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Phthalate metabolites related to infertile biomarkers and infertility in Chinese men☆

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ABSTRACT

Although *in vitro* and *in vivo* laboratory studies have demonstrated androgen and anti-androgen effects on male reproduction from phthalate exposures, human studies still remain inconsistent. Therefore, a case-control study (n = 289) was conducted to evaluate the associations between phthalate exposures, male infertility risks, and changes in metabolomic biomarkers. Regional participants consisted of fertile (n = 150) and infertile (n = 139) males were recruited from Nanjing Medical University' affiliated hospitals. Seven urinary phthalate metabolites were measured using HPLC-MS/MS. Associations between levels of phthalate metabolites, infertility risks, and infertility-related biomarkers were statistically evaluated. MEHHP, one of the most abundant DEHP oxidative metabolites was significantly lower in cases than in controls ($p = 0.039$). When using the 1st quartile range as a reference, although statistically insignificant for odds ratios (ORs) of the 2nd, 3rd, and 4th quartiles (OR (95% CI) = 1.50 (0.34–6.48), 0.70 (0.14–3.52) and 0.42 (0.09–2.00), respectively), the MEHHP dose-dependent trend of infertility risk expressed as OR decreased significantly ($p = 0.034$). More interestingly, most of the phthalate metabolites, including MEHHP, were either positively associated with fertile prevention metabolic biomarkers or negatively associated with fertile hazard ones. Phthalate metabolism, along with their activated infertility-related biomarkers, may contribute to a decreased risk of male infertility at the subjects' ongoing exposure levels. Our results may be illustrated by the low-dose related androgen effect of phthalates and can improve our understanding of the controversial epidemiological results on this issue.

1. Introduction

Phthalates are inexpensive synthetic chemicals and have been widely used as plasticizers in a broad range of industrial and commercial products (Barr et al., 2003; Guo et al., 2011). The most commonly used phthalates are di-(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), diethyl phthalate (DEP), and benzylbutyl phthalate (BzBP). In all, more than 75% of DEHP produced worldwide is used in plastic products. The other phthalates are largely used in personal care products like foams, shampoos, dyes, lubricants, and food packaging materials (Guo et al., 2014a). Humans are often exposed to these ubiquitous chemicals directly or indirectly through multiple pathways including food, water, air, dust and consumer products (Guo and Kannan, 2013; Han et al., 2014; Joensen et al., 2012). Due to their short biological half-lives, phthalates are readily metabolized to one or more derivatives in humans. Following hydrolysis to their corresponding monoesters, the remaining long chains can be further metabolized through oxidation (Katsikantami et al., 2016). Monoesters, along with oxidative metabolites, are bound to glucuronide before excretion

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via urine and/or feces (Lorber et al., 2009). The vast majority of phthalate metabolic products are excreted in urine within 48 h; therefore, urinary phthalate metabolites have been used extensively as exposure biomarkers in human studies (Guo et al., 2011). In totality, the metabolites can be used to reconstruct exposure to the parent compounds.

Several phthalates (e.g., DEHP, DBP, and BBzP) are suspected endocrine disruptors in humans and wildlife. Both *in vitro* and *in vivo* toxicology studies have demonstrated their endocrine disrupting potential in model organisms, with endpoints such as antiandrogen effects, reproductive abnormalities, testicular lesions and reduced sperm production (Meeker and Ferguson, 2014). However, a recent US EPA review of epidemiological studies of phthalate exposures and health outcomes did not reach a conclusion about their reproductive effects on humans (ACEIII, 2013). However, dose ranges used for traditional reproductive toxicological studies were much higher than those observed in human epidemiological studies. Therefore, it is not surprising that these studies do not entirely align with the human studies. Controversially, from a recent review of *in vitro* and *in vivo* toxicology studies (Hughes-Lock, 2015), low exposures to phthalates were linked to decreased semen quality and male infertility in animals, as well as to decreased androgen production and steroidogenesis (Bao et al., 2011; Bloom et al., 2015; Fan et al., 2010; Gunnarsson et al., 2008; Hu et al., 2013; Li et al., 2016; Louis et al., 2014; Savchuk et al., 2015; Xi et al., 2013).

