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Elevated phthalates' exposure in children with constitutional delay of growth and puberty

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ABSTRACT

Background: Phthalates have been proven to be antiandrogenic, which may interfere with the timing of puberty. Children with Constitutional Delay of Growth and Puberty (CDGP) typically display short stature and pubertal delay. This study investigated whether phthalate's exposure was associated with CDGP, and evaluated the potential mediator role of testosterone.

Methods: In this case-control study, a total of 167 boys, including 57 boys with CDGP (cases) and 110 controls were enrolled. We measured six major phthalate metabolites in urine samples using high-performance liquid chromatography and tandem mass spectrometry (LC–MS/MS). The serum testosterone level was determined by radioimmunoassay.

Results: Children in the CDGP group were determined to have significantly elevated urinary phthalates concentration compared with control subjects (total phthalates median: case, 107.00 ng/ml; control, 62.22 ng/ml, $p = 0.001$). After adjustment for BMI and other confounding factors: mono-n-butyl phthalate (MBP), monoethyl phthalate (MEP) and total phthalate concentrations were significantly negatively associated with serum testosterone level (MBP: $\beta = -45.7$, $p = 0.017$; MEP: $\beta = -31.6$, $p = 0.022$; total phthalates: $\beta = -24.6$, $p = 0.011$); MBP, MEP, mono (2-ethylhexyl) phthalate (MEHP) and total phthalates were significantly associated with CDGP (odds ratio: MBP: 8.30, $p = 0.002$; MEP: 5.43, $p = 0.002$; MEHP: 3.83, $p = 0.017$; total phthalates: 9.09, $p = 0.001$). Serum testosterone level acted as a mediator of the association between phthalates' exposure and CDGP ($p = 0.002$) (proportion mediated: 34.4%).

Conclusions: In this case–control study, elevated phthalates' level was detected in children with CDGP in Shanghai, China and phthalate level was associated with CDGP, which appeared to be mediated by circulating testosterone level.

1. Introduction

Changes in the timing of puberty, a marker of general public health, may result in later physical and psychological disease (Lee et al., 2001; Sun et al., 2012). Children with Constitutional Delay of Growth and Puberty (CDGP) are short for their genetic height potential, and display biological immaturity as determined by a delayed bone age (BA) and sexual delay (Ghai et al., 1995; Moreira-Andrés et al., 1998). CDGP is the most common cause of delayed puberty within the general population, and is most frequently encountered in boys (Sedlmeyer and Palmert, 2002). The timing of puberty is influenced by genetic, metabolic, and environmental factors (Ojeda et al., 2010a, 2010b; Palmert and Boepple, 2001; Tena-Sempere, 2006). Animal and human data have suggested perturbations in pubertal onset with exposures to endocrine-disrupting compounds (EDCs) (Jacobson-Dickman and Lee, 2009). Recent evidence has indicated subtle effects of pesticides, dioxins, and PCBs on delaying onset of puberty (Den Hond et al., 2002; Grandjean et al., 2012; Guo et al., 2004; Korrick et al., 2011).

Phthalates are a family of plasticizers that have been used for a variety of purposes (Hauser and Calafat, 2005; Shea, 2003), and can enter human body via multiple routes including dietary intake, dermal penetration and inhalation (Wittassek et al., 2011). A growing body of literature showed phthalates' anti-androgenic properties, such as reducing androgen production and other reproductive hormones, and decreasing semen quality (Akingbemi et al., 2001; Duty et al., 2003; Jönsson et al., 2005; Kim et al., 2003; Meeker et al., 2009; Pan et al., 2006; Stroheker et al., 2005). The link between exposure to phthalates and pubertal onset has been explored, although the results were inconsistent. One study showed an association between prenatal and childhood phthalates' exposure reduced odds of adrenarche and pubarche in a longitudinal design (Ferguson et al., 2014). However, adolescents with neonatal di-(2-ethylhexyl) phthalate (DEHP) exposure through extracorporeal membrane oxygenation

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(ECMO) showed no significant effects on testicular volume and the phallic length (Rais-Bahrami et al., 2004).

Due to the anti-androgenic effect of phthalate, we herein hypothesized that there is a difference of phthalates' exposure between boys with CDGP and controls. Therefore, we designed this case–control study to investigate phthalate's exposure on the risk of CDGP, and to explore whether testosterones mediate the association between phthalates' exposure and CDGP.

2. Methods

2.1. Subjects recruitment

We recruited 60 CDGP cases and 120 normal boys aged 8–15 years old from Fudan University Affiliated Children Hospital from June 2013 to early 2014. Cases were diagnosed CDGP patients and the controls were age-matched prepubertal (Tanner stage 1) and early pubertal (Tanner genital stage 2) boys from department of general surgery or department of orthopedics excluding endocrinopathy (n = 120). The diagnosis of CDGP was made on clinical grounds in children whose bone age (BA) was at least 1.75 years below their chronological age (Moreira-Andrés et al., 1998), or entry into puberty at >14 years (Gill et al., 1999). Pubertal assessment was undertaken according to methods of Tanner and Whitehouse (1976). Gonadotropin releasing hormone (GnRH) stimulation tests, nuclear magnetic resonance and physical examinations were performed to exclude clinical, radiological or biochemical evidence of congenital hypogonadism syndrome, endocrinopathy, chronic disease or skeletal dysplasia. We excluded 13 boys with alcohol abuse or tobacco smoking (Peck et al., 2011). Our subjects comprised 57 cases and 110 controls. Of note, all subjects were reared in Shanghai and above 90% were born in urban districts. Informed content was signed by the participants' parents. This study was conducted in accordance with protocols approved by Fudan University's Human Studies Committee.

