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In utero exposure to the endocrine disruptor di(2-ethylhexyl) phthalate targets ovarian theca cells and steroidogenesis in the adult female rat

Deborah Meltzer [a,b,1], Daniel B. Martinez–Arguelles [a,c,1], Enrico Campioli [a,c], Sunghoon Lee [a,b], Vassilios Papadopoulos [a,b,c,d,*]

[a] *Research Institute of the McGill University Health Centre, Montreal, Quebec H3G 1A4, Canada*
[b] *Department of Biochemistry, McGill University, Montreal, Quebec H3G 1A4, Canada*
[c] *Departments of Medicine, McGill University, Montreal, Quebec H3G 1A4, Canada*
[d] *Department of Pharmacology & Therapeutics, McGill University, Montreal, Quebec H3G 1A4, Canada*

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP) is an endocrine disruptor used in industry as an additive to polyvinyl chloride-based products. Pregnant dams were gavaged with oil, 1, 20, 50, or 300 mg of DEHP/kg/day from gestational day 14 until birth in order to characterize the effects of DEHP in the adult female offspring. *In utero* exposure to DEHP resulted in reduced estrogen levels at proestrus. Theca cell layer thickness was decreased starting at 50 mg DEHP/kg/day dose. Follicle-stimulating hormone levels were significantly increased at proestrus and estrus. F1 reproduction using a known breeder was not affected. F3 generation showed a decreased pregnancy rate and weight, and increased litter size in the animals exposed to 20 mg DEHP/kg/day. The data presented herein suggest that *in utero* exposure to DEHP targets the theca cell layer and decreases the estrus cycle steroid surge, but despite these effects, does not cause infertility.
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1. Introduction

Di(2-ethylhexyl) phthalate (DEHP[1]) is a plasticizer commonly introduced to polyvinyl chloride (PVC) products to increase their flexibility and transparency [1]. DEHP is ubiquitously found in the environment because of its large-scale use in industrial, consumer, and medical products [2]. Exposure to DEHP occurs mainly through oral ingestion [3], dermal contact [4], and inhalation [5]. Once ingested, lipases generate the active metabolite, mono-2-ethylhexyl phthalate (MEHP[2]), which is ten times more potent than the parent compound [6,7]. DEHP and its metabolites were found in several bodily fluids [8] including amniotic fluid [9], umbilical cord blood [10], and breast milk [11]. In addition, baby formulas were found to be contaminated with phthalates [12,13], raising concerns for fetal and neonatal development. Human exposure to DEHP is in the range of 1.7–52.1 μg/kg/day [14,15], and it has been proposed that newborns and infants are at greater risk of exposure due to their low surface area and increased contact with DEHP-contaminated products [1,16]. DEHP has a short half-life [17] and its metabolites can be readily detected in urine [18–20]. Urine levels of DEHP and its metabolites reflect recent contact and are frequently used to estimate phthalate exposure [21,22].

DEHP is an endocrine disruptor and reports correlating DEHP levels with negative endocrine effects in females are increasing [23]. In humans, studies have shown an association between high levels of DEHP and endometriosis [24], decreased gestational times [25], and increased rate of early miscarriages [26]. DEHP was also reported to be associated with premature thelarche in Puerto Rican girls [27]. In addition, occupational exposure to DEHP has been associated with hypoestrogenism and anovulation in middle-aged women [28]. Exposure to DEHP or MEHP has been found to target steroidogenesis, follicle development, and has resulted in decreased fertility [29]. Davis et al. [30,31], initially showed that acute exposure to high doses of DEHP (2 g/kg/day) targeted the ovary, resulting in decreased estradiol (E2) levels, the absence of the LH surge, anovulation, and a prolonged estrus cycle. Effects in estrogen-dependent tissues were reported in rats that were

* Corresponding author at: Research Institute of the McGill University Health Centre, Montreal General Hospital, 1650 Cedar Avenue, Room C10-148, Montréal, Québec H3G 1A4, Canada. Tel.: +1 514 934 1934x44580; fax: +1 514 934 8439.
E-mail address: vassilios.papadopoulos@mcgill.ca (V. Papadopoulos).
[1] These authors equally contributed.

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exposed to MEHP where there was delayed estrus, the shortening of reproductive life, and mammary hyperplasia [32]. In addition, *in utero* and lactational exposures with high doses of DEHP were shown to delay puberty in the female offspring [33]. *In vitro* models mainly focused on ovarian follicle cultures because the granulosa and thecal cell layers are retained. Using these models, studies have shown that acute exposure to MEHP targets follicular development and induces apoptosis of granulosa cells [34,35].

Until now, most of the research has focused on acute or prolonged exposures to DEHP, and little is known regarding the long-term effects of *in utero* exposure to DEHP in the female offspring. The various dose regimens and time points used in DEHP studies make the data difficult to compare but, in general, earlier exposures are associated with long-lasting effects such as epigenetic modifications and post-pubertal serum steroid level decreases [23]. We characterized the effects of DEHP in the testes and adrenal glands of male offspring exposed to DEHP from gestational day (GD) 14 until birth [36–40]. We reported that DEHP exposure during early development results in reduced testosterone and aldosterone levels mediated by a mechanism independent of the classic steroidogenic pathway [36,37,39]. Moreover, we reported that 17β-estradiol (E2) levels were decreased in the female offspring exposed to 300 mg of DEHP/kg/day at PND60 [37].

