Environment International 119 (2018) 447–454

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

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

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ARTICLE INFO

Handling editor: Heather Stapleton

Keywords:
Exposure assessment
Diisopentyl phthalate
Diisoamyl phthalate
Endocrine disruption
Testosterone production

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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https://doi.org/10.1016/j.envint.2018.06.042
Received 11 April 2018; Received in revised form 13 June 2018; Accepted 30 June 2018
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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 epidydimal 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 mono-isopentyl 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,

Göttingen Germany): 3-hydroxy-monoisopentyl phthalate (3-OH-MiPeP) and 4-hydroxy-monoisopentyl phthalate (4-OH-MiPeP). The newly synthesized compounds had a purity > 95% determined by ^{1}H NMR. For quantification by isotope dilution we obtained the isotope labelled analogues D4-MiPeP (CDN Isotopes, Augsburg, Germany; chemical purity 99%) and D4-4OH-MnPeP (Dr. Belov, Max Planck Institute for Biophysical Chemistry, Göttingen Germany; chemical purity > 95%). These D4 isotope labelled internal standards had no detectable levels of the non-labelled analogues. Mass-spectrometry parameters for the above compounds are provided in Supplementary Table 2. Precision data from repeat measurements of self-prepared quality control samples (pooled native urine, at two concentration levels) are presented in Supplementary Table 3. Recovery data from spiking eight different urine samples (creatinine content between 0.206 g/L and 2.59 g/L) at two concentration levels (between 1 and 10 μg/L) are presented in Supplementary Table 4. In short, recoveries for all three biomarkers at both concentration levels were between 85.4 and 108%, with no dependency on the creatinine content of the individual sample. Within-series imprecision ($n = 8$) was < 6% for all three metabolites and < 8.4% in the spiking experiments for MiPeP and 3OH-MiPeP (18.3% for 4OH-MiPeP). Based on a signal to noise ratio of 9, the limits of quantification (LOQs) were 0.2 μg/L for all three DiPeP metabolites. Laboratory blanks and field blanks (distilled water collected together with participants' samples) did not contain detectable levels of any of the DiPeP metabolites.

2.3. Animal study

The study protocol was approved by the Animal Ethical Committee of the Federal University of Paraná (Curitiba, Brazil) under the number 1024, in accordance with guidelines of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). We used Wistar rats from the conventional animal facility of the Federal University of Paraná, which were kept in polypropylene cages with wood shavings bedding under controlled conditions of light (12 h light/dark cycle) and temperature (21 ± 2 °C), and receiving tap water and standard rodent food (Nuvilab CR-1, Quimtia, Colombo, Brazil) *ad libitum*. Adult female rats (mean weight 208 g; range 180–234 g) were mated daily with adult males during 3 h in the dark until a sufficient number of pregnant rats were obtained. The day of sperm detection in vaginal smears was set as gestation day 0 (GD0) and dams were randomly assigned to the treatment groups. A total of seven experimental groups ($n = 8$–9 dams/group) were established and the rat dams were treated daily by oral gavage with canola oil (Sigma Aldrich, Darmstadt, Germany), used as vehicle for phthalate administration, three doses of DiPeP (99%, CAS 84777–06-0, Petrom – Petroquímica Mogi das Cruzes SA, Brazil) or three doses of di-n-butyl phthalate (DnBP 99%, CAS 84-74-2, Sigma-Aldrich, Darmstadt, Germany, Product number 524980). Sample size was based on Furr et al. (2014) and on sample size calculations considering a reduction of 45% in testosterone production in relation to control values of a pilot study (mean = 3.3 ng/testis/3 h; standard deviation = 0.85). According to Gray Jr. et al. (2016) a reduction in fetal testis testosterone production of 45% or above is related to the induction of reproductive tract malformations in rats. Dams were treated from GD14 to 18, which represents the masculinization programming window (MPW) in rats, when testicular testosterone production peaks prenatally to induce differentiation and set maximum growth potential of genitals and reproductive organs (Macleod et al., 2010; Welsh et al., 2008). The DiPeP used in our study (CAS 84777-06-0), donated by Petrom SA, is a mixture of esters of 3-methyl, 1-butanol (85%) and 2-methyl, 1-butanol (15%). The dose levels of DiPeP and DnBP were 125, 250, and 500 mg/kg body weight/day and were selected based on previous DnBP studies showing that this range of doses can induce dose-dependent reductions in fetal testicular testosterone production in rats (Furr et al., 2014; Howdeshell et al., 2008). We used DnBP as the reference phthalate, because prenatal DnBP exposure can induce the full spectrum of the rat phthalate syndrome and it is commonly used as an animal model of reproductive disorders that comprise the human testicular dysgenesis syndrome (Fisher et al., 2003; Macleod et al., 2010; van den Driesche et al., 2012). Furthermore, both DnBP and DiPeP are made up of 4 carbon atoms in the alkyl chain backbone. The volume of administration was 2 mL/kg body weight/day. Pregnant rats were rapidly euthanized by decapitation under sevoflurane anesthesia on GD 18, 2–4 h after the last oral administration. All fetuses were removed from the gravid uterus starting from the left ovarian end in a clockwise direction, individually weighed, and decapitated. We also determined the total number of implantations by adding the number of live and dead fetuses with the number of resorptions. The fetal viability (% alive) was determined by dividing the number of live fetuses by the total number of implantations. Fetal sex was determined under a dissecting scope (Olympus BX41, Tokyo, Japan) by visualization of the gonads and the first three male fetuses had their left testes removed and immediately placed individually into wells of a 24-well plate containing 500 μl of M199 media without phenol red (Vitrocell, Campinas, Brazil) and incubated for 3 h at 37 °C with gentle rocking (Furr et al., 2014; Wilson et al., 2004). Following incubation, the media were collected, centrifuged at 4 °C and 2000*g* for 10 min and the supernatant frozen at −20 °C until testosterone analysis. All necropsies were conducted within a strict interval of 2 h, between 8:00 AM and 10:00 AM, to avoid interference of fetal growth. Testosterone concentrations were measured in the media by enzyme immunoassay using antibodies anti-testosterone and the respective horseradish peroxidase-conjugate obtained from Coralie Munro at the University of California (Davis, CA, USA), and according to the procedures described by Brown et al. (2004). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was used as substrate in the presence of hydrogen peroxide. All samples were evaluated in duplicate, and intra and inter-assay coefficients of variation were < 10%.

