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Reproductive development and function of female rats exposed to di-η-butyl-phthalate (DBP) *in utero* and during lactation

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ABSTRACT

Phthalates are environmental contaminants used in the production of plastics, cosmetics and medical devices. Studies on the effects of phthalates on female reproductive health are particularly sparse and mostly restricted to high-dose exposure in rats. In the present study, pregnant rats were treated with 100 mg/kg-d of di-η-butyl-phthalate (DBP) or only the vehicle (control group), from GD 12 to GD 20 for evaluation of reproductive outcomes and fetal gonads analysis (F0), and from GD 12 to PND 21 to evaluate reproductive development and function on F1 female offspring. Results showed that all parameters were comparable between groups, although there was a significant increase in the fetal weight after DBP exposure. However, the body weight at birth was normal. Based on these data we can conclude that, in these experimental conditions, DBP did not disturb the reproductive development or function of female rats.

1. Introduction

Humans are constantly exposed to a wide range of environmental contaminants from industrial processes, through air, food, water or contact with a variety of consumer products. In recent years, one class of chemicals used as plasticizers called phthalates have attracted special attention from the scientific community and the general public due to their high production volume, in millions of tons annually, and variety of applications. In addition, they are suspected of acting as endocrine disruptors which signifies that they have the potential to modify normal endocrine function [1–4]. Phthalate esters are liquid plasticizers widely used in flexible polyvinyl chloride (PVC) products, personal care products, cosmetics (perfume, lotions and nail polish), paints, coatings, some pesticide formulations, and pharmaceutical products [5,6] and thus are ubiquitous low-level environmental contaminants [7,8].

Di-η-butyl-phthalate (DBP) is a phthalic acid ester used extensively as a plasticizer in many products including medical devices [9], flexible plastics (children's toys, beverage bottles and feed bottles) and some cosmetic formulations [10–12].

Human exposure occurs primarily through contaminated food, especially high-fat foods, which may have been in contact with plastic, adhesives, or other packing materials that contain DBP. Herbal preparations and nutritional supplements may also incorporate phthalates in their formulations. This is especially true for di(2-ethylhexyl) phthalate (DEHP) and DBP. These types of products are typically taken by pregnant women. Pharmaceutical formulations also result in significant human exposure, because various plasticizers are used to coat medicines such as antibiotics, antihistamines and laxatives [13].

Most of the studies related to phthalates are restricted mainly to high-dose exposure [3,14–17], in which the effects are more pronounced. Human and animal exposures to low concentrations of phthalates usually occur after the release of phthalate esters from plastic products and utensils because phthalates are not permanently bound to the polymer matrix [12,18–21]. Phthalate metabolites were detected in the urine of children and adults, in serum, seminal fluid, amniotic fluid, breast milk and saliva [22–27]. Estimates for human exposure to DBP range from 0.84

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to 113 μg/kg-d [5,28], but the most critical population comprises women of reproductive age in whom exposure may be up to 20 times greater than in the average person from the general population. In this case, the highest exposure estimates were above the federal safety standard [5,28,29].

Studies that report effects of phthalates on female reproductive health are particularly sparse and mostly restricted to high-dose exposure in rats. The present study aims to evaluate the effects on female reproductive development and function, in rats whose mothers were exposed to DBP at a dose of 100 mg/kg-d (LOAEL for male rats) during pregnancy and lactation.

2. Materials and methods

2.1. Animals

Adult female (60 days of age, $n = 20$) and male (90 days of age, $n = 10$) Wistar rats were supplied by Central Biotherium of State University of São Paulo (Unesp) and were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1 °C) and lighting conditions (12:12-h photoperiod). Rat chow and filtered tap water were provided *at libitum*. Two non-gravid female rats were mated with one male, during the dark portion of the lighting cycle, and the day of sperm detection in the vaginal smear was considered day 0 of gestation (GD 0). The gravid females were randomly assigned between the experimental groups and housed individually in cages. At birth, the offspring were weighed and reduced to eight pups per litter, in order to maintain the female ones. The experimental protocol followed the Ethical Principles in Animal Research of the Brazil College of Animal Experimentation and was approved by the Bioscience Institute/UNESP Ethics Committee for Animal Experimentation (protocol no. 60/06-CEEA).

2.2. Treatment

Gravid rats ($n = 5$/group) were administered either di-η-butyl-phthalate (DBP—Sigma Chemical Co., St. Louis, MO) at 100 mg/kg bw-d, considered the LOAEL dose for male reproductive effects [10], by gavage (oral route), or corn oil (vehicle) from GD 12 until GD 20 when the dams were sacrificed and caesarean section was performed. Reproductive performance data were assessed and the gonads of one female offspring (F1) from each litter were also analyzed. Two additional groups of gravid rats (C = 9; T = 10) were exposed to either 100 mg/kg bw-d or vehicle from GD 12 to the end of lactation (post-natal day 21—PND 21) to evaluate the possible effects of the treatment on the female offspring. The beginning of the mothers' treatment coincided with the critical period of reproductive system development, which continues after birth [30,31].

2.3. Maternal body weight

Dams were weighed on alternate days from GD 0 until sacrifice on GD20 or the end of lactation (PND 21) to adjust the volume of DBP to be administered and to evaluate possible maternal toxicity. Pups were weaned on PND 21 and the respective mothers were sacrificed by decapitation on the following day.

