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Short term exposure to di-n-butyl phthalate (DBP) disrupts ovarian function in young CD-1 mice

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ABSTRACT

Di-*n*-butyl phthalate (DBP) is present in many beauty and medical products. Human exposure estimates range from 0.007–0.01 mg/kg/day in the general population and up to 0.233 mg/kg/day in patients taking DBP-coated medications. Levels of phthalates tend to be higher in women, thus, evaluating ovarian effects of DBP exposure is of great importance. Mice were given corn oil (vehicle) or DBP at 0.01, 0.1, and 1000 mg/kg/day (high dose) for 10 days to test whether DBP causes ovarian toxicity. Estrous cyclicity, steroidogenesis, ovarian morphology, and apoptosis and steroidogenesis gene expression were evaluated. DBP exposure decreased serum E_2 at all doses, while 0.1DBP increased FSH, decreased antral follicle numbers, and increased mRNA encoding pro-apoptotic genes (*Bax*, *Bad*, *Bid*). Interestingly, mRNAs encoding the steroidogenic enzymes *Hsd17b1*, *Cyp17a1* and *Cyp19a1* were increased in all DBP-treated groups. These novel findings show that DBP can disrupt ovarian function in mice at doses relevant to humans.

1. Introduction

Di-*n*-butyl phthalate (DBP) is a phthalate ester produced by the reaction of *n*-butanol with phthalic anhydride [1]. DBP is an environmental endocrine disruptor of interest because it is present in many products including latex adhesives, cellulose acetate plastics, dyes, personal care products, and in the coating of some oral medications [1–3]. DBP may not only be released to the environment during its production and incorporation into products, but it may also be released from products as they are used and disposed [1,4].

Humans are widely exposed to DBP as evidenced by the presence of its metabolites in spot urine samples from subjects in the National Health and Nutrition Examination Survey (NHANES; [2]). According to exposure estimates the largest source of DBP exposure to the general population is food and has been estimated to range between 7 and 10 μg/kg/day [4,5]. Notably, patients taking medications coated with DBP and workers exposed occupationally have exposure estimates that exceed those in the general population. Specifically, it is estimated that patients taking medications coated with DBP are exposed to 1–233 μg/kg/day and individuals are exposed occupationally to 0.1–76 μg/kg/day [3,6]. Interestingly, human studies have also pointed out that among all age groups, women of reproductive age tend to have higher urinary levels of phthalate metabolites than older women or than men [2].

The reproductive and developmental toxicities of DBP have been reported previously by expert panels [4,7]. Most studies previously reviewed were done in rats, used mostly high doses, and built on the original observation that DBP produced testicular atrophy [4]. Most of these high-dose chronic exposure studies concluded reproductive toxicity in males only. Although few, some reports on the reproductive toxicity of DBP have been recently reviewed by Kay et al. [8] and include increased mid-term abortions in treated rats (500 mg/kg/day), reproductive tract malformations (250 and 500 mg/kg/day; [7]), delayed vaginal opening and onset of estrous cyclicity [9], and decreased lordosis quotient [10] in the female offspring from rats treated during pregnancy. DBP treatment decreased ovarian (1250 and 1500 mg/kg/day) and uterine (750–1500 mg/kg/day) weight in pseudopregnant rats [11] and in immature female rats [12]. Furthermore, no studies on short-term and/or low dose exposures to DBP in non-pregnant animals have focused on ovarian function.

It is important to understand how exposure to DBP could directly affect the ovary because it is critical for reproductive function in females. Ovarian follicles are the functional units of the

Abbreviations: DBP, di-n-butyl phthalate; E_2, 17β-estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; CL, corpus luteum/corpora lutea; HPO, hypothalamic–pituitary–ovarian axis; MEHP, mono-2-ethylhexyl phthalate; PPAR, peroxisome proliferator-activated receptor; AHR, aryl hydrocarbon receptor.

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ovary and contain the oocyte (egg) for ovulation. Ovarian follicles exist in various stages of development including primordial, primary, secondary, and antral. The most mature follicles, antral follicles, are capable of ovulation and production of 17β-estradiol (E_2) in cycling animals [13,14]. Toxic damage to ovarian follicles may result in blocked ovulation and estrogen deficiency, which in turn may lead to infertility [15]. Also, estrogen deficiency may increase a woman's risk for developing disorders such as osteoporosis, cardiovascular disease, and depression [16–18]. A previous study identified the ovary as a potential target for DBP. Specifically, DBP was shown to inhibit the growth of mouse antral follicles in vitro by altering expression of cell cycle and apoptosis genes, and causing cell cycle arrest and follicular death [19]. However, an in vivo study has not been conducted in mice. Thus, to begin eliminating these gaps in our understanding about DBP, we designed the present work to test whether a short-term exposure to low levels of DBP causes ovarian toxicity in non-pregnant mice. We accomplished this objective by evaluating the effect of a 10-day exposure to DBP on estrous cyclicity, steroidogenesis, folliculogenesis, and on expression of steroidogenesis and apoptosis-related genes.

2. Materials and methods

2.1. Animals

Female CD-1 mice (28 days old) were purchased from Charles River Laboratories (Charles River, CA). Animals were housed four mice per cage at the University of Illinois College of Veterinary Medicine Central Animal Facility. Animals were given food and water ad libitum and subjected to 12L:12D cycles with temperature set at 22 ± 1 °C. All animals were allowed to acclimate to the animal facilities for 48 h before starting the experiments. All experiments and methods involving animals were approved by the University of Illinois Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Experimental Animals [20]. Animals were euthanized by carbon dioxide (CO_2) inhalation followed by cervical dislocation and exsanguination by cardiac puncture.

2.2. DBP dosing

On postnatal day 35, animals (n = 8) were randomly assigned to receive tocopherol-stripped corn oil (vehicle; MP Biomedicals, Solon, OH) or dibutyl phthalate (dissolved in vehicle, 99.6% purity, Sigma–Aldrich, St. Louis, MO) at 0.01, 0.1, and 1000 mg/kg/day. Animals were weighed and dosed daily for 10 consecutive days. All doses were administered orally by placing a pipette tip containing the dosing solution into the mouth past the incisors and into the cheek pouch. Doses were selected to approximate the tolerable daily intake (0.01 mg/kg/day; European Food Safety Agency) and the oral reference dose for DBP (0.3 mg/kg/day; [21]), as well as, a high dose (1000 mg/kg/day) to model toxicity at higher concentrations. DBP dosing did not cause overall toxicity as determined by the lack of differences in body and main organ weights between vehicle- and DBP-treated mice. A slight decrease in liver/body weight ratio in the absence of body weight change was observed in animals treated with DBP at 0.01 mg/kg/day (see Supplementary Table 1). Animals showed no other signs or symptoms indicative of overall toxicity. Most animals (all groups) were in the stage of diestrus at euthanasia, thus, only tissues from mice in diestrus were used for subsequent hormone and gene expression analyses.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2015.02.012.

2.3. Estrous cyclicity

Estrous cyclicity was monitored by daily vaginal cytology starting on postnatal day 30 and throughout the study. Vaginal cytology was assessed as previously reviewed [22], with minor modifications. Briefly, animals were restrained gently and 20 μL of sterile-filtered PBS was used to perform a vaginal washing. Vaginal washings were placed on microscope slides and evaluated unstained under an inverted microscope without knowledge of treatment. Percentage of days in proestrus/estrus and metestrus/diestrus was determined by dividing the total number of days spent in each stage by the total number of days in the study and multiplying that number by 100 [23,24].

2.4. Hormone assays

Blood was collected at euthanasia and allowed to clot before centrifugation at 14,000 rpm for 15 min to obtain serum. Serum samples were shipped to The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core and levels of circulating 17β-estradiol (E_2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined without knowledge of treatment.

2.5. Follicle and corpora lutea counts

Following euthanasia, one ovary per animal (n = 7–8 ovaries per treatment) was fixed and processed for histological classification and enumeration of ovarian follicles and corpora lutea. Briefly, ovaries were fixed in 4% formalin (overnight at 4 °C), transferred to 70% ethanol, and embedded in paraffin. Paraffin-embedded ovaries were serially sectioned at 5 μm thickness and mounted in glass slides and processed for hematoxylin and eosin staining. Oocyte-containing follicles with visible nuclear material and corpora lutea were counted on every 20th section without knowledge of treatment by two experienced individuals using criteria previously described [23]. Specifically, follicles were classified as primordial if they consisted of a single oocyte surrounded by a single layer of squamous granulosa cells, primary if the oocyte was surrounded by a single layer comprised of ≥50% cuboidal granulosa cells, secondary if the oocyte was surrounded by two or more layers of cuboidal granulosa cells and a theca layer, and antral if the oocyte was surrounded by multiple layers of cuboidal granulosa cells, theca cells and contained an antrum. Data are presented as number of follicles or CL per ovary.

2.6. Real-time PCR

Following euthanasia, one ovary per animal was snap frozen and stored at −80 °C for subsequent real-time PCR analysis as previously described [19]. Briefly, total RNA was extracted from individual ovaries (n = 4–5 mice per treatment) using AllPrep DNA/RNA/Protein Mini kits (Qiagen, Valencia, CA) and incubated with DNAse (Qiagen; 15 min) to eliminate potential genomic DNA contamination. RNA concentration was determined at 260 nm using a Synergy H1m microplate reader equipped with a Take3 micro-volume plate (Biotek, Winooski, VT). RNA samples (1 μg) were reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Each cDNA sample was diluted 1:5 with nuclease-free water prior to analysis. All qPCR reactions contained 1 μL of cDNA, 1 μL of gene-specific primers (500 nM; Integrated DNA Technologies, Coralville, IA), 3 μL of nuclease-free water, and 5 μL of SsoFast EvaGreen Supermix (Bio-Rad) for a final volume of 10 μL. All reactions were done in triplicate on a CFX Connect Real-time System (Bio-Rad) using the qPCR program suggested by the manufacturer and previously published in Craig et al. [19].