During the fetal necropsies of the current study, we noted that testes of DiPeP and DnBP exposed fetuses were located near the kidneys in a higher position than testes of control rats. Based on this observation we decided to measure the distance between the bladder and the lower pole of the testis (bladder-testis distance) using the distance of the bladder to the lower end of the kidney (bladder-kidney distance) as a reference (Imajima et al., 1997). These parameters were obtained for the right testes and right kidneys using a dissecting scope (Olympus BX41, Tokyo, Japan) with an eyepiece micrometer and reported as a ratio of the two measures × 100 (normalized bladder-testis distance). This endpoint is available for only a limited number of animals ($n = 3$–9 fetuses from 1 to 3 litters/group) because the measurements were initiated at a later time point in our study.

2.4. Statistical analysis

We obtained descriptive statistics for sociodemographic characteristics of study participants and urinary concentrations (μg/L) of the primary monoester metabolite of DiPeP, MiPeP, and two secondary oxidized metabolites, 3OH-MiPeP and 4OH-MiPeP. The DiPeP metabolite concentrations were calculated for samples collected in early pregnancy ($n = 50$) as well as the gestational pool ($n = 44$). These concentrations were not normally distributed even after log10 or cube root transformation. Values below LOQ were replaced by LOQ/2. We also calculated pair-wise Spearman's correlation coefficients between each of these metabolites in all measured samples, *i.e.*, early pregnancy and gestational pool samples combined ($n = 94$), and plotted their relationships in a log-log scale. Analyses were conducted with IBM SPSS software version 21.0 (Armonk, NY, USA). Testicular testosterone production in fetal rat testis was analyzed by ANOVA followed by Tukey's test. Dose-response curves of testosterone production were analyzed as a percent of control and log10 of phthalate doses in a nonlinear regression model with variable slope with GraphPad Prism 6.01 Software (La Jolla, CA, USA). We estimated the effective doses

which inhibited testosterone production by 50% of control (ED_{50}) for both DiPeP and DnBP. In the animal study the litter was used as the statistical unit and results are reported as litter means, except for bladder-testis distance, where results are means of individual fetuses.

