# Interspecies Approach to the Assessment of Endocrine Disrupting Chemicals in Low Dose or Complex Mixtures

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## ABSTRACT

The *in utero* and perinatal hormonal environment is critical for proper development of the male reproductive system. Low-dose exposures to endocrine disrupting chemicals may predispose the male to reproductive tract abnormalities leading to altered reproductive function. Phthalate esters are ubiquitous chemicals used to impart flexibility in plastics. Exposure (of dams) to low doses of di-(n-butyl) phthalate (DBP) in the rat exhibit an anti-androgenic effect through alterations in genes involved in testicular steroidogenesis, in addition to a significant reduction in fetal testicular testosterone. Given the presence of various phthalate esters in different plastics, exposure to these mixtures complicates the assessment of human susceptibility. The development of a rodent host bioassay using human fetal testicular grafts will provide essential information relevant to human risk evaluation of toxicant-induced suppression of *in utero* steroidogenesis.

## INTRODUCTION

The *in utero* and perinatal hormonal environment is critical for proper development of the male reproductive system. *In utero* exposure to endocrine disrupting chemicals (EDCs), has been shown to impact normal development of the male reproductive system, potentially leading to altered reproductive function. In an attempt to explain the increasing incidence of falling sperm counts, hypospadias, cryptorchidism and testis germ cell cancer over the past 50 years, Skakkebaek has proposed a "testicular dysgenesis syndrome"[1], suggesting that the developing male reproductive tract may be impacted due to the hormonally-sensitive nature of the underlying developmental events.

Phthalate esters are chemicals used to impart flexibility to rigid plastics. As these esters are noncovalently linked to the polymer, they leach from plastics through normal use[2]. Di-(n-butyl)phthalate (DBP) is widely used in plastic manufacturing and has been shown to exhibit anti-androgenic effects in the rat when exposed during a critical *in utero* developmental window. These effects include cryptorchidism, hypospadias, reduced sperm counts, decreased anogenital distance, retained nipples and lowered testosterone levels as a result of decreased expression at the mRNA and protein levels of genes associated with cholesterol transport and steroid metabolism[3-8]. Unlike the rat, mice do not exhibit a phthalate-induced suppression of fetal testicular steroidogenesis, and show no change in fetal testicular testosterone content. This striking difference in species sensitivity invites speculation as to human susceptibility to phthalate exposure.

Considering the widespread presence of phthalate esters, environmental exposure is of serious concern. In particular, the developing fetus and neonate represent a subpopulation which may have considerable exposure through medical devices such as blood bags and tubing during a critical window of development. Exposure to different types of plastics with various concentrations and analogs of phthalate esters further complicates the risk characterization process. Experiments in sensitive (rat) and resistant (mouse) species provide a comparative biology approach to extrapolate to human risk of toxicant-induced suppression of *in utero* steroidogenesis.

## RESULTS

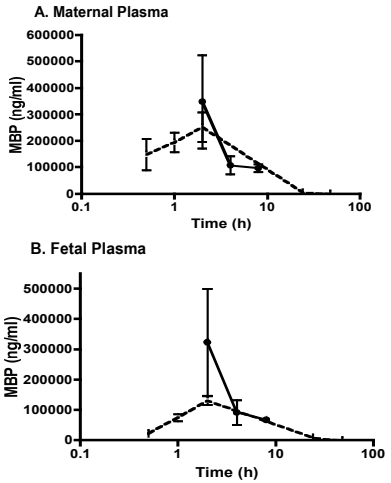


Figure 1. Plasma concentration of MBP (ng/ml) in maternal plasma (A) or pooled fetal plasma (B) following administration of DBP to mice (solid line, gd 18) or rats (dashed line, gd 19) at various times following a single dose of 500 mg/kg DBP. Symbols represent the mean concentration ± SD (n=4, note fetal blood was pooled so n=Dam). Rat results from Kremer et al 2005[9]. Mouse results from Gaido et al 2007[10].

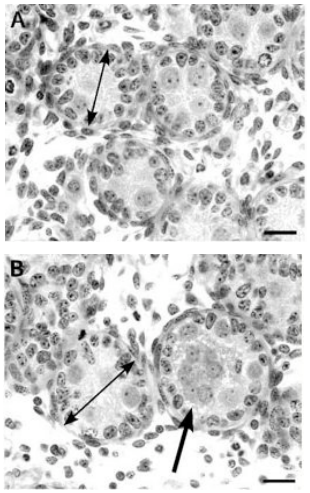


Figure 2. Mouse fetal testis histopathology at gd 19 following corn oil (A) or 250 mg/kg DBP (B) exposure of the dam on gd 16 – gd 18 from Gaido et al 2007[10]. Note the DBP-induced increase in the seminiferous cord diameter (double-headed arrow), and the multinucleated gonocyte (B, arrow). Bar, 20 µm; H&E staining.

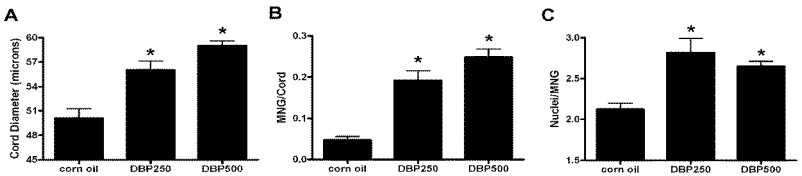


Figure 3. The gd 19 fetal testis cross sections were quantitatively assessed for seminiferous cord diameter (A), number of MNG per cord cross section (B), and the number of nuclei per MNG (C) from Gaido et al 2007[10]. Exposure of the dam to either 250 mg/kg or 500 mg/kg DBP on gd 16 – gd 18 significantly altered each of these endpoints. The bars represent the mean concentration ± S.E.M. Asterisk (*), significantly different from corn oil control, $p < 0.05$.

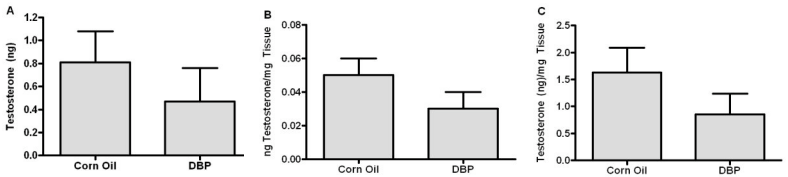


Figure 4. Fetal (gd 17) rat testes were implanted under the kidney capsule of a syngeneic castrated rat host, dosed with 250 mg/kg DBP gd 18-20 and assessed for mean testicular testosterone content (A), expressed per mg of testicular tissue (B) and per mg of protein (C). Bars represent the mean concentration ± S.E.M. Corn oil (n=4); DBP (n=6).

## MATERIALS AND METHODS

*Animals*
Pregnant time-mated CD-1 mice were purchased from Charles River Laboratories, Inc (Raleigh, NC) and housed at the AAALAC-accredited animal facility at CIIT Centers for Health Research in temperature and humidity controlled vivarium with a 12-h alternating light-dark cycle. Rodent diet NIH-07 (Ziegler Brothers, Gardner, PA) and reverse-osmosis water were provided ad libitum. This study was approved by the Institutional Animal Care and Use Committee at CIIT Centers for Health Research and followed federal guidelines for the care and use of laboratory animals. Pregnant time-mated C57Bl/6J mice, pregnant time-mated Fisher rats and castrated Fisher rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and housed in the same conditions as above, but at animal facility at Brown University. All animals were housed in community cages with free access to water and Purina Rodent Chow 5001. The Brown University Institutional Animal Care and Use Committee approved all experimental animal protocols in compliance with the National Institute of Health guidelines.

*MBP Quantification*
Plasma quantification of monobutylphthalate (MBP) was performed by liquid chromatography/mass spectrometry (LC-MS/MS) (Applied Biosystems, Foster City, CA) using the method described previously[8]. Sample preparation and analysis were performed using selected reaction monitoring of precursor-product ion transitions at m/z 221.1-77.1 for MBP and 225.1-79.1 for 13C4-MBP (internal standard added to plasma samples).

*Testosterone Radioimmunoassay*
Fetal testis testosterone concentrations were determined using a method modified from vom Saal (vom Saal et al 1990) as described previously[10].

*Histopathology*
Seminiferous cord development and multinucleated gonocyte (MNG) formation was assessed following dosing of pregnant mouse dams with 250 or 500 mg/kg DBP gd 16 to 18 with collection on gd 19. Testes were collected and fixed in buffered 4% formalin. Tissue was dehydrated and embedded in glycol methacrylate (Technovit 7100, Heraeus Kulzer GmBH, Wehrheim, Germany), sectioned (3 µm) and stained with hematoxylin and eosin. Photographs were taken using a Zeiss Axiovert 35 microscope equipped with a Spot Diagnostic Digital camera and RT software (Sterling Heights, MI). Images were analyzed with METAMorph Image series 6.1 software (Molecular Devices Corp., Sunnyvale, CA). Photographs of fetal testis xenotransplants in kidney were taken with an Aperio Scanscope and visualized with ImageScope software (Aperio Technologies, Vista, CA)

*Fetal Testis Xenograft*
Pregnant Fisher rat females at gd 17 were euthanized by an overdose of Isoflurane, USP (Baxter Healthcare Corp., Deerfield, IL). Embryos were dissected, and testes were removed from the males and immediately transferred to ice cold Hank's Balanced Salt Solution (Invitrogen Corporation, Grand Island, NY). Castrated Fisher rat hosts were anesthetized with Isoflurane and a fetal testis was implanted in the renal subcapsular space. A total of three implants were performed in each kidney. The abdominal cavity was then sutured closed and stapled. Hosts were then dosed for three days (gd 18-20) with 250 mg/kg DBP or corn oil. On gd 20, hosts were dosed and euthenized 6 hours later by an overdose of Isoflurane. Testes were dissected from the capsule, fixed in modified Davidson's solution, and processed for plastic embedding as described above.

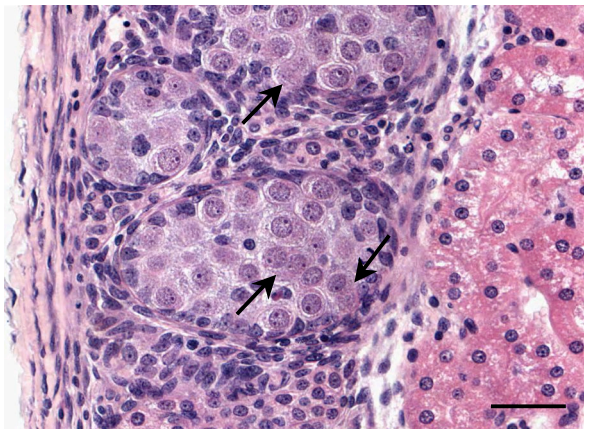

Figure 5. Fetal (gd 17) rat testis were implanted under the kidney capsule of a castrated rat host, dosed with 250 mg/kg DBP gd 18-20. Note the formation of multinucleated gonocytes (arrows). Tissue of the kidney cortex is present on the right, kidney capsule on the left margin. Bar, 50 µm; H&E staining.

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