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Follicular fluid and urinary concentrations of phthalate metabolites among infertile women and associations with *in vitro* fertilization parameters

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ABSTRACT

Evidence from toxicological studies has demonstrated that phthalates can lead to reduced fertility through effects on folliculogenesis, oocyte maturation and embryonic development, but human data are limited. Concentrations of eight phthalate metabolites in 110 follicular fluid (FF) and urine samples collected from 112 women attending an infertility clinic in Wuhan, China were quantified, and correlations between paired matrices were explored. Associations between metabolite concentrations and *in vitro* fertilization (IVF) parameters were evaluated with multivariable models. Six metabolites were detected in >72.73% of the FF samples. MEHP and MBP were the dominant metabolites with a median level of 2.80 and 2.05 ng/mL, respectively. Significant correlations between the two matrices, urine and FF, were found for MEP (rs = 0.44), and MBP (rs = 0.22). FF and urinary metabolite concentrations were not associated with any IVF parameters. However, given the prevalence of phthalates exposure, further work is needed to elucidate the potential hazard on female reproduction.

1. Introduction

Phthalates are a class of man-made industrial chemicals which have been ubiquitously applied to manufacture polyvinyl chloride products, such as construction materials, food packaging and children's toys, mainly based on their ability to impart flexibility. They are also commonly used as solvents in cosmetics and personal care products, or as excipients, such as coatings of medications and dietary supplements [1–3]. Owing to the extensive use of phthalates, as well as their non-covalent conjugation with the products, the general population has been pervasively exposed to these compounds [1]. Once the phthalates enter the human body via various routes, primarily through ingestion, they can interfere with the endocrine systems, thus so called "endocrine-disrupting chemicals (EDCs)" [1,4]. To date, detectable levels of phthalates and their metabolites have been found in a wide range of biological fluids including human urine, serum, semen, amniotic fluid, umbilical cord blood and breast milk [5–8].

Prevalent exposure to phthalates has aroused growing public health concern based on their endocrine-disrupting potency. The adverse effects of phthalates on male fertility have been well documented and extensively studied[9]. In females, on the other hand, the influence of phthalates on ovarian and reproductive functions remains not well understood. However, there is accumulated evidence from experimental animal studies suggesting that phthalates exert reproductive toxicity by targeting the ovary [10,11]. Exposure to phthalates has been demonstrated

Abbreviations: AFC, antral follicle count; BMI, body mass index; DEHP, di(2-ethylhexyl) phthalate; EDCs, endocrine-disrupting chemicals; E_2, estradiol; FF, follicular fluid; FSH, follicle-stimulating hormone; HCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; LOD, the limits of detection; MBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MEP, monoethyl phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MMP, monomethyl phthalate; MOP, mono-*n*-octyl phthalate; %MEHP, the percentage of DEHP metabolites excreted as MEHP; 2PN, two pronuclei.

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to disrupt folliculogenesis, steroidogenesis, oocyte maturation and embryonic development [11–13], thus lead to reduced fertility. The effect of di(2-ethylhexyl) phthalate (DEHP) [prototype of mono(2-ethylhexyl) phthalate (MEHP)] on ovarian follicles and oocyte cellular structures was evidenced by depleted primordial follicle pool, increased atresia of antral follicles and observed spindle abnormalities which may result in aneuploidy, miscarriages and congenital defects [14,15]. A significant decrease of estradiol (E_2) production in a dose related pattern was observed in primary cultured granulosa cells after exposure to MEHP [16,17]. Laboratory studies using *in vitro* maturation systems showed that MEHP impeded the progression of oocyte meiosis and inhibited developmental potential of embryos in different animal models [13,18].

Investigations regarding reproductive toxicity of phthalates on females conducted to date have been through animal or *in vitro* studies, whether they can execute comparable effects in humans remains to be elucidated, especially, when the high dosages used in laboratory experiments were considered. The hitherto published epidemiological literature focusing on phthalates exposure and female fertility is scarce. Moreover, most previous studies measured urinary concentrations of phthalate metabolites as internal exposure biomarkers. Although urinary metabolites are reliable indicators of individual exposure, they may not effectively reflect the actual exposure status of the ovary. Follicular fluid (FF), the micro-environment that oocyte and its surrounding somatic cells directly contact with, is critical for oocyte health [19]. Any contamination with endocrine disruptors may affect developmental competence of the oocyte. Therefore, FF may serve as a more suitable and biological relevant matrix to assess ovarian phthalate exposure. Additionally, studies examining other EDCs in FF samples obtained from women undergoing IVF have demonstrated that exposure to environmental chemicals can result in detectable concentrations in FF [20,21]. Some of these EDCs found in FF were even suggested to associate with adverse reproductive outcomes including elevated risk of fertilization and implantation failure [22,23]. Findings from the above studies underscored the need to identify whether phthalates would present in the FF and may cause similar effects observed in experimental animal studies.