3. Results

3.1. Human biomonitoring

In our study population of pregnant women, we could detect and quantify MiPeP and 3OH-MiPeP in all 44 investigated gestational pool samples and in 49 out of 50 early pregnancy urine samples indicating omnipresent exposure to DiPeP. 4OH-MiPeP was quantifiable in 42 samples of gestational pool and 46 samples of early pregnancy urine. Within the spectrum of the three metabolites, the simple monoester MiPeP was generally excreted at highest concentrations, but closely followed by 3OH-MiPeP and 4OH-MiPeP. Side chain oxidized metabolites have recently been proposed and successfully used as biomarkers of exposure for other short-chain phthalates such as DnPeP, DiBP and DnBP as additional, contamination free metabolites for confirmation and for complementation of the exposure biomarker profile (Koch et al., 2012; Lorber et al., 2017; Silva et al., 2011; Weschler et al., 2015). Median urinary concentrations in the early pregnancy samples were 3.65 µg/L for MiPeP, 1.70 µg/L for 3OH-MiPeP and 1.00 µg/L for 4OH-MiPeP. Median concentrations in the gestational pool samples were very similar, with 3.15 µg/L, 1.70 µg/L and 1.00 µg/L, respectively. Maximum concentrations observed in this study were up to 30-times higher. A detailed summary of the biomonitoring data is depicted in Table 1. We observed very strong and significant correlations (r = 0.89 to 0.99) between all DiPeP metabolites (Fig. 1). Example chromatograms from a calibration standard and a representative urine sample are shown in Fig. 2. Results for the full set of 21 phthalate metabolites will be presented and discussed in detail elsewhere, but in principle reflected the broad bandwidth of phthalate body burden known from other studies. Mono-n-pentyl phthalate (MnPeP), the primary metabolite of Di-n-pentyl phthalate (DnPeP), the straight chain isomer of DiPeP was detected only in one of the 94 urine samples investigated, at a very low concentration of 0.2 µg/L.

Table 1
Maternal urinary concentrations (µg/L) of DiPeP metabolites in early pregnancy and gestational pool samples from CARES.

Variable	Early pregnancy samples			Gestational pool		
	MiPeP[a]	3OH-MiPeP[b]	4OH-MiPeP[c]	MiPeP	3OH-MiPeP	4OH-MiPeP
N	50	50	50	44	44	44
LOQ[d]	0.20	0.20	0.20	0.20	0.20	0.20
N > LOQ	49	49	46	44	44	42
% > LOQ	98	98	92	100	100	95.5
Min	0.10	0.10	0.10	0.80	0.20	0.10
Max	115.0	25.10	12.50	111.0	48.00	27.80
P5[e]	0.36	0.20	0.10	0.90	0.42	0.15
P25	2.18	1.17	0.50	1.83	0.90	0.60
P50	3.65	1.70	1.00	3.15	1.70	1.00
P75	6.90	2.85	1.63	4.60	2.30	1.38
P95	25.81	10.64	6.13	61.47	26.67	13.65
Mean (SD)[f]	7.74 (16.52)	3.00 (4.16)	1.67 (2.18)	7.56 (19.56)	3.53 (8.46)	2.00 (4.71)
GM[g]	3.78	1.68	0.95	3.32	1.66	0.95

[a] MiPeP = monoisopentyl phthalate.
[b] 3OH-MiPeP = 3-hydroxy-monoisopentyl phthalate.
[c] 4OH-MiPeP = 4-hydroxy-monoisopentyl phthalate.
[d] LOQ = limit of quantification; values below LOQ were replaced by LOQ/2.
[e] P = percentile.
[f] Mean (SD) = arithmetic mean and standard deviation.
[g] GM = geometric mean.

3.2. Animal study

The results of the *ex vivo* testicular testosterone production in rats following *in utero* exposure to DiPeP and DnBP are shown in Figs. 3 and 4. DiPeP and DnBP reduced testosterone production in a dose-responsive manner, with significant reductions at all three dose levels tested for DiPeP and at 250 and 500 mg/kg/day for DnBP. The ED_{50} (95% confidence interval) for DiPeP and DnBP was 93.6 mg/kg/day (62.9–139.3 mg/kg/day) and 220.3 mg/kg/day (172.9–280.7 mg/kg/day), respectively. At the selected doses, oral administration of DiPeP or DnBP to pregnant rats did not induce overt maternal or fetal toxicity as measured by maternal body weight gain and fetal weight and viability, respectively (Supplementary Table 5). Maternal DiPeP and DnBP exposure also induced a dose-related increase in the bladder-testis distance (Fig. 5). No statistical analysis was performed for bladder-testis distance due to the small sample size for this endpoint. In Table 2 we show the ED_{50s} for the inhibition of testosterone production following maternal exposures to DiPeP, DnBP, DnPeP, and DEHP obtained from different rat studies.