2.2. Clinical information and specimen collection

A questionnaire was administered to participants to obtain information on socio-demographic characteristics, medical history, nutrition, health conditions and toxicant exposure history. The questionnaire was completed by children under their parents' direction. Left hand and wrist radiography was performed to evaluate BA. Testicular volume detection was conducted using an ultrasonic instrument and nuclear magnetic resonance by skillful male technicians. Measurements of weight, height were based on standardized clinical techniques. Five milliliter blood and 15 ml morning urine (complete void urine, stirred and completely mixed) for each subject were obtained, immediately enclosed in glass containers, subsequently transferred on ice to the Key Lab of Public Health Safety of the Ministry of Education, and stored at –80 °C.

2.3. Serum hormone analyses

Prior to analysis, approximately 2 ml of serum was isolated from each subject's blood sample by centrifugation. The serum was immediately transferred and used for the determination of testosterone level by radioimmunoassay (SRL Inc., Tokyo, Japan) in a commercial laboratory. The reference values for the determinations provided by the laboratory were 14–40 pg/ml, the limits of detection (LOD) was 0.6 pg/ml and the reference range was 3.3–21.3 pg/ml.

2.4. Phthalates measurement

Six phthalate metabolites, including mono-n-butyl phthalate (MBP) (total of mono-n-butyl phthalate (MnBP) and mono-i-butyl phthalate (MiBP)); mono-methyl phthalate (MMP); monoethyl phthalate (MEP); mono (2-ethylhexyl) phthalate (MEHP); mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); and mono (2-ethyl-5-oxohexyl) phthalate (MEOHP), were analyzed in 0.5 ml urinary sample as described previously (Guo et al., 2011; Silva et al., 2004).

Briefly, the determination of phthalates and metabolites in urine (0.5 ml) included enzymatic deconjugation (β-glucuronidase enzyme from *Escherichia coli*) of the metabolites, solid-phase extraction, separation with high-performance liquid chromatography (HPLC), and detection by tandem mass spectrometry (MS/MS). Samples were enzymatically hydrolyzed and purified by solid-phase extraction (SPE). Phthalate and its metabolites extracted from samples were detected by an Agilent 1100 Series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) and detected by an API 2000 electrospray triple quadrupole mass spectrometer (ESI–MS/MS; Applied Biosystems, Foster City, CA). $^{13}C_4$-labeled internal standards and conjugated internal standards were used to increase the precision of the measurements. Procedural blanks, and two sets of standards were analyzed along with unknown urine samples. Due to the glucuronidation of phthalate metabolites in the liver and its elimination by active tubular secretion, creatinine correction may not be appropriate for urine phthalate metabolite concentration (Hauser et al., 2004; Hines et al., 2009). For this reason, we used specific gravity to correct for urinary dilution by a hand-held refractometer (Atago PAL 10-S, Tokyo, Japan) (Hauser et al., 2004). The correction formula was $Pc = P \times [(1.024 - 1)/(SG - 1)]$, where Pc is the specific gravity-corrected phthalate metabolite concentration (ng/ml), P is the experimental phthalate metabolite concentration (ng/ml), and SG is the specific gravity of the urine sample. LOD was 0.25–0.5 μg/l for urine. The values below LOD were substituted for LOD divided by 2.

2.5. Statistical analysis

We performed the statistical analyses using SAS 9.2. Basic characteristics were tabulated and compared between CDGP cases and controls using Mann–Whitney U-test and chi-square test. We used Spearman correlation to explore the intercorrelation among urinary metabolites. We used Mann–Whitney U test (some of data still followed non-normal distribution) and t-test to analyze the difference of log-translated phthalate metabolites concentrations between cases and controls. We summed molar concentrations of MEHP, MEHHP, and MEOHP represented as ∑DEHP. We calculated total phthalate concentration as the summed values of MBP, MMP, MEP, MEHP, MEHHP and MEOHP.

Unconditional logistic regression models were used to simulate the associations of SG-corrected phthalate metabolite concentration (categorical data) and the risk of CDGP. Participants were categorized into tertiles based on each metabolite concentrations to facilitate logistic model fitting. Covariates included body mass index (BMI was calculated as weight (kg) divided by height (m) squared) because data from literature reveal that BMI has effects on CDGP (El-Eshmawy et al., 2010; Gill et al., 1999). In addition, since metabolites of phthalates were related with each other, urine phthalate metabolite concentrations were also included as covariates (Table S1).

Likewise, we conducted stepwise multiple linear regression models to explore the associations of phthalate metabolite concentration (continuous variable using log-translation) and serum testosterone level. We chose the following covariates a priori: phthalate metabolites, age, BMI, daily sleeping time, and exercise intensity, important in predicting the serum testosterone level. The significance levels for entry and inclusion in the model were $p < 0.05$ and $p < 0.10$, respectively.

Finally, the association of serum testosterone level and CDGP was explored using two unconditional logistic regression models. One