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Prenatal exposure to phthalates is associated with decreased anogenital distance and penile size in male newborns

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Abstract

Reproductive effects from phthalate exposure have been documented mostly in animal studies. This study explored the association between prenatal exposure to phthalate metabolites, anogenital distance and penile measurements in male newborns in Toluca, State of Mexico. A total of 174 pregnant women provided urine samples for phthalate analysis during their last prenatal visit, and the 73 who gave birth to male infants were included in the study. The 73 male newborns were weighed and measured using standardized methods after delivery. After adjusting for creatinine and supine length at birth, significant inverse associations were observed between an index of prenatal exposure to total phthalate exposure and the distance from the anus to anterior base of the penis ($\beta = -0.191$ mm per 1 $\mu\text{g/l}$, $P = 0.037$), penile width ($\beta = -0.0414$, $P = 0.050$) and stretched length ($\beta = -0.2137$, $P = 0.034$); prenatal exposure to mono-2-ethylhexyl phthalate exposure was associated with a reduction in the stretched length of the penis ($\beta = -0.2604$, $P = 0.050$). Human exposure to phthalates is a public health concern, and the system most vulnerable to its potential effects seems to be the immature male reproductive tract.

Keywords

phthalates; anogenital distance; penile measurements; prenatal exposure

Introduction

Phthalate acid esters, commonly called phthalates, used for a variety of purposes including the widely used plasticizer di-(2-ethylhexyl) phthalate (DEHP), are components of many plastic products, such as food wraps, toys and some medical devices.¹ DEHP is loosely held with the plastic polymer and can be easily released into the environment. While exposure via house dust is extensive, at least for some phthalates (e.g. DEHP), foodstuff and, to a lesser extent, the use of oral drugs present the major uptake pathways.²

Because phthalates are found ubiquitously in the environment, humans have chronic and repetitive exposure to phthalates. Phthalates have a short life in the body; however, exposure assessment can be based on a single urine sample in epidemiological studies since exposure is relatively constant over time.^{3–5}

Experiments on animal models have demonstrated that diverse phthalate isomers cause reproductive toxicity in both prenatal and postnatal stages of development, particularly for dibutyl phthalate, di-2-ethylhexyl phthalate, benzylbutyl phthalate and di-isononyl

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Conflicts of Interest

None.

phthalate.⁶ Through their metabolites, phthalates act as endocrine disruptors during the developmental stage in male animals through an anti-androgen pathway, causing damage to the reproductive system.⁷ Some effects on development include reduction in androgen-dependent tissues, such as seminal vesicles, epididymis and prostate, and reduction in the anogenital distance (AGD).^{8–10}

Studies of phthalate exposure in humans are recent and scarce; however, results suggest that the stages of development affected by phthalates may be consistent throughout the species. AGD is an indicator sensitive to the hormonal effects of chemical tests. A study conducted by Swan *et al.* in the United States reported a reduction in AGD with higher phthalate exposure in children younger than 2 years of age.^{11,12} More recent research found that urinary mono-2-ethylhexyl phthalate (MEHP) was inversely correlated with AGD in a Japanese population.¹³

Given that phthalates have acted as endocrine disruptors during the developing stage in male animals,^{7,8,10} and in young children in the United States and Japan,^{11,13,14} we sought to evaluate the relationship between prenatal phthalate exposure and AGD measurements in a group of Mexican male newborns to evaluate the consistency of this association and added penile and anthropometric measurements in this evaluation.

Methods

Study design and population

We conducted a hospital-based cohort study among Mexican pregnant women during their last prenatal visit at the Hospital Materno Infantil of the Instituto de Seguridad Social del Estado de México y Municipios. As we aimed to assess antiandrogenic effects, only male newborns were included in this analysis.

The women in this study were all 18 years of age or older (mean age of 29.5 years), nonsmokers, in their last trimester of pregnancy, middle class, employees of the state of México with health insurance coverage and residents of the city of Toluca and surrounding areas. Only women with singleton pregnancies were included in the study, and most of the women had had three or more previous full-term pregnancies. Women with serious health problems (e.g., diabetes, hypertension, preeclampsia and renal or hepatic pathologies) were excluded from participation in the study.

The study was approved by the Investigation and Ethics Committee of the Hospital Materno Infantil. After the study was explained in detail and women signed the informed consent form, women were asked to donate a urine sample and participate in a personal interview using a baseline standardized questionnaire.