4. Discussion

In this study, we report on the ubiquitous presence and quantification of DiPeP metabolites in urinary samples of pregnant Brazilian women participating in the pilot phase of our cohort, Curitiba Reproductive and Environment Study (CARES). Determination of DiPeP exposure *via* specific urinary metabolites and authentic analytical standards has not been included as a routine parameter in biomonitoring studies. However, relevant mass transitions have been covered by the routine analyses of the monoester (MnPeP) of the straight chain isomer DnPeP. This straight chain monoester metabolite of DnPeP has only rarely been detected in population studies, and if at all, at very low concentrations (Correia-Sa et al., 2018; Kasper-Sonnenberg et al., 2012; Koch et al., 2017; Silva et al., 2011). Similarly, in the CARES samples we also could detect MnPeP in only one urine sample. However, unlike in any other population study performed by us, we consistently detected a peak neighboring MnPeP indicating to the presence of its isomer MiPeP. At the same time, this finding was confirmed by a recent study of Rocha et al. (2017) who reported the ubiquitous detection of MiPeP in urine samples of 300 Brazilian children from states of all five geographic regional divisions of Brazil. For verification of these findings and a reliable quantification, we therefore decided to include three potential DiPeP metabolites (the simple monoester MiPeP and two oxidized metabolites, 3OH-MiPeP and 4OH-MiPeP) in our existing phthalate method. The median urinary MiPeP concentrations reported in our study (3.65 µg/L and 3.15 µg/L) are remarkably similar to the median levels reported in Brazilian children (6.58 µg/L) by Rocha et al. (2017). Also, maximum levels reported are very similar with 115 µg/L in our pregnant women, and 261 µg/L in the children. These numbers confirm both the omnipresent DiPeP exposure in Brazil and a considerable bandwidth of exposures spanning several orders of magnitude.

The interpretation of simple (primary) monoester metabolite levels alone, in terms of absolute exposure, is difficult, because we know from other phthalates, that the shares of the simple monoester metabolite excreted can differ dramatically from phthalate to phthalate. For example, approximately 80% of an oral DnBP or DiBP dose is excreted in urine as the simple monoester, while only 6% of DEHP is excreted as the monoester MEHP. DiPeP, like DnBP and DiBP belongs to the group of low molecular weight phthalates, for which we assumed that oxidized metabolites make up only a relatively small share of the total dose. Our results do not confirm this assumption. Although MiPeP is the major DiPeP metabolite, the two oxidized metabolites 3OH-MiPeP and 4OH-MiPeP taken together represent almost a similar share of the dose excreted *via* urine. The confirmatory power of these oxidized metabolites is additionally illustrated by the very strong correlation. Taken this

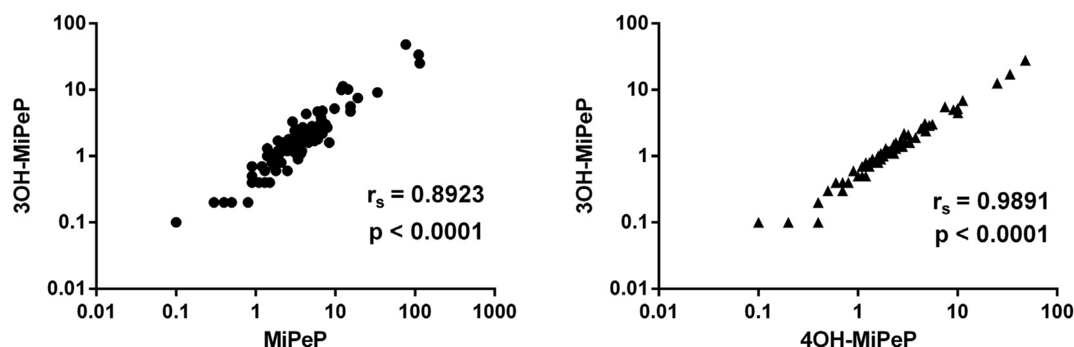

Fig. 1. Correlations between $\log_{10}$ urinary concentrations of DiPeP metabolites. r_s = spearman correlation coefficient. MiPeP = monoisopentyl phthalate; 3OH-MiPeP = 3-hydroxy-monoisopentyl phthalate; 4OH-MiPeP = 4-hydroxy-monoisopentyl phthalate.

information on DiPeP metabolite distribution together, we estimate that the fraction of the parent phthalate dose that is excreted as primary monoester (MiPeP) in urine is approximately 35–40%, which is half of the urinary fraction of DnBP/DiBP primary monoesters. This means that similar primary monoester metabolite levels (MiPeP and MnBP/MiBP) probably indicate to DiPeP doses that are at least twice as high as DnBP/DiBP. Because of ethical constraints, it is unlikely that we will derive more robust human metabolite conversion factors for DiPeP in a similar manner as previously obtained for DiBP, DnBP or DEHP. Animal studies designed to quantify urinary DiPeP metabolites following oral administration may be useful to estimate the excretion fractions of primary and oxidized metabolites. The exemplary chromatogram of the native urine sample in Fig. 2 illustrates additional, interesting findings. The MiPeP peaks in native urine samples are somewhat malformed by a so-called shoulder, which indicates to some other potential isomer contributing to the MiPeP peak. From the DiPeP used in Brazil, we know that it is probably composed of 85% 3-methyl-1-butanol and 15% 2-methyl-1-butanol (see methods of the animal study section above). Thus, this shoulder in the MiPeP peak is probably caused by another (other) isomer(s) of the isopentyl side chain. This is also supported by other, minor peaks in the chromatograms of the hydroxylated metabolites (labelled “unknown” in Fig. 2) that could represent hydroxylated metabolites of isopentyl isomers next to the 3-methyl butyl isomer. Furthermore, as has been shown by Silva et al. (2011) for straight-chain isomer DnPeP, other oxidized metabolites carrying oxo- or carboxy-modifications are also likely to be formed, which have not been included in our quantitative method.

DiPeP, also known as diisoamyl phthalate (DiAP), is synthesized by the esterification of phthalic anhydride with isoamyl alcohol, which is a side product of sugar fermentation during the production of ethanol. In Brazil, ethanol and its side products are largely available due to the national strategy of replacement of fossil fuels by sugarcane ethanol. Therefore, the easy access to isoamyl alcohol in Brazil may have favored the industrial production of DiPeP by the local plasticizer industry. The isoamyl alcohol used in the synthesis of DiPeP in Brazil is usually a mixture of 3-methyl-1-butanol (85%) and 2-methyl-1-butanol (15%) obtained from fusel oil, a byproduct of alcoholic fermentation. DiPeP has been previously detected in soils receiving wastes of a Brazilian plastic manufacturing plant (Ferreira and Morita, 2012) and in landfill leachate samples (do Nascimento Filho et al., 2003), but to the best of our knowledge there are no plastic or plasticizer industries in the area where our study participants were recruited. The sources of

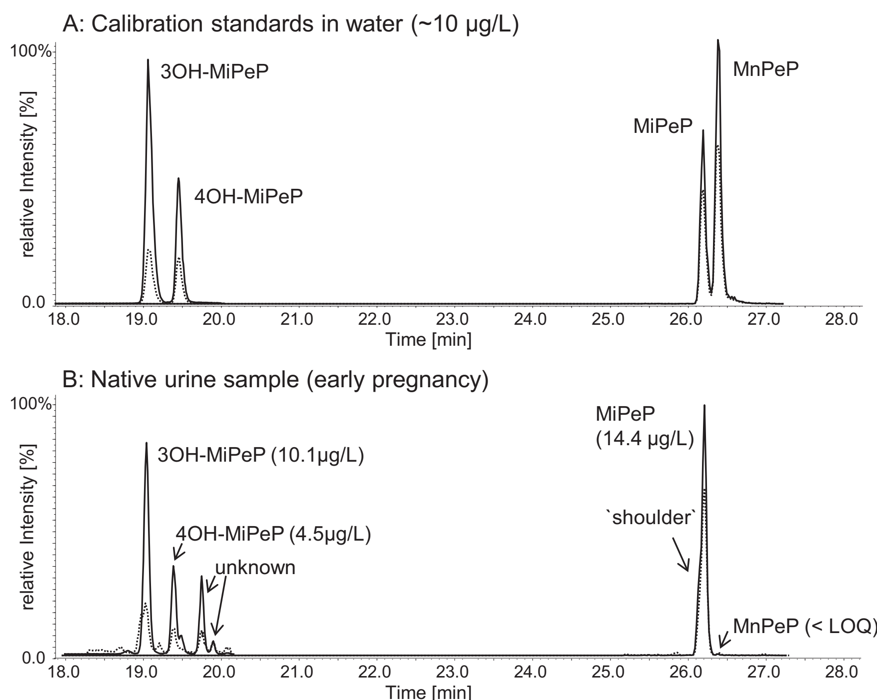

Fig. 2. Reconstructed ion chromatogram of a calibration standard in water (A) and an exemplary urine sample from the study population (B). Mass transitions for quantification in are shown bold lines, confirmation traces are shown in dotted lines. Respective concentrations are depicted in each chromatogram.

