ABSTRACT

BARLOW, NORMAN JAMES. Antiandrogens and Development of the Male Rat Reproductive Tract. (Under the direction of Paul M.D. Foster and Talmage T. Brown).

Di(*n*-butyl) phthalate (DBP) is an antiandrogen with known human exposure. The objectives of this thesis were to investigate the development of male reproductive tract malformations secondary to *in utero* DBP exposure from the fetus to the adult, to characterize the effects of DBP on fetal testicular gene expression for the steroidogenic enzymes, and to further explore DBP's potential for inducing Leydig cell adenomas following gestational exposure. *In utero* DBP exposure led to a characteristic set of fetal testicular lesions including large aggregates of fetal Leydig cells, multinucleated gonocytes, and increased numbers of gonocytes. In addition to the testicular effects, DBP also caused maldevelopment of the epididymides. During the early postnatal period the fetal testicular lesions became less apparent while decreased numbers of spermatocytes were observed. Underdeveloped epididymides noted in fetuses remained small or failed to fully develop resulting in epididymides with missing components. Malformed epididymides were fully manifest in the adult with absent portions observed both unilaterally and bilaterally. Testicular atrophy with loss of spermatocytes became more severe as gestationally exposed animals matured. Gene expression for the steroidogenic enzymes was examined in testes exposed to DBP *in utero*. Gene expression was decreased for P450 side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase, and P450c17; while mRNA expression for 17β-hydroxysteroid dehydrogenase, which catalyzes the final

step in testosterone biosynthesis, was not altered. *In utero* exposure to DBP failed to induce an increased incidence of classical Leydig cell adenomas. However, a dysgenetic lesion composed of numerous poorly differentiated Leydig cells surrounding immature seminiferous tubules was identified. Testicular dysgenesis was observed with a similar incidence between age groups in mature rats, which supports *in utero* induction by DBP rather than development over time. Together these data provide insight into the molecular mechanisms underlying the induction of DBP-initiated male reproductive tract malformations.

ANTIANDROGENS AND DEVELOPMENT OF THE MALE RAT REPRODUCTIVE TRACT

by
NORMAN JAMES BARLOW

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

COMPARATIVE BIOMEDICAL SCIENCES

Raleigh

2003

APPROVED BY:

Dr. C. Lee Robinette	Dr. Philip L. Sannes Minor Representative
Dr. Paul M.D. Foster Co-Chair of Advisory Committee	Dr. Talmage T. Brown Co-Chair of Advisory Committee

DEDICATION

I dedicate this dissertation to my grandmother, Emma Milke. She wanted me to be a doctor, lawyer, or the President of the United States. Grandma, I have succeeded.

BIOGRAPHY

Norman James Barlow was born December 18, 1969 in South Bend, Indiana. He was primarily raised in Niles, Michigan, where he attended Edwardsburg Public Schools, graduating from Edwardsburg High School in 1988.

Norman received his Bachelor of Arts degree, majoring in biology, from Kalamazoo College in 1992. He attended veterinary school directly after receiving his undergraduate degree, graduating from Michigan State University as a Doctor of Veterinary Medicine in 1996. Norman then entered a residency program in veterinary anatomic pathology at North Carolina State University, passing the certifying examination of the American College of Veterinary Pathologists and becoming a diplomate of the college in 1999. He entered graduate school at North Carolina State University in 1998, carrying out his graduate research at what was then known as the Chemical Industry Institute of Toxicology. Under the direction of Dr. Paul M.D. Foster Norman examined the effects of antiandrogens on the developing male reproductive tract. Norman will join Aventis Pharmaceuticals in Bridgewater, New Jersey as a research pathologist upon fulfillment of the requirements for his Doctor of Philosophy degree.

ACKNOWLEDGMENTS

First and foremost I would like to acknowledge the support of my family and friends throughout my academic career. Going to college was never an option for me; I just do not think my mother envisioned me staying in school for 15 years. I thank my mother for her unwavering support and for always encouraging me to take the next step. I am especially indebted to three particular friends, Drs. Beth J. Chittick, Michael P. DeLorme, and Barry S. McIntyre. These three provided support to me "above and beyond the call of duty" during all phases of my graduate program and constantly reminded me that it could be done. I would have completed the degree without their support, but I would have been miserable. I can only hope to some day return the favor.

Simply said, there is no mentor better than Dr. Paul M.D. Foster. We are colleagues and we are friends. In my opinion there is no better combination and I look forward to future scientific and personal interactions. I would also like to thank the rest of my graduate advisory committee, Drs. Talmage T. Brown, C. Lee Robinette, and Philip L. Sannes, for their guidance and support throughout my career as a graduate student. I would especially like to thank Dr. Brown for encouraging me to have lofty goals and for helping me fulfill them. Research at the CIIT Centers for Health Research is a team effort so I would like to thank Drs. Christopher J. Bowman, Kevin Gaido, Madhabananda Sar, Katie J. Turner, and Katrina Waters for their assistance with my studies. I'd also like to thank Kathy Claypoole, Kim Lehmann, Suzanne Phillips, and Duncan Wallace for their assistance and untiring support of our research program. My work as a pathologist leaves me indebted to the necropsy and histology laboratories at CIIT and I thank Betsy Gross-Bermudez and her

staff, including Corrie Dunn, Donald Joyner, Otis Lyght, Renee Thacker, David Weil, and Delorise Williams, for their expert assistance and devotion to their work. Our toxicology research could not be completed without the use of animals and an experienced animal care staff, for which I thank Paul Ross, Carol Bobbitt, Kathy Bragg, Steve Butler, Richard Masney, Jessica Ramsberger, Tim Shepherd, Israel Uzoma, and Felicia Wolfe. I would also be remiss if I did not thank the computer support team of Eric Anderson and Dwight McFarland, whom I've probably bugged more over the last several years than anyone else.

I would also like to express my gratitude to Dawn Dugan for her patience with me and for always being a friend. I extend a special thanks to my buddy Aaron Keatley for his invaluable assistance with the "é" symbol. Just because I was a friend to Mike DeLorme didn't mean his wife had to accept me, but Holly welcomed me and all of my antics and I really appreciated feeling like one of the family. The list of others to thank is far too long for this dissertation. I hope that anyone reading this realizes I did not complete my degree on my own.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AGD	anogenital distance
AR	androgen receptor
BBP	benzylbutyl phthalate
C-kit	stem cell factor tyrosine kinase receptor
BC	*bulbocavernosus* muscle
DBP	di(*n*-butyl) phthalate
DDT	dichlorodiphenyltrichloroethane
DEHP	di(2-ethylhexyl) phthalate
DES	diethylstilbestrol
DHT	dihydrotestosterone
DLP	dorsolateral prostate
DPP	dipentyl phthalate
FSHR	follicle stimulating hormone receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GD	gestation day
H&E	hematoxylin and eosin
HDL	high-density lipoprotein
HPT	hypothalamus-pituitary-testis
HSD	hydroxysteroid dehydrogenase
Insl3	insulin-like 3 gene
LA	*levator ani* muscle

LC	Leydig cell
LHR	luteinizing hormone receptor
MBP	monobutyl phthalate
MIS	Mullerian inhibiting substance
NOAEL	no observed adverse effect level
NTP	National Toxicology Program
OP	os penis
P450scc	P450 side-chain cleavage
PCNA	proliferating cell nuclear antigen
PGC	primordial germ cell
PND	postnatal day
ROC	receiver operator characteristic
SCF	stem cell factor
SER	smooth endoplasmic reticulum
SF-1	steroidogenic factor 1
SRB1	scavenger receptor B-1
StAR	steroidogenic acute regulatory protein
T	testosterone
TP	testosterone propionate
TRPM-2	testosterone-repressed prostate message-2
VP	ventral prostate
WT1	Wilms' tumor 1

CHAPTER ONE

General Introduction

Endocrine disrupters and their effects on males

Environmental contaminants may affect adults of a species by altering their endocrine status, and, more importantly, may alter the sexual differentiation and development of offspring of exposed adults by disrupting the endocrine milieu of the fetus. Alterations due to *in utero* exposures may be permanent and manifest as different phenotypes in exposed adults (31). Additionally, altered fetal development may lead to changes that will not be manifest until adulthood. Endocrine disrupters are compounds, whether environmental contaminants or pharmaceutical agents, which alter the endocrine status of an organism such that normal physiological processes controlled by hormones are altered. This disruption may result from decreased synthesis or transport of hormones, altered metabolism of hormones, blockade of hormone receptors (receptor antagonists), binding and activation of receptors (receptor agonists), or decreased expression of hormone receptors (14, 109, 158). In the case of the developing fetus, especially the male, which is extremely sensitive to changes in levels of endogenous hormones, altered endocrine status may be detrimental to sexual differentiation and development (152).