The questionnaire elicited information on the participating women's sociodemographic, reproductive and medical histories, phthalate exposure (e.g., utilization of plastics in daily life, use of cosmetics and perfumes, etc.), smoking and alcohol use and phytoestrogen consumption. All the urine samples were collected at a doctor's office during the third trimester of pregnancy. All samples were frozen at -20°C until processing. Because the urine samples were collected at different times of the day, all the samples were adjusted for creatinine during the statistical analyses. We measured four primary phthalate metabolites – mono-2-ethylhexyl phthalate (MEHP), mono-benzyl phthalate (MBzP), monoethyl phthalate (MEP) and mono-butyl phthalate (MBP). In addition, we summed up the four phthalates measured in an index called total prenatal phthalate levels.

Anthropometric and genital measurements

Newborns were weighed and measured at birth (supine length and head, thoracic and abdominal circumference), and three AGD (anoscrotal distance, anus to posterior and anterior base of penis) and two penile measurements (width and stretched length) were obtained between 24 and 48 h after birth. AGD and penile measurements were taken with a plastic Vernier caliper (Swiss Precision Calimax) from the Bel Art (Pequannock, NJ, USA) product line. To prevent skin lesions on the newborn, the edges of the calipers were removed.

All AGD measurements were taken by two trained nurses following the methodology used by Salazar-Martínez *et al.*¹⁵ in a study conducted among newborns in México. Briefly, newborns were placed in a dorsal decubitus position; one nurse flexed both of the infant's hips and exerted light pressure on the infant's thighs, and the other nurse measured the distance from the center of the anus to the junction of the smooth perineal skin and the rugated skin of the scrotum, and from the center of the anus to the posterior base of the penis and to the anterior base of the penis. Penile width, penile circumference and stretched penile length were each measured in duplicate. The first measurement was the most reliable, as penile erection and scrotal sac retraction usually occurred during consecutive measurements in response to temperature changes when infants remained uncovered for prolonged periods; thus, penile width was used in this analysis. The method proposed by Capurro *et al.*¹⁶ was used to estimate gestational age.

Analytical method for phthalate analysis

Phthalates were identified and quantified at the Laboratorio de Toxicología, Facultad de Química of the Universidad Autónoma del Estado de México in Toluca by gas chromatography–mass spectroscopy (GC–MS), using a modification of the high-performance liquid chromatography (HPLC)–tandem MS (MS/MS) method reported by Albro *et al.*¹⁷ The rationale for modifying the methodology proposed by Albro *et al.*¹⁷ was twofold: first, our laboratory did not possess an HPLC–MS/MS, and second, we wanted to develop a more accessible and inexpensive method for phthalate analysis. For the purpose of avoiding contamination during the extraction process, the modifications proposed by Blount *et al.*¹⁸ were incorporated, and a derivatization step was included to make the metabolites more volatile and conducive to GC–MS. A VARIAN gas chromatograph was used with the following conditions: temperature program: 60°C for 3 min, increased to 300°C at 10°C/min and held for 10 min; injection volume 2 µl using a split injection (split ratio 20:1); injection port temperature 280°C; and carrier gas (He) flow 1 ml/min. The mass spectrometer was operated in the negative ion mode with electron impact ionization, and full scan spectra were obtained (from 50 to 400 amu).

To determine the validity of the method, the following method characteristics were determined: linearity, limits of detection (LOD), limits of quantification (LOQ), precision and relative recovery (spiked recovery). For this purpose, standard curves were made for a mixture of MEP, MBP, MEHP and MBzP phthalate metabolites, preparing solutions with final concentrations of 3, 5, 10, 20, 30 and 50 µg/l each.

To determine the analytical precision, an analysis of the concentration of the metabolite mixture (20 µl/l) was performed in triplicate by two analysts on two different days.

The standard deviation of the measurements divided by the mean provided a relative standard deviation (RSD) that we could use to evaluate the precision of the method. The relative recovery was made adding the same concentration of a solution of deionized water, phosphate buffer with a pH of 6.8 and 0.1% of sodium chloride, extracted and quantified

using GC–MS. The RSD for extracting the tested metabolites and LODs and LOQs were determined using standard formulas.¹⁹

Metabolite extraction from urine samples

One hundred milliliters of urine collected from each study participant was used to carry out the metabolite extraction. As phthalate metabolites are excreted as conjugates of glucuronides or sulfates, it was necessary to liberate the monoesters by adding 50 µl of a solution of βglucuronidase (12 µl/ml; *Escherichia coli*) to the urine samples that had been previously adjusted to a pH of 6.8 with a 0.2 M phosphate buffer. The samples were then incubated in an oven at 37°C for 12 h. After incubation, the hydrolysates were adjusted to pH 2 using concentrated HCl then 0.1 g of NaCl and 1 ml of HPLC grade MeOH were added to facilitate the extraction of the monoesters. The phthalate metabolites were extracted via liquid–liquid extraction using 300 ml of dichloromethane (equivalent to three times the urine volume). The extract was then passed in a bed of anhydrous sodium sulfate (0.5 g) to eliminate excess water and particulates, and then it was evaporated at ambient temperature to a volume of 5 ml.