To date, potential biological mechanisms of phthalates on reproductive system have not been clearly established and inconsistent. Multiple hypotheses were proposed to explain the mechanisms by which phthalates result in human reproductive toxicity such as changes in semen parameters, hormone, metabolic function and oxidative stress. Phthalates have mostly shown the antiandrogen effect on testicular function during steroid formation (Dees et al., 2001; Dobrzynska et al., 2011; Fiandanese et al., 2016; Wolff et al., 2008). Several *in vitro* or *in vivo* studies also showed that phthalates, as well as their metabolites (e.g., DEHP/MEHP, DBP/MBP) have stimulatory effects at low doses through inducing the production of progesterone, testosterone, steroidogenesis-related proteins and gene expression (Fan et al., 2010; Gunnarsson et al., 2008; Hu et al., 2013; Li et al., 2016; Savchuk et al., 2015; Xi et al., 2013). Indeed, more studies are needed to improve our understanding of the effects of low-dose exposures among humans, especially on reproductive outcomes.

Human metabolomic profiles have been used to gain insight into biological responses to chemical and non-chemical exposures or diseases. The changes in urine and blood metabolic profiles are easily observed and thus can be used to diagnose certain diseases or their progression (Zhang et al., 2012). We recently reported that aberrant physiological reproductive function led to altered metabolic profiles among infertile men (Shen et al., 2013). We also reported that the fertile and infertile men with oligozoospermia or normozoopspermia could be discriminated effectively using their urinary metabolic profiles (Zhang et al., 2014a, 2014b). These profiles directly pointed to certain biological pathways such as oxidative stress and endocrine disruption (Guo et al., 2014b), both of which may play a huge role in the etiology of male infertility.

Currently, inconsistent associations from limited epidemiological studies are not adequate to confirm the deleterious effects of phthalate exposure on male reproductive endpoints. We conducted this case-control study using a combination of urinary phthalate metabolite data and metabolic biomarkers with the ultimate goal of better understanding the association of phthalate exposures with male infertility.

2. Method and materials

2.1. Participant recruitment and urine sample collection

This study was performed according to the Declaration of Helsinki, and the procedures employed were approved by the local ethics committee. Our case-control study participants (n = 289) were volunteers from hospitals affiliated with Nanjing Medical University (NJMU) in Nanjing, China. Participants, after giving informed consent, were enrolled from April 2007 to September 2010. The study participation rate was 93.2%, with the participation rate for cases (n = 139) being 7–8% higher than controls (n = 150). The cases included in this study were infertile patients with diagnosed idiopathic male infertility (Ding et al., 2010). They were unable to conceive for at least 12 months in the absence of any fertility problems in their female partners; their female partners were also rigorously screened by complete historical and physical examinations. The cases with known causes of male infertility such as a history of orchitis, cryptorchidism, varicocele, vas deferens obstruction, and Y chromosome microdeletions were excluded (Xia et al., 2008, 2009).

The control subjects were healthy, fertile men who were recruited in the early pregnancy registry at the same hospitals as cases; their partners were in their first trimester of pregnancy and gave birth to healthy babies 6–8 months later (Xia et al., 2009). All participants claimed that their lifestyles, diet, and environment had not been changed for several months prior to sample collection. The questionnaires, including demographics and anthropometric measures (e.g., age, height and weight), smoking and drinking habits, education, as well as other medical history, were collected by trained interviewers. All participants provided a spot morning urine sample. The urine samples were initially stored at −20 °C and then transported on dry ice to the Key Laboratory of Environmental Health, Institute of Environment, Chinese Academy of Sciences, Xiamen, where they were stored at −80 °C until analysis.

2.2. Semen collection and analysis

Semen samples were collected from each participant on the same day of urine sample collection. Samples were collected following masturbation into a sterile wide-mouth glass container and were liquefied at 37 °C for 30 min before analysis. Analysis details for semen parameters have been previously described (Xia et al., 2008, 2009). Semen analysis including examination of sperm motility, semen volume, and sperm concentration was conducted using computer-aided semen analysis (CASA, WLJY 9000, Weili New Century Science and Tech Dev). Sperm count was counted by multiplying the semen volume and sperm concentration. Semen quality parameters such as sperm concentration (million/mL), sperm count (million), and sperm motility (A + B motile sperm), were recorded. In addition, three principal parameters for the vigor and pattern of sperm motion were also recorded as straight-line velocity (VSL), curvilinear velocity (VCL), and linearity (LIN = VSL/VCL × 100). External quality controls were established by the semen laboratory analysis according to the WHO guidelines (Organisation, 1999).

2.3. Urinary phthalate metabolite concentrations

The following seven urinary phthalate metabolites were measured: monomethyl phthalate (MMP), monoethyl phthalate (MEP), monobutyl phthalate (MBP), monobenzylphthalate (MBzP), mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), and mono-2-ethyl-5-oxohexyl phthalate (MEOHP). All standards, including their labeled analogs,