17.6	13.2	56.4	800	56.7	244	1725
31-35 (n = 290)	0.79	3.13	24.1	ND	0.38	6.5	9.30	87.0	995	0.48	4.33	26.4	1.67	7.30	35.2	0.79	4.33	17.7	0.50	3.60	16.9	12.9	52.9	818	47.7	256	1567
≥ 36 (n = 144)	0.51	2.87	25.2	ND	0.56	6.9	3.08	82.4	912	0.20	4.00	18.5	0.90	8.64	33.3	0.58	4.12	15.8	0.20	3.67	32.1	12.2	55.1	605	45.1	263	1242
Race																											
White (n = 726)	0.70	3.21	23.7 *	ND	0.41	5.63 **	9.08	80.9	825 **	0.57	4.72	26.7 *	1.63	7.53	29.9 **	0.72	4.25	15.5 *	0.53	3.80	20.2	13.4	59.8	765 **	51.7	250	1392
African American (n = 42)	0.87	2.24	10.7	ND	0.60	8.06	20.5	183	2688	0.26	3.23	20.2	2.65	10.1	68.2	0.54	5.85	31.6	0.87	4.62	14.0	8.44	36.8	264	53.0	263	2771
American Indian or Alaska Native (n = 25)	0.56	3.26	10.4	ND	0.78	14.9	4.40	106	1384	0.36	4.12	29.5	0.90	6.57	65.0	0.80	4.37	17.4	0.12	4.10	84.6	7.62	32.8	1086	23.2	342	1493
Asian (n = 18)	0.82	4.46	–[d]	ND	0.70	–	8.65	103	–	1.62	4.46	–	5.90	13.6	–	2.40	7.09	–	1.34	3.28	–	19.1	54.6	–	113	270	–
Native Hawaiian or other Pacific Islander (n = 2)	2.38	3.00	–	ND	0.08	–	48.5	94.5	–	3.13	3.95	–	9.88	12.5	–	4.11	5.22	–	3.90	5.39	–	18.3	30.5	–	123	152	–
Other (n = 70)	0.52	2.78	17.5	ND	1.01	7.08	12.7	96.5	1468	0.38	3.92	36.0	1.29	7.03	61.2	0.92	4.63	27.5	0.48	3.72	13.5	12.9	43.1	689	60.8	198	1901
Smoking																											
No (n = 776)	0.74	3.11	22.3	ND	0.48	5.85	9.27	83.9	871	0.57	4.61	29.2 **	1.79	7.69	34.5	0.83	4.39	17.6	0.56	3.81	18.7	13.7	56.7	720 *	53.9	249	1498
Yes (n = 115)	0.53	3.26	25.1	ND	0.39	5.52	8.18	105	1154	0.31	3.54	13.9	0.97	7.93	34.3	0.40	4.53	16.0	0.45	4.16	20.9	6.52	42.2	509	41.6	253	1386

[a] 5th, 50th, and 95th percentile. [a] ND: concentrations were lower than 0.01 μg/g creatinine. [c] * = $p < 0.05$. ** = $p < 0.01$. [d] – = not available.

dx.doi.org/10.1021/es5024898 | Environ. Sci. Technol. 2014, 48, 9804–9811

Table 2. Correlations between Concentrations of 8-OHdG and Urinary Phthalate Metabolites in Couples in Texas and Michigan Planning Pregnancy, 2005−2009, the LIFE Study

	mMP	mEP	mCPP	mBP	miBP	mBzP	mEHP	Σ_5mEHP	Σ_{14}phthalate
females (n = 454)									
correlation	−0.045	0.141[a]	−0.031	−0.015	−0.046	−0.095[b]	0.129[a]	0.091	0.165[a]
sig (two-tailed)	0.347	0.003	0.522	0.753	0.333	0.047	0.007	0.056	0.001
males (n = 439)									
correlation	0.059	0.030	−0.022	0.032	0.104[b]	0.047	0.591[a]	0.083	0.088
sig (two-tailed)	0.226	0.533	0.643	0.505	0.033	0.338	0.000	0.087	0.070

[a]Correlation is significant at the 0.01 level (two-tailed). [b]Correlation is significant at the 0.05 level (two-tailed).

mBP were significantly higher for Asians (13.6 and 7.09 μg/g creatinine) than for other races. It has been reported that the concentrations of mEP for non-Hispanic Blacks and Mexican Americans were significantly higher than those for non-Hispanic Whites (254, 212, and 165 μg/g creatinine, respectively).[29] In our previous study, we reported elevated concentrations of mBP and miBP in urine from Asians.[36] These results suggest differences in sources of exposure to phthalates among various ethnic groups, which may be related to lifestyle and cultural practices. Significantly higher concentrations of mCPP and Σ_5mEHP were found in nonsmokers (4.61 and 56.7 μg/g creatinine) than in smokers (3.54 and 42.2 μg/g creatinine).

