NJDEP Exposure of Infants to Endocrine Disruptors-
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INTRODUCTION

Phthalates and bisphenol A (BPA) are used in many products that are ubiquitous in the urban environment. Phthalates are present in lubricants, cosmetics, construction materials, wood finishers, adhesives, floorings, and paints. High molecular weight phthalates, such as di(2-ethyl-hexyl) phthalate (DEHP), are used as plasticizers in the manufacture of polyvinyl chloride, which is in consumer products such as perfumes, nail polish, automobile interiors, vinyl shower curtains, wall and floor coverings, food contact applications, and medical devices (1). In addition to exposure directly via manufactured goods, increasing concentrations of phthalates have been identified as environmental contaminants. For example, significant quantities have been documented in snowmelt, wastewater, and household dust (2-4). Moreover, some phthalate metabolites may persist in the environment due to variable mechanisms and rates of degradation (5). In its 3rd National Report on Human Exposure to Environmental Chemicals, the CDC collected urine samples from a randomly selected sample US subjects and found elevated levels of mono-(2-ethylhexyl) phthalate (MEHP), the primary monoester metabolite of DEHP in humans. They found a level of 3.5 µg/l urine at the 10th percentile and 13.6 µg/l urine at the 90th percentile of the population distribution (6). Two oxidative DEHP metabolites, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), were also present in high concentrations in urine and are indicative of exposure to DEHP (7). Lower molecular weight phthalates, such as di-n-butyl phthalate (DBP) and diethyl phthalate (DEP) are also ubiquitous in the environment. They are used as solvents and plasticizers, and are present in lacquers, varnishes, perfumes, lotions, cosmetics, and coatings.

There is also widespread exposure to BPA and its metabolites in the U.S population (8). BPA is used in the manufacture of polycarbonate, epoxy resins, and other plastics in printed circuit boards, composites, and adhesives. Polycarbonates are used for food-contact use, such
as in microwave oven-ware, milk and juice containers, baby bottles, and the interior coating of cans, thus posing a substantial risk for exposure to the public (9). Bisphenol A diglycidyl ether (BADGE) is the lowest molecular weight oligomer, used in commercial liquid epoxy resins. Like phthalates, significant levels of BPA have been found in wastewater, drinking water, air, and dust (10).

The fetal and neonatal periods represent a particularly vulnerable period of susceptibility to adverse effects of environmental exposures. Therefore, the recent finding that the levels of several phthalate metabolites are elevated in maternal urine, collected at the time of delivery, are of concern (11). In our institution, urinary concentrations of MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), three metabolites of DEHP, were found to be 5-20 times higher in mothers prior to elective cesarean deliveries than the U.S. general and female population based on NHANES 2001–2002 data. This suggests either that pregnant females are more exposed to these substances than the general population, or that the background level of exposure in central New Jersey exceeds that in the U.S. as a whole (11). Phthalate metabolites in the maternal urine are thought to be more sensitive biomarkers of exposure than those measured in maternal or cord serum (11-12). Our group has also found that hospitalized infants excrete much higher concentrations of MEHP, MEHHP, and MEOHP in their urine than in a US population (NHANES) sample (13). Similarly, BPA has been found in follicular and amniotic fluid, umbilical cord blood, and placental tissue (14-15). Several groups have shown that BPA is present in maternal serum or urine, amniotic fluid, and/or fetal serum at concentrations comparable to levels known to interfere with normal development in animals. Mean levels in maternal plasma have been reported from 0.43-3.1 μg/L, with some subjects as high as 22.3 μg/L. Mean amniotic fluid and fetal serum levels are lower (0.5-8.3 and 0.64-2.3 μg/L, respectively) (15-19). These levels may affect reproductive and fetal health, constituting an important public health concern.
Some previous studies have suggested that phthalates and BPA may exert anti-androgenic effects and shorten gestation (20-25). For example, males exposed prenatally to phthalates may have decreased anogenital distance (AGD), penile volume, and scrotal size (20-21). BPA and its metabolites have a high affinity for the androgen receptor and exhibit marked anti-androgenic activity (22), and also have been shown to have estrogenic activity in vitro (23). Both phthalates and BPA can also induce inflammatory activity, potentially shortening gestation by triggering parturition (24-25). In our investigation, we quantified maternal exposure to phthalate and BPA metabolites in a high-risk obstetrical population. In contrast to earlier studies, these measurements were made at the final prenatal clinic visit, reflecting ongoing exposure in the home environment, rather than the hospital environment after admission for delivery of the infant. In addition, multiple urine samples were obtained longitudinally during the pregnancy at successive clinic visits, and these may be analyzed in future investigations. AGD measurements were made in the immediate neonatal period, prior to discharge from the hospital, in order to decrease variability related to postnatal age. We hypothesized that elevated levels of phthalates and BPA in this population of high-risk pregnancies are associated with an increased risk for preterm delivery and alterations in genital development.

