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Unexpected, ubiquitous exposure of pregnant Brazilian women to diisopentyl phthalate, one of the most potent antiandrogenic phthalates

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ABSTRACT

Background: Human exposure to phthalates and other non-persistent chemicals in developing countries is largely unknown. A preliminary analysis of urinary samples from pregnant Brazilian women revealed the presence of metabolites of Diisopentyl phthalate (DiPeP).

Objectives: Reliably quantify DiPeP metabolites in human urine and investigate the potential antiandrogenic activity of this phthalate in rats.

Methods: We initiated a pilot pregnancy cohort in Curitiba, Brazil, to examine phthalate exposure in urine samples collected in early pregnancy (n = 50) or pooled samples from early, mid and late pregnancy (n = 44). Our well established phthalate method was modified to include the primary DiPeP metabolite, monoisopentyl phthalate (MiPeP), and two additional secondary oxidized metabolites, 3OH-MiPeP and 4OH-MiPeP. In a parallel approach, we orally exposed pregnant rats to DiPeP or Di-n-butyl phthalate (DnBP; reference phthalate) at 0, 125, 250, and 500 mg/kg/day from gestation day 14 to 18 and measured *ex vivo* fetal testis testosterone production.

Results: We were able to detect and quantify specific DiPeP metabolites in nearly all (98%) of the early pregnancy urine samples and in all gestational pool samples with a median concentration for MiPeP of 3.65 and 3.15 µg/L, respectively, and for the two oxidized metabolites between 1.00 and 1.70 µg/L. All three urinary DiPeP metabolites were strongly correlated (r = 0.89 to 0.99). In the rat model, the effective dose (mg/kg/day) inhibiting fetal testosterone production by 50% (ED_{50} [95% confidence interval]) was 93.6 [62.9–139.3] for DiPeP which was significantly lower than for DnBP (220.3 [172.9–280.7]), highlighting the strong antiandrogenic potency of DiPeP within the spectrum of the phthalates.

Conclusions: We unveiled and confirmed the exposure of pregnant Brazilian women to DiPeP *via* specific urinary metabolites. This unexpected and ubiquitous DiPeP exposure indicates to unique DiPeP exposure sources in Brazil. These exposures spark considerable concern because DiPeP is one of the most potent antiandrogenic phthalates.

1. Introduction

Phthalates, dialkyl or alkyl/aryl esters of phthalic acid, are high production volume chemicals used as additives and plasticizers in a wide variety of industrial products. This class of chemicals has attracted significant attention from the scientific community, regulatory agencies and the public alike, because certain phthalates have the potential to act as antiandrogenic endocrine disruptors and reproductive toxicants. However, the toxicological profile of phthalates is largely related to their chemical structure, in particular the size and branching of the

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alcohol that makes up the alkyl side chain of the ester molecule (Furr et al., 2014; Gray Jr. et al., 2000; Lioy et al., 2015; Wittassek et al., 2011). Structure-activity relationship studies indicate that anti-androgenic phthalates have three to seven (or eight) carbons in the linear portion of the alkyl side chain (linear carbon backbone) (Furr et al., 2014; Gray Jr. et al., 2000; Lioy et al., 2015; Saillenfait et al., 2011). In laboratory rats, *in utero* exposure to these "active phthalates" results in a set of male reproductive tract abnormalities, the rat phthalate syndrome, characterized by undescended testes, hypospadias, small or malformed sex accessory glands, short (feminized) anogenital distance, and epididymal and testicular alterations (Foster, 2006). These reproductive disorders are downstream consequences of abnormal fetal testis development and associated hormonal disturbances, particularly testosterone insufficiency (Gray Jr. et al., 2016; Martino-Andrade and Chahoud, 2010). The highest potency for reducing fetal rat testosterone production has been reported for di-n-pentyl phthalate (DnPeP), a compound with five carbon atoms in the alkyl side chain, which is about three to eight-fold more potent than the commonly used counterparts di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DnBP) for this effect (Furr et al., 2014; Hannas et al., 2011a; Howdeshell et al., 2008; Howdeshell et al., 2015). In human studies, maternal exposure to certain phthalates has been associated a.o. with reduced anogenital distance in male newborns, an external marker of prenatal androgen deficiency (Martino-Andrade et al., 2016; Swan et al., 2005; Swan et al., 2015).

Human biomonitoring has been regarded as the best alternative for assessment of cumulative phthalate exposure not only for representing an integral measure of exposure from multiple sources and routes but also for not being affected by external contamination, since the secondary oxidized metabolites measured in urine are exclusively formed *in vivo* (Koch and Calafat, 2009). In Western Europe and in the United States, biomonitoring programs have consistently shown widespread human exposure to phthalates over the last years. Also, these programs have been advantageous to indicate temporal, geographic and demographic differences in exposure that reflect changes in phthalate production/regulation, use and lifestyle habits (Helm, 2007; Koch and Calafat, 2009; Koch et al., 2017). However, there is a lack of biomonitoring data from developing countries, such as BRICS (Brazil, Russia, India, China, and South Africa), which are important producers and consumers of industrial chemicals.

