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Effect of phthalates exposure during perinatal period on hormonal profile in Mexican males during their first months of life

Lilia Patricia Bustamante-Montes [a,*], Víctor Hugo Borja-Aburto [b], María Hernández-Valero [c], María Magdalena García-Fábila [d], Patricia Borja-Bustamante [b], Rafael González-Álvarez [a]

[a] Universidad Autónoma de Guadalajara, Decanato de Ciencias de la Salud, Guadalajara, Jalisco, Mexico
[b] Instituto Mexicano del Seguro Social (IMSS), Coordinacion de Vigilancia Epidemiologica, Ciudad de Mexico, Mexico
[c] College of Acupuncture & Oriental Medicine, Houston, TX, USA
[d] Universidad Autónoma del Estado de México, Facultad de Química, Toluca, Estado de México, Mexico

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ABSTRACT

Phthalates affect development of male reproductive system acting as an antiandrogenic agents. We sought to explore if perinatal exposure to phthalates could alter male hormone levels in humans during the first months of life. A cohort of 83 pregnant women and their male infants were studied. Five phthalate metabolites were measured in the mother's urine during the first, second, and third trimesters of pregnancy and during the first, third, and sixth months of life in the infants. Luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone and inhibin B were analyzed. Association between phthalate exposure and hormone variation was assessed using regression models for longitudinal data. Mono-butyl phthalate reduced FSH concentration (ß = –0.0012 international units [IU]/L, $p < 0.01$), mono-ethylhexyl phthalate reduced inhibin B (ß = –0.0094 pg/mL, $p = 0.02$), monoethyl phthalate reduced testosterone (ß = –0.0071 ng/L, $p = 0.07$), mono-ocytl phthalate reduced LH (ß = –0.0041 IU/L, $p = 0.13$). No effects were observed for exposure to mono-methyl phthalate. Our results are consistent with the findings in animal and human studies. Special precaution should be taken when measuring phthalate exposure in susceptible populations such as pregnant women and infants.

1. Introduction

Phthalates are plasticizers which provide flexibility to polyvinyl chloride (PVC) and are widely used worldwide. PVC is used in the manufacturing of blood bags, food packaging, electrical components, pesticides, insect repellents, perfumes, make-up, soaps, detergents, dyes, lacquers, lubricating oils and adhesives. Phthalates also are found in paper, photographic film, toys, bottles and pacifiers. Phthalates as plasticizers are not polymerized within the plastic matrix, instead they are held to the matrix by Van der Waals interactions; thus, they can become dislodged with time and use and released into the environment at which point human exposure can occur [1].

Phthalates affect development of male reproductive system acting as an antiandrogenic agents [2]. Phthalate toxicity is caused by their metabolites. The primary metabolite (monoester) is more toxic than the original compound [3]. The first study of phthalate toxicity in humans was published in 2000 [4]. The following studies showed that phthalates affect semen parameters [5], DNA damage [6], hormonal changes [7], reduction of pregnancy time [8], as well as bronchial asthma [9]. To the date, there are many reports about phthalate effects on human health, such reproductive, metabolic, gynecologic, hormonal, cardiovascular, growth, development and others [10,11].

In infants, sexual hormones levels such as testosterone, gonadotropins and inhibin B are used as biomarkers to test the phthalate reproductive toxicity and their effects in later years. Male hormones are found in all stages of reproductive life of males, from development to adulthood. In humans, the hypothalamic-hypophysis- gonads axis is active since early months of postnatal life, it allows us measure sexual hormone levels and determine if there were phthalate exposure [12,13]. Phthalates acts as endocrine disruptors. Many studies show that exposure

* Corresponding author at: Universidad Autónoma de Guadalajara, Decanato de Ciencias de la Salud, Av. Patria 1201, Lomas del Valle, 45129, Zapopan, Jalisco, Mexico.
E-mail addresses: patricia.bustamante@edu.uag.mx (L.P. Bustamante-Montes), victor.borja@imss.gob.mx (V.H. Borja-Aburto), mariangehernandez@yahoo.com (M. Hernández-Valero), mmgarciaf@uaemex.mx (M.M. García-Fábila), p.borjabustamante@gmail.com (P. Borja-Bustamante), gonzalezrafael@edu.uag.mx (R. González-Álvarez).

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during pregnancy and infancy may affect the newborn's health and development [11,14]. Thus, in this study we sought to explore if perinatal exposure to phthalates could alter male hormone levels in humans during the first months of life.

