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Urinary phthalate monoesters and endometriosis in infertile Japanese women

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ABSTRACT

Phthalates may act as an estrogen and are a potential risk factor for estrogen-related diseases such as endometriosis. We assessed the association between phthalate exposure and endometriosis in 166 consecutive women who presented at a university hospital for consultation regarding infertility. The subjects were interviewed and provided a urine specimen prior to a laparoscopic diagnosis of endometriosis. They were then categorized by the severity of endometriosis as controls (stages 0–I) and cases (stages II–IV). Urinary concentrations of the phthalate metabolites monoethyl phthalate, mono-n-butyl phthalate, monobenzyl phthalate, mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-oxohexyl) phthalate, and mono(2-ethyl-5-hydroxyhexyl) phthalate were measured in 57 cases and 80 controls using high-performance liquid chromatography isotope-dilution tandem mass spectrometry. Adjusted odds ratios for endometriosis in relation to dichotomized individual phthalate metabolites (standardized for creatinine) were calculated. No significant association between endometriosis and any urinary creatinine-adjusted phthalate monoester was seen. Adjusted odds ratio (95% confidence interval) for higher dichotomized MEHP by endometriosis was 1.57 (0.74–3.30). No monotonic trend was seen in urinary creatinine-adjusted concentration of phthalate metabolites by endometriosis stage ($p = 0.23$–0.90). Our results do not support the hypothesis that higher urinary concentrations of phthalate metabolites are associated with the risk of endometriosis in infertile Japanese women.

1. Introduction

Phthalates, a group of multifunctional chemicals used in various plastics as plasticizers and in a variety of common consumer and personal care products such as cosmetics, perfumes, lotions and deodorants, may act as an estrogen and be a potential risk factor for estrogen-related diseases such as endometriosis. Phthalates have shown estrogenic or anti-estrogenic properties in a number of experimental studies (Lovekamp-Swan and Davis, 2003). Among them, di-n-butyl phthalate (DnBP) and butyl-benzyl phthalate (BBzP) showed estrogenic activity in an estrogen receptor-dependent E-screen assay (Soto et al., 1995); mono(2-ethylhexyl) phthalate (MEHP), the active metabolite of di(2-ethylhexyl) phthalate (DEHP), decreased granulose cell aromatase RNA messaging and protein levels in vitro in a dose-dependent manner (Lovekamp-Swan and Davis, 2003); and DEHP dosed at 2 g/kg caused a decrease in serum estradiol levels, prolongation of estrous cycle, and anovulation in adult, cycling rats (Lovekamp-Swan and Davis, 2003).

Scientific and public concern has therefore focused on whether phthalate exposure is associated with estrogen-related diseases such as endometriosis. Three epidemiological studies have reported a positive association between several phthalates and endometriosis (Cobellis et al., 2003; Reddy et al., 2006a,b). Reddy et al. found that plasma DEHP concentrations were higher in patients with endometriosis than in controls, and that higher plasma concentrations of DnBP, BBzP, DEHP, and di-n-octyl phthalate were associated with a higher endometriosis stage (Reddy et al., 2006a,b). Cobellis et al. also found higher plasma concentrations of DEHP, but not MEHP, in endometriosis patients than in controls (Cobellis et al., 2003).

Given that ingested phthalate diesters are usually absorbed from the gastrointestinal tract after hydrolysis by pancreatic lipase into a monoester form (WHO, 1992), it remains unclear why phthalic acid diesters were detected in blood specimens in these studies. Phthalate diesters in human blood are known to be unstable, with short half-lives (28 min for DEHP) (ATSDR, 2002). It is also unclear whether the Italian and Indian studies collected blood specimens prior to surgery (Cobellis et al., 2003; Reddy et al., 2006a,b): because blood bags are made of polyvinyl chloride, which contains DEHP as a plasticizer, the possibility of intravenous exposure to phthalates during blood sampling should also be considered (ATSDR, 2002). Moreover, the susceptibility of sampling and analysis of trace amounts of phthalate diesters to contamination from the laboratory environment is well

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known. On these bases, blood phthalate diesters would usually be considered less appropriate biomarkers of phthalate exposure.

Phthalates are quickly metabolized and largely excreted in urine. Urinary concentrations of phthalate metabolites have been used as biomarkers of exposure to precursor phthalate diesters. Given that most urinary phthalate metabolites are stable, relatively free from contamination by the laboratory environment, believed to be the active toxicants as opposed to the parent diesters, and detectable even in urine specimens from low-exposure general populations (Silva et al., 2004), most epidemiologic studies of the association between various endocrine-related endpoints and exposure to phthalates to date have evaluated individual levels of exposure by measuring urinary monoesters (Duty et al., 2004, 2005, 2003a,b; Hatch et al., 2008; Hauser et al., 2006, 2007; Hoppin et al., 2004; Jönsson et al., 2005; Pan et al., 2006; Stahlhut et al., 2007; Swan et al., 2005), in all cases using high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

Endometriosis, a common benign disease in women of childbearing age, is characterized by the ectopic growth of endometrial-like tissue leading to the painful condition of dysmenorrhea and other reproductive disorders (Cramer and Missmer, 2002). In one study, the prevalence of largely asymptomatic endometriosis found in women undergoing tubal ligation was about 4%, ranging from 1% to 7% (Missmer and Cramer, 2003). Although little is known about the pathological mechanism of endometriosis, epidemiological studies have suggested an association with several estrogen-dependent factors, namely early menarche, shorter menstrual cycle length, and lower parity (Cramer and Missmer, 2002; Missmer and Cramer, 2003; Vigano et al., 2004). Based on this, endometriosis is likely to be a sensitive detector of the effect of xenoestrogens in humans.

Here, to test the possible association between phthalate exposure and endometriosis, we investigated urinary concentrations of phthalate monoesters as biomarkers for exposure to phthalates in Japanese women who had complained of infertility. Our analysis targeted six urinary phthalate metabolites, monoethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP). MEP, MnBP and MBzP are the main metabolites of the corresponding phthalate diesters, diethyl phthalate (DEP), DnBP and BBzP, respectively, while the other three monoesters are the major metabolites of DEHP. To our knowledge, this is the first study to assess the association of endometriosis with phthalates using their urinary metabolites as biomarkers for exposure.

2. Subjects and methods

2.1. Subjects

Subjects were recruited from among 166 consecutive female patients aged 20 to 45 years who had consulted the Department of Obstetrics and Gynecology of the Jikei University School of Medicine for treatment of infertility. A total of 148 women provided written informed consent to participate. Women who had previously given birth ($n=1$) or who had lactated, and those who had undergone surgery for endometriosis ($n=1$) or had a history of miscarriage at greater than three months' gestational age ($n=2$) were excluded. One woman of non-Japanese ethnicity and a second who lived abroad were also excluded, finally leaving 142 women eligible. Of these, 140 who submitted an eligible spot urine specimen and underwent laparoscopic examination between January 2000 and December 2001 were available for analysis. Although all women consulted the Jikei University School of Medicine for treatment of infertility, as expressed by their attendance at the Infertility Clinic, nine selected the answer "no" from among alternatives (yes, no, or unknown) to a questionnaire item on whether the purpose of their consultation for the treatment of infertility, but were nevertheless included to increase statistical power. Three subjects were excluded from analysis because of the inadequate amount of urine available (<50 μL), leaving 137 subjects for final analysis. The study was approved by the Institutional Review Boards of the Jikei University School of Medicine and the National Cancer Center (Tokyo, Japan).

The severity of endometriosis was diagnosed using laparoscopy and classified into five stages based on the revised American Fertility Society classification as stages 0 ($n=59$), I ($n=21$), II ($n=10$), III ($n=23$), and IV ($n=24$) (American Fertility Society, 1985), and then categorized into controls (stage 0 or I, $n=80$) and cases (stages II−IV, $n=57$) (Tsukino et al., 2005, 2006).

Participants were interviewed before laparoscopic examination by a single trained interviewer using a structured questionnaire to collect information on demographic factors, age, height, weight, personal and family medical, reproductive and menstrual history, oral contraceptive use, food and alcohol consumption, and smoking history. The questionnaire and participant profile have been described in detail elsewhere (Tsukino et al., 2005, 2006).

Participants also collected first morning urine into a paper cup, which was then transferred into a plastic tube, before laparoscopic examination. Three spot urine specimens were not first morning urine but were included. All urine samples were stored at −80 °C for about 6 years until analysis but were thawed and refrozen several times during this period. An aliquot of each sample was shipped to a commercial clinical examination laboratory (SRL, Tokyo, Japan) for measurement of individual creatinine concentrations using an enzymatic method.

2.2. Laboratory analysis

Most standard substances were obtained as reported in our previous study (Itoh et al., 2007). We newly purchased standard substances MEHHP and MEOHP and their respective labeled internal standards, MEHHP-$^{13}C_4$ and MEOHP-$^{13}C_4$ from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Water was supplied using a Milli-Q Gradient A10 (Millipore Corporation, Bedford, MA, USA). Spatulas, glassware and plastic tubes were previously rinsed with acetonitrile three times and dried, except unused vials, septa and inserts. We analyzed 0.2-mL aliquots of urine by a modification of existing SPE methods (Fujimaki et al., 2006; Yoshimura et al., 2006). Frozen urine was thawed at 4 °C overnight, sonicated for 5 min and vortex-mixed. A 200-μL aliquot was decanted into a 2-mL silanized glass vial, to which a 50-μL mixed solution of labeled phthalate monoesters (200 μg/L), 2-μL β-glucuronidase solution (200 units/mL, from *Escherichia coli* K12) and 1 M ammonium acetate solution (0.1 mL) were added. This specimen was mixed well and then incubated at 37 °C (90 min). A SPE cartridge (OASIS MAX, 60 mg/3 mL, Waters, Milford, MA, USA) was preconditioned with 4-mL acetonitrile followed by 2-mL water. After dilution with 25%-ammonia water (1 mL), the urine specimen was vortex-mixed, and then loaded onto this SPE column. The SPE column was then washed with 1-mL water followed by acetonitrile (2 mL). Analytes were finally eluted from the SPE column using 1-mL solvent (acetonitrile:formic acid = 1:0.01, v/v). This SPE eluate was collected into a prerinsed plastic tube (1.1 mL) and dried using a vacuum centrifuge (45 min). The residue was then reconstituted with acetonitrile:water = 1:1 (v/v; 200 μL) and the resulting solution was decanted into a silanized glass insert. For specimens with an inadequate amount of urine, this analytical procedure was scaled down according to the amount (50–100 μL).

The phthalate monoesters were separated using a high-performance liquid chromatograph (Alliance 2795, Waters, Milford, MA, USA) equipped with a C_{18} column (RS Pro C_{18}, 2.1 mm i.d. × 100 mm, 3 μm, YMC, Kyoto, Japan), and detected and measured using an electrospray negative ionization tandem mass spectrometer (Quattro Ultima Pt, Micromass, Manchester, UK). This LC-MS/MS procedure has been detailed previously (Itoh et al., 2007). Limits of detection (LODs) were calculated in two ways, as follows. First, the instrumental LOD in