Metabolite derivatization

One milliliter of recently prepared diazomethane (from diazonium salts) and 1 ml of HPLC grade methanol were added to convert the nonvolatile phthalate monoester metabolites into volatile methylated metabolites. The extract was then evaporated to dryness at ambient temperature and resuspended with 100 µl of methanol, and then transferred to an amber vial and stored at –20°C until analyzed.

Creatinine determination

Creatinine in the urine samples was determined using the RANDOX brand colorimetric immunoassay kit, as recommended by Adibi *et al.*²⁰

Method characteristics

Table 1 includes the LODs, LOQs for each analyte and demonstrates the linearity of the analytical method throughout the calibration range. All r^2 values were >0.98 and the confidence intervals for the gradient and for the ordinate at the source complied with the recommended acceptance criteria by García *et al.*¹⁹ After combining the data from the two analysts, the RSD of the method was <2% and a standard deviation of <1%.

Statistical analyses

All analyses were performed with the statistical software Stata (version 8.0, 2003, Stata Corp., College Station, Texas). Intra- and inter-observer anthropometric and genital measurement reliability tests were conducted on all measurements by three standardized observers, and results were not statistically different. Because phthalate concentration is a left-censored independent variable owing to the limit of detection (LOD), to assess linear regression models we imputed values for women below the LOD with LOD/square root of 2.

Linear regression models were first fit to evaluate the association between all the measurements under study and mean phthalate exposure levels, adjusted for creatinine. In addition, linear regression models were also fit to evaluate the association between prenatal phthalate exposure levels and the measurements adjusted for creatinine level and supine length at birth, as the length of the newborn may influence the AGD and penile measurements.¹³

Results

The majority of the 73 women included in the analysis resided in the city of Toluca (75.4%). The mean age was 30.0 ± 5.4 years. The mean number of years of education was 12.2, which correspond to a middle-upper educational status or a technical career in México. More than half of the women were homemakers (54.4%); the next most common occupations were university professor (21.7%) and civil service occupations (19.9%; not shown).

The primary phthalate metabolite and total phthalate levels obtained during the last trimester of pregnancy among the 73 women who delivered male infants are summarized in Table 2. The exposure level was highest for MEP and lowest for MBP. However, only a few women had exposure to MBzP, MEP and MBP above the detection levels. Therefore, the statistical models only assessed the association with AGD and penile measurements with MEHP and total phthalate male newborns' AGD and penile measurements, adjusted for creatinine levels and supine length of the newborns at birth. We also assessed models with other variables not shown (i.e. consumption of foods rich in phytoestrogens, exposure to pesticides, tobacco and alcohol use) and found no statistically significant effects. Exposure to MEHP was associated with a reduction in the stretched length of the penis ($\beta = 520.2604 \text{ mm/1 } \mu\text{g/l}$, $P = 0.050$). Total phthalate prenatal exposure level was inversely associated with anus to anterior base of penis, penile width and stretched length.

Discussion

In this study, we assessed the relationship between prenatal exposure to phthalates and AGD and genital and anthropometric measurements in Mexican male newborns. Exposure levels are consistent with reports from Mexican populations.^{21,22} Our findings are consistent with two previous reports by Swan^{11,12} who found inverse association between prenatal maternal urinary DEHP metabolites, AGD and penile measurements, and a more recent report by Suzuki *et al.*¹³ However, Huang *et al.*¹⁴ did not find this association for male newborns. Our results also provide new insights into the relationship between prenatal phthalate exposure and the male reproductive system, as we also observed inverse association between prenatal phthalate exposure and penile width and length among the newborns.

In a previous study conducted on newborns in Chiapas, México,²³ the mean penile length (27.1 mm) was similar to the one observed in our study (24.8 mm). The newborns in our study had significantly shorter penises than non-Mexican newborns. Mexican in our study 24.8 mm v. Chinese 30 mm, Caucasians 34 mm, Indians 36 mm,²⁴ Argentinians 33 mm,²⁵ French 36.5 mm,²⁶ Turks 36.5 mm,²⁷ Saudis 35.5 mm²⁸ and Japanese 29mm.²⁹

Given that the newborns in our study had smaller penises than non-Mexican newborns, we further investigated whether any of the penises observed in our newborn population could be classified as micropenises. A micropenis is a very small penis with a normal configuration and is considered a sign and not a diagnosis. The etiologies of micropenis are classified as hypogonadotropic hypogonadism, primary hypogonadism, androgen insensitivity or idiopathic. According to the formula set by Lee *et al.*³⁰ a micropenis is classified as a penis smaller than the population mean minus 2.5 standard deviations (s_d). For a Mexican newborn to be classified as having a micropenis, the penile length would have to be 13.9mm ($24.8\text{mm} - 2.5 s_d$). The smallest penile length in our newborn population was 14.3 mm, and it was observed among only 1% of the newborns (not shown). In addition, in our study we used the same technique to measure penile length as the one used previously by Romano-Riquer *et al.*²³ (measured at the anterior base of the penis, where the penis meets the pubic area) among Mexican male newborns in Chiapas, México, and the findings in their study

were similar to ours (ours: 10.2 ± 0.9 mm; Chiapas (Romano-Riquer *et al.*²³: 10.5 ± 0.9 mm). These findings indicate that the small penile length observed in the two Mexican newborn populations may not be indicative of a micropenis, as micro penis is usually associated with hypothalamic disorders.³¹

It is also possible that the observed differences in penile measurements between newborns from different countries may be because of differences in measurement techniques, measurement instruments or the environments where the newborns were measured. Nevertheless, the findings from the study conducted by Swan *et al.*¹¹ among American newborns and toddlers and the findings from our study demonstrate that phthalates can affect AGD and penile measurements.

Available scientific evidence on phthalate exposure in humans, although limited, has generated great concern because of the possible adverse effects on masculine gonads from development until adulthood. This concern continues to increase as results from recent studies have associated phthalate exposure with adverse health effects in children.¹¹

As male rodent models^{8,33–36} can be applied to humans, it is plausible that the same biological mechanisms observed in rodents can act as endocrine disruptors in humans. In rodents, perineal development is determined by the androgens (dihydrotestosterone) during sexual differentiation.³³ Ema and Miyawaki⁸ demonstrated that testosterone metabolites are reduced by prenatal exposure to MBP, suggesting an antiandrogenic effect. In addition, other in vitro studies have shown that phthalates exert a negative influence on Leydig cells,³⁴ disrupting testosterone production and increasing cell proliferation. These effects potentially contribute to testicular dysgenesis syndrome, which includes a variety of conditions that involve the male reproductive system, including undescended testes, hypospadias (an abnormality of the penis in which the urethra opens on the underside), changes in the timing of puberty, testicular cancer and reduced fertility.³⁵

As previously stated, the study conducted by Swan *et al.*¹¹ demonstrated that phthalate exposure was associated with reduced AGD but not reduced penile size, as we demonstrated in our study. In contrast to the methodology used by Swan, the newborns in our study were measured within 24–48 h of birth. We also did not utilize an AGD index but rather performed a reliability analysis on each of the AGD measurements under study. In addition, we adjusted for the supine length of the newborns at birth, as AGD was associated with the length of the male infants at birth in a previous study conducted in México.¹⁵ Nonetheless, residual confounding may still exist in our study as we only obtained a single urine sample, during the third trimester of pregnancy; moreover, urine was not collected during the first trimester, the window of greatest risk for phthalate effects.

Conclusions

Human exposure to phthalates is a public health concern, and the system most vulnerable to its potential effects seems to be the immature male reproductive tract. As studies on phthalate exposure in humans are few and recent, especially among vulnerable populations, additional epidemiologic, basic and clinical research is needed to further elucidate the toxic effects of these chemicals and to improve environmental management and policy decision making. In addition, one of the goals of environmental health programs worldwide is to identify and evaluate environmental health risks among vulnerable populations, especially children, as owing to their size and developmental stage children are more susceptible than adults to the effects of environmental toxins.³⁷

This is the first study conducted in México to explore the toxic effects of phthalates in male newborns using AGD and penile measurements as markers of reproductive toxicity. Even though our findings are consistent with those observed by Swan *et al.*^{11,12} in United States and Suzuki *et al.*¹³ in Japanese infants, it is important to further investigate whether the observed effects will be maintained over time or whether mechanisms of compensation exist among populations with higher phthalate levels. To further confirm our study findings, we suggest conducting a larger cohort study using the same parameters of reproductive toxicity among male newborns whose mothers are occupationally exposed to phthalates.