2.4. Evaluation of maternal endpoints and analysis of fetal gonads

Control ($n = 5$) and treated ($n = 5$) pregnant dams (F0) were killed by decapitation on GD 20. After the uterus and ovaries were removed, the number of corpora lutea were examined by gross morphology, and the number of implantation sites, resorptions and live and dead fetuses were recorded. From female fetuses (one per litter) the ovaries were collected, fixed in Karnovsky (2.5% glutaraldehyde, 8% paraformaldehyde), included in historesin, processed for histological analysis and stained with hematoxylin and eosin. Germ cells were counted, 3 sections per animal with 50 μm of distance among them, from control and treated ovaries and expressed as number of cells per unit area (no. germ cells/mm^2).

2.5. Anogenital distance and number of nipples/areola

On PND 4, the anogenital distance (AGD, the distance between the anus and the genital tubercle) was measured in female pups, using a pachymeter. On PND 13, the number of areolas was recorded. Observations were scored based on the presence or absence of a nipple bud or a discoloration of the skin surrounding the nipple [10].

2.6. External signs of puberty onset

Beginning on PND 30, all females (F1) were evaluated daily for vaginal opening (VO). The day of complete VO was recorded. From the day of VO, daily vaginal smears were collected to detect the day of first estrus, characterized by the predominance of cornified epithelial cells.

2.7. Estrous cycle

On PND 60, in treated ($n = 45$ females/10 litters) and control ($n = 36$ females/09 litters) groups, the estrous cyclicity of female rats (F1) was assessed on cells from daily vaginal smears, collected over a period of 15 days, as described by Marcondes et al. [32]. Every morning 10 μL of 0.9% saline was instilled into the vagina and subsequently aspirated. The material was observed under light microscopy and the estrous cycle phase was determined by cytology: predominance of nucleated epithelial cells (proestrus); predominance of cornified epithelial cells (estrus); the presence of cornified and nucleated epithelial cells and leukocytes (metaestrus); predominance of leukocytes (diestrus). The total frequency of each phase for every rat observed in this period was used to calculate the total length of the proestrus, estrus, metaestrus and diestrus (in days) and the estrous cycle length.

2.8. Analysis of reproductive organs

At PND 60, ovaries and uteri were collected from rats (F1) in estrus phase ($n = 5$ in each group), weighed on precision balance, fixed in Alfac's solution, dehydrated in ethanol and embedded in paraplast. Three sections (5 μm) per animal, with 50 μm of distance among them, were obtained, mounted on glass slides and stained with hematoxylin and eosin.

In each ovary, ovarian follicles and corpora lutea were counted in 3 sections per animal and expressed as number per unit area (mm^2). Follicles were classified according to Borgeest et al. [33] and Talsness et al. [34]. Primordial and primary follicles were enumerated together; oocytes surrounded by a single layer of either squamous or cuboidal epithelial cells were included. Follicles were classified as pre-antral when containing 2–4 layers of granulosa cells with no antral space. Antral follicles were classified when containing three or more layers of granulosa cells and a clearly defined antral space. Characteristics of atretic follicles included pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte and detachment from the basement membrane. In the uterus, the endometrial height was measured, in 3 sections per animal using a light microscope. In each section, five different regions were analyzed, resulting in a total of 15 measurements per animal.

At PND 75, 8 F1 rats per group in estrus were sacrificed for the determination of reproductive organ weights (ovaries and uterus) on precision balance and hormonal analysis.

2.9. Hormonal analysis

Female pups (C = 8; T = 8) were sacrificed on PND 75 (F1), during the estrus phase, between 8:00 and 10:00 a.m. After decapitation, trunk blood was collected and allowed to clot on a refrigerator (4 °C) for 30 min. Serum was collected after centrifugation and stored at −20 °C until analysis. Serum FSH, LH and progesterone concentrations were measured using a double-antibody radioimmunoassay (RIA) kit (National Institute of Arthritis, Diabetes and Kidney Diseases–NIADDK, USA). All the samples were analyzed at the same assay to avoid inter-assay variability.

2.10. Sexual behavior

On the first estrus after PND 80, control ($n = 10$) and treated ($n = 10$) female rats from the F1 generation were used for the mating test. Rats were maintained under controlled temperature conditions on an inverted 12-h light–dark cycle, for at least seven days, with food and water *ad libitum*. For the evaluation of female sexual behavior, sexually experienced males were allowed ten mounts on the female and the presence of lordosis was measured. Results were expressed as the lordosis quotient (LQ, number of lordosis/10 mounts × 100). All females were used only once.

2.11. Fertility and reproductive performance of F1 female rats

This analysis was performed through natural mating. Rats from control ($n = 14$) and treated ($n = 15$) groups were placed with sexually experienced males (1:1), until 3 sexual cycles (3 estrus), in the beginning of the morning, during a dark period of the cycle. At the end of afternoon, males and females were separated and vaginal smears were collected, in which initial sperm detection was determined to be GD 0. On GD 20 females were killed by decapitation. After collection of the uterus and ovaries, the number of corpora lutea (by gross morphology), implantation sites, resorptions, live fetuses and fetal weights were determined. From these results, the following were determined—gestation rate: number of pregnant females/number of inseminated females × 100; implantation rate (efficiency of implantation): implantation sites/corpora lutea × 100; pre-implantation loss rate: number of corpora lutea − number of implantations/number of corpora lutea × 100; post-implantation loss rate: number of implantations − number of live fetuses/number of implantations × 100; sex ratio: number of male fetuses/number of female fetuses × 100.

2.12. Statistics

Values are expressed in mean ± SEM and medians (Q_1–Q_3). For comparison of results between the experimental groups, Student's *t*-test and Mann–Whitney test were utilized. Differences were considered significant when $p < 0.05$.