Relationship of 8-OHdG and Phthalate Metabolites. The mean and median concentrations of 8-OHdG in urine were 6.02 and 3.13 μg/g creatinine, respectively; the corresponding unadjusted mean and median concentrations were 4.21 and 3.30 ng/mL, respectively. In leaner individuals (i.e., low BMI), significantly higher levels of 8-OHdG were found in urine from females than from males and from Asians (Table 1). Previous studies have reported higher concentrations of 8-OHdG in females than in males.[20] As a biomarker of oxidative DNA damage, urinary levels of 8-OHdG can be affected by aging and cigarette smoking.[20] A significantly higher concentration of 8-OHdG was found in the blood of people exposed to environmental tobacco smoke.[20] Nevertheless, no significant differences were observed among various age groups or between smokers and nonsmokers in the present study. This observation may be explained by the narrow age range (reproductive age) or limited number of smokers (n = 115) in this study. However, a trend of increasing levels of 8-OHdG with age was found in females, with respective median values of 3.19, 3.99. 3.76, and 4.26 μg/g for the age groups ≤25, 26−30, 31−35, and ≥36 years (Table 1).

The relationship between urinary concentrations of 8-OHdG and phthalate metabolites was investigated (Table 2). For females, significant relationships were found between 8-OHdG and mEP, mEHP, Σ_5mEHP, and Σ_{14}phthalates. For males, significant associations were found between 8-OHdG and miBP and mEHP concentrations. A recent study showed that occupational exposure to DEHP among male workers in plastic waste recycling industries increased urinary levels of 8-OHdG.[29] Laboratory rat studies showed elevated levels of 8-OHdG in urine following exposure to DBP or butyl benzyl phthalate (BzBP)[37] and mBP;[38] nevertheless, a significant positive relationship was only found between urinary 8-OHdG and mEHP or miBP levels in our study. However, it should be noted that statistically significant relationships were observed between 8-OHdG and urinary mMP, mEP, mBP, mBzP, mEHP, Σ_5mEHP, and Σ_{14}phthalate concentrations in females, and urinary mMP, mCPP, miBP, mBzP, and Σ_{14}phthalates in males, when the levels were not normalized for creatinine (Table S3, Supporting Information). Our results suggested that exposure to phthalates, especially DEP, DBP, diisobutyl phthalate (DIBP) and DEHP, increases oxidative stress. Oxidative stress can cause a decline in the levels of critical cell cycle factors that are related to reproduction and fertility and directly damage multiple intracellular components of the oocyte, such as lipids, proteins, and DNA.[39] A recent study showed that high concentrations of 8-OHdG in urine were significantly correlated with lower sperm count, lower sperm mobility, and abnormal sperm morphology.[40] We reported earlier that select phthalate exposures (mEP, mBP, and mBzP) in males from this study were negatively associated with time to achieve pregnancy.[23] Although several lifestyle factors, including dietary preferences, can contribute to oxidative stress that would affect fertility and fecundity, this study indicated the possibility that phthalate exposures can result in oxidative stress, which eventually effects reproductive performance in couples.

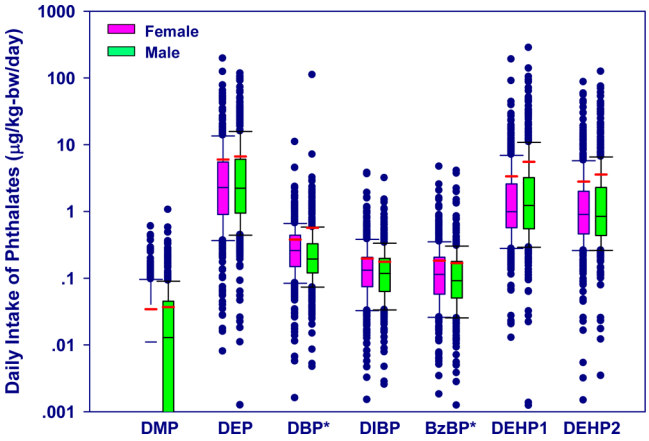


Figure 1. Estimated daily intake (μg/kg-bw/day) of phthalates for males and females (from the urinary metabolite concentrations) in couples in Texas and Michigan planning pregnancy, 2005−2009. Horizontal lines represent the 10th, 50th, and 90th percentiles, and the boxes represent the 25th and 75th percentiles. Outliers are shown as individual points. The short horizontal line represents mean concentrations. DEHP1 and DEHP2 are DI of DEHP estimated from the concentrations of mEHHP and mEOHP, respectively. Legends with an asterisk indicate significant differences between females and males.