To test our hypothesis, we first aimed to quantify eight phthalate metabolites, including monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), monobenzyl phthalate (MBzP) and mono-*n*-octyl phthalate (MOP), in FF and urine samples from women undergoing *in vitro* fertilization (IVF). These metabolites were chosen to determine because their parent compounds, i.e., dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), butylbenzyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), and di-*n*-octyl phthalate (DOP), were among the most commercially significant phthalates with large volumes of production and widespread uses [24]. The US Environmental Protection Agency (USEPA) has also identified the six chemicals as priority pollutants. In order to investigate whether it was possible to predict concentrations of metabolites in the FF based on urinary measurements, we went further by studying the correlations between levels in the two matrices. Finally, the associations of metabolite concentrations with IVF parameters, comprising peak E_2 levels and oocyte yielding, both reflecting ovarian response to hyperstimulation [25,26], as well as early reproductive outcomes, such as the number of matured and fertilized oocytes, and Day 3 embryo quality, were explored. These early reproductive end-points which could be only observed in an IVF laboratory reflect stages of reproduction that are critical to form a fully competent embryo and predict pregnancy outcomes [27,28].

2. Materials and method

2.1. Study population and data collection

In this prospective cohort study, 112 women seeking infertility treatment at the Reproductive Medicine Center of Tongji Hospital, Wuhan, China, between July, 2014 and August, 2014 were recruited. Among couples attending the infertility clinic, the female partners aged 20–45 years, diagnosed with infertility (not being pregnant without protected intercourse for more than one year), and with indications for IVF or ICSI, were eligible for the study. Patients who received oocyte donation (n = 3) or used cryo-thawed oocytes (n = 2) were not included in the study. During the studying period, the long gonadotropin-releasing hormone (GnRH) agonist and GnRH antagonist protocols were the main stimulation regimens used in the clinic, which accounted for nearly 90% of the treatment cycles. The clinical pregnancy rates per transfer cycle at the clinic maintained at around 55%. All enrolled patients were followed up until they withdrew from the study because of achieving a live birth or discontinuing the treatment due to other reasons.

At the time of recruitment, each participant signed an informed consent and completed a questionnaire collecting information about demographics, life-style, and medical and reproductive histories. Their clinical data on antral follicle count (AFC), serum peak E_2 concentrations, and IVF outcomes, etc. were obtained from electronic medical charts. AFC was determined through transvaginal ultrasound on the third day of a menstrual cycle as a routine infertility evaluation at the clinic. The study was approved by the Institutional Review Board of Tongji Hospital.

2.2. Ovarian stimulation

Patients underwent either a long GnRH agonist or a GnRH antagonist protocol, according to their ovarian response. A long GnRH agonist regimen was offered to patients with a normal ovarian response, and pituitary suppression was achieved by daily injection of triptorelin acetate (Decapeptyl; Ferring) initiating from the mid-luteal phase prior to the stimulation cycle. While among women with a diminished ovarian reserve or a known poor response, a flexible GnRH antagonist protocol was conducted by administrating 0.25 mg Cetrorelix Acetate (Cetrotide; Merck-Serono) when at least one follicle reached 14 mm. Recombinant follicle-stimulating hormone (FSH) (Gonal-F, Serono or Puregon, MSD) was given to induce ovarian stimulation starting with 150 IU/d and adjusted based on the ovarian response, as previously described [29]. Ovulation was triggered by Recombinant human chorionic gonadotropin (HCG) (250 mg; Ovidrel; Serono) when a mean diameter of 18 mm was reached by at least two leading follicles. Ovum pick-up was performed transvaginally 34–36 h after trigger with HCG.

2.3. IVF procedures

Details on the IVF procedure and embryo culture have been previously described [30]. Briefly, the oocytes were evaluated for maturity 2–4 h after ovum pick-up, as evidenced by extruding the first polar body, and fertilized either by conventional insemination or intracytoplasmic sperm injection (ICSI). Fertilization was checked 16–18 h after insemination, and normal fertilization was defined as the appearance of two pronuclei (2PN). The count of 2PN relative to the amount of retrieved oocytes was referred to as fertilization rate. Embryo consisted of seven to nine cells without multinucleation and had less than 20% fragments on day 3 after oocyte pick-up were considered as a good-quality embryo [31]. The percentage of optimal embryos among the total number of cleavage embryos was calculated as good-quality embryo rate. Usually, fewer than two embryos of good quality were chosen for transfer