Interest in endocrine disrupters has dramatically increased over the last decade. Declining semen quality over the last 50 years was reported in 1992. (27). In 1993, Sharpe and Skakkebaek (153) speculated that the cause of decreased sperm counts and increased incidence of testicular cancer, cryptorchidism, and hypospadias was due to a common

mechanism related to increased exposure to environmental estrogens (17, 172). In fact, much of the initial fervor over endocrine disrupters was centered on estrogens (31). Reports in the literature on the potential effects of antiandrogens did not begin to surface until the mid 1990s (62, 97, 98). While the initial report by Carlsen et al. (1992) indicated a decrease in sperm counts, additional reports indicated that this decrease might be regional with different areas of countries or the world more significantly altered, while in other areas, sperm counts have remained static (5, 47, 48, 87, 167). The cause of the regional differences in sperm counts and the increased incidence of testicular cancer, cryptorchidism, and hypospadias is not easily explained but changes in lifestyle, diet and exposure to endocrine disrupters are speculated to all be contributing (9, 153, 172).

Wildlife populations may be good sentinels for environmental health and alterations in sexual development and reproductive dysfunction in these populations may be an indicator of exposure to environmental endocrine disruptors (108). Numerous examples of the effects of endocrine disrupters on wildlife are in the literature (108, 175). One of the first to be described was the estrogenicity of river water downstream of sewage treatment plants in the United Kingdom (163). Male fish caged at different locations in the river had varied increases in vitellogenin, an estrogenic responsive egg protein not normally present in male fish. The amount of estrogenicity of the water was determined to be due to the amount of effluent that entered the river at that particular point (163). A second well-publicized wildlife population affected by endocrine disrupters was the male alligators in Lake Apopka in Florida (69). This lake experienced a dramatic decrease in the alligator population in the early 1980s that continues to this day. In 1980, a large chemical spill of dicofol occurred, which contained up to 15% dichlorodiphenyltrichloroethane (DDT) and

its metabolites. Male alligators surveyed from Lake Apopka were found to have decreased penis size and markedly decreased levels of plasma testosterone (T), while there was an overall decrease in fertility of the alligators in this lake (69). These effects were attributed to environmental exposure to the dicofol and DDT metabolites. The question of whether wildlife populations are indicative of potential alterations in human reproduction and development remains. The general conclusion is that sufficient empirical evidence for effects in wildlife and humans is available to implicate endocrine disrupting compounds as a legitimate cause of alterations in reproductive development and fertility. To that end the United States Environmental Protection Agency was mandated by the Food Quality Protection Act and the Safe Drinking Water Act to develop testing methods to identify potential endocrine disrupters (53, 61).

Brief review of antiandrogens studied

Antiandrogens are compounds capable of disrupting androgen signaling via a variety of mechanisms and may subsequently alter reproductive differentiation and development if male fetuses are exposed during critical periods of gestation (96). These antiandrogen-induced changes during differentiation of the male reproductive tract may lead to irreversible developmental abnormalities that manifest in the adult male. Chemicals or pharmaceuticals may alter androgen-mediated development by antagonizing the androgen receptor (AR), by disrupting 5α-reductase, the enzyme necessary for conversion of T to dihydrotestosterone (DHT), the androgen necessary for development of the urogenital sinus and external genitalia, or by causing decreased synthesis of T. Androgen receptor antagonists may block the action of androgens via two mechanisms, by binding to the hormone receptor but failing to bind to DNA response elements or by binding to the

receptor and subsequently binding to DNA but failing to initiate transcription (96). Examples of antiandrogens that antagonize the AR include flutamide, linuron, and fenitrothion.

Flutamide, a pharmaceutical compound developed for the treatment of benign prostatic hyperplasia and prostate cancer, is a potent AR antagonist (23, 144, 146). When male rats are exposed *in utero* to flutamide, they exhibit decreased anogenital distance (AGD) and increased areolae retention, which are morphologic indications of altered fetal androgen signaling (115). When males exposed to flutamide during gestation are examined as adults they have decreased accessory sex gland weights and lesions in the reproductive tract such as cryptorchidism and malformations, predominantly of DHT-dependent tissues (84, 115).

Linuron is an herbicide that induced an increased incidence of Leydig cell (LC) adenomas in rats following a two-year exposure (16, 44). Further studies utilizing a multigenerational protocol showed increased serum LH while levels of T were not increased (32). The authors of this study also demonstrated the ability of linuron to bind the AR. Linuron induction of LC adenomas was proposed to occur through disruption of the hypothalamus-pituitary-testis (HPT) axis (32). Several other studies confirmed the ability of linuron to competitively antagonize the rat and human AR; and in the Hershberger assay, this herbicide significantly reduced organ weights of androgen-dependent tissues (106, 118). The Hershberger assay is an *in vivo* test of the ability of a compound to bind to the AR. Males are castrated and supplemented with the synthetic androgen testosterone propionate (TP) while being exposed to the test chemical. If the compound competitively binds the AR, organ weights will be decreased in androgen-dependent tissues. The

supplemental testosterone ensures that androgen is available regardless of the effects of the test compound on the testes or the HPT axis. Studies of linuron using *in utero* and transgenerational exposures revealed the ability of linuron to cause decreased AGD and increased areolae retention (two DHT-dependent end points) and to induce male reproductive tract malformations (63, 116, 118).

Fenitrothion is an organophosphate insecticide that is structurally similar to flutamide (168). It was tested *in vitro* in a human AR binding assay and was found to competitively antagonize the AR (157, 168). Although low doses (0–3 mg/kg/day) of fenitrothion failed to reduce male reproductive organ weights in the Hershberger assay (164), doses of 15 and 30 mg/kg/day significantly decreased ventral prostate, seminal vesicle, and *levator ani bulbocavernosus* weights (168), which supports the *in vitro* AR binding data that fenitrothion is an AR antagonist. Two separate Hershberger assays using higher doses of TP (157, 164) failed to concur with the findings of the Tamura (2001) Hershberger study, as higher doses of TP were used in both of these negative studies making it more difficult for fenitrothion to antagonize the AR (157, 164, 168, 174). An *in vivo* study in which male fetuses were exposed to fenitrothion on gestation days (GD) 12 to 21 showed that high doses of fenitrothion caused decreased AGD on postnatal day (PND) 1 and increased areolae retention on PND 13; no significant effects were seen on male reproductive organ weights, nor was there any dose-responsive pathology in the male reproductive tract (174).

Another class of antiandrogens can exert their effects on male reproductive differentiation and development through inhibition of type II 5α-reductase. Competitive inhibition of this enzyme leads to reduced formation of DHT while having little to no effect

on T synthesis and T-mediated development (30, 58). Finasteride effectively inhibits type II 5α-reductase in the developing male fetus when pregnant dams are exposed during gestation. This inhibition leads to decreased AGD on PND 1 (18, 30, 58, 82) and increased nipple retention (18, 83). Malformations of the external genitalia, hypospadias (18, 30, 82, 84), and improper testis descent (18) also occurred following *in utero* exposure. One of the most significant findings following gestational exposure to finasteride is decreased prostatic budding (30, 58), which manifests as decreased prostatic size (84) or complete absence of the prostate (18) in adulthood.