In this regard, we established a pregnancy cohort in Curitiba, Brazil, the Curitiba Reproductive and Environment Study (CARES), to determine the exposure of pregnant women to phthalates and other endocrine disruptors, predictors of exposure, and possible health outcomes. In the pre-screening of urine samples of this study, sparked by an unknown peak in the vicinity of the Di-n-pentyl phthalate (DnPeP) monoester metabolite peak, we found several peaks with mass transitions indicative of likely metabolites (monoester and oxidized) of diisopentyl phthalate (DiPeP) in nearly all samples investigated. The detection of possible DiPeP metabolites in this Brazilian study population was in contrast to any other European study population previously investigated by our group. In other international human biomonitoring publications we also found no hints on possible DiPeP exposures, except in a very recent publication by Rocha et al. (2017) who reported the ubiquitous presence of the primary monoester metabolite monoisopentyl phthalate (MiPeP) in urine samples of Brazilian children. Triggered by these findings, we immediately incorporated the quantification of MiPeP and two secondary oxidized metabolites (3OH-MiPeP and 4OH-MiPeP) using authentic analytical standard substances in our long-running phthalate method (Koch et al., 2017). Based on the known structure-activity relationship for phthalates we predicted that DiPeP, the branched isomer of DnPeP, would act as an antiandrogen with a similar potency of other C4-C5 phthalates. Therefore, in parallel to the quantification of DiPeP exposure, we conducted an animal study examining the ability and potency of DiPeP to inhibit the fetal testicular testosterone production in rats.

2. Materials and methods

2.1. Study Population and urine samples

We established a pilot pregnancy cohort study in the city of Curitiba, Brazil, the Curitiba Reproductive and Environment Study (CARES), designed to investigate exposure of pregnant women to nonpersistent chemicals and reproductive outcomes in mothers and newborns. Pregnant women were recruited between January and June 2015 at three public health care centers in Curitiba. All women visiting the three participating centers who were < 16 weeks pregnant, 18–40 years old, living in Curitiba, and whose pregnancies were not medically threatened, were eligible. Our study population ($n = 50$) consisted overall of married women (90%), white (76%), with basic or high school education (88%). Supplementary Table 1 shows the general sociodemographic characteristics of this population. Our study population has similar ethnic composition, but somewhat lower education in comparison with the general population of Curitiba, where 79% of the population is white and 24% have college degree (12% in our study) (IBGE, 2010). The mean gestational age of recruitment, when the first urinary samples were collected, was 10.2 ± 3.4 weeks (mean ± standard deviation). Participants provided urine samples and completed lifestyle questionnaires at three gestational periods, corresponding approximately to early (< 16 weeks), mid (16–28 weeks) and late pregnancy (> 28 weeks). At each gestational period, up to three spot urine samples were collected with an interval of approximately 1–2 weeks between samples, totaling up to nine urine specimens for each participant, whenever possible. Urinary phthalate metabolites were measured in the first spot samples collected in early pregnancy from all study participants ($n = 50$). In addition, from all participants that collected at least three urine samples during the study ($n = 44$), a pooled urine sample of the first, mid and last spot urine collected was used. The mean (± standard deviation) gestational age of urine collection of the first, mid and last spot samples used in the pools ($n = 44$) was 10.2 ± 3.4, 22.5 ± 5.7, and 33.8 ± 6.6 weeks, respectively.

CARES study protocols were approved by institutional review boards at the Federal University of Paraná and Curitiba Health Department and subjects provided signed informed consent before starting any study activities.

2.2. Chemical analysis of urinary DiPeP metabolites

The novel analysis of three potential urinary DiPeP metabolites was included in our long-running phthalate method analyzing 21 primary and secondary phthalate metabolites (Kasper-Sonnenberg et al., 2012; Koch et al., 2003; Koch et al., 2017; Preuss et al., 2005). In this method, in short, urine samples are submitted to enzymatic deconjugation with an arylsulfatase free beta-glucuronidase from *E. coli* K 12 (Roche Diagnostics Mannheim, Germany) and analyzed by on-line multidimensional liquid chromatography coupled to tandem mass spectrometry (LC/LC–MS/MS) with quantification by isotope dilution. Limits of quantification (LOQ) for all metabolites ranged from 0.2 to 1.0 µg/L. Because initial sample analyses showed a chromatographic peak close to the retention time of mono-n-pentyl phthalate (MnPeP), the monoester metabolite of the straight chain isomer di-n-pentyl phthalate (DnPeP, included in the previously established method), we suspected the possible presence of monoisopentyl phthalate (MiPeP), the monoester metabolite of the branched chain isomer diisopentyl phthalate.

Consequently, we obtained mono-(3-methyl) butyl phthalate (Campro Scientific GmbH, Berlin, Germany; chemical purity 99.5%), which represents the most important alkyl chain of DiPeP (see composition of technical DiPeP used in animal dosing study). Additionally, based on this 3-methyl butyl alkyl side chain, we obtained two secondary, hydroxylated metabolites (omega- and omega-1) by custom synthesis (Dr. Belov, Max Planck Institute for Biophysical Chemistry,