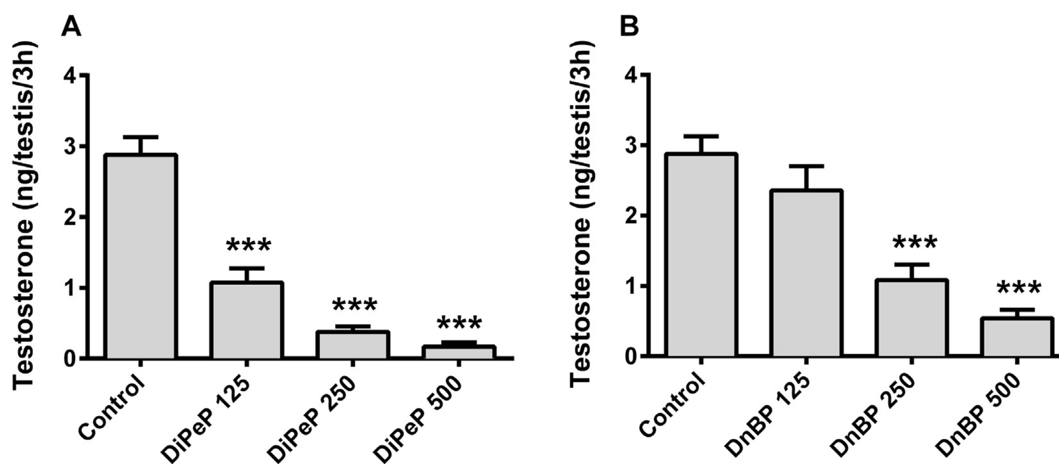

Fig. 3. Fetal testicular testosterone production in rats following *in utero* exposure to DiPeP (A) or DnBP (B) from gestation day 14 to 18. Values are litter means ± standard error ($n = 8$ litters/group, except for DnBP 125 mg/kg/day ($n = 9$)). *** $p < 0.001$ (ANOVA/Tukey).

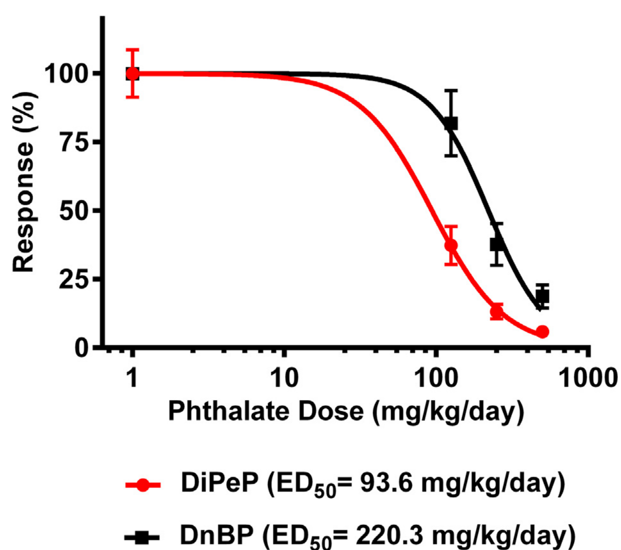

Fig. 4. Dose-response relationship for fetal testicular testosterone production in rats exposed *in utero* to DiPeP or DnBP. Data were analyzed as percent of control and log10 of phthalate doses in a nonlinear regression model with variable slope. ED_{50}: effective dose which inhibited testosterone production by 50% of control.

DiPeP human exposure are largely unknown, but due to its structural and physicochemical properties, it may be used as a plasticizer or additive of polyvinyl chloride (PVC) plastics and in many other niche industrial applications (ECHA, 2012). The use of DiPeP and other critical phthalates in cosmetics and personal care products was banned in Brazil in 2016 (ANVISA, 2016), but there is the potential of DiPeP use in other applications. In the European Union, DiPeP has been registered for use in the manufacture of propellants used in the production of ammunition. However, DiPeP metabolites have never been reported in biomonitoring studies in Europe.