The incidence rate of prematurity has been increasing in the U.S. during the last two decades. Given that the complications of even moderate prematurity are associated with substantial morbidity, mortality, and health care costs, it is important to identify high-prevalence environmental exposures that may increase the risk of these adverse outcomes.

**METHODS**

**Study population.** We recruited a population of 72 mothers, over the age of 18 and expecting singleton infants, enrolled in the High-Risk Obstetric Clinic at Robert Wood Johnson University Hospital, a major central New Jersey teaching hospital in New Brunswick. It serves both urban and suburban populations for a large portion of the state. All subjects provided
informed consent prior to participation in the study which was approved by the Institutional Review Board (IRB) at the University of Medicine of New Jersey-Robert Wood Johnson Medical School. A detailed medical history, information on household product use, occupation, hobbies, diet, demographic variables, and ethnicity were collected as a part of the study.

**Urinary phthalate and BPA metabolite concentrations.** Urine samples were collected in phthalate and BPA-free containers at the beginning of each prenatal clinic visit, resulting in 1 to 13 successive samples per subject. In the current study, only the samples from the last prenatal clinic visit prior to delivery were analyzed. Samples were stored in coolers with ice packs and delivered to the Neonatology Laboratory, located in the same building. No preservatives were added prior to sampling. All samples were stored at -70°C until transferred on dry ice to the Analytical Core Facility at the Center for Environmental Exposures and Disease (CEED) NIEHS Center of Excellence of the Environmental and Occupational Health Sciences Institute (EOHSI) of Rutgers/UMDNJ. They were maintained at -70°C until analyzed, using a modification of a high-performance liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry method first described by Silva et al (26). The following metabolites were quantified: MEHP, monomethyl phthalate (mMP), monoethyl phthalate (mEP), monobutyl phthalate (mBP), monocyclohexyl phthalate (mCHP), mEHHP, mono-3-methyl-7-methyloctyl phthalate (isodecyl, mDP), mEOHP, mono-n-octyl phthalate (mOP), mono-3-methyl-5-dimethylhexyl phthalate (iso-nonyl, mNP), BPA total, BPA sulfate, and BPA glucuronide. The detection limits for this method range from 0.1 to 0.5 ng/ml, with recovery efficiency of 100 ± 12% (26). All LC/MS measurements were made using Thermo Fischer scientific ion trap mass spectrometers, and normalized to urine specific gravity, measured concurrently. BPA and its glucuronide and sulfate metabolites were isolated by liquid/liquid extraction using MTBE/hexane, and quantified by LC/MS/MS using an electrospray interface operated in the negative ionization mode.
**Physical examination and anogenital index.** Male and female infants were examined in the nursery at < 72 hrs of age. Anogenital distance (AGD) was measured by tape measure from the center of the anus to the posterior base of the scrotum in males, and to the posterior edge of the vagina in females (27). Anogenital index (AGI) (mm/kg) was calculated by normalizing AGD (mm) to weight (kg). The presence or absence of other genital abnormalities, including hypospadias or incomplete testicular descent in boys, was also noted. Gestational age and birth weight were recorded, and data obtained on other medical diagnoses, medications, and interventions at the time of examination. Gestational age was determined based on the best obstetric estimate in the medical record, using either sonographic dating or date of implantation.