Estimated Daily Intake of Phthalates from Urinary Markers. For the estimation of human exposure doses of phthalates, a back-calculation method that involves extrapolation of concentrations determined in urine was used.[41] Based on the measured concentrations of urinary phthalate metabolites (both unadjusted and creatinine adjusted), daily intake (DIs) doses of phthalates were estimated by following equations as employed by Koch et al.[42]

$$\mathrm{DI} = \frac{C_{\mathrm{cu}} \times F_{\mathrm{cre}}}{\mathrm{BW} \times F_{\mathrm{ue}} \times 1000} \times \frac{\mathrm{MW_d}}{\mathrm{MW_m}} \quad (1)$$

dx.doi.org/10.1021/es5024898 | Environ. Sci. Technol. 2014, 48, 9804−9811

Table 3. Estimated Daily Intake of Phthalates in Couples in Texas and Michigan Planning Pregnancy, 2005–2009, the LIFE Study (Median, μg/kg-bw/day)

	mMP		mEP		mBP		miBP		mBzP		mEHHP		mEOHP	
	Cre[a]	unadj	Cre	unadj	Cre	unadj	Cre	unadj	Cre	unadj	Cre	unadj	Cre	unadj
Total (*n* = 894)	0.01	0.02	2.25	3.06	0.22	0.32	0.13	0.18	0.10	0.15	1.10	1.76	0.87	1.35
Site														
Michigan (n = 173)	0.01**	0.01**[b]	1.84*	2.51**	0.20**	0.30	0.08	0.12*	0.11	0.15	1.11	1.80	0.78	1.50
Texas (n = 721)	0.01	0.02	2.32	3.17	0.23	0.32	0.13	0.21	0.10	0.15	1.10	1.75	0.90	1.35
Gender														
females (n = 454)	0.01	0.02	2.27	3.03	0.26**	0.39**	0.13*	0.19	0.11	0.17*	1.00	1.73	0.90	1.40
males (n = 440)	0.01	0.02	2.22	3.17	0.19	0.27	0.12	0.17	0.09	0.13	1.23	1.82	0.84	1.33
Body mass index														
<25.0 (n = 284)	0.01	0.02	2.74	2.89	0.29**	0.35	0.15**	0.20	0.11**	0.15	1.30**	1.71	1.14**	1.43
25.0–29.9 (n = 306)	0.01	0.02	2.17	2.98	0.22	0.30	0.13	0.18	0.10	0.15	1.12	1.68	0.85	1.31
≥30.0 (n = 305)	0.01	0.02	1.94	3.52	0.17	0.31	0.09	0.16	0.08	0.15	0.95	1.87	0.74	1.35
Age (years)														
≤25 (n = 100)	0.02	0.03	2.15	4.21	0.25	0.42	0.13	0.23	0.13	0.18	0.98	1.63	0.80	1.22
26–30 (n = 360)	0.01	0.02	2.19	3.18	0.23	0.32	0.13	0.18	0.10	0.15	1.11	1.74	0.91	1.40
31–35 (n = 290)	0.01	0.01	2.33	2.82	0.21	0.29	0.12	0.16	0.09	0.15	1.06	1.85	0.86	1.35
≥36 (n = 144)	0.01	0.02	2.29	3.13	0.22	0.41	0.12	0.20	0.11	0.15	1.27	1.72	0.96	1.33
Race														
white (n = 726)	0.01**	0.01**	2.15**	2.82**	0.22*	0.30**	0.12**	0.17**	0.10**	0.14	1.15**	1.80	0.95**	1.40
African American (n = 42)	0.01	0.03	3.34	7.56	0.26	0.61	0.15	0.31	0.11	0.24	0.57	1.36	0.45	0.99
American Indian or Alaska native (n = 25)	0.02	0.03	3.03	4.97	0.18	0.38	0.13	0.19	0.12	0.23	0.74	1.87	0.51	1.10
Asian (n = 18)	0.03	0.03	3.30	5.46	0.49	0.73	0.23	0.45	0.09	0.17	1.31	2.12	1.48	1.89
Native Hawaiian or other Pacific Islander (n = 2)	0.00	0.00	2.71	3.38	0.43	0.67	0.18	0.28	0.17	0.28	0.81	1.33	0.57	0.92
other (n = 70)	0.02	0.05	2.32	3.78	0.19	0.36	0.15	0.27	0.10	0.17	1.06	1.51	0.76	1.05
Smoking														
no (n = 776)	0.01	0.02	2.23	3.09	0.22	0.32	0.13	0.18	0.10	0.15	1.14	1.79	0.94**	1.40
yes (n = 115)	0.01	0.02	2.40	3.23	0.21	0.34	0.13	0.19	0.10	0.16	0.92	1.46	0.61	1.03

[a]Median value: cre, calculated from creatinine adjusted phthalate concentration; unadj, calculated from concentration values without creatinine adjustment. [b]* = $p < 0.05$, ** = $p < 0.01$.