Although our sample size is small, the fact that we detected primary and secondary DiPeP metabolites in urinary samples of all 50 women recruited in our pilot study raises concerns and points out to the need of additional exposure assessment and epidemiological studies in Brazil and other developing countries. Also, the detection of human exposure to DiPeP is a cause of concern given the known structure-activity relationships for phthalates. DnPeP, the linear isomer of DiPeP, is considered the most potent antiandrogenic phthalate, but to the best of our knowledge, there are no published studies on the endocrine disruptive effects of DiPeP. According to the EU regulation (EC) No 1272/2008, DiPeP is classified as toxic for reproduction category 1B (presumed human reproductive toxicant). A developmental toxicity study reported that DiPeP administered to pregnant rats from gestation day 6 to 15 at 1000 mg/kg/day caused 100% resorption rate, a more severe effect than that produced by the same dose level of DEHP (Hellwig et al., 1997). However, no endocrine sensitive endpoints were examined in

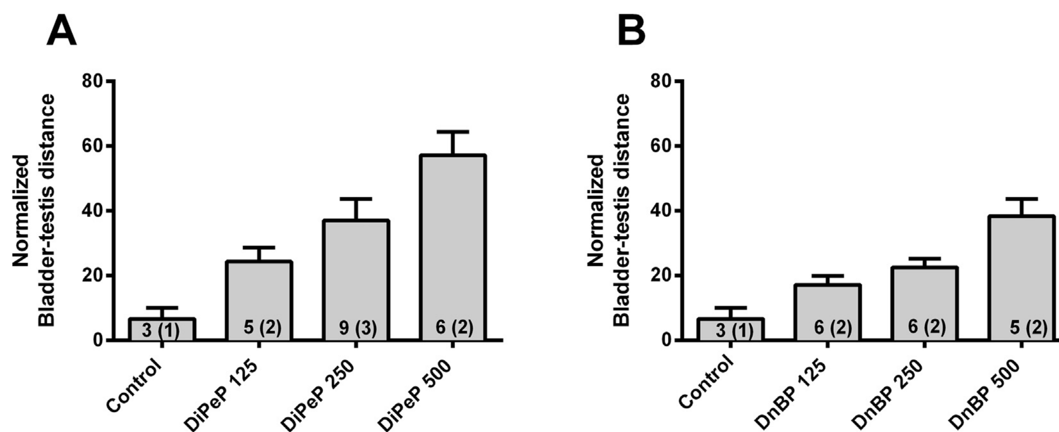

Fig. 5. Normalized bladder-testis distance in fetuses exposed *in utero* to DiPeP (A) or DnBP (B). Data represent the % ratio of the distance between the bladder and the lower pole of the testis (bladder-testis distance) and distance of the bladder to the lower end of the kidney (bladder-kidney distance). Values are means ± standard error of individual fetuses. Numbers inside bars indicate the numbers of fetuses (litters).

Table 2
Comparison of ED_{50}[a] values for reductions in fetal testicular testosterone production for different phthalate esters.

Phthalate[b]	ED_{50}	Rat strain	Exposure window[c]	Reference
DiPeP	93.6	Wistar	GD14-18	This study
DnBP	220.3	Wistar	GD14-18	This study
	398.5	Sprague-Dawley (Charles-River)	GD8-18	Howdeshell et al. (2008)
	337.1	Sprague-Dawley (Charles-River)	GD14-18	Furr et al. (2014)
	157.9	Sprague-Dawley (Harlan)	GD14-18	Furr et al. (2014)
DnPeP	130.7	Sprague-Dawley (Charles-River)	GD8-18	Howdeshell et al. (2008)
	47.7	Sprague-Dawley (Charles-River)	GD14-18	Hannas et al. (2011a)
	81.6	Sprague-Dawley (Charles-River)	GD14-18	Furr et al. (2014)
	35.6	Sprague-Dawley (Harlan)	GD14-18	Furr et al. (2014)
DEHP	383.4	Sprague-Dawley (Charles-River)	GD8-18	Howdeshell et al. (2008)
	347	Wistar	GD14-18	Hannas et al. (2011b)
	426	Sprague-Dawley (Charles-River)	GD14-18	Hannas et al. (2011b)
	359.8	Sprague-Dawley (Charles-River)	GD14-18	Furr et al. (2014)
	121.2	Sprague-Dawley (Harlan)	GD14-18	Furr et al. (2014)

[a] ED50 (mg/kg/day) = effective dose which inhibited fetal testicular testosterone production by 50% in relation to control values.
[b] DiPeP = Diisopentyl phthalate; DnBP = Di-n-butyl phthalate; DnPeP = di-n-pentyl phthalate; DEHP = di-2ethylhexyl phthalate.
[c] GD = gestation day.

the study by Hellwig et al. (1997).