**Umbilical cord blood inflammatory cytokine levels.** Samples of umbilical cord blood (8-10 ml) were obtained from placentas of study subjects at the time of delivery, when available. Blood was spun at 3000 x g for 10 min in 1.5 ml Eppendorff tubes in a microcentrifuge. The serum supernatants were removed, placed in a new tube, and frozen at -70 for batched analysis. Sera were then incubated with premixed flex set beads coated with antibodies to macrophage inflammatory protein-1β (MIP-1β), IL-8, IL-6, IL-1β, vascular endothelial growth factor (VEGF), TNF-α and Regulated upon Activation, Normal T-cell Expressed, and Secreted protein (RANTES) in 96-well filtration plates. Premixed phycoerythrin-labeled detection reagent was added to the wells and incubated in the dark for 2 hr. The bead-protein complexes were analyzed for fluorescence intensity using a BD FACS Array Bioanalyzer. Data were analyzed using BD FCAP software (v 2.0).

**Statistical Analysis.** We calculated descriptive statistics for gestational age and AGI, as well as each urinary phthalate metabolite, BPA sulfate, and total BPA concentration. We
used separate linear regression models to estimate the change in gestational age associated with each interquartile range (IQR) increase in the metabolite (BPA sulfate, MEP, MEHHP, MEOHP, MCHP, MEHP, and MBP) concentration. We first fit unadjusted bivariate linear regression models with each of the metabolites, as well as parity, race, and other predictors of gestational age including maternal education, maternal race, gestational age group, parity, gravid, maternal employment, paternal employment, fast food consumption, maternal age, birth country (US vs. outside of US). We then adjusted the models of individual metabolites by adding parity and maternal race, those variables that were significant in univariate models (here defined as p<0.15). Finally, we re-ran the same models after stratifying by gender (n= 40 for boys and 32 for girls). We used a similar set of models with AGI as the outcome, but included gestational age (preterm birth [<37 weeks] versus term births [≥ 37 weeks]) and maternal birth country (outside US versus US) in the adjusted models.

To evaluate our assumption of a linear relationship between our continuous, independent variables (the metabolites) and the response variables, we log transformed each outcome and classified the metabolite concentrations into quartiles with the first quartile as the reference and then re-ran the regression models. We separately tested for trend by including an ordinal quartile term (0, 1, 2 or 3) in the model, instead of the indicator variables. All statistical analyses were done using SAS V.9.2 (© SAS Institute, Inc., Cary, NC).

Separate exploratory analyses regressing gestational age on the two metabolites, MEHHP and total BPA, that showed some significant association in the above models, were performed with and without individual umbilical cord blood cytokine measurements to determine if the relationship of the metabolite and gestational age was mediated by the cytokine. This was repeated individually for each cytokine. Due to the small sample size of subjects with umbilical blood specimens for cytokines (N=38) and the exploratory nature of this analysis, we performed these regressions without the other independent covariates that were used to adjust for modeling the effects of the metabolites on gestational age without cytokine data.
RESULTS

The characteristics of the 72 pregnant woman enrolled in the study are shown in Table 1. Indications for enrollment in the High-Risk Obstetric Clinic included previous preterm birth (36%), febrile illness (18%), hypertension (19%), diabetes (14%), urinary tract infection (10%), and preterm labor (6%). Forty infants were male (56%), and 32 female (44%). Of these 72 infants, 7 were preterm (32-33 weeks gestation), 14 late preterm (34-36 weeks), and 51 term (37-42 weeks). Table 2 summarizes newborn measurements and gestational age at birth. Gestational age was 37.47 +/- 2.41 weeks (mean +/- SD), with birth weights 3073.35 g +/- 754.91 g. The distribution of phthalate and BPA metabolite concentrations in maternal urine, standardized to specific gravity, is shown in Table 3. The DEHP metabolites MEHP, MEHHP, and MEOHP, as well as mEP, mCHP and mBP were present in measurable quantities, while mMP, mOP, mNP, and mDP were present at low concentrations or were not detectible. Therefore, we did not further analyze urinary mMP, mOP, mNP, or mDP. BPA and BPA sulfate were also detected in maternal urine. Table 4 shows the correlation matrix among metabolites: MEOHP correlated highly with mEHP (r=0.80) and MEHHP was moderately correlated with mEOHP (r= 0.76) and MEHP (r= 0.50). Moderate correlations were also noted for mBP with mCHP and mEHP (r=0.46 and 0.31 respectively), for BPA with mEHHP and mEOHP (r=0.34 and 0.32 respectively), and for BPA with BPA sulfate (r= 0.30).