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Table 1

LOD and LOQ for each metabolite under study and linearity of the analytical method

Metabolite	Concentration	RSD (%)	Relative recovery (%)	Straight line equation	r ²	LOD (µg/l)	LOQ (µg/l)
MEP	19.69 ± 0.12	0.63	98.45	$y = 1116.4x + 315.83$	0.9944	0.10	0.40
MBP	19.69 ± 0.08	0.43	98.45	$y = 8875.9x - 17367$	0.9902	0.01	0.03
MEHP	19.64 ± 0.12	0.60	98.20	$y = 5445x - 21109$	0.9893	0.07	0.20
MBzP	19.62 ± 0.09	0.47	98.15	$y = 9797.2x - 35342$	0.9869	0.01	0.03
MOP	19.85 ± 0.21	1.07	99.25	$y = 42783x - 22294$	0.9985	0.30	0.90

LOD, limits of detection; LOQ, limits of quantification; MBzP, mono-benzyl phthalate; MEP, mono-ethyl phthalate; MBP, mono-butyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MOP, mono-n-octyl phthalate.

Initial metabolite concentration = 20 µg/l; y = area under the curve obtained from the chromatogram; x = metabolite concentration in µg/l and r² = determination coefficient.

Table 2

Mean prenatal phthalate metabolite concentrations among 73 Mexican women with male newborns

Phthalate metabolites	n above detection level	Mean (μ /l)	S.D.	Range
MEHP	49	4	4.2	0.4–19.7
MBzP	10	0.56	0.15	0.39–0.74
MEP	8	7.63	12.66	0.27–26.48
MBP	9	0.65	0.49	0.25–1.61

MEHP, mono-2-ethylhexyl phthalate; MBzP, mono-benzyl phthalate; MEP, mono-ethyl phthalate; MBP, mono-butyl phthalate.

Table 3

AGD, penile and anthropometric measurements among 73 Mexican male newborns

Measurement	Mean $\pm$ S.D.	Percentile		CI
		25 th	75 th	
AGD (mm)				
Anoscrotal distance ^a	12.4 $\pm$ 2.0	11.0	14.0	11.9—12.9
Anus to posterior base of penis	42.4 $\pm$ 4.4	39.8	45.4	41.4—43.5
Anus to anterior base of penis	48.1 $\pm$ 4.3	45.5	51.5	47.2—49.2
Penile (mm)				
Width	10.2 $\pm$ 0.9	9.7	10.8	10.0—10.4
Stretched length	24.8 $\pm$ 4.4	22.3	27.8	23.9—25.9
Anthropometric				
Supine length (cm)	48.8 $\pm$ 1.85	47.9	50.1	48.4—49.3
Weight (g)	3093.0 $\pm$ 414.7	2760.0	3380.0	2996.3—3189.8
Cephalic perimeter (cm)	34.5 $\pm$ 1.4	33.6	35.2	34.1—34.8
Thoracic perimeter (cm)	33.0 $\pm$ 1.7	31.9	34.1	32.6—33.39
Abdominal perimeter (cm)	30.4 $\pm$ 2.0	29.0	31.7	30.0—30.9

AGD, anogenital distance.

^aDistance from center of anus to junction of smooth perineal skin and rugated skin of scrotum.

AGD, penile and anthropometric measurements among 73 Mexican male newborns

Table 4

Linear regression models of the association between prenatal MEHP and total phthalate exposure levels ($\mu\text{g/l}$), and AGD, and penile measurements, adjusted for creatinine^a and supine length at birth^b, among 73 Mexican male newborns

Measurements	MEHP		Total levels	
	β (1 $\mu\text{g/l}$)	P-value	β (1 $\mu\text{g/l}$)	P-value
AGD (mm)				
Anoscrotal distance ^c	−0.0049	0.943	−0.0355	0.475
Anus to posterior base of penis	−0.0733	0.063	−0.1742	0.095
Anus to anterior base of penis	−0.0252	0.840	−0.1914	0.037
Penile (mm)				
Width	−0.0383	0.183	−0.0414	0.050
Stretched length	−0.2604	0.050	−0.2136	0.034
Circumference	−0.1219	0.200	−0.1272	0.072

AGD, anogenital distance; MEHP, mono-2-ethylhexyl phthalate.

^aTo compensate for urine collection at different times of the day.

^bThe size (length) of the newborn could be related to the AGD and penile measurements of the newborns.

^cDistance from center of anus to junction of smooth perineal skin and rugated skin of scrotum.