In our rat study, DiPeP reduced the fetal testicular testosterone production in a dose-responsive manner, with greater potency than the reference compound DnBP. We selected high DiPeP doses to compare its effects with the typical toxicological doses used with other phthalates to induce suppression of testosterone production. The ED_{50} for DiPeP on fetal rat testicular testosterone production was lower than that calculated for DnBP. When compared to values reported in the literature, the ED_{50} calculated for DiPeP was lower than those estimated for DnBP and DEHP and comparable to those reported for its linear isomer, DnPeP. Considering that the estimated ED_{50} for DiPeP (93.6 mg/kg/day) is below the lowest dose tested in the current study (125 mg/kg/day), there may be an imprecision in this estimate. Despite this extrapolation, it is clear that DiPeP potency is closer to that of DnPeP than of DnBP. Inhibition of fetal testicular testosterone production is a key event in the adverse outcome pathway of phthalates which has been used to accurately predict compounds that can induce the rat phthalate syndrome. According to Hannas et al. (2011a), fetal testicular testosterone production is more sensitive than other endpoints such as expression of steroidogenic enzymes and early postnatal endpoints like reduced anogenital distance and nipple retention. In the current protocol, the anogenital distance of fetal rats was not assessed because of the early gestational age at necropsies (gestation day 18) and the need to collect the fetal testes in a short time window interval to avoid the effects fetal/testicular growth and development (Furr et al., 2014). However, apical studies designed to examine the early and late reproductive consequences of *in utero* DiPeP exposure are needed to fully characterize its endocrine disrupting effects. In our rat model, we also observed that DiPeP increased the bladder-testis distance, an effect consistent with impairment of testicular descent mediated by inhibition of insulin-like factor 3 (INSL-3) production, which is commonly observed in rats prenatally exposed to active phthalates (Foster, 2006; Wilson et al., 2004). Although the small sample size precluded statistical analyses of bladder-testis distance data, the obtained results could be considered biologically relevant given the observed dose-related effects and that undescended testis is a typical feature of the rat phthalate syndrome.

The inhibition of testosterone production and the induction of the phthalate syndrome in rats usually occur at high treatment doses, which are not typically relevant for the human exposure scenario. However, it is important to recognize that humans are exposed to mixtures of phthalates and other chemicals that can affect common target tissues. The exposure of pregnant women to a potent antiandrogenic phthalate such as DiPeP is a cause of concern because it may contribute to the cumulative risk for reproductive disorders. Several studies have demonstrated that endocrine disrupting chemicals that target similar tissues *via* dissimilar modes of action can act in a dose-additive fashion to induce adverse reproductive effects in rats (Howdeshell et al., 2008; Rider et al., 2008; Rider et al., 2010). In an effort to clarify the risks associated with DiPeP alone and in the context of environmentally relevant chemical mixtures, we will conduct a larger version of CARES to assess the exposure of pregnant Brazilian women to phthalates and other endocrine disruptors, as well as toxicity studies on DiPeP and phthalate mixtures in rats, including the analysis of several reproductive and behavioral endpoints following developmental exposures.

5. Conclusions

In this study, we demonstrated ubiquitous exposure of pregnant Brazilian women to DiPeP. Brazil, so far, is the only country for which such ubiquitous DiPeP exposures have been reported. These findings highlight the need to investigate exposures to less commonly used, but potentially toxic phthalates, also in other parts of the world. The combined evidence of human exposure and toxicity data from this study warrants a comprehensive analysis of reproductive risks of DiPeP, especially considering that we detected exposures in pregnant women and that DiPeP exhibited higher potency than other phthalate esters for inhibition of fetal testicular testosterone production in rats.

Acknowledgments

We thank PETROM S/A (Brazil) for donating the DiPeP used in the animal study.

Funding

This work was supported by PROAP-CAPES (Programa de Pos-graduacao em Fisiologia UFPR) and by Fundação Araucária Edital PPSUS (052/2017 – 48.2020). MTP, KBM and ACV are fellowship recipients from CAPES (Brazil). PRD and AJMA are fellowship recipients from CNPq (Brazil).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.06.042.

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