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Impact of gestational and lactational phthalate exposure on hypothalamic content of amino acid neurotransmitters and FSH secretion in peripubertal male rats

Silvia Carbone [a,b], Berta Szwarcfarb [b], Osvaldo Ponzo [a,*], Roxana Reynoso [a], Nancy Cardoso [a], Laura Deguiz [a], Jaime A. Moguilevsky [a,c], Pablo Scacchi [a,b]

[a] Departamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 7° P (C1121 CABG), Buenos Aires, Argentina
[b] CONICET, Buenos Aires, Argentina
[c] Facultad de Postgrado, Universidad Favaloro, Buenos Aires, Argentina

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ABSTRACT

This study investigated the effect of the pre- and perinatal exposure to di-(2-ethylhexyl) phthalate (DEHP) on the neuroendocrine parameters that regulate reproduction in peripubertal male rats. DEHP at dose of 3 and 30 mg/kg bw/day was administered orally to female rat since pregnancy onset until weaning. The male litters were sacrificed at 30 days of age to determine gonadotropin serum level and the hypothalamic contents of the amino acids aspartate and gamma-aminobutyric acid. No changes in gonadotropin, aspartate and gamma-aminobutyric acid levels were detected at the low dose. DEHP 30 mg/kg bw/day reduced testis weight and serum FSH, in correlation with a significant increase in the inhibitory GABAergic tone and a reduction in the stimulatory effect of aspartate on gonadotropin level. This study provides unknown data regarding changes in the hypothalamic contents of the amino acid neurotransmitters, which are involved in the neuroendocrine regulation of reproductive axis, in peripubertal male rat offspring from dams exposed to DEHP during gestational and lactational periods. This could be related with the gonadotropin modifications also here described.

1. Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is the most widely used phthalate to convey flexibility and transparency to numerous plastic products made of polyvinyl chloride (PVC) (Latini et al., 2003; NTP-CERHR, 2006). Humans are daily exposed to this chemical through ingestion, inhalation and by dermal contact (Moore et al., 2001; NTP-CERHR, 2000). DEHP is not covalently bound to the polymer and therefore it can leach from plastic products into foods, beverages or directly into body fluids (Moore et al., 2001; Lovekamp-Swan and Davis, 2003). Moreover, also occupational and medical exposure (e.g., tubing, catheters and intravenous delivery sets) increase body burden levels (Latini et al., 2003; Doull et al., 1999; Calafat et al., 2004; Faouzi et al., 1999; Calafat and McKee, 2006; Koch et al., 2006), reaching much higher concentrations in this population.

Pre- and postnatal oral exposure to DEHP may be associated in animals with male reproductive development and function alteration. DEHP in rats is rapidly hydrolyzed in the gut to mono-(2-ethylhexyl) phthalate (MEHP), which pass into breast milk and cross the placental barrier (Latini et al., 2003; Stroheker et al., 2005). MEHP interact with fetal sexual steroids and produce testicular atrophy (Albro, 1987; Parks et al., 2000; Borch et al., 2005; Andrade et al., 2006), alteration in Leydig cell development and reduce serum testosterone levels in rats (Akingbemi et al., 2001). In uterus DEHP exposure produces abnormalities in androgen-dependent processes (e.g., undescended testis, retained nipples), malformations (e.g., ventral prostate, seminal vesicle, gubernacular cord and the epididymis) and sexual behavior alterations in male rat offspring (Andrade et al., 2006; Gray et al., 2000; Dalsenter et al., 2006). These alterations could be directly associated with epidemiological evidence indicating that boys born from women exposed to phthalates during pregnancy have an increased incidence of inborn genital malformations and spermatogenic dysfunction (Hu et al., 2009; Ge et al., 2007; Main et al., 2006).

It has been proposed that DEHP acts in an anti-androgenic manner, which appears to result from an androgen receptor-independent mechanism of anti-androgenicity (Latini et al., 2003; Parks et al., 2000; Gray et al., 2000; Akingbemi et al., 2004; Foster et al., 2001; Bonefeld-Jorgensen et al., 2001). Others mechanisms as reduction in the expression of steroidogenesis related factors and in nuclear receptors that regulate cholesterol transport could explain the suppressive effect of DEHP on testosterone levels (Howdeshell et al., 2007; Borch et al., 2006; Wilson et al., 2008).

* Corresponding author. Tel.: +54 011 5950 9500x2147.
E-mail address: oponzo@fmed.uba.ar (O. Ponzo).

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The hypothalamic amino acid system appears to play an important role in the neurotransmission pathway that regulates gonadotropin releasing hormone (GnRH) neurons and gonadotropin secretion during sexual development in rats (Moguilevsky and Wutke, 2001). The excitatory amino acid aspartate (ASP) stimulates GnRH release (Brann and Mahesh, 1994). Previously we have demonstrated that *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate receptors of the excitatory amino acids system stimulate the release of Gn-RH from hypothalamic fragments in vitro and also increase serum gonadotropin levels during maturation in male rats (Carbone et al., 1996). Conversely, gamma-aminobutyric acid (GABA) has an important role in the gonadotropin inhibitory control in adult and prepubertal male rats (Feleder et al., 1996). Testosterone exerts a negative feed back effect on gonadotropin release in male rats (Ojeda and Urbanski, 1994). It is also demonstrated that the regulatory effects of amino acid neurotransmitters system on GnRH neurons are modulated by sexual hormones acting on different receptor-subtypes of these neurotransmitters in the anterior preoptic and medial basal areas (APOA-MBH) of the hypothalamus (Carbone et al., 1996; Szwarfarb et al., 1994).

Taking into account that the amino acid neurotransmitters are involved in the neuroendocrine regulation of the reproductive function during sexual maturation, this work was designed to study the impact of DEHP on gonadotropin levels and on the hypothalamic content of the amino acid neurotransmitters ASP and GABA, in 30-day-old immature male rats offspring from DEHP-exposed dams during gestational and nursing periods. An oral route of DEHP administration was chosen for this study to mimic the most likely route of exposure to the compound in humans and wildlife.

2. Materials and methods

2.1. Animals and drug

Wistar rats used for this work were provided by the Department of Physiology, School of Medicine, University of Buenos Aires. They were allowed at minimum 7-day acclimatization period and observed for signs of illness before experimental procedures took place. Animals were raised under light (lights on from 7 am to 7 pm), temperature (22–24 °C) and humidity controlled conditions. Rats were fed with balanced food and water ad libitum until time of sacrifice and were weighed daily. Adequate measures were taken to minimize pain or discomfort in accordance with protocols of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Approval to conduct the study was granted by the Animal Care and Ethics Committee of the University of Buenos Aires.

DEHP (99% pure, Cat D 20,115-4, Aldrich Chemical Company, Inc. Milwaukee, WI, USA) were used.

2.2. Verification of DEHP dose

The doses of exposure to dams were 3 and 30 mg/kg bw/day of DEHP in drinking water. The doses and the administration pathway were chosen based on those previously used by Arcadi et al. (1998). Also, a preliminary study was undertaken to confirm that the selected experimental doses of DEHP were not overtly toxic. For this purpose, four pregnant Wistar rats were exposed orally to DEHP at the low and high doses (two rats per group) during gestational and lactational periods. Two solutions of DEHP at the concentrations of 32.5 and 325 μl/l were made up fresh daily by sonicating for 30 min that ensure a permanent and homogenized solution. Each dam was weighed daily and the liquid ingested was measured to adjust the volume of DEHP solution that it was necessary to add in the corresponding glass bottle to reach the dose chosen. The assessments assume that all the DEHP solutions lost from the bottle were consumed by the animal. They do not account for possible leakage or evaporation of the solution or for potential loss of DEHP activity during the 24-h period. According to the study of Rubin et al. for bisphenol A exposure in drinking water, the level of DEHP affecting the foetuses during gestation or that was ingested postnatally by the offspring during the period of lactation, was not estimated in this work (Rubin et al., 2001). The doses of DEHP 3 and 30 mg/kg bw/day were used for the main part of the study as no overt signs of toxicity. These doses were less and greater than 3.7 and 14 mg/kg bw/day, respectively, which represent the range of the no-observed-adverse-effect level (NOAEL) determined by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR 2006) for testis/developmental effects of DEHP by oral route in rodents (NTP-CERHR, 2006).

2.3. Experimental design

Ten Female young adult rats (with weights from 240 to 250 g on the day after mating) received DEHP or vehicle during pregnancy and lactation. For that purpose, each female was put to mate with a male in individual metallic cages for 4 days (one estrous cycle). The animals were daily examined for mucous plug. The presence of copulatory plugs in the cage was taken as evidence of mating and designated as first day of gestation (GD1). At that time each mated rat was separated from the male. Pregnant rats ($n = 9$) were randomly assigned (3 per group) to one of the three experimental groups (Control; DEHP 3 mg/kg bw/day and DEHP 30 mg/kg bw/day). The control group received water without DEHP added. Dams exposed to DEHP with dosage of 3 or 30 mg/kg bw/day in drinking water every day from GD1 to weaning. During the treatment each dam was weighed and DEHP solution consumption was measured to determine the dosage to be given. The number of male litters was not altered by maternal DEHP treatment (4–5 males per dam in DEHP and control groups).

On 21th perinatal day pups of the three groups were removed from their mothers' cage and then housed by sex and treatment. The male litters that were born from each of the three groups of pregnant rats were assigned randomly to sacrifice at 30 days of age with a body weight from 70 to 90 g. Males with undescended testes were not used for hormone and amino acids assay. Ten males per group were used.

2.4. Tissue collection

When the target age was reached the male animals were weighed and sacrificed by decapitation at 16.00–17.00 h. Blood was collected from the trunks and the samples centrifuged for 10 min at 2500 rpm, the serum separated and stored at −20 °C until gonadotropin determination. Testes were also dissected and weighed immediately after sacrifice.

Brains were rapidly removed and hypothalami dissected out with a single razor blade and weighed. Hypothalamic samples containing the anterior preoptic and medial basal areas (APOA-MBH) were dissected with the help of a stereomicroscope. The hypothalamic samples were obtained at a depth of 3–4 mm and were bordered laterally by the hypothalamic sulci; rostrally 3 mm anterior to the optic chiasma and caudally by the mammillary bodies. The samples were less than 2 mm thick and no significant differences in weight were observed.

2.5. Hypothalamic homogenization and amino acid measurement

Each APOA-MBH was weighed and homogenized in $HClO_4$ acid 0.6 N, glass Potter homogenizer refrigerated with ice. The