dx.doi.org/10.1021/es5024898 | Environ. Sci. Technol. 2014, 48, 9804–9811

$$DI = \frac{C_u \times V_u}{BW \times F_{ue}} \times \frac{MW_d}{MW_m} \quad (2)$$

where DI is the daily intake of phthalates (mg/kg/day), C_{uc} (μg/g creatinine) and C_u (μg/L) are creatinine adjusted and unadjusted concentration of urinary phthalate metabolite, F_{cre} is the creatinine excretion rate (mg/day), V_u is the daily urine volume excreted (L/d), BW is the body weight (kg), F_{ue} is the molar fraction of the excreted metabolite in relation to the ingested parent phthalate molecule, and MW_d and MW_m are the molar weights of the parent phthalate and the corresponding metabolite. For F_{cre}, values of 1.0 g/day for females and 1.7 g/day for males were applied, as reported previously;[43] for V_u, 2 L/d was applied as a conservative estimate, and for F_{UE}, 0.69 for mMP, mEP, mBP, and miBP,[41] 0.73 for mBzP, 0.23 for mEHHP, and 0.15 for mEOHP[44] were applied.

The highest estimated DI values were found for DEP, followed by DEHP, DBP, DIBP, BzBP, and dimethyl phthalate (DMP) (Figure 1). The median DIs of phthalates calculated from creatinine-adjusted urinary concentrations were slightly lower than the values calculated from unadjusted urinary data, and the values were 0.01, 2.25, 0.22, 0.13, 0.10, and ~1.0 μg/kg-bw/day for DMP, DEP, DBP, DIBP, BzBP, and DEHP, respectively (Table 3). The mean DI values were 2 to 3 times higher than the median DI values for all phthalates and were 0.04, 6.31, 0.47, 0.19, 0.18, and ~4 μg/kg-bw/day for DMP, DEP, DBP, DIBP, BzBP, and DEHP, respectively. These estimated DI values were lower than the oral reference doses (RfDs) recommended by the U.S. EPA, which are 100, 200, 800, and 20 μg/kg-bw/day, for DBP, BzBP, DEP, and DEHP, respectively.[45,46] Nevertheless, the DIs estimated for DEHP for 39 individuals (4.4% of the population) (calculated from mEHHP) were above the RfD of 20 μg/kg-bw/day.

For the U.S. general population, it was reported that the DIs for DEP and DEHP were 5.4−11.4 and 2.1−6.5 μg/kg-bw/day (geometric mean), respectively.[41] In another study, the median DIs of DEP, BzBP, DBP, DIBP, and DEHP for pregnant women in the U.S. were estimated to be 6.64, 0.50, 0.84, 0.12, and 1.32 μg/kg-bw/day, respectively.[45] For the general population in Europe, the median DIs of DEP, DBP, BzBP, and DEHP were reported to be 1.43/1.15, 3.53/3.61, 0.27/0.31, and 2.54/2.85 μg/kg-bw/day for females/males, respectively.[47] For the population in China, the estimated median DIs for DMP, DEP, DBP, and DEHP were 0.6, 1.1, 8.5, and ~2 μg/kg-bw/day, respectively.[27] The DIs of phthalates estimated for the LIFE study cohort are similar to those reported in other studies, but the DIs estimated for DEHP for 39 individuals (4.4% of the population) (calculated from mEHHP) were above the RfD of 20 μg/kg-bw/day.

No significant difference in the DIs of phthalates was found among the four age groups or between smoking habits, except for significantly higher intakes of DEHP among nonsmokers (Table 3). The estimated DIs of DMP, DEP, and DBP were higher for Texas than for Michigan couples, the DIs of DBP and BzBP were higher for females than males, and the DIs of DBP, DIBP, BzBP, and DEHP were higher in leaner individuals (low BMI) than in obese individuals.

We examined the relationship between 8-OHdG and DI values calculated for phthalates. Significant positive relationships were found between the estimated DIs of DMP, DEP, DBP, and BzBP for females and DMP, DIBP, and BzBP for males (calculated from unadjusted urinary concentrations) and 8-OHdG concentrations in urine. The results further indicate that exposure to phthalates induces oxidative stress, which is a factor associated with reduced fertility and fecundity.

■ ASSOCIATED CONTENT

Supporting Information

Results of quality assurance and quality control measurements (QA/QC), phthalate concentrations on a volumetric basis (ng/mL), and distribution of phthalate metabolites in subgroups with different body mass index; correlations between phthalate concentrations or daily intakes and 8-OHdG levels. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was funded by the Intramural Research Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health (NICHD; contract nos. N01-HD-3-3355, N01-HD-3-3356, NOH-HD-3-3358, HHSN27500001).

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