After adjusting for parity and maternal race, each interquartile range increase in urinary MEHHP concentration was associated with a significant 4.2 day decrease in gestation (95% confidence interval = -7.9, -0.4) (Table 5). Similarly, each interquartile range increase in total BPA was associated with a significant 1.1 day decrease in gestation (95% CI = -2.0, -0.1). Although not statistically significant, we observed decreased gestation associated with each interquartile range increase in MEOHP (-0.6 days), MCHP (-0.9 days), MBP (-2.1 days) and BPA sulfate (-0.5 days), but not with MEP or MEHP (Table 5).
Next, we stratified by gender, and found that larger and more consistent reductions in gestation were associated with phthalate metabolites in male infants. Gestational effects for BPA total were nearly identical in both genders although statistically significant only for the males. We observed significant reductions in gestation associated with IQR increases in MEHHP (-5.1 days, 95% CI = -9.6, -0.6) and BPA total concentrations (-1.1 days, 95% CI = -2.1, -0.1), and non-significant reductions in all other phthalates and metabolites. Conversely, for female infants, we observed generally smaller and non-significant decreases in gestation associated with MEHHP (-1.4 days, 95% CI = -8.4, 5.7), MCHP (-0.4 days, 95% CI = -7.4, 6.7), MBP (-0.5, 95% CI = -6.2, 5.1), and BPA total (-1.6, 95% CI = -4.1, 1.0), and no such decreases in gestation associated with MECP, MEOHP, MEHP, or BPA sulfate.

For AGI, after adjusting for parity and maternal race, we found no statistically significant associations with interquartile range increase in urinary concentration any of the metabolites for all infants combined as well as for female only infants. For boys, there was one statistically significant association of MEP with AGI, but this was of borderline clinical significance (-0.2 mm, 95% CI = 0.0, -0.4) (Table 6).

Next, we evaluated whether our inference would change if we repeated our analyses after log-transforming gestational age and using indicator variables for the quartiles of phthalate/BPA metabolite in our models, instead of a continuous phthalate/BPA metabolite concentration variable. Generally, gestation decreased with increasing quartile of MEHHP (Figure 1) and BPA total (Figure 2). For boys only, gestation decreased with each increasing quartile of maternal urinary MEHHP concentration (Figure 3) and BPA total concentration (Figure 4).

MIP-1β, IL-8, IL-6, IL-1β, VEGF, TNF-α and RANTES were all detected in measurable quantities in umbilical cord blood sera (Table 7). The cytokines themselves did not appear to have any significant effect on gestational age with the exception of RANTES (beta = -0.0002, p=.05 and .06) when modeled with MEHHP and total BPA respectively. IL-1β had an
association of increasing gestational age (beta = 0.05, p=.36 and beta= 0.07, p= 0.21) when modeled with MEHHP and total BPA, respectively. However, there were no changes in the effect estimates of MEHHP or total BPA on gestational age when the regressions were done with or without the addition of the individual cytokines (Table 8).

DISCUSSION

We found that each IQR increase in maternal urinary MEHHP was associated with a 4.2 day shorter gestational age. This effect was observed primarily in males (5.1 days/21.9 ng/mL) but not in female infants. This is consistent with some previous animal studies demonstrating spontaneous abortion and preterm delivery in animals exposed to phthalates (ATSDR 2001). In humans, one recent study suggested that MEHP-positive newborns had lower mean gestational age than controls (28). In another study in an inner-city population, gestational age was shorter by 1.1 days (95% CI: 0.2-1.8 days) for each 1-logarithmic unit increase in specific gravity-adjusted MEHP concentrations, and averaged 5.0 days (95% CI: 2.1-8.0 days) less among subjects with the highest versus lowest quartile concentrations. Results were similar and statistically significant for the other DEHP metabolites (25). MEHHP is of particular interest because it is one of the most abundant of DEHP metabolites. In one recent cohort, it was found in measurable quantities in the urine of 100% of women and was associated with exposure to body lotions and bottled water (29).

