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Effect modification by apoptosis-related gene polymorphisms on the associations of phthalate exposure with spermatozoa apoptosis and semen quality☆

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ABSTRACT

Background: Human studies indicate that phthalate exposure is associated with adverse male reproductive health, and this association may be modified by genetic polymorphisms.
Objectives: We investigated whether apoptosis-related gene polymorphisms modified the associations of phthalate exposure with spermatozoa apoptosis and semen quality.
Methods: In this Chinese population who sought for semen examination in an infertility clinic, we measured 8 phthalate metabolites in two urine samples to assess the individual's exposure levels. Apoptosis-related gene (Fas, FasL, and caspase3) polymorphisms were performed by real-time PCR. Spermatozoa apoptosis and semen quality parameters were evaluated by Annexin V/PI assay and computer-aided semen analysis, respectively.
Results: We found that Fas rs2234767, FasL rs763110, and caspase3 rs12108497 gene polymorphisms significantly modified the associations between urinary phthalate metabolites and spermatozoa apoptosis. For example, urinary monobutyl phthalate (MBP) associated with an increased percentage of Annexin V^+/PI^- spermatozoa of 25.11% (95% CI: 4.08%, 50.53%) were only observed among men with CT/TT genotype of FasL rs763110. In addition, we found that caspase3 rs12108497 gene polymorphisms significantly modified the associations of urinary mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) with decreased sperm concentration and sperm count (both p-values for interactions = 0.02).
Conclusion: Our results provided the first evidence that apoptosis-related gene polymorphisms might contribute to the effects of phthalate exposure on male reproductive health.

1. Introduction

Phthalates, as a family of man-made chemicals, are widely used as additives, solvents in personal care products, medical devices, and plasticizers. As a result of their non-covalent conjugation with the products, phthalates can be easily released and distributed into the environment. Human may be exposed to phthalates through dermal absorption, inhalation, and ingestion (Heudorf et al., 2007). Once exposure, phthalates are rapidly metabolized to hydrolytic monoesters and mainly excreted in urine (Koch et al., 2005, 2003). The measurements of urinary phthalate monoesters have been common monitoring approach for human exposure to environmental phthalates (Barr et al., 2003; Calafat and McKee, 2006). Certain phthalate metabolites such as mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono (2-ethyl-5-oxohexyl) phthalate (MEOHP) have been detected in almost 100%

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of human urine samples (Johns et al., 2016; Wu et al., 2017). The ubiquity of human exposure to phthalates is an increasing concern worldwide due to their potential adverse health effects.

Testicular toxicity of phthalates has been documented in experiment studies. *In vivo* studies have observed that di(2-ethylhexyl) phthalate (DEHP) and di(n-butyl) phthalate (DBP) can adversely affect spermatogenesis, resulting in decreased semen quality (Hannas et al., 2011; Hsu et al., 2016; Kwack et al., 2009). Evidence from human studies have also indicated that exposure to phthalates such as dibenzyl phthalate (DBzP), dimethyl phthalate (DMP), and diethyl phthalate (DEP) is associated with adverse male reproductive health, though the conclusion remains inconsistent (Axelsson et al., 2015; Bloom et al., 2015; Han et al., 2014; J Nsson et al., 2005). Misclassification of exposure assessment in previous studies relying on a single urine measurement may partially contribute to the discrepancy (Hauser et al., 2004; Wang et al., 2015). Recently, given the high intra-individual variability of urinary phthalate metabolites (Hauser et al., 2004; Preau et al., 2010), we measured phthalate metabolites in two urine samples to improve the exposure assessment. We found adverse effects of phthalate exposure on male reproduction such as increased spermatozoa apoptosis and decreased semen quality (Wang et al., 2015, 2016). However, the potential biological mechanism remains poorly characterized.

Apoptosis pathway is one of the pivotal mechanisms in regulation of normal spermatogenesis (Print and Loveland, 2000). As the initiators of apoptosis pathway, Fas and FasL have been considered to be key regulators of testicular germ-cell apoptosis (Lee et al., 1997). Several single-nucleotide polymorphisms (SNPs) in Fas and FasL genes can change their expressions (Huang et al., 1997; Sibley et al., 2003; Wu et al., 2003), resulting in apoptosis in spermatocytes and spermatids (Kim et al., 2007; Pentikainen et al., 1999). The downstream regions of Fas include multiple caspases, of which, caspase3 has been implicated in the pathogenesis of multiple andrological pathologies, such as abnormal testicular torsion, sperm DNA damage, and semen parameters (Said et al., 2004). Furthermore, animal studies have shown that up-regulation of Fas, FasL, and caspase3 genes after phthalate exposure increase incidence of germ cell apoptosis in the testis (Dalgaard et al., 2001; Lee et al., 1999; Richburg et al., 2000).

In this study, we hypothesized that men with apoptosis-related gene variants may have different susceptibility to spermatozoa apoptosis and semen quality posed by phthalate exposure. We measured urinary phthalate metabolites in two urine samples to assess the individual's exposure levels, and investigated the effect modification by Fas, FasL and caspase3 gene polymorphisms on the associations of urinary phthalate metabolites with spermatozoa apoptosis and semen quality.

2. Materials and methods

2.1. Study subjects

This study is part of a cross-sectional investigation of exposure to environmental chemicals and human reproductive health in China, which has been described in detail previously (You et al., 2015). In brief, the study participants who sought for semen analysis in an infertility clinic (Reproductive Center of Tongjing Hospital in Wuhan, China) were included in the study. Each men was asked to provide two urine samples, a blood sample, and a semen sample if they agreed to participant in the study. All subjects completed a questionnaire that included information on general lifestyle, demographic factors, reproductive history, and occupational exposure. Of the original study of 1247 men, 6 had history of occupational exposure to synthetic materials (e.g., polyvinyl chloride and dyes), 22 missed urine sample, and 179 reported the history of adverse reproductive health (e.g., azoospermia, orchiditis, and undescended testicle). After these exclusions, a total of 1040 men were finally included in the study. The current analysis was based on the 473 men with full data on urinary phthalate metabolites, semen parameters, and blood DNA sample. Among them, 213 men had the data on spermatozoa apoptosis measurement. The ethical approval was attained by the Ethics Committee of Tongji Medical College. All participants provided informed consent before participation.

2.2. Semen collection and analysis

Each man provided a semen sample by masturbation in a private room of hospital. Self-reported abstinence time since previous ejaculation was recorded. Two well-trained technicians at the Reproductive Center of Tongji Hospital conducted the semen analysis according to the World Health Organization (WHO, 2010) guidelines as detailed previously (Yang et al., 2017). In brief, sperm concentration and sperm motility were analyzed by a microcell slide and computer-aided semen analysis. Semen volume was measured using a serologic pipette. Total sperm count was calculated according to the following equation: semen volume $\times$ sperm concentration.

After aliquots of semen sample for semen parameters measurements, the remaining fresh sample was immediately transported to our laboratory for the spermatozoa apoptosis assay. The spermatozoa apoptosis parameters were measured according to the detailed methods we previously reported (You et al., 2015). In short, a 20-μL sperm sample was washed with phosphate-buffered saline and re-suspended in a buffer solution. Then, the buffer solution was stained with 5-μl A-FITC solution and 10-μl propidium iodine (PI). The mixture was incubated at room temperature for 5 min. Subsequently, flow cytometry was used to quantitatively determine percentage of Annexin V^-/PI^- spermatozoa (viable cells), Annexin V^+/PI^- spermatozoa (apoptosis cells), and PI^+ spermatozoa (dead cells) of sperm cells.

2.3. Exposure assessment

Two urine specimens (mean duration ± standard deviation: 4.47 ± 3.7 h) from each participant were collected in polypropylene tubes, transported with ice packs, and stored at −40 °C in the laboratory. Eight urinary phthalate metabolites [monomethyl phthalate (MMP), monoethyl phthalate (MEP), monobutyl phthalate (MBP), monobenzyl phthalate (MBzP), mono(2-ethylhexyl) phthalate (MEHP), MEHHP, MEOHP, and mono-*n*-octyl phthalate (MOP)] were measured based on the method described previously (Wang et al., 2015). In brief, 1-mL urine was digested with β-glucuronidase. The target compounds were purified by solid-phase extraction, separated by high-performance liquid chromatography, and detected by a triple quadrupole tandem mass spectrometry. Each analytic run included a full procedure blank and two quality control samples of low and high concentration to monitor for precision and accuracy. The average recovery for eight urinary phthalate metabolites ranged from 88.06% to 110.93%, and relative standard deviation was less than 10.00%. The limit of detection (LOD) for MMP, MEP, MBP, MBzP, MEHP, MEHHP, MEOHP, and MOP was 0.03, 0.02, 0.01, 0.01, 0.02, 0.01, 0.01, and 0.04 ng/mL, respectively. Concentrations below the LOD were substituted as a value of the LOD divided by the square root of 2. To adjust for urine dilution, urine creatinine was measured at the laboratory using Jaffe's colorimetric method.