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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 E₂ 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.

Keywords

phthalate; ovary; follicle; steroidogenesis; apoptosis

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].

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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 7–10 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 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 (E₂) 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 $22 \pm 1^\circ\text{C}$. 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.

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 were 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 $5\ \mu\text{m}$ 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 ($1\ \mu\text{g}$) 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 $1\ \mu\text{L}$ of cDNA, $1\ \mu\text{L}$ of gene-specific primers (500 nM; Integrated DNA Technologies, Coralville, IA), $3\ \mu\text{L}$ of nuclease-free water, and $5\ \mu\text{L}$ of SsoFast EvaGreen Supermix (Bio-Rad) for a final volume of $10\ \mu\text{L}$. 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., 2013 [19]. Primers were designed using PrimerBLAST software [25] or purchased ready to use (see Table 1 for sequence information). Primer specificity was assessed as described in Craig et al., 2013 [19]. β -actin (*Actb*) was found to not differ between treatments and, thus, was used as a reference gene. Expression data were generated using the Gene Study function of Bio-Rad's CFX Manager Software which utilizes the mathematical model for relative quantification of real-time PCR data developed by Pfaffl [26]. Reported data consist of mean relative mRNA expression ratios from four to five separate ovaries per treatment.

2.7 Statistical Analysis

Estrous cyclicity, hormone, and follicle and CL count data were analyzed using SPSS Statistics 22 software (IBM, Chicago, IL). Gene expression data were compared using the gene expression analysis feature of CFX Manager software (Bio-Rad). Comparisons between all treatment groups were done by ANOVA or Kruskal-Wallis non-parametric test as appropriate. *Post hoc* comparisons between vehicle and DBP-treated groups were conducted using Dunnett's or Mann-Whitney non-parametric test as appropriate. For all comparisons, statistical significance was assigned at $p \leq 0.05$ and marked using asterisks (*) on their respective graphs.

3. Results

3.1 Effect of DBP exposure on estrous cyclicity

To determine if DBP exposure disrupts estrous cyclicity, we compared the percentage of time that animals spent on average in the stages of proestrus and estrus, as well as, metestrus and diestrus between treatments (Figure 1). There were no differences in the percentage of time spent in proestrus/estrus (VEH: $41.7 \pm 3.5\%$; 0.01DBP: $40.9 \pm 2.7\%$) and the time spent in metestrus/diestrus (VEH: $56.7 \pm 3.3\%$; 0.01DBP: $57.1 \pm 2.9\%$) between mice treated with vehicle (0 mg/kg/day) and 0.01 mg/kg/day. On the other hand, mice treated with DBP at 0.1 and 1000 mg/kg/day spent less time in proestrus/estrus (0.1DBP: $29.2 \pm 5.3\%$; 1000DBP: $25.8 \pm 6.9\%$; $p \leq 0.05$) and more time in metestrus/diestrus (0.1DBP: $67.5 \pm 6.2\%$; 1000DBP: $72.5 \pm 6.9\%$) than controls, but this difference was only statistically significant in mice treated with DBP at 1000 mg/kg/day ($p \leq 0.05$).

3.2 Effect of DBP exposure on circulating 17β -estradiol (E_2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels

We compared circulating levels of E_2 , FSH, and LH between vehicle and DBP-treated mice to determine whether DBP exposure affects the hypothalamic-pituitary-ovarian axis (HPO). All DBP-treated mice, including those treated with DBP below the oral reference dose, had decreased circulating E_2 when compared to vehicle-treated controls (VEH: 8.7 ± 0.8 pg/mL, 0.01DBP: 6.4 ± 0.5 pg/mL, 0.1DBP: 5.8 ± 0.6 pg/mL, 1000DBP: 4.6 ± 0.8 pg/mL; $n=5-7$ mice per treatment; $p \leq 0.05$; Figure 2). We observed that overall DBP-treated mice had higher circulating FSH than control but, that this difference was statistically significant only in mice treated with DBP at 0.1 and 1000 mg/kg/day (VEH: 5.4 ± 0.9 ng/mL, 0.01DBP: 7.7 ± 1.5 ng/mL, 0.1DBP: 10.8 ± 2.1 ng/mL, 1000DBP: 8.7 ± 1.1 ng/mL, $n=5-6$ mice per treatment; $p \leq 0.05$; Figure 3A). In turn, only mice treated with DBP at 0.1 mg/kg/day showed a trend for increased circulating LH when compared to controls (VEH: 0.31 ± 0.13 ng/mL, 0.01DBP: 0.37 ± 0.06 ng/mL, 0.1DBP: 0.77 ± 0.27 ng/mL, 1000DBP: 0.25 ± 0.10 ng/mL; $n=5-7$ mice per treatment; $p=0.06$; Figure 3B).