Biologically plausible mechanisms for the induction of shorter gestation by phthalates include the induction of inflammation, which can trigger parturition. Several phthalate metabolites, that are present in a high proportion of urine samples from the general U.S. population, are associated with increased serum C-reactive protein and γ-glutamyltransferase, which are markers of inflammation and oxidative stress, respectively (30). Both DEHP and MEHP have been shown to bind the transcription factor PPAR-γ (31-34), potentially blocking important anti-inflammatory pathways and resulting in inflammatory signaling and possible
triggering of labor. In the current studies, we could not demonstrate a change of effect of the metabolites by adding key inflammatory cytokines into the models suggesting the absence of either a confounding or mediating effect. However, the sample size of available cytokines was small for this analysis. There was some evidence of an independent association of RANTES blood levels with a decrease in gestational age. In the range of the values seen in our 38 subjects, a change in 10,000 units of this cytokine with a beta of .0002 (change in gestational age in weeks per unit increase of RANTES) translates to a decrease in gestational age of 14 days, a very significant clinical effect. An association of high RANTES levels with shortened gestation is biologically plausible because RANTES, also known as CCL5, is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. It is produced “late” (3-5 days) after T-lymphocyte activation, consistent with a possible contribution to sustained inflammatory stimuli triggering labor. However, this should be considered a very preliminary finding since it was not an a priori hypothesis. This finding could be a type 1 error or it could be due to a combination effect of other cytokines that are correlated with RANTES. This does indicate the need for further research in this area. An Expert Panel Update from the Center for the Evaluation of Risks to Human Reproduction has confirmed that there is an urgent public health need for more data on the toxicity of phthalates in human neonates, including the potential roles of PPAR signaling and preterm labor on these outcomes (35).

We also found that total BPA was associated with shortened gestational period (by 1.1 days/180.1 ng/mL), and that this effect was also stronger in males. This is consistent with several recent studies showing that BPA and BADGE exert pro-inflammatory effects. Serum levels as low as 2.59 ± 5.23 µg/L have been associated with recurrent miscarriage (36). Recently, studies in Mexico and China have suggested dose-related effects on the risk of delivery at less than 37 weeks of gestation (24, 37) and low birth weight (38). BADGE is a competitive inhibitor of PPAR-γ signaling in vitro (39). Thus, BADGE abrogates the induction of
mRNA for the adipose triglyceride lipase by rosiglitazone (40) and blocks the activation of PPAR-γ by ω-3 PUFAs (41). In vivo, BADGE blocks the protective effects of rosiglitazone in carageenan-induced edema and pleurisy, resulting in increased histologic indicators of inflammatory injury (42). BADGE also reverses the protective effects of the PPAR-γ agonists, ciglitazone and PGJ2, in experimental allergic encephalomyelitis (43). Prenatal exposure of animals to BPA results in decreased immune tolerance to protein allergens (44), as well as reduced numbers of regulatory T cells in mice, promoting increased Th1 responses (45). BADGE has been shown to have direct cytotoxic effects, including alterations in cell morphology, adhesion, and F-actin depolymerization in cultured cells in vitro (46).

The increased susceptibility of males to the induction of prematurity by phthalates is consistent with epidemiologic data suggesting that males are more likely to deliver prematurely, in general (47). In one study, there was a 7.2% excess of males among white singleton preterm births, distributed uniformly from 20-37 weeks of gestation (48). Several plausible biologic mechanisms have been proposed for the higher incidence of preterm labor by male infants. These include the action of androgen precursors, which are involved in the production of estrogen and are elevated in male relative to female fetuses (49). Alternatively, induction of labor may be promoted by interleukin (IL)-1 in males, who have lower levels of IL-1 receptor antagonist in amniotic fluid than females (50-51).

Currently, only limited or inadequate data exist on the relationship between phthalate exposure and developmental anomalies in humans. In our investigation we did not identify any association between phthalate levels and AGI. Some studies have previously suggested that phthalates may act as developmental and reproductive toxicants in experimental animals and humans. The commonly observed anomalies include reduction in androgen-dependent tissue weights, and increased incidence of malformations of the external genitalia such as hypospadias. Males exposed prenatally to phthalates may have decreased AGD, penile volume, and scrotal size, as well as increased incidence of incomplete testicular descent (20-
Significantly, the median exposures to DEHP and other phthalate metabolites associated with reduced anogenital distance were lower than the current U.S. EPA reference doses (RfD) for these chemicals, which are based on increased relative liver weight and therefore not directly applicable to their endocrine disruption effects (21). In one recent study, exposure to phthalates was associated with shortened AGD, but frank genital anomalies were not seen (52). In that data set, MEP, MBP, and three DEHP metabolites (MEHP, MEOHP, and MEHHP) were inversely related to AGD (1). We only observed a significant decrease in AGI for MEP, but not other metabolites, in boys. The imprecision of these outcomes and their inconsistency may be partially explained by inaccurate measurements and non-differential misclassification as well as small sample size. This imprecision does not rule out clinically significant effects as some of the confidence interval boundaries are consistent with clinically important effects.

Like phthalates, elevated urinary BPA has been linked to reduced levels of gonadotrophic hormones in males (53), and BPA and its metabolites have estrogenic activity in vitro (23). These compounds also have a high affinity for the androgen receptor and exhibit marked anti-androgenic activity (22). BPA initiates rapid alterations in cell membranes via estrogen receptors at concentrations that are below those demonstrated in maternal and fetal tissues (54-55). In vivo exposure of pregnant rats to BPA results in decreased spermatogenesis and lower plasma testosterone levels in male offspring (56). BPA may also interfere with normal development of the prostate gland, increasing the susceptibility to carcinogenesis. Alterations in sexually dimorphic patterns of behavior after low doses of BPA have been noted (57-58). However, no previous studies have demonstrated developmental effects of BPA in humans (16), and in our study we also did not observe any alterations in AGD.

Our study has several limitations, most notable being the small sample size. We also examined multiple metabolites, so it is possible that some of our associations occurred by chance. Our tool for measuring AGI was crude (tape measure of length between genitalia and center of the anus), thereby increasing the chance of nondifferential outcome misclassification
resulting in a bias towards the null. We were limited to one urine sample per mother, and thus we were only able to obtain 1 narrow, point-in-time measurement of metabolites. Therefore, we were unable to assess the variability of each metabolite across the pregnancy. The one-time urine measurement does not reflect the variable and overall, cumulative exposure to these metabolites in utero. It is not known at what point(s) in gestation an infant is most potentially vulnerable to these compounds. Analyzing the other urine samples from earlier time points in the pregnancy in further studies may be helpful in addressing this question. This would help to provide a measure of the variability of exposure to these compounds in the mother’s environment, as well as a more longitudinal and nuanced characterization of the exposure to the developing fetus. Finally, the analytic technique was not sensitive enough to measure several of these phthalate metabolites at the low end of the level found in urine (mMP, mOP, mNP, and mDP).

Our study focused on high-risk pregnancies so it is possible that our results may not be directly generalizable to a more general population of pregnant women. For example, our mean gestational age and birth weight was 38.5 weeks and 3255 grams respectively, as compared to that of New Jersey with means of 37.5 weeks and 3073 grams. However, we do not feel that these differences are significant enough to consider that the associations we describe would not also be seen in the more general population. The strengths of this study are that the study group was a very diverse population of high-risk pregnancies, and our measurements of BPA and phthalates were obtained prospectively and blindly with respect to anatomic measurements. Gestational age was established by sonographic dating or date of implantation, thus providing an accurate accounting of this outcome.
REFERENCES