2. Materials and methods

2.1. Population and study design

Longitudinal cohort study of pregnant women and their male newborns was conducted in Toluca metropolitan area, a city in central Mexico located in west of Mexico City. Pregnant women were recruited during their first prenatal visit between the seventh and thirteenth week of pregnancy. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Instituto Nacional de Salud Pública (INSP) under de number CI-056.

Eligibility criteria were pregnant women free of any medication, diabetes or any others chronic diseases. Participants must live in Toluca area for more than 2 years and sign an informed consent. Then a baseline questionnaire was applied by personal interview to address socio demographic information, reproductive, occupational and dietary histories, especially the consumption of dairy products. The same questionnaire was applied in the second and third trimesters of pregnancy to update any information. In each visit a urine sample was obtained to measure phthalates exposure.

After delivery, the infants were appointed during their first, third and sixth month of life to measure their hormone levels and urinary concentration of phthalates. In each visit, infant's saliva and urine samples were obtained to measure testosterone, gonadotropins, and phthalates, respectively. A questionnaire was applied to elicit information on breast feeding practices and the use of oral infant products.

235 pregnant women met inclusion criteria and they were invited to participate. Fifteen women refused to participate, thus the final cohort was of 220 pregnant women. 8 of whom had spontaneous abortions and 7 were lost to follow-up due to change of address or refusal to continue in the study. Of the total births, 46 % were boys and 54 % girls. Ten of the infants were lost to follow-up due to parental refusal, death, or change of address; in two of these cases no biological samples were obtained.

2.2. Samples collection

To detect their presence across the time it was necessary perform repeated measurements in urine samples from the mothers and newborns. In the mothers, approximately 250 mL of first morning urination from the pregnant women was obtained in prewashed glass flasks that were previously analyzed and found to be free of contamination. The first urine sample was obtained between the seventh and tenth week of pregnancy to evaluate exposure as near as possible to the period at which male sexual differentiation is produced, in addition to identifying possible changes in exposure during the pregnancy. In the infants, 100 mL of urine sample were collected in phthalate-free urine culture bags.

2.3. Phthalates quantification

The primary phthalate metabolites identified were mono-methyl phthalate (MMP); mono-butyl phthalate (MBP); mono-ethyl phthalate (MEP); mono-octyl phthalate (MOP), and mono-ethylhexyl phthalate (MEHP); these corresponded to dimethyl phthalate (DMP), dibutyll phthalate (DBP), diethyl phthalate (DEP), dioctyl phthalate (DOP), and di(2-ethylhexyl) phthalate (DEHP), respectively, which are the most used phthalates as plasticizers in commercial products.

From the mothers, urine sample was collected in each trimester of pregnancy. For the male newborn, urine samples were collected at the first, third and sixth month of life. 50 μL of glucoronidase E solution from E coli were added to urine samples, then pH was adjusted at 6.8 using 0.2 M phosphate buffer. Samples were incubated at 40 °C for twelve hours, at the end of the time pH was adjusted to 2 using concentrated HCl, subsequently 0.1 g of NaCl and 1 mL of HPLC grade methanol were added. Then metabolites extraction was carried out with three portions of dichloromethane. Organic phase was passed through an anhydrous sodium sulfate bed and evaporated at room temperature to final volume of 0.5 mL. The extracted metabolites were derivate using clorometil silane and the volume was adjusted to 0.5 mL with methanol. The extracts were injected into the mass gas chromatograph and quantified with the respective calibration curves. The results were reported as μg/mL adjusted to one g of creatinine [15]. Phthalate measurements were done by triplicate in mothers and male newborns.

2.4. Sexual hormones quantification

Gonadotropins (FSH and LH) were quantified from urine, testosterone in saliva and inhibin B in blood samples. All samples were stored at −20 °C until processing.

Testosterone and gonadotropins (FSH and LH) were quantified by immunofluorometry assay (Delfia, Wallac, Finland) with detection limits of 0.03 nmol/L and 0.05 U/L, respectively. Inhibin B was quantified by enzyme-linked immunosorbent assay (ELISA) (Inhibin-B Assay, Bio-innovation, Ltd., Oxford, UK), with detection limits of 18 pg/mL. Immunoflurometry and ELISA assays were performed using the manufacturer's recommendations. FSH, LH, and testosterone assays were made by triplicate, while inhibin B assay was performed only once.

2.5. Statistical analysis

Statistical analysis included the phthalates and hormone concentrations at each point of their measurement expressed by geometric means and 95 % confidence intervals since hormone concentrations did not follow normal distribution. To evaluate associations, hormone values were transformed to logarithmic scales.

The co-variables associated with hormone levels were: (1) age, (2) levels of other hormones from the hypothalamic-hypophysis-gonad axis, and (3) creatinine present in the urine. To test the association between hormones and phthalates we used Generalized Estimating Equations (GEE) models to longitudinal data [16]. To correct for urine dilution, creatinine was included in the multivariate model as an independent variable following the recommendations by Barr 2005 [17]. All data were analyzed using the Stata Statistical Software (StataCorp 2003) [18].

3. Results

A total of 83 children were included in the study; 80 % were born vaginally, while 20 % were born via cesarean section. 38 weeks was the average of gestation time, with a standard deviation (SD) of 1.8. The American Pediatric Gross Assessment Record (APGAR) at birth was 7–10 in 95 % of the children, while < 5% obtained an APGAR score < 7. The average birth weight was 3090 g (SD, 487 g) and the mean maternal age was 24 years old (SD, 4.7 years).

Table 1 shows the quantity of the 5 primary phthalate metabolites during the pregnancy and postnatal periods. In the pregnancy, MEHP levels were the highest during the first and third pregnancy trimesters; during second trimester MEP had the highest level. While MOP showed the lowest levels at the first trimester and MMP and MBP for the second and finally MMP for the third trimester. There is not any special or obvious reason that explains this result. During the first months of life, MBP levels were the highest, followed by MEHP and MEP; however, at 6 months of age MEHP concentrations increased notably compared to the MPP levels. Finally, at 27th week MBP showed the highest value being 3.5 times higher than MEP, MEHP and MOP and 20 times higher than

Table 1
Geometric mean levels of five phthalate metabolites measured in Mexican pregnant women and their male infants by the exposure period.

Exposure period	Week*	(N)	Phthalate metabolites (μg/mL) Geometric mean (95 % CI) MMP**	MEP**	MBP**	MEHP**	MOP**
Prenatal (Pregnant women)	11	(83)	0.335 (0.222, 0.504)	0.462 (0.292, 0.730)	0.461 (0.275, 0.771)	0.552 (0.382, 0.796)	0.328 (0.209, 0.515)
	22	(78)	0.194 (0.159, 0.236)	0.608 (0.401, 0.922)	0.194 (0.159, 0.236)	0.544 (0.368, 0806)	0.501 (0.299,0.838)
	34	(78)	0.190 (0.154, 0.237)	0.650 (0.391, 1.079)	0.370 (0.222, 0.615)	0.678 (0.409, 1.124)	0.232 (0.163, 0.330)
Post-natal (Infants)	4	(77)	0.211 (0.164, 0.272)	0.439 (0.304, 0.633)	0.766 (0.431, 1.333)	0.692 (0.459, 1.044)	0.264 (0.187, 0.372)
	14	(79)	0.216 (0.156, 0.299)	0.790 (0.480, 1.30)	2.136 (1.040, 4.383)	2.522 (1.398, 4.528)	0.173 (0.140, 0.213)
	27	(79)	0.428 (0.250, 0.734)	2.303 (1.233, 4.301)	7.845 (3.402, 18.086)	2.056 (1.061, 3.983	0.219 (0.151, 0.317)

MOP = Mono-Octyl Phthalate.
Nono-ethylhexyl phthalate (MEHP) levels being higher during the three pregnancy trimesters, followed by mono-ethyl phthalate (MEP) and mono-butyl phthalate (MBP).
During the first months of life, MEHP levels were higher, followed by MBP; however, at 6 months of age MBP concentrations increased notably compared to the MEHP levels.
* Week when sample was obtained.
** MMP = Mono-Methyl Phthalate, MEP = Mono-Ethyl Phthalate, MBP = Mono-Butyl Phthalate, MEHP = Mono-Ethyl-Hexyl Phthalate.

MMP.

Table 2 shows hormone at 4th, 14th and 27th week. Testosterone and LH levels were higher at 1 month of age (4th week) than the 14th and 27th weeks. FSH increased between the second and third measurements. The mean inhibin B level obtained during the 14th week was 522.1 pg/mL.

Table 3 includes the association between phthalate and hormone levels. In general, all hormones reduced their levels, although not all coefficients were statistically significant. FSH levels were reduced by MBP (β = –0.0012, $p = 0.001$), inhibin B by the MEHP (β = –0.0094, $p = 0.02$), testosterone by MEP (β = –0.0071, $p = 0.07$), and LH by MOP (β= –0.0041, $p = 0.13$). In these cases, the coefficient was negative and the *p*-values were statistically significant for the associations of MBP and FSH, and MEHP and inhibin B. MBP reduces FSH levels. MEHP reduces inhibin B levels. No hormonal changes were observed with MMP.

Table 4 shows a comparison of urinary phthalates metabolite median levels in the NHANES and among pregnant women from New York City (NYC), Denmark, Canada, several United States (US) cities*, Boston, Mexico and Greece.

4. Discussion

There is a paucity of research on phthalate exposure in human populations and even fewer studies have evaluated the mother-child binomine in Latin America. Our study found lower phthalate concentration levels than those reported by Adibi 2003 [19] in four populations of pregnant women in New York and in Krakow, Poland; in China [20,21] and in South Korea [22]. These studies showed variations in phthalates concentrations in pregnancy woman. Such differences may be due to feeding habits given that diet is the principal source of phthalate

Table 2
Mean testosterone, FSH, LH and Inhibin ß levels in Mexican infants during the first six months of life (in weeks).

Hormones	Week	(N)	Mean std test for deviation trend (SD)	*P*-value
Testosterone (nmol/l)	4	(78)	1.81 (3.56)	
	14	(79)	0.76 (1.50)	
	27	(75)	0.38 (0.61)	<0.01
FSH* (u/L)	4	(75)	1.43 (1.93)	
	14	(80)	1.19 (1.50)	
	27	(76)	1.59 (2.34)	0.23
LH* (u/L)	4	(75)	1.59 (2.32)	
	14	(80)	1.15 (1.14)	
	27	(76)	0.69 (0.89)	<0.01
Inhibin ß (pg/mL)	14	(68)	522.1 (342.8)	

* FSH = Follicle-stimulating hormone, LH = Luteinizing hormone.

Table 3
Regression coefficients for the association between testosterone, FSH*, LH*, Inhibin β and phthalate metabolite average levels adjusted by exposure period** (weeks), creatinine and hormonal levels.

Phthalate metabolites***	Testosterone log	FSH log	LH log	Inhibin β log
MBP β (95 % CI)	0.0001 (−0.0007, 0.0010)	−0.0012 (−0.0019, −0.0005)	0.0047 (0.0002, 0.0012)	0.0002 (−0.0004, 0.0008)
P-value	0.078	0.001	0.18	0.56
MEHP β (95 % CI)	−0.0070 (−0.0320, 0.0180)	0.0067 (−0.0288, 0.0155)	0.0061 (−0.0263, 0.0141)	0.0094 (−0.0173, −0.0013)
P-value	0.58	0.55	0.55	
MEP β (95 % CI)	−0.0071 (−0.0147, 0.0005)	0.0022 (−0.0043, 0.0088)	−0.0033 (−0.0094, 0.0027)	0.0034 (−0.0032, 0.0101)
P-value	0.07	0.50	0.28	0.31
MOP β (95 % CI)	0.0033 (−0.0010, 0.0034)	0.0037 (−0.0021, 0.0096)	−0.004 (−0.0094, 0.0013)	0.0043 (−0.0007, 0.0093)
P-value	0.33	0.21	0.13	0.09
MMP (95 % CI)	−0.0048 (−0.0118, 0.0022	−0.0040 (−0.0103, 0.0023	0.0039 (−0.0018, 0.0110)	−0.0010 (−0.0059, 0.0038)
P-value	0.18	0.21	0.18	0.67

MMP = Mono-Methyl phthalate.
Cut off value of p was ≤ 0.05.
* FSH = Follicle-stimulating hormone, LH = Luteinizing hormone.
** Pre and post-natal periods.
*** MBP = Mono-Butyl phthalate, MEHP = Mono-Ethylhexyl phthalate, MEP = Mono-Ethyl phthalate, MOP = Mono-Octyl phthalate.

Table 4
Comparison of urinary phthalates metabolite median levels in the NHANES and among pregnant women from New York City (NYC), Denmark, Canada, several United States (US) cities*, Boston, Mexico and Greece.

Phthalate Metabolites **	NHANES 1988-1994	NHANES 1999-2000	NYC Adibi et al. (2003) [19]	Denmark Toft et al. (2012) [27]	NY Kobrosly et al. (2014) [28]	Canada Arbuckle et al. (2014) [29]	Several US cities** Serrano et al. (2014) [30]	Boston Braun et al. (2014) [31]	Canada Fisher et al. (2015) [32]	Mexico Bustamante-Montes et al. (2013) [15]	Crete, Greece Katsikantami et al. (2020) [11]
MBP***	41.0	26.0	42.6	225.1	13.61	–	–	77.8	23	11.23	28.1
MEHP***	2.7	3.2	4.60	16.2	3.65	2.24	2.54	–	2.26	3.48	6.1
MEP***	305.0	164	236.0	405.8	81.01	32.02	35.63	309.5	42.5	34.88	–
MBzP***	21.2	17.0	12.1	20.3	6.59	5.20	4.28	–	10.7	–	46.7

* Minneapolis, Rochester, Seattle and San Francisco.
** μg/L, μg/g of creatinine.
*** MBP = Mono-Butyl Phthalate, MEHP = Mono-Ethyl-Hexyl Phthalate, MEP = Mono-Ethyl Phthalate, MBzP = Mono-Benzyl Phthalate.

exposure. Our study population came from lower-middle socio economic status, with low feeding habits of dairy, meat, and other packaged products. Phthalate levels observed in our study were lower than those reported by national health surveys in the U.S. in children among 4–6 years old [23] and those reported in our previous study in newborns hospitalized in Toluca, Mexico [24]. Our study shows two finding: first, phthalate concentration in newborns is higher than in the mothers; second, in newborns phthalate concentration was increasing as new born grew up. These findings could be explained because newborn phthalate concentration still increasing as newborn put through to exposition phthalate sources such as feeding bottle and cleanliness products.

The hormonal levels observed in this study were like those reported by Andersson 1998 [25], who reported high hormonal levels at the 1st month of age, and then notably decreasing by the 6th month. Testosterone measures in our study were composed of free testosterone; thus, our levels were less than the total testosterone levels measured in the Andersson study.

Animal studies have shown that mono-(2-ethylhexyl) phthalate affects Sertoli cells [26]. To evaluate this finding, we quantified inhibin B levels, a specific biomarker of healthy of these cells. Our result agreed with the animal studies performed by Ferguson 2014 [12], who studied Mexican children between 8 and 14 years old. They found a reduction levels in sexual hormones due to pre-and postnatal phthalate exposure. They showed that prenatal phthalates exposure was associated with decreased levels of dehydroepiandrosterone sulfate (DHEAS), inhibin B, total and free testosterone, and increased levels of sex hormone binding globulin (SHBG).

Duty 2005 [7], reported an association between phthalate exposure and the sexual hormone levels in adult males. Monobutyl phthalate increased inhibin B levels and monobenzyl phthalate reduced FSH levels. We did not evaluate the effect of monobenzyl phthalate on the hormonal profiles. We cannot discard that phthalate susceptibility is different in children and adults. Our findings were like Duty and Ferguson ones, in which all the phthalate metabolites reduced the hormonal levels [7,12]. Our findings suggest that phthalate exposure during prenatal period may affect the hormonal levels of male infants. Nevertheless, the significance of our observations regarding human health requires further investigation due to the uncertainty of whether the fetus and the infant can regulate lesser changes by means of compensation mechanisms in the endocrine environment.

As in all observational studies, interpretation of the results should bear in mind the possibility of selection and/or information biases. Nonetheless, our longitudinal study design and repeated exposure evaluations allowed us to reduce these possible biases. Selection bias was reduced by obtaining sociodemographic information on all the women who refused to participate in the study and by analyzing the samples of the women who after enrollment decided to withdraw from the study. No major differences between the two groups of women were observed. The women who refused to continue in the study were followed three times and documentation obtained on the reason for their refusal. The major reason given by the women had to do with the acquisition of a blood sample from their infants, usually the extra blood sample needed for the measurement of inhibin B; hence, our lower number of samples for inhibin B determination. Whenever a mother was not able to go to the hospital for their appointment; we either provided the transportation to the hospital, or a member of the research team conducted a home visit to collect the urine samples from the infant and conduct the personal interviews with the mother.

Phthalates effects are still under investigation, too much effects are found on human health; however, some results are controversial, so more studies are needed, particularly in the toxicokinetics in susceptible populations such as pregnant women and their products.

Author contributions

LPBM Funding Acquisition; VHBA, MHV, MMGF and PBB Methodology; RGA Data Curation.

Declaration of Competing Interest

The authors report no declarations of interest.

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