Herein, we used a window of treatment known to affect steroidogenesis in the male offspring to characterize the post-pubertal outcomes of *in utero* exposure to DEHP on steroidogenesis and reproductive function in the female offspring.

2. Methods

2.1. DEHP treatment and animal care

A first round of timed pregnant Sprague Dawley rats ($n = 4$) were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and they were gavaged daily with corn oil (vehicle) or with 1, 20, 50, or 300 mg of DEHP/kg/day (Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) from GD14 until postnatal day 0. The gavage method was selected to accurately dose the pregnant dams, which were weighed every 2 days and the doses were adjusted accordingly. Litters were reduced at PND3 to 6 pups, 2 male and 4 female per dam. Offspring of all treatment groups were euthanized between PND60 and PND68. Female offspring were staged using the crystal violet staining method of vaginal lavage [41] and euthanized between 1:00 PM and 3:00 PM. Tissue was collected at proestrus, estrus, metestrus, and diestrus. Blood was collected for quantification of estradiol, progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Ovaries were snap-frozen in liquid nitrogen and kept for gene and protein quantification or fixed in 4% formaldehyde for morphological analysis. We performed a second round of treatment with time pregnant dams ($n = 8$) gavaged with corn oil, 20, or 300 mg DEHP/kg/day for reproductive studies and tissue collection at proestrus. The estradiol profile at proestrus between the first and second round in control and treated animals was the same. Blood was collected and the levels of inhibin A, inhibin B, and activin A were measured. Pituitary and hypothalamus were collected for quantification of genes modulating FSH or LH synthesis, and ovaries were collected in optimal cutting medium (OCT) for the staining of lipid droplets with Oil Red O. A third round was carried out with dams ($n = 8$) gavaged with corn oil, 20, or 300 mg DEHP/kg/day for transgenerational studies. Male offspring from either of the first two rounds was not collected in this study. We refer the reader to our previous work on the effect of *in utero* exposure to DEHP in the adult male offspring [36,37,39,42]. Of note, no animal toxicity was observed at birth or in the adult females. Animals were handled according to the protocols approved by the McGill University Animal Care and Use Committees.

The doses of DEHP used in our study correspond to human-equivalent doses. This is based on a formula that suggestively converts substance exposure across species by taking into account many relevant features, including body surface area and metabolism, which are thought to be more reliable conversion factors when compared to body mass [43]. Based on our calculations, human exposure corresponds to rat exposures of 0.18–62.5 mg of DEHP/kg/day. Our methods comprise DEHP doses ranging from 1 to 300 mg of DEHP/kg/day; thus, the effects reported herein fall within the window of environmentally relevant doses for humans.

2.2. Serum measurements

Blood was collected by percutaneous cardiac puncture and the serum was separated and stored until further use. Enzyme-linked immunosorbent assays (ELISAs) from USCN Life Science Inc. (Wuhan, Hubei 430056, PRC) were used to quantify circulating levels of E2 (Cat# E90461Ge), LH (Cat# E90441Ra), progesterone (P4; Cat# E90459Ge), FSH (Cat# E90830Ra), inhibin B (Cat# CEA760Ra), inhibin A (Cat# SEA395Ra), and activin A (Cat# CEA001Ra).

2.3. RNA extraction

Tissue extraction to measure gene expression using quantitative polymerase chain reaction (Q-PCR) was performed, as previously described [37]. Briefly, total ribonucleic acid (RNA) was extracted from snap-frozen ovaries and livers using an RNAeasy kit (Qiagen, Limburg, Netherlands), which included an in-column DNase step, according to the manufacturer's instructions. QuantiTect Reverse Transcription kit (Qiagen) was used to convert 800 ng of total RNA into complementary deoxyribonucleic acid (cDNA) according to the manufacturer's instructions.

2.4. Quantitative real-time PCR analysis

Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as an endogenous control to normalize the gene targets obtained from one female offspring from each of three to four litters per treatment, with each sample processed in duplicate. Multiplex Q-PCR mix consisted of 10 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 2 μl cDNA, 0.7 μl VIC-labeled *Gapdh* and 1 μl FAM-labeled gene target TaqMan probes in a final volume of 20 μl. Supplemental Table T1 contains a list of the TaqMan probes used with the TaqMan gene expression master mix (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). The delta Ct method was used to express the results relative to the reference gene. Q-PCR measurements were carried out in LightCycler 480 (Roche Diagnostics; Hoffman-La Roche Ltd., Basel, Switzerland).

2.5. Morphometry, histology, hematoxylin and eosin, and Oil Red O staining

Ovaries fixed in 4% paraformaldehyde were embedded in paraffin and subsequently sectioned (4 μm). Slides were stained with hematoxylin and eosin. Two sections per ovary spaced 700 microns apart were assessed with two antral follicles per section, which were selected to measure theca and granulosa cell layer thickness in three different areas.

Staining of lipid droplets was carried out by Cytochem (Montreal, QC, Canada) using Oil Red O staining. Tissues embedded in cold OCT (Tissue-Tek®; Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) were frozen in isopentane at −40 °C and cut into 8 μm sections with a Hacker/Bright OTF cryostat, and

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