3.3 Effect of DBP exposure on ovarian follicle and corpus luteum numbers

We classified and counted the ovarian follicles present in the ovaries of vehicle and DBP-treated mice to determine whether the effects on estrous cyclicity and circulating E_2 were also accompanied by disruptions in folliculogenesis. Ovaries from vehicle and all DBP-treated groups had similar numbers of primordial and primary follicles. There was a trend

for fewer secondary follicles in ovaries from mice treated with DBP at 0.01 mg/kg/day (Figure 4A) and mice treated with DBP at 0.01 and 0.1 mg/kg/day had fewer antral follicles than vehicle-treated controls (Figure 4B). Interestingly, this difference in antral follicle numbers was statistically significant only in the 0.1 mg/kg/day group (VEH: 9.5 ± 1.2 follicles; 0.01DBP: 5.9 ± 1.4 follicles; 0.1DBP: 5 ± 1.1 follicles; 1000DBP: 12.1 ± 3.0 follicles; $n=7-8$ ovaries; $p=0.05$). No differences were observed for antral follicle numbers in mice treated with DBP at 1000 mg/kg/day.

We also determined the number of corpora lutea present in the ovaries of vehicle and DBP-treated mice to determine whether DBP exposure disrupted ovulation. Numbers of corpora lutea did not differ between mice treated with vehicle and DBP at 0.01 and 0.1 mg/kg/day; however, mice treated with DBP at 1000 mg/kg/day had fewer CL ($p=0.05$) than vehicle controls (VEH: 5.1 ± 0.9 CLs; 0.01DBP: 4.7 ± 1.0 CLs; 0.1DBP: 5.1 ± 1.1 CLs; 1000DBP: 2.4 ± 1.1 CLs; $n=7-8$ ovaries; Figure 5).

3.4 Effect of DBP exposure on apoptosis gene expression

We hypothesized that decreased antral follicle numbers could be due to an increase in apoptosis signaling in the ovary. Thus, we determined the effect of DBP exposure on expression of key regulators of ovarian apoptosis including BCL2-associated agonist of cell death (*Bad*), BCL2-associated X protein (*Bax*), BH3 interacting domain death agonist (*Bid*), and BCL2-related ovarian killer (*Bok*; Figure 6). DBP exposure at 0.01 and 0.1 mg/kg/day resulted in increased expression of *Bax* and *Bad* mRNA ($n=4$; $p=0.05$ vs. control). Similarly, *Bid* mRNA was up-regulated by DBP treatment, but this change was only statistically significant in the 0.01 mg/kg/day dose group ($n=4$; $p=0.05$). Finally, the expression of *Bok* mRNA was decreased by DBP when given at 0.1 mg/kg/day ($n=4$; $p=0.05$).

3.5 Effect of DBP exposure on steroidogenic enzyme expression

We hypothesized that decreased circulating E_2 levels in DBP-treated mice could be due to altered expression of the enzymes responsible for its synthesis (Figure 7) and/or metabolism (Figure 8). We compared the expression of mRNAs encoding steroidogenic acute regulatory protein (*Star*), cholesterol side-chain cleavage enzyme (*Cyp11a1*), 3 β -hydroxysteroid dehydrogenase (*Hsd3b*), 17 α -hydroxylase/17,20-desmolase (*Cyp17a1*), 17 β -hydroxysteroid dehydrogenase (*Hsd17b*), aromatase (*Cyp19a1*), and cytochrome P450 1B1 (*Cyp1b1*). Consistent with decreased CL numbers, animals treated with DBP at 1000 mg/kg/day had decreased expression of *Star* and *Hsd3b1* mRNA. Compared to vehicle control, ovaries of all DBP-treated mice had increased expression of *Hsd17b1*, *Cyp17a1*, and *Cyp19a1* ($n=5$; $p=0.05$ vs. vehicle). Interestingly, a significant increase in *Hsd3b1* and *Cyp1b1* mRNAs was only observed in ovaries of mice treated with DBP at 0.01 mg/kg/day ($n=5$; $p=0.05$ vs. vehicle).

4. Discussion

We have used a rodent model to evaluate the effects of a 10-day exposure to DBP given at two doses that have relevance to humans. Here, we show that DBP exposure at doses below its current oral reference dose (0.3 mg/kg/day) is capable of disrupting reproductive

processes in young female mice. Our main findings show that at these doses, DBP leads to decreased circulating E₂, increased serum FSH, decreased antral follicle numbers, and altered mRNA expression of genes involved in apoptosis and steroidogenesis. Interestingly, the effects of a high-dose exposure to DBP (1000 mg/kg/day) differed from those observed with the doses below the oral reference dose mainly in that these animals had altered estrous cyclicity and reduced number of CL in their ovaries. Animals treated with the high doses also showed disruptions in steroidogenic enzyme expression, but lacked alterations in antral follicle number and disruptions in apoptosis gene expression. These findings suggest that DBP exposure can cause various detrimental effects and that these effects largely depend on the dose of DBP received.

We evaluated circulating levels of E₂, FSH, and LH to determine whether DBP disrupts the integrity of the HPO axis. DBP exposure resulted in decreased E₂ in all DBP-treated groups, increased FSH in mice treated with DBP at 0.1 and 1000 mg/kg/day, and a trend for increased LH only in mice treated with DBP at 0.1 mg/kg/day. Although the present study is the first to evaluate the effect of DBP exposure on sex steroid hormone and gonadotropin levels in adult mice, other studies have evaluated similar endpoints following longer exposure periods and greater doses in rats. Ex vivo ovarian steroidogenesis was evaluated in Long Evans rats chronically exposed to DBP at 250, 500, and 1000 mg/kg/day [27]. In contrast to our findings, the ovaries from DBP-treated dams produced more E₂ than those from control-treated rats in that study. However, when serum FSH and LH were compared between control and DBP-treated rats, results were similar to ours in that DBP-treated rats showed increased levels of these hormones at DBP exposures of 500 and 1000 mg/kg/day [27]. Another study evaluated the effects of DBP exposure from late gestation to weaning on various reproductive parameters in the F₁ offspring including pituitary weights and pituitary FSH- and LH-positive cell numbers [28]. DBP exposure altered pituitary weights in females on postnatal weeks 11 and 20 at DBP intakes ranging from 14.4–1371.8 mg/kg/day. At weaning, DBP intake ranging from 14.4–28.5 mg/kg/day resulted in decreased number of FSH-positive cells and increased LH-positive cells in the offspring. On the other hand, offspring from dams with DBP intakes ranging between 712.3–1371.8 mg/kg/day showed increased FSH-positive cell numbers in the absence of any changes in the LH-positive cell population. Unfortunately, that study did not measure serum E₂ to obtain a complete assessment of the HPO axis [28]. Despite the lack of directly comparable studies, our results agree with previous work in rats showing disruptions in gonadotropin levels. Furthermore, our results provide evidence that these effects also occur after short exposures at low doses. Whether DBP directly targets E₂ production in the ovary or via altering gonadotropin release by the pituitary has not been directly tested following adult exposure to DBP. However, DBP has been shown to decrease in vitro E₂ production in isolated antral follicles [19]. That finding supports the idea that DBP-induced reduction in E₂ production may not be the result of impaired gonadotropin release. Further studies will be needed to directly test this hypothesis using in vivo approaches.

Based on the hormone data, we predicted that animals treated with DBP would show altered estrous cyclicity. In agreement with the hormone data, mice treated with 1000 mg/kg/day showed disrupted estrous cyclicity. The effects of DBP on estrous cyclicity have been evaluated previously in large studies focused on evaluating outcomes from maternal

exposures. For example, maternal exposure to DBP did not alter estrous cyclicity in the F₁ generation when CD rats were treated with DBP (0, 250, 500, and 750 mg/kg.bw/day) throughout pregnancy and lactation until their offspring were at postnatal day 20 [29]. Another study evaluated the reproductive toxicity of DBP in CD Sprague-Dawley rats treated (0, 80, 385, and 794 mg/kg/day) during a continuous-breeding and crossover mating study and found no effects on estrous cycle length in the dams and no effect on ante-mortem estrous cyclicity in the F₁ generation [30]. Finally, another continuous breeding study in CD-1 mice treated with DBP at 53, 525, and 1750 mg/kg/day revealed no effects on estrous cyclicity [31, 32]. It is not surprising that our results differ from those reported in these studies. Previous studies have been focused on evaluating higher doses and understanding the effects of DBP exposure on prenatal and neonatal development following maternal exposure, while the present study evaluated effects of short-term exposure to low doses of DBP using non-pregnant mice. Also, lack of effects on estrous cyclicity in previous studies may not necessarily mean that ovulation was not impaired because normal cyclicity can still occur in the absence of ovulation when luteinization of unruptured follicles occurs [22].

We next tested whether DBP exposure resulted in altered folliculogenesis and number of corpora lutea. DBP exposure did not affect the numbers of small follicles (primordial, primary, and secondary) in DBP-treated mice compared to controls. However, mice treated with DBP at 0.01 and 0.1 mg/kg/day had decreased numbers of antral follicles, whereas no change was observed in mice treated with the high dose of DBP. It was previously shown that a 96-h in vitro exposure to DBP results in decreased growth and increased atresia in mouse antral follicles [19]. Consistent with the idea that DBP induces antral follicle death, we observed that expression of three of four pro-apoptotic genes (*Bax*, *Bad*, and *Bid*) was increased in treatment groups with decreased antral follicle numbers. Interestingly, expression of the pro-apoptotic gene *Bok* was decreased in the ovaries of mice treated with DBP at 0.01 mg/kg/day whereas it was unchanged by other treatments. A lack of effect on *Bok* mRNA expression following in vitro DBP treatment [19] and its down-regulation by another phthalate, MEHP, in mouse antral follicles have also been reported recently [33]. Overall, these observations suggest that low dose DBP alters apoptosis gene expression and leads to increased antral follicle loss. In turn, increased loss of antral follicles can explain decreased serum E₂ in DBP-treated animals because the antral follicle is the main site of E₂ production in females [14]. However, it is important to highlight that E₂ has been shown to protect follicles from atresia by preventing activation of apoptosis [13, 14] and that follicle death could be due to decreased availability of E₂ in circulation.

Based on altered estrous cyclicity and decreased antral follicle numbers, we hypothesized that DBP-treated mice would also have fewer ovulations as evidenced by decreased number of CL in their ovaries. Only mice treated with a high dose of DBP showed decreased number of CL in their ovaries despite of no effect on antral follicle number. These observations suggest that DBP interfered with ovulation. This is the first study to report the effects of DBP on ovulation and numbers of corpora lutea.

We evaluated the mRNA expression of enzymes involved in the synthesis and breakdown of E₂ in the ovary. In agreement with our data showing decreased serum E₂ and decreased number of CL in the ovaries of mice treated with DBP at 1000 mg/kg/day, we observed that

these ovaries also had decreased expression of *Star* and *Hsd3b1* mRNAs. Most work on the effects of DBP on steroidogenesis has been done in males. Our observations are consistent with studies showing that in utero exposure to DBP results in decreased testicular expression of *Star* and *Hsd3b1* among other genes that encode key steroidogenic enzymes [34–37].

Based on our serum E₂ results and publications by others on the fetal testis, we expected DBP-treated mice to have decreased expression of *Cyp17a1*, *Cyp19a1*, and *Hsd17b1* in their ovaries. However, all DBP-treated mice showed increased expression of *Cyp17a1*, *Cyp19a1*, and *Hsd17b1* whereas those treated with the lowest dose level also showed up-regulation of *Cyp11a1* and *Hsd3b1*. Although DBP decreases the expression of these genes in the fetal testis, two studies reported increased expression of these enzymes (except *Cyp19a1*) in the testes of Sprague Dawley rats treated with DBP during the postnatal period (versus gestational exposure; [38, 39]). Thus, it appears that age and route of exposure are important determinants of the effects of DBP exposure of steroidogenic enzyme gene expression. Nonetheless, the observations in our study can also be explained by the elevated levels of FSH in serum observed in these animals because FSH, via the FSH receptor, signals to increase the expression of these enzymes. Perhaps, elevated FSH is causing the remaining antral follicles to increase the expression of these enzymes to compensate for the decreased E₂ levels in circulation. Finally, we observed that the expression of the E₂ breakdown enzyme, *Cyp11b1*, was increased by DBP at the lowest exposure level in this study. This is interesting because *Cyp11b1* is regulated by the aryl hydrocarbon receptor (AHR), an important transcription factor in the response to xenobiotics and development and function of the female reproductive system [40, 41]. It will be interesting to determine the involvement of AHR signaling in DBP-induced ovarian toxicity.

In summary, the present study provides evidence of ovarian toxicity following a short low-dose exposure to DBP in young non-pregnant CD-1 mice. DBP exposure altered serum E₂, LH and FSH levels, reduced antral follicle numbers, and disrupted ovarian gene expression at doses at or below its RfD (0.1 mg/kg/day). Based on the effects of low and high dose exposures to DBP, it appears that DBP-associated disruptions on ovarian function strongly depend of the level of exposure. Although, how DBP causes dose-specific effects remains to be elucidated, based on previous work with other endocrine disruptors, potential mechanisms include receptor non-selectivity at high doses and differential regulation of receptors and essential signaling factors at low vs. high doses (reviewed in [42]). DBP disrupted the expression of genes involved in apoptosis and steroidogenesis, thus, future work will be aimed at elucidating how DBP may interact with transcription factor pathways such as the PPARs and AHR to cause these gene expression changes. Finally, understanding how short-term exposures to DBP might affect reproductive health will provide valuable information to better predict risks associated with women's exposure to DBP and other phthalates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DBP	di-n-butyl phthalate
E₂	17 β -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
PPARα	peroxisome proliferator-activated receptor alpha
AHR	aryl hydrocarbon receptor
HDAC6	histone deacetylase 6
MYC	myelocytomatosis oncogene

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HIGHLIGHTS

- A 10-day exposure to DBP disrupted reproductive processes in CD-1 mice
- DBP exposure resulted in decreased circulating 17β -estradiol
- Antral follicle numbers and apoptosis gene expression were altered at low doses
- Estrous cyclicity and corpora lutea counts were altered at a high dose
- DBP exposure resulted in altered steroidogenesis gene expression

Figure 1. Effect of DBP exposure on estrous cyclicity

CD-1 mice were weighed and given daily oral doses of tocopherol-stripped corn oil (vehicle) or DBP at 0.01, 0.1 and 1000 mg/kg for 10 consecutive days (n=8/group, total n=32). Vaginal smears were taken to monitor estrous cyclicity daily throughout the study as described in Materials and Methods. Data are presented as mean percentage of time spent in the stages of proestrus/estrus and metestrus/diestrus $\pm$ SEM. Asterisks indicate statistical differences versus vehicle ($p < 0.05$).

Figure 2. Effect of DBP exposure on circulating levels of estradiol

CD-1 mice were weighed and dosed as described in Materials and Methods. Blood samples were collected and serum obtained and processed for E₂ assays as described in Materials and Methods. Data are presented as mean ± SEM. Asterisks indicate statistical differences versus vehicle (p < 0.05).

Figure 3. Effect of DBP exposure on circulating levels of FSH and LH

CD-1 mice were weighed and dosed as described in Materials and Methods. Blood samples were collected and serum obtained and processed for a FSH/LH multiplex assays as described in Materials and Methods. FSH (**A**) and LH (**B**) level data are presented as mean $\pm$ SEM. Asterisks (*) indicate statistical differences versus vehicle ($p < 0.05$) and hashtags (#) denote trends where p was greater than 0.05 but less than 0.10.

Figure 4. Effect of DBP exposure on ovarian follicle numbers

CD-1 mice were weighed and dosed as described in Materials and Methods. Ovaries were dissected and processed to generate histological sections for ovarian follicle classification and enumeration as described in Materials and Methods. Primordial, primary, and secondary follicle (**A**) and antral follicle (**B**) count data are presented as mean $\pm$ SEM number of follicles per ovary. Asterisks (*) indicate statistical differences versus vehicle ($p < 0.05$) and hashtags (#) denote trends where p was greater than 0.05 but less than 0.10.

Figure 5. Effect of DBP exposure on corpora lutea numbers

CD-1 mice were weighed and dosed as described in Materials and Methods. Ovaries were dissected and processed to generate histological sections for enumeration of corpora lutea as described in Materials and Methods. Data are presented as mean $\pm$ SEM number of CL per ovary. Asterisks (*) indicate statistical differences versus vehicle ($p < 0.05$).

Figure 6. Effect of DBP exposure on apoptosis gene expression

CD-1 mice were weighed and dosed as described in Materials and Methods. Ovaries were dissected and processed for qPCR analysis as described in Materials and Methods. Whole ovary cDNA samples were subjected to qPCR to determine the expression of the pro-apoptotic genes *Bax*, *Bad*, *Bid*, and *Bok* using *Actb* as a reference gene (see Materials and Methods). Relative gene expression data are presented as mean $\pm$ SEM. Asterisks (*) indicate statistical differences versus vehicle ($p < 0.05$) and hashtags (#) denote trends where p was greater than 0.05 but less than 0.10.

Figure 7. Effect of DBP exposure on steroidogenesis gene expression

CD-1 mice were weighed and dosed as described in Materials and Methods. Ovaries were dissected and processed for qPCR analysis as described in Materials and Methods. Whole ovary cDNA samples were subjected to qPCR to determine the expression of *Star*, *Cyp11a1*, *Cyp17a1*, and *Cyp19a1* using *Actb* as a reference gene (see Materials and Methods).

Relative gene expression data are presented as mean $\pm$ SEM. Asterisks (*) indicate statistical differences versus vehicle ($p < 0.05$).

Figure 8. Effect of DBP exposure on ovarian *Cyp1b1* expression

CD-1 mice were weighed and dosed as described in Materials and Methods. Ovaries were dissected and processed for qPCR analysis as described in Materials and Methods. Whole ovary cDNA samples were subjected to qPCR to determine the expression of *Cyp1b1* using *Actb* as a reference gene (see Materials and Methods). Relative expression data are presented as mean $\pm$ SEM. Asterisks (*) indicate statistical differences versus vehicle ($p < 0.05$).

Table 1

Accession No.	Gene name	Abbreviation	Forward	Reverse
NM_007393	actin, beta	<i>Actb</i>	ATGCCGGAGCCGTTGTC	GCGAGCACAGCTTCTTTG
NM_007522.2	BCL2-associated agonist of cell death	<i>Bad</i>	AAGTCCGATCCCGGAATCC	GCTCACTCGGGCTCAAACTCT
NM_007527.3	BCL2-associated X protein	<i>Bax</i>	TGAAGACAGGGGCTTTTGG	AATTCGGCGGAGACACTCG
NM_016778.2	BCL2-related ovarian killer protein	<i>Bok</i>	CTGCCCCCTGGAGGACGCTTG	CCGTCAACCAACAGGCTCCGAC
NM_007544.3	BH3 interacting domain death agonist	<i>Bid</i>	AGCAAAATGTTCCCTCCGCTTCTGT	GTAGGCTGTGGCGGCTCGTG
NM_009994.1	cytochrome P450, family 1, subfamily b, polypeptide 1	<i>Cyp1b1</i>	TCCTCTTTACCAGATACCCGGA	GACATATGGCAGGTGGGCT
NM_019779.3	cytochrome P450, family 11, subfamily a, polypeptide 1	<i>Cyp11a1</i>	GGTTCCACTCTCTCAAAGCCA	GCCACCTGTACCAAAGTCTTG
NM_007809.3	cytochrome P450, family 17, subfamily a, polypeptide 1	<i>Cyp17a1</i>	GGAGAGTTTGGCCATCCCGAA	GATCTAAGAAAGCGCTCAGGCA
XM_006510809.1	cytochrome P450, family 19, subfamily a, polypeptide 1	<i>Cyp19a1</i>	ACACTGTTGTGGGTGACAGA	GCATGACCAAGTCCACAACA
NM_008293.3	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	<i>Hsd3b1</i>	CAGGAGAAAGAACTGCAGGAGTGC	GCACACTTGTCTTGAACACACAGGC
NM_010475.1	hydroxysteroid (17-beta) dehydrogenase 1	<i>Hsd17b1</i>	ACTGTGCCAGCAAAGTTGCG	AAGCGGTTCTGTGGAGAAAGTAG
NM_011485.4	steroidogenic acute regulatory protein	<i>Star</i>	ATTTTGGGGAGATGCCGGAG	GCCACCCCTTTCAGGTCAATAC