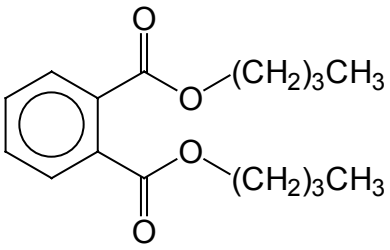


Figure 1.1. Di(*n*-butyl) phthalate.

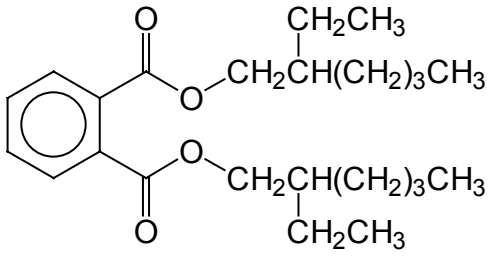


Figure 1.2. Di(2-ethylhexyl) phthalate.

Di(*n*-butyl) phthalate (DBP) (Figure 1.1) and di(2-ethylhexyl) phthalate (DEHP) (Figure 1.2) are phthalate esters of different chain lengths that have been shown to selectively alter male reproductive differentiation and development through decreased T production by the fetal testes when exposed *in utero* (107, 123, 138, 154). The two compounds also caused decreased AGD on PND 1 and increased areolae retention on PND 13 following gestational exposure (63, 138). The increased incidence of areolae retention is a permanent change, with adult males exhibiting retained nipples (63). In addition to alteration of DHT-dependent end points, AGD and areolae retention, both phthalates

induced a suite of male reproductive tract lesions following *in utero* exposure (63, 121, 122, 124). These lesions are found predominantly in T-dependent tissues, the Wolffian duct derivatives.

Reproductive development in the male rodent

Reproductive development in the male rodent is similar to that occurring in most mammals and can be divided into closely interrelated molecular, morphological, and hormonal events. A cascade of molecular signals begins the process that leads to the morphologic development of the testis and subsequently to the differentiation of the male reproductive tract and regression of the ducts that would become the female reproductive tract. Several hormones, including T, Mullerian inhibiting substance (MIS), and *insulin-like 3* (*Insl3*) gene product (relaxin-like factor), are directly involved in progression of male reproductive tract development and regression of the female tract (36, 88, 92, 127, 128). Disruption of any aspect of this intricately choreographed process may cause altered reproductive differentiation and permanent manifestations leading to reproductive tract malformations, infertility, and increased risk for the development of testicular neoplasia (60, 61, 129, 156, 162).

At the molecular level, sexual differentiation and development are a multiple-stage processes involving the formation of an undifferentiated gonad that is morphologically similar regardless of genetic sex (94, 112, 119, 182). Under the influence of a cascade of gene products this gonad develops into the testis, which then secretes hormones responsible for differentiation and development of the male reproductive tract and regression of primordial female reproductive tract structures (Figure 1.3) (119, 127). In a crucial experiment in 1947, Alfred Jost discovered that if one removes the undifferentiated gonads

of a developing fetus, these animals all develop as phenotypic females regardless of genetic sex (93). This led him to the hypothesis that a "testis-determining factor" existed that caused differentiation of the testis and development of the male reproductive organs. This factor, named *Sry* in the mouse, was not isolated and identified until 1990 (68, 103, 110). That a testis determining factor exists implies that a set of genes codes for development of the undifferentiated gonad regardless of sex and that an additional set of genes codes for proteins causing the development of the male and female phenotypes.

The formation of the undifferentiated gonad is initiated by activation of primordial germ cells (PGC) by bone morphogenic protein 4 (119). Stem cell factor (SCF) receptor (*c-kit*) is a PGC surface protein that acts as a receptor for *steel* or SCF. SCF is present on somatic cells along the migratory pathway, helping to guide leading the PGCs to the genital ridge (119, 147). Expression of steroidogenic factor 1 (SF-1) is essential for establishment of the bipotential gonad (112, 119, 147). SF-1 knockout mice fail to develop gonads, either testes or ovaries; however, morphologically they possess the internal reproductive structures of a female (111, 150). Expression of SF-1 first occurs in mice on GD 9–9.5 and continues to be expressed at high levels in Sertoli cells of the testis throughout gestation (137). Steroidogenic factor-1 may have a regulatory role in steroidogenesis and may be the signal that initiates steroidogenesis in the fetal rat (Figure 1.3) (102). Wilms' tumor 1 (WT1) is another gene expressed early in development and appears to be directly responsible for gonad formation (Figure 1.3) (125). WT1 knockout mice failed to develop kidneys or gonads (147, 166). The expression pattern and timing for WT1 is similar to SF-1 and are localized in undifferentiated mesenchymal cells, which may be Sertoli cell precursors since WT1 is also expressed in adult Sertoli cells (147). Another set of genes

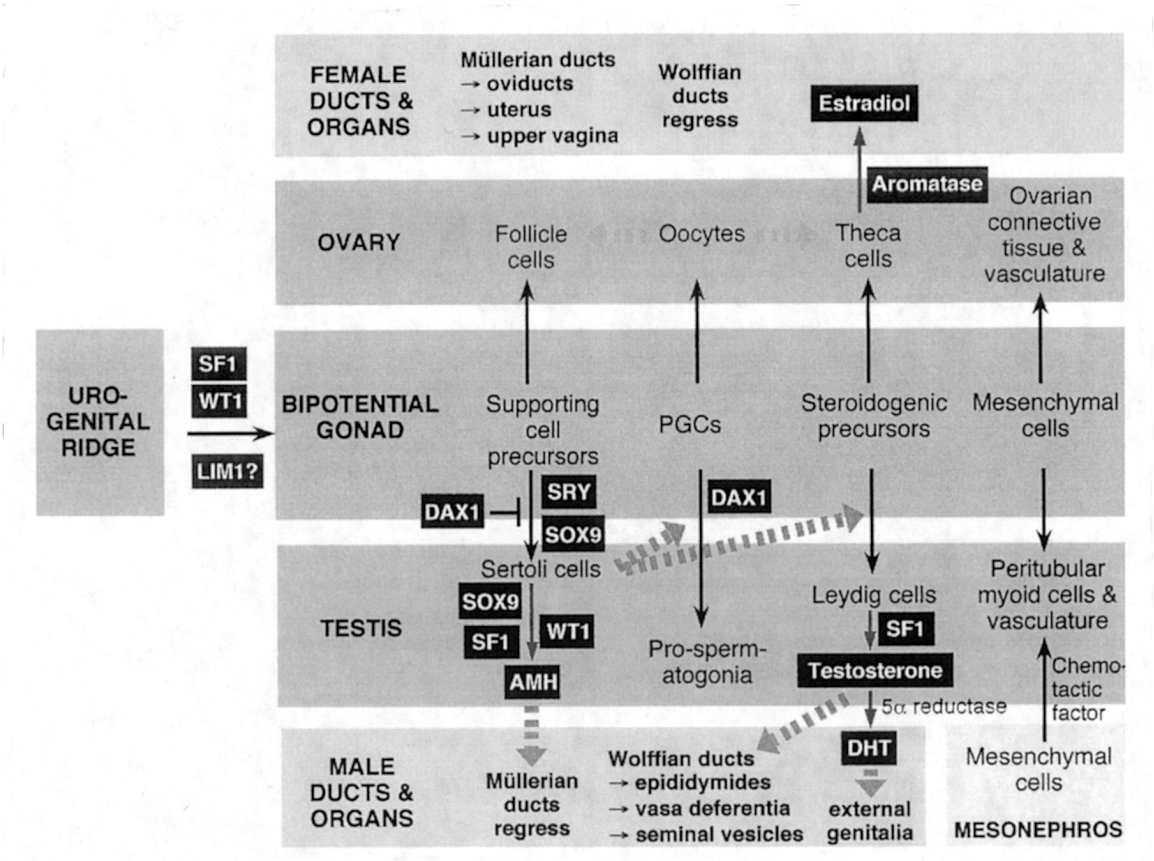


Figure 1.3. Schematic of genes involved in reproductive development and morphological changes occurring in the male and female reproductive tracts (102).

implicated in development of the gonad are Lim-1, Lim-9, and Emx2. Knockout mice for these genes fail to develop gonads, among other morphological structures, but their specific role in formation of the undifferentiated gonad is currently unclear (26). A host of other genes whose expressions have been noted in the undifferentiated gonad but whose roles are currently unknown.

Sry, the sex-determining region Y chromosome, is the gene that Jost believed to be responsible for the formation of the testis (Figure 1.3) (68, 103, 110). Sry is a transcription factor whose function, aside from generically determining testis formation, is not completely understood (26). Evidence suggests that SRY acts by repressing repressors of

testis formation and other evidence to suggest that it represses genes necessary for ovarian formation (119). Regardless, Sry is the one single protein that allows for formation of the testis, even in the genetic female. Sry is expressed early, at approximately the same time as SF-1 and WT1. Although the definitive role for Sry has not been determined, it is believed to be involved in the induction of Sertoli cell differentiation (119) and that this is a critical step in testis development . Expression of Sry is transient and is closely followed by Sox9, also in Sertoli cells (Figure 1.3) (99, 119). Expression of Sox9 is implicated in further differentiation of Sertoli cells. Other genes responsible for formation of the seminiferous cords and differentiation of the LCs have not been elucidated.

While the molecular aspects of early sexual differentiation are still being discovered, morphological events leading to development of the male reproductive tract are well characterized. All fetuses initially develop bipotential gonads and two sets of primordial ducts, the Wolffian ducts develop into the male reproductive tract and the Mullerian ducts differentiate into the female tract (Figure 1.4). As noted above, the formation of the bipotential gonad begins in the genital ridge where PGCs have migrated into a site of somatic cells derived from the coelomic epithelium (127). The PGCs originate in the epiblast of the gastrulating embryo and migrate on approximately GD 8.5 in the rat (112). They migrate from the extraembryonic mesoderm to the mesoderm of the primitive streak and then to the dorsal mesentery via the developing hindgut and allantois before ending their migration in the genital ridges (119). During migration the PGCs are proliferating and have reached a population of 2,500 to 5,000 cells by GD 11 (59). The Sertoli cell is the first somatic cell type to develop, initiating testis development (166). The Sertoli cells aggregate around PGCs and begin to form cords, which isolate the developing

gonocytes from the interstitium and the developing LCs (119). Gonocytes continue to proliferate until shortly before birth, at which time they become quiescent until the early postnatal period (55, 112, 166).

The Wolffian duct is derived from the mesonephros, one of the nephric structures to develop in male and female embryos (Figure 1.4) (166). The first nephric structure to develop is the pronephros, which leads to the formation of the mesonephros and metanephros and eventually regresses (59). The metanephros, the third nephric structure, eventually develops into the kidney. The mesonephros is adjacent to the developing genital ridge and eventually forms the excurrent ducts, the rete testis, for the testes (59, 166). Differentiation of the Wolffian duct occurs prior to formation of the Mullerian ducts, or paramesonephros, and is recognizable before formation of the gonad (22). The Mullerian duct, which develops from infolding of the coelomic epithelium, forms subsequent to the Wolffian duct and develops cranially to caudally following the course of the Wolffian ducts (36). Upon stimulation by androgens, the Wolffian ducts develop into the epididymides, *vasa deferentia*, and seminal vesicles, while the Mullerian ducts, which form the oviducts, uterus, and cranial portion of the vagina in females, regress secondary to release of MIS from Sertoli cells (Figure 1.4) (22, 59). The Wolffian ducts of females regress due to lack of androgens with passive maintenance of the Mullerian ducts (22).

Three essential hormones are necessary for proper development of the male reproductive tract. Androgens are necessary for stabilization of the Wolffian duct, testicular descent, and virilization of the external genitalia (127). MIS, produced by the Sertoli cells, causes regression of the Mullerian duct, inhibiting development of the female reproductive tract (127). The *Insl3* gene product of Leydig cells is necessary for the transabdominal

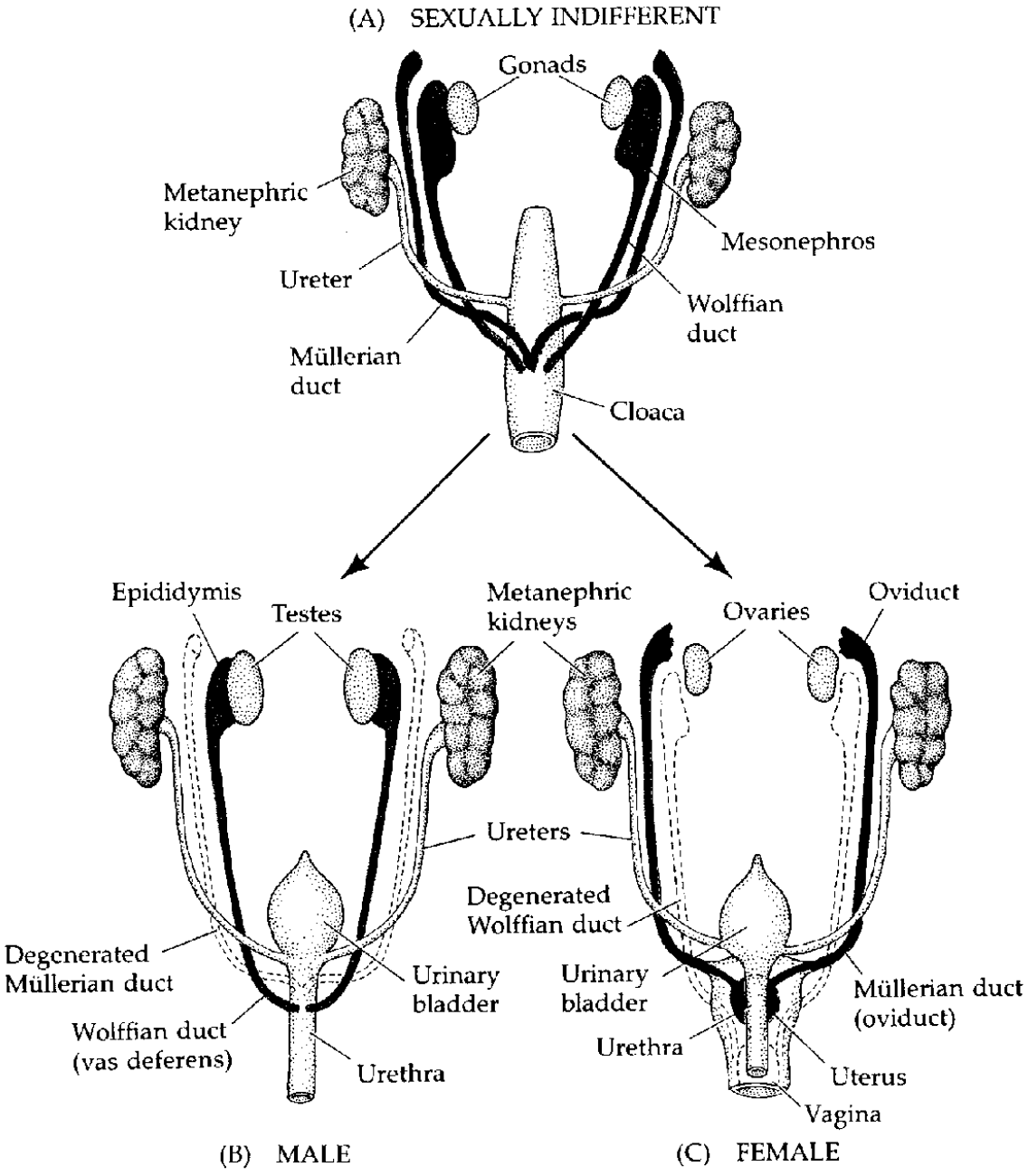


Figure 1.4. Male and female reproductive tract development from the Wolffian and Mullerian ducts (59).

phase of testicular descent (88). Gene expression for MIS in Sertoli cells begins approximately on GD 11.5 in the mouse and persists throughout the rest of the fetal period (127). Its receptor, MIS type II receptor, is expressed in the mesenchymal cells surrounding the Mullerian duct (78, 127). Mullerian duct regression occurs in a cranial to caudal fashion with apoptosis of the mesenchymal cells and degeneration of epithelial cells. Both the Wolffian and Mullerian ducts have developed in male MIS knockout mice making them male pseudohermaphrodites. The rest of the male reproductive tract develops normally, but the animals are infertile due to interference of the female ducts with sperm release (127).

Formation of androgens in the fetal testis occurs only in LCs (70). The steroidogenic pathway in the fetus is similar to the adult; in fact, T levels in the fetus are nearly the same as in adult males (80, 178). The formation of T begins around GD 14.5–15 and reaches its peak around GD 18–19 with a decline that continues until after birth (80, 170). The high-density lipoprotein (HDL) receptor on the cell surface (scavenger receptor class B-1) (SRB1) is responsible for uptake of cholesterol (3, 25, 173). In the fetus, large amounts of cholesterol esters are stored in large lipid vacuoles that are largely absent in adult Leydig cells (145). Cholesterol is carried within the cell by sterol carrier protein, which delivers the cholesterol to the mitochondria (120, 160). The first enzymatic step in the formation of androgens occurs on the inner membrane of mitochondria. Steroidogenic acute regulatory protein (StAR) is responsible for delivery of cholesterol across the outer membrane of mitochondria to P450 side-chain cleave enzyme (P450scc) on the inner membrane (72, 160, 161). In the rat, P450scc converts the cholesterol to pregnenolone, which is then carried to the smooth endoplasmic reticulum (SER), the most abundant organelle in fetal LCs (134, 145). Within the SER, pregnenolone is converted to

progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) (Figure 1.5) (49, 134, 176). Progesterone is subsequently acted on by P450c17, which catalyzes two reactions leading to the formation of 17-hydroxyprogesterone and androstenedione. Androstenedione is converted in the final enzymatic step to T by 17β-hydroxysteroid dehydrogenase (17β-HSD) (49, 134, 176).

Testosterone from the fetal testis is crucial for male reproductive differentiation and development. This androgen is responsible for stabilization of the Wolffian duct and formation of the epididymis, *vas deferens*, and seminal vesicles. Additionally, T mediates the inguino-scrotal portion of testicular descent. The gubernaculum contains large numbers of androgen receptors and androgen-responsive alterations in the gubernaculum may allow for increased abdominal pressure to push the testes into the scrotum (43, 81, 127). T is metabolized by 5α-reductase to the more potent androgen DHT. While both T and DHT bind to the same site of the AR, DHT has increased affinity for the receptor and is therefore the more potent androgen (13, 127, 165). DHT is responsible for development of the external genitalia including lengthening of the perineum (AGD) and androgen-induced apoptosis of the nipple anlagen (29, 30, 83, 84). DHT also mediates closure of the urethral folds of the developing penis so disruption in androgen synthesis, blockade of the AR, or inhibition of 5α-reductase may lead to malformations of the penis, termed hypospadias.

Testicular descent occurs in two stages. The testis develops in the abdominal cavity near the kidney. The testis must migrate across the abdomen to the inguinal region, where it passes through the inguinal canal to descend into the scrotum (81). While T mediates the inguino-scrotal phase of testicular descent, the *Insl3* gene product mediates the transabdominal phase (127, 184). This protein is responsible for relaxation of the cranial

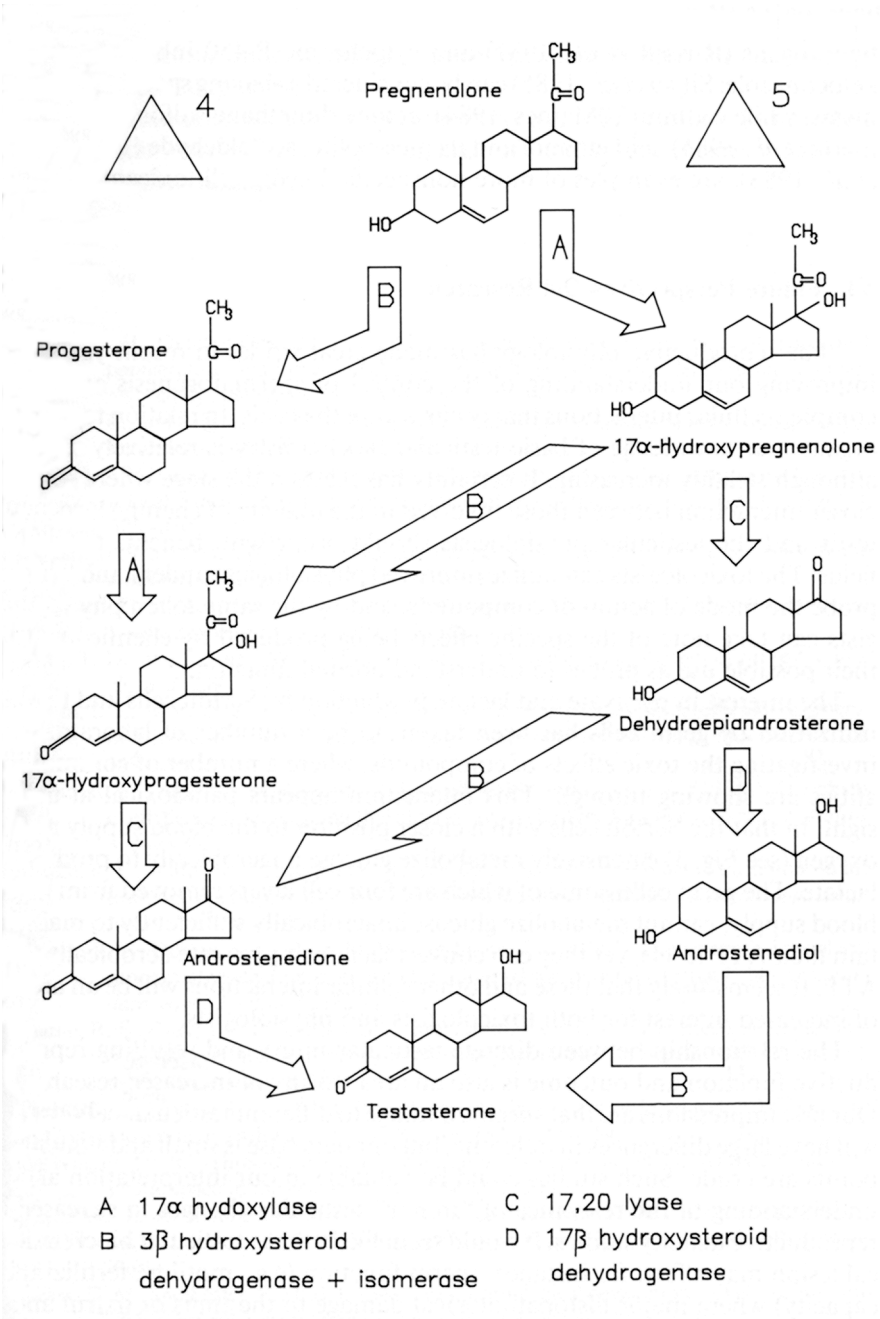


Figure 1.5. Testosterone biosynthetic pathway (49).

suspensory ligament, which normally holds the testis near the kidney, and outgrowth and contracture of the gubernaculum, which essentially pulls the testis towards the inguinal canal (184). The testis begins its decent from the kidney on GD 16 to 17 in the rat and

reaches the inguinal canal just before birth. Male knockout mice for *Insl3* are bilaterally cryptorchid (184). Analysis of these animals reveals that the gubernaculum is thin and elongated with improper development of the mesenchymal and muscle components and resembles a normal female gubernaculum.

The rat is the predominant species utilized in reproductive toxicology studies. This stems from several factors, including litter size and historical database. Although litter size varies among strains of rats, the average Sprague-Dawley rat in our laboratory has 12 to 14 fetuses or pups with a 1:1 sex ratio. This number of fetuses or pups gives a large sample for which assessment of toxicologic end points can be made. Although mice also have large litters, the pups are much smaller making morphologic evaluation of the reproductive tract difficult. Other species such as guinea pigs and rabbits bear larger young, but their litter sizes are typically smaller. Since the rat has been used for so long, there is a large historical database on this species for most end points in reproductive toxicology. This database aids in the interpretation of results for which there is a minor or rare change, for example, upon gestational exposure to a compound a 3% incidence of LC adenomas in 90-day-old rats is observed. This change is significant in the Sprague-Dawley rat, as the lifetime incidence for this neoplasm in this strain of rat is only 4–5% and they generally do not develop this neoplasm until late in life.

Although the rat is widely used in reproductive toxicology studies, differences between this species and humans should be considered before extrapolating rodent data to humans. One of these is the timing of differentiation and development of the male reproductive tract. In the rat, these occur during the last half of gestation with organogenesis of most of the reproductive organs during the last third of gestation. This is

in contrast to the human where most of reproductive differentiation of the male occurs during the first trimester (79, 129). The PGCs in humans are first identified around week four of gestation, and the fetal testis is formed by gestation week 8 with completion of the formation of the reproductive organs by week 16 to 20, the first half of gestation.

Two pathways for T synthesis, the δ4 and δ5 pathways, are present in rodents and humans (Figure 1.5). The rat preferentially synthesizes T via the δ4 pathway, with the formation of progesterone from pregnenolone; in the human, the δ5 pathway is preferential, with the formation of 17-hydroxypregnenolone before the synthesis of progesterone (139). In addition, the primary source of cholesterol for T synthesis in the human is low-density lipoprotein, while the preferential source of cholesterol in the rat fetus is HDL (3, 120). T levels do not reach their zenith in the rat until only a few days prior to birth, GD 18–19, while T levels in humans peak between gestation weeks 10 and 12 (79). Another aspect of male reproductive development that differs between humans and rats is the timing of testicular descent. In the human, the testes have completed descent by about gestation week 35; thus the testes are descended at birth (127). In the rat, the gubernacular bulb is at the inguinal canal on GD 21, and testicular descent occurs shortly after birth. The differences between human and rat developmental chronology must be taken into consideration when evaluating toxicology and exposure data from the rat and attempting to extrapolate the data to humans.

Phthalate esters and human exposure

Phthalate esters are ubiquitous environmental contaminants that have been identified in the soil, water, fish, wild and domestic animals, and humans (75, 85, 86, 89, 90, 113, 126, 149, 183). They are commercially synthesized from phthalic anhydride and

an alcohol of the appropriate carbon length (171). The designation "phthalate ester" is generally reserved for the *ortho* form versus the *meta* and *para* forms (Figures 1.1 and 1.2) (100). Phthalate esters are high-production-volume chemicals with DEHP comprising over 50% of the total of all phthalates produced, in excess of 1,000,000 tons per year (85). Even though it is also considered a high-production-volume chemical, only 11,400 tons of DBP were produced in the United States in 1987 (86). Phthalates are widely used, especially DEHP, in the production of polyvinyl chloride, to impart flexibility to the final product. DBP uses include latex adhesives, cellulose plastics, and solvent for dyes. DBP is also used in personal care products as a perfume solvent and fixative, as a suspension agent for solids in aerosols, as a skin emollient, and as a plasticizer in nail polish and hair spray (86, 95, 136).

Phthalate esters are not covalently bound to the final manufactured goods into which they are incorporated, and therefore they have a tendency to leach from the final product during use or following disposal (86, 95). The predominant route of exposure to humans is through ingestion of food containing phthalates that have leached out of containers or wrappings. Phthalates are hydrolyzed to their monoester forms by nonspecific hydrolases in the intestinal epithelium and liver (105, 148, 179). The monoester is more rapidly absorbed following ingestion, most likely because of its increased water solubility (180). Phthalates are rapidly metabolized and excreted in the urine within 48 hours following a single oral dose in the rat (169). Similar to excretion in the rat, DEHP infused intravenously into humans and primates is rapidly metabolized and predominantly excreted in the urine (2, 140–142).

Exposures of the general population to phthalates have been estimated by the International Programme on Chemical Safety, the United Kingdom Ministry of Agriculture, Fisheries, and Food, Health Canada, and the United States Agency of Toxic Substances and Disease Registry. People may be exposed via inhalation, dermal absorption, ingestion, or intravenously due to leaching of DEHP from polyvinyl chloride bags and tubing (74, 85, 86, 143, 149). The daily estimated exposure of DBP from all sources for the general population is 2–10 μg/kg/day (86). However, several studies have recently shown that exposure to phthalates for certain segments of the population appears to be higher than originally thought. A recent study by the Centers for Disease Control and Prevention found higher levels of phthalate metabolites than previously estimated in a general population study of 289 adult humans (15). Since phthalates are ubiquitous contaminants of laboratory equipment, a method was developed whereby only specific metabolites of phthalates were detected, eliminating potential contamination by parent diesters (20). Levels of metabolites for individual phthalates did not correlate with their production volumes, that is, DBP is a low-production-volume phthalate, and yet the second highest concentration of a phthalate metabolite in urine from this population was for DBP. Additionally, women of child-bearing age(20–40 years old) had six of the eight highest levels of DBP metabolites. Similar levels of urinary phthalate metabolites were found in a separate study of African-American women (77). In a small study of children in California, levels of metabolites for DBP, DEHP, and benzylbutyl phthalate (BBP) were higher than the 50^{th} percentile of the Blount (2000) adult study, suggesting higher exposure of children to these phthalates (19). While these studies measure urinary metabolite levels, correlating these levels with actual exposures has been difficult. Two separate investigators modeled

Blount's data (2000) using creatinine excretion ratios and achieved similar proposed phthalate exposures (34, 101). However, these figures were estimated based on pharmacokinetics in rodents and primates and corrected for creatinine excretion. A study in the United Kingdom using human volunteers exposed people to known amounts of phthalates and measured the urinary metabolites (4). The data from this study are invaluable since the metabolites produced from known phthalate exposures were measured; thus future studies utilizing urinary metabolite levels will be able to give a more accurate estimate of phthalate exposure.

Effects of phthalates on the male reproductive system

Most of the work on phthalate esters as a class has been performed on adult animals. This research dates back to the early 1940s when the first abstract and manuscripts were being published on the effects of DEHP (76, 151). Since then a plethora of data has been published on the effects of phthalates of different chain lengths, species and age sensitivity to phthalates, and general effects on the adult male reproductive tract.

As previously described, phthalate esters of different chain lengths, including straight and branched chains, can be synthesized. Multiple studies have shown that phthalate esters of medium chain length, C4, C5, and C6 di-*n*-alkyl phthalates, are toxic to the male reproductive system, with dipentyl phthalate (DPP) being the most toxic (54). Dibutyl, DPP, and dihexyl phthalates have been shown to produce testicular lesions similar to DEHP, while no lesions were seen with dimethyl, diethyl, dipropyl, diheptyl, or dioctyl phthalate (C1–C3 and C7–C8) (64). In addition, different isomers of the phthalate ester mono-*n*-butyl phthalate may also affect the toxicity profile (52). Similar to Foster et al. (1980), Oishi et al. found decreased testis weight and testicular histologic lesions following

treatment with DBP, di(iso-butyl) phthalate, and DEHP (132). In addition, these phthalates caused increased intratesticular T levels. In a separate study, Oishi et al. found that treatment with mono(*n*-butyl) phthalate (MBP), mono(iso-butyl) phthalate, and mono(2-ethylhexyl) phthalate (MEHP) (the monoesters from the previous experiment) caused decreased testicular and body weights (133). Interestingly, in a 1966 study, increased molecular weight of phthalates caused a decrease in acute toxicity (LD_{50}), with DEHP having the lowest acute toxicity, even though it is one of the most toxic phthalates to the adult male reproductive tract (24). Most of the toxic phthalates caused decreased testicular and liver zinc concentrations and increased urinary excretion of zinc (54, 132, 133). Whether the effects on zinc were a cause or an effect of phthalate-induced testicular lesions was not clear (50). Overall, lesions secondary to phthalate exposure in the adult rat are confined to the testes with decreased weights of this organ and decreased zinc concentrations. Severity of the lesions and the amount of decrease in weights were directly related to the dose of the phthalate and the duration of treatment (24, 50, 52, 54, 64, 76, 132, 133, 151).

Several critical studies have identified species differences in sensitivity to phthalate esters (50, 67). In one experiment, testicular weights and histopathology indicated that the rat was the most sensitive of species followed closely by the guinea pig (67). The mouse was also sensitive to the effects of phthalates but had only mild decreases in testicular weights and minimal testicular lesions. The hamster was almost completely refractory to phthalate-induced testicular pathology. Whereas DBP, MBP, DEHP, MEHP, and DPP all cause severe testicular degeneration in the rat, only MEHP caused this lesion in the hamster, which was minimal in comparison with the rat (67). In addition, increased urinary

excretion of zinc and decreased concentrations of testicular zinc occurred in the rat, while there were no effects on zinc levels in the hamster. A study was performed by Foster et al (1982) to elucidate the mechanism of species differences. They found increased levels of free MBP and DBP, versus the glucuronidated form, in the rat along with higher levels of MBP in rat urine, implicating higher levels of the toxic moiety in the rat and possibly the reason for the more severe testicular lesions compared with the hamster. In addition, β-glucuronidase activity in the rat testis was much higher than in the hamster, the amount of free DBP and MBP, the toxic phthalate moiety, was present in the hamster testis was much smaller.

Since phthalates act as peroxisome proliferators, there was speculation that the testicular toxicity attributed to phthalates would not be relevant to humans, that is, phthalate toxicity would be rodent-specific. A peroxisome-proliferator-activated-receptor-α (PPARα) knockout mouse was generated that lacked the receptor to which phthalates bound (177). In a study of phthalate exposure using this mouse, testicular lesions still occurred implicating a PPARα-independent mechanism of testicular toxicity. Supporting this was the development of testicular lesions in guinea pigs, a nonperoxisome-proliferator-responsive species (67, 73), and ferrets, which are nonrodents (104). Together, these data indicate that humans may be susceptible to the male reproductive effects of phthalates.

In addition to species differences, marked differences in age sensitivity to phthalate esters have also been observed (35, 66, 155). Sjoberg et al. (1986) exposed rats to DEHP for 14 days starting on PND 25, 40, and 60. They found that body weights and testicular weights were markedly decreased at both a high and low dose on PND 25, were reduced only at the high dose on PND 40 and were unaffected at either dose on PND 60. Testicular

lesions included germinal epithelial degeneration in all tubules of the PND 25 rats and milder damage in PND 40 rats (155). Sertoli and germ cells of the seminiferous tubules were hypothesized to be more sensitive in younger rats. However, measurements of MEHP in plasma and urine showed that young animals had higher levels of both, implicating increased absorption and metabolism of DEHP and therefore increased exposure to the toxic metabolite (155). In this study, animals were also dosed intravenously for approximately 6 days starting on PND 25 and 40 with relatively few effects and no difference between the younger and older animals. The reason for the lack of response following intravenous administration was not completely understood but is likely due to differences in metabolism between oral and intravenous dosing (155).

A similar study by Gray et al. (1986) also showed that younger animals were more sensitive to the effects of phthalates. DEHP caused decreased testicular, seminal vesicle, and prostatic weights at 4 weeks of age while animals at 10 weeks of age only had decreased weights of seminal vesicles and prostates and no effects on organ weights at 15 weeks of age. This study also showed that the immature rats had decreased fluid secretion from the testes and that androgen binding protein production per testis was decreased (66). Dostal et al. (1988) also showed that younger animals were more sensitive to the effects of DEHP and that the Sertoli cells appeared to be the primary target since the number of Sertoli cells was decreased in animals treated at one week of age. Following a recovery period, however, the number of Sertoli cells returned to normal with only minor effects on spermatogenesis (35).

It is well documented that exposure of immature and mature animals to phthalates leads to decreased body weight, decreased testicular weights, increased liver weight,

decreased weight of the accessory sex glands, and testicular lesions (1, 28, 54, 57, 65, 66). However, these alterations are dependent on age of the animals, species, the particular phthalate ester, dose, and duration of treatment. Although the chronic testicular lesions of phthalate exposure were well characterized, acute lesions caused by exposure to di-*n*-pentyl phthalate remained unknown (33, 51). Studies by Foster and Creasey (33, 51) identified the Sertoli cell as the initial target of phthalate toxicity. Three hours after a single dose of DPP, perinuclear vacuolation of the SER was observed in approximately 20% of the tubules (33). This vacuolation increased in severity over the next couple of hours so that by six hours postdosing Sertoli cell cytoplasmic vacuolation of the apical membrane was present in all of the tubules (33, 51). At this time, early degeneration of spermatocytes and spermatids had also occurred. There was extensive degeneration of the germinal epithelium 24 hours following the dose of DPP. An unexpected lesion at 6 hours was an acute inflammatory infiltrate, but this had resolved by 24 hours after the dose (33). Following daily dosing over the course of four days, there was germinal cell degeneration leaving seminiferous tubules lined by Sertoli cells only (33, 51).

Di(*n*-butyl) phthalate

The testicular lesions observed following exposure of adults to DBP were similar to those described in the section on general phthalate toxicity of the male reproductive system. There were decreased testicular weights and marked degeneration of the germinal epithelium (56, 159). While a significant amount of research has been performed with DBP on postnatal animals, relatively few investigators have examined the effects of DBP on the developing male reproductive tract. Ema et al. have performed a series of studies with DBP, BBP, and MBP using an *in utero* exposure regimen with examination of fetuses on

the last day of gestation. Experiments utilizing these phthalates showed that all three compounds caused similar teratogenic effects including skeletal malformations, cleft palate, and undescended testes when dosed on similar days (38, 39, 41). While mono(*n*-benzyl) phthalate and MBP are both formed from the metabolism of BBP, MBP is produced in higher quantities and is the more stable metabolite in the rat (37, 38). However, this is not true in the human, as the monobenzyl metabolite is the predominant form due to preferential cleavage of the butyl ester link of BBP (4). At doses of 555 and 661 mg/kg/day of DBP on GD 11 to 21 Ema et al. (40) observed undescended testes but no other lesions in the male reproductive tract . When dosed early in gestation increased pre- and postimplantation loss occurred (42). However, these losses occurred at doses of 750 mg/kg/day and higher with maternal toxicity. When animals were dosed with 500, 1000, or 1500 mg/kg/day on GD 12 to 14, 15 to 17, or 18 to 20, the most susceptible window of exposure was found to be GD 15 to 17, at which time an increased incidence of undescended testes over the other time points was noted (41). In a final study by Ema et al. (39), fetuses were dosed with 250, 500, and 750 mg/kg/day of MBP on GD 15 to 17 . Undescended testes were observed down to 250 mg/kg/day, while fetal toxicity was noted only at 750 mg/kg/day. Ema's studies illustrated that MBP appears to be the toxic metabolite of DBP and that undescended testes could be induced at doses as low as 250 mg/kg/day during a three-day gestational window.

DBP was studied by the National Toxicology Program (NTP) in their Reproductive Assessment by Continuous Breeding protocol (131, 181). In that study, it was shown for the first time that animals exposed to DBP *in utero* developed more severe lesions of the male reproductive tract than animals exposed as adults only. Of the animals examined,

50% exposed gestationally, lactationally, and during maturation to 794 mg/kg/day of DBP had lesions of the epididymides described as absent or markedly underdeveloped. Males exposed to 385 and 794 mg/kg/day had seminiferous epithelial degeneration. In addition, of the 20 matings that occurred using F_1 males, only one litter was produced versus 20 matings and 20 litters produced by the exposed parenteral generation. The most significant finding from this study was that the males exposed *in utero* were more sensitive to the effects of DBP than immature or adult animals.

The results of the studies by the NTP led Mylchreest et al. to perform a series of studies further evaluating *in utero* exposure to DBP. Their protocol included *in utero* exposures followed by maturation to adulthood to allow for examination of reproductive tract development following gestational exposure (121). Gestational and lactational exposure to DBP at 250, 500, and 750 mg/kg/day led to decreased male AGD on PND 1, hypospadias, absent or malformed epididymides, decreased reproductive organ weights, absence of male accessory sex glands, ectopic or absent testes, and marked seminiferous tubular epithelial degeneration (121). While a small number of alterations in the female reproductive tract were observed, they were only in the high-dose group, and there were no alterations in estrogen-dependent end points such as time to vaginal opening or estrous cyclicity. Taken together, these data support an antiandrogenic mechanism of action for DBP rather than an estrogenic mechanism as had been proposed by others (71, 91). In addition, *in utero* and lactational exposure alone could lead to abnormal development of the male reproductive tract (121).

In a second study (122) utilizing *in utero* exposure alone on GD 12 to 21 with examination of the males as adults, the effects of DBP at 100, 250, and 500 mg/kg/day

were compared with the potent AR antagonist flutamide. Di(*n*-butyl) phthalate produced the same lesions as described with gestational and lactational exposure (121). In addition, concentrations of DBP as low as 250 mg/kg/day were found to decrease AGD on PND 1 and induce retained areolae on PND 13 (122). 500 mg/kg/day also induced a low incidence of LC adenomas. The finding of LC adenomas in 90-day-old rats is unprecedented, especially in a strain (Sprague-Dawley) that has a low lifetime incidence of this neoplasm. The findings secondary to *in utero* DBP exposure contrasted to flutamide exposure in that DBP induced a low incidence of cryptorchidism while flutamide at 100 mg/kg/day induced a high incidence of ectopic testes. A difference in the incidence of hypospadias, with DBP inducing hypospadias in approximately 40% of the animals exposed to 500 mg/kg/day of DBP while flutamide exposure induced nearly a 100% incidence, was observed. Also, the incidence of prostatic and epididymal lesions was much higher with flutamide exposure. Whereas DBP acted as an antiandrogen when male rats were exposed *in utero*, the pattern of lesions and incidence was different from flutamide. Although both compounds acted as antiandrogens, their different mechanisms led to separate adult phenotypes following *in utero* exposure.

Since a no-observed-adverse-effect level (NOAEL) was not identified in the first two Mylchreest studies (1998 and 1999), a further dose-response study was performed. Animals were dosed with 0.5, 5, 50, 100, and 500 mg/kg/day of DBP on GD 12 to 21 and necropsied as adults (124). AGD was decreased only at 500 mg/kg/day while areolae were retained at 100 mg/kg/day and higher. All other alterations noted were similar to those previously described and occurred only in the 500 mg/kg/day dose group (121, 122, 124), thus establishing a lowest-observed-adverse-effect level at 100 mg/kg/day and a NOAEL at

50 mg/kg/day, the lowest observed NOAEL for DBP at that time. Similar to the 1999 Mylchreest study, a LC adenoma was identified in a young adult male following *in utero* exposure to 500 mg/kg/day of DBP.

The DBP-induced developmental lesions in tissues derived from the Wolffian ducts and the knowledge that these tissues are T-dependent led Mylchreest et al. (123) to study the fetal rat testis to determine whether pathological changes following gestational exposure to DBP were present and also to determine the levels of T in fetal testes . Male fetuses were exposed to 500 mg/kg/day of DBP on GD 12 to 21 and were examined on GD 14, 16, 18, and 21. No lesions were identified on GD 14, while areas of LC hyperplasia were described on GD 16, 18, and 21 . These areas of hyperplasia contained large numbers of LCs that were AR-positive and variably positive for 3β-HSD. On GD 18 and 21, the testes were visibly smaller but contained enlarged seminiferous tubules with increased numbers of gonocytes and multinucleated gonocytes. Epididymides on these same gestation days had decreased coiling of the epididymal duct. Analysis of fetal intratesticular T levels showed that T was markedly decreased to 34 and 26% of control on GD 18 and 21. DBP induced morphological changes in fetal LCs and germ cells and caused a decrease in intratesticular T at a time when fetal T levels are supposed to be at their highest. Mylchreest et al. (123) speculated that this decrease in fetal T led to abnormal development of the tissues derived from the Wolffian ducts .

Gray et al. (63) conducted several studies examining the effects of *in utero* exposure to DBP and determined whether DBP had any *in vivo* estrogenic effects . In a uterotropic and sex behavior assay, DBP did not have any *in vivo* estrogenic effects. In a multigenerational study, the F_1 animals were exposed only during gestation and lactation.

These animals exhibited decreased fertility and had a low incidence of male reproductive tract malformations, including hypospadias and cryptorchidism. In a transgenerational exposure study utilizing Long-Evans hooded rat, dams were treated with 500 mg/kg/day of DBP on GD 16 to 19. This exposure led to decreased AGD, increased areolae retention that was also seen during adulthood (i.e., it was a permanent effect) and decreased weights of androgen-dependent tissues. In a second transgenerational study, Sprague-Dawley rats were exposed to DBP from GD 14 to PND 3. Findings in this study were similar, but the effects were more pronounced because of the longer dosing period, 12 days versus 4. These results of these studies are similar to those of the NTP study and Mylchreest et al., while this was the first report of permanently retained nipples in male rats exposed gestationally to DBP (63).

Given the morphological alterations and decreased T in the fetal testis, Shultz et al. designed a study to investigate potential molecular alterations in the fetal testis and to determine whether changes in gene expression could be attributed to *in utero* DBP exposure. Male fetuses were exposed to DBP from GD 12 to GD 16, 19, or 21. Testicular RNA from a single fetus from each of three dams (n = 3 fetuses examined) was used for microarray analysis, and an additional three fetuses were used for RT-PCR. Microarray analysis on GD 16 was not possible due to marked variability on the blots. On GD 19 and 21, multiple genes were found to be altered by DBP exposure. Genes involved in steroidogenesis that were upregulated included SRB1, testosterone-repressed prostate message-2 (TRPM-2), and leutinizing hormone receptor. These genes and several others in the steroidogenic pathway, including StAR, P450scc, and P450c17, were examined with RT-PCR to confirm the findings of the microarrays. Decreased gene expression for SRB1,

StAR, P450scc, and P450c17 was observed. These genes are directly involved in T synthesis, and therefore alteration of gene expression may have led to decreased production of T or possibly DBP-induced decrease in T production may have led to end-product inhibition of gene expression for these steroidogenic proteins.

CHAPTER TWO

Experimental Hypotheses and Objectives

Hypothesis 1: Decreased fetal T induced by *in utero* DBP exposure leads to altered morphogenesis of the male reproductive tract that begins in the fetus and is ultimately manifest in the adult male reproductive tract.

Extensive studies by Mylchreest et al. (1998, 1999, 2000) and Gray et al. (1999) have led to the understanding that *in utero* exposure to DBP leads to gross malformations in the tissues of the male reproductive tract accompanied by histological changes in the germinal epithelium of the testis. Limited studies by Mylchreest et al. (2002) and Shