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Urinary phthalate metabolites and male reproductive function parameters in Chongqing general population, China

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ABSTRACT

This study was designed to investigate the phthalates exposure levels in general population in Chongqing City of China, and to determine the possible associations between phthalate exposure and male reproductive function parameters. We recruited 232 general men through Chongqing Family Planning Research Institute and Reproductive Center of Chongqing. In a single spot urine sample from each man, phthalate metabolites, including mono-butyl phthalate (MBP), mono-ethyl phthalate (MEP), mono-(2-ethylhexyl) phthalate (MEHP), mono-benzyl phthalate (MBzP), phthalic acid (PA), and total PA were analyzed using solid phase extraction and coupled with high-performance liquid chromatography and detection by tandem mass spectrometry. Semen parameters were dichotomized based on World Health Organization reference values. Sperm DNA damage were analyzed using the alkaline single-cell gel electrophoresis assay. Reproductive hormones were determined in serum by the radioimmunoassay kit. We observed a weak association between urinary MBP concentration and sperm concentration in Chongqing general population. MBP levels above the median were 1.97 times (95% confidence interval [CI] 0.97–4.04) more likely to have sperm concentration below the reference value. There were no other associations between phthalate metabolites and reproductive function parameters after adjusted for potential risk factors. Our study suggested that general population in Chongqing area of China exposure to the environmental level of phthalate have weak or without adverse effects on the reproduction.

Introduction

Phthalates are a class of artificial chemicals which have a wide range of industrial applications (Agency for Toxic Substances and Disease Registry (ATSDR) 1995, 2001, 2002). Phthalates are not covalently bound to the products and can easily release into the external environment and be ingested, inhaled, dermal absorbed by human (Swan, 2008). Experimental data indicate that some phthalates and their metabolites are associated with reproductive and developmental toxicity (Foster et al., 2001; Gray et al., 2000; Martino-Andrade and Chahoud, 2010; Philippat et al., 2012). Testicular toxicity as malformations, including hypospadias, cryptorchidism, and reduced anogenital distance (AGD) have been observed (Foster et al., 2001; Skakkebaek et al., 2001). A critical decrease in testosterone biosynthesis involving damage to Leydig cells and altered cholesterol metabolism have also been observed (Akingbemi et al., 2004; Hallmark et al., 2006; Hu et al., 2009).

Exposure to some phthalates results in severe disorders of the developing reproductive system especially in male animals, raising the possibility that phthalate exposures could be the leading cause of the reproductive disorders in humans (reviewed by Hauser, 2008; Thompson et al., 2009). Some investigators, but not all, have found that the increase of phthalate exposure concentration was associated with the declining of male reproductive function parameters (Table 6). The most frequently found association was that urinary MBP concentration was negatively associated with sperm concentration (Liu et al., 2012; Toshima et al., 2012; Hauser et al., 2006; Duty et al., 2003a). Significantly associations were also found between MBP and sperm motility (Duty et al., 2003a; Hauser et al., 2005), MBP and serum FSH concentration (Duty et al., 2005), MBP

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and serum testosterone concentration (Duty et al., 2003b), MEP and sperm DNA damage (Hauser et al., 2007; Duty et al., 2003b), MEP and sperm concentration, MEHP (after adjusted for oxidative metabolites) and sperm DNA damage (Hauser et al., 2007), MBzP and sperm concentration (Hauser et al., 2006), as well as between MBzP and serum FSH concentration (Duty et al., 2005). However, all of these significant associations were analyzed in subfertility clinical populations. There are very few study focused on the association between phthalate exposure and male reproductive outcomes in general population. The unique study came from a Swedish study of military service recruits which found that no clear pattern of associations between urinary phthalate metabolites and any of the semen quality parameters (Jönsson et al., 2005).

Chongqing is a highly industrialized city and suffers from significant pollution. In our earlier study, we found that polycyclic aromatic hydrocarbons (PAHs) and phthalic acid esters (PAEs) were the main contaminants in water sources of Chongqing (Cui et al., 2009; Tian et al., 2003). In another cross-section study (Li et al., 2009), we found that 61.1% of male subjects from the general population in this area had one or more semen parameters below the World Health Organization (1999) reference values. So, we are concerned the potential toxicity of organic pollutants in the rivers of this city on human reproductive health. We have explored the relationships between PAHs exposure and male reproduction, and found there were no associations between PAHs metabolites and semen parameters, but significant associations between PAH metabolites and DNA damage outcomes (Han et al., 2011). So, we are interesting that whether environmental exposure to PAEs contributes to the alteration in semen quality. We conducted this study to investigate the relationship between concentrations of phthalate metabolites in human urine detected in Chongqing of China and male reproductive function parameters, including semen quality, sperm DNA damage and serum hormones.

Materials and methods

Study population

This investigation was carried out in 2007, The volunteers were recruited by Chongqing Institute of Science and Technology for Population and Family Planning (CISTPF) to take part in an ongoing cross-section study on semen quality in general population in Chongqing (Li et al., 2009). Among the 1346 investigated subjects, 232 participants came from the urban area of Chongqing City were included in this study and their demographic categories have been described previously in details (Han et al., 2011). Briefly, Subjects were between 20 and 40 years old, had no reproductive or urological diseases or special occupational exposure to PAEs. The participants were informed of the purpose of the study, possible benefits and risks of participating in the study, and were instructed to abstain from ejaculation for 2–7 days before producing the semen samples. Written informed consent was obtained from all participants. All the participants were asked to complete a questionnaire by items concerning full sociodemographic data, smoking, alcohol, nutrition and other lifestyle factors. The study proposal was reviewed and approved by the Ethical Committee of the Third Military Medical University.

Analysis of urinary phthalate metabolites

Urine specimens were collected and aliquoted into 20.0 mL samples on the same day of the semen samples collected. Urine samples were stored at −20 °C until analysis. Five hydroxylated phthalate metabolites (MEP, MEHP, MBP, MBzP and PA) in human urine were measured in this study. Total phthalic acid (total PA) produced by hydrolysis of urinary phthalate metabolites was used to assess the total phthalate body burden (Kato et al., 2005). All tests were performed in a blinded fashion by an experienced analytical chemistry technician. MEP, MBP, MBzP, MEHP, (>99.9%) were purchased from AccuStandard, Inc. (New Heaven, CT, USA), their ^{13}C-labeled internal standards (>99.9%) were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA, USA). PA (>98%) were purchased from Sigma–Aldrich Laboratories, Inc (St. Louis, MO, USA).

The analytical approach was based on existing methods (Silva et al., 2004; Kato et al., 2005) with slight modifications. Briefly, 950 μL urine samples were spiked with a mixture of labeled internal standards of ^{13}C-labeled phthalate monoesters (50 μL, 1 μg/L) and buffered with ammonium acetate and followed by hydrolysis with β-glucuronidase enzyme (Sigma–Aldrich, Inc., St Louis, MO, USA). The mixture was incubated at 37 °C for 90 min to deconjugate the phthalate metabolites from glucuronidated form. The hydrolyzed urine samples were extracted using solid phase extraction (SPE) cartridges (ABS ELUT-NEXUS, Varian, Harbor City, CA, USA) conditioned with acetonitrile (1 mL) and with phosphate buffer (1 mL, 0.14 M NaH_2PO_4 in 0.85% H_3PO_4, pH 2). The urine was diluted with pH 2 phosphate buffer (1 mL), and loaded onto the SPE cartridge at a rate of 1 mL/min. The column was then rinsed with 2 mL 0.1 M formic acid and 1 mL water, after which it was dried by passing air for 0.5 min. The analytes were then eluted with 1 mL acetonitrile followed by 1 mL ethyl acetate. The elute was evaporated to dryness under a stream of nitrogen gas. The residue was resuspended in 200 μL 1:9 acetonitrile: water and then analyzed by high-performance liquid chromatography (HPLC)–electrospray-ionization (ESI) tandem mass spectrometry (MS/MS). To detect the total PA, the deconjugated urine was treated with HCl and reacted for 1.5 h at 90 °C. After cooling to room temperature, the hydrolyzed urine sample underwent SPE and HPLC-ESI-MS/MS.

HPLC-ESI-MS/MS was performed using a Waters Quattro Micro system (Waters Co., Milford, MA, USA) equipped with Kromasil 100-3.5-C18 column (150 mm × 2.1 mm, 3.5 μm). The injection volume was 20 μL; the column temperature was maintained at 40 °C; the flow rate was 0.3 mL/min. The linear gradient program was as follows: (phase A: 0.1% acetic acid in water; phase B: 0.1% acetic acid in acetonitrile), 70%A/30%B (0 min), 70%A/30%B (0.2 min), 52%A/48%B (7 min), 0.0%A/100%B (7.1 min), 0.0%A/100%B (8.3 min), 70%A/30%B (8.4 min) and 70%A/30%B (15 min). Analyses were performed in the negative ion multiple reaction monitoring mode, and the following *m/z* ion combinations were monitored: *m/z* 165 → 77 (PA), *m/z* 193 → 77(MEP), *m/z* 197 → 79(^{13}C-MEP), *m/z* 221 → 77 (MBP), *m/z* 225 → 79(^{13}C-MBP), *m/z* 255 → 77 (MBzP), *m/z* 259 → 107 (^{13}C-MBzP), *m/z* 277 → 134(MEHP), *m/z* 281 → 137(^{13}C-MEHP). Two quality control samples (human urine spiked with phthalates, 200 ng/mL) were analyzed with the unknown samples in each batch.

The calibration graphs (peak area ratios of the internal standard versus sample concentration) were used for determination ($R^2 > 0.999$). The limit of detection (LOD) was 0.6 μg/L for MBP and MBzP, 0.3 μg/L for MEP, 0.8 μg/L for MEHP and 1.5 μg/L for PA. The average recovery for MEP, MEHP, MBP, MBzP and PA (at 7.81, 62.5 and 250 μg/L) in urine samples ranged from 95.2% to 108.2% ($n = 10$). The average intraday variability for MEP, MEHP, MBP, MBzP and PA was 4.73%, 5.6%, 5%, 4.2% and 3.2%, respectively. The day to day variability for phthalate metabolites ranged from 2.8% to 10.5%.

Semen analysis

Routine semen analyses, including pH value, sperm volume (mL), concentration ($\times 10^6$ mL^{-1}), count, progressive motile spermatozoa (grade [A + B]%) and rapid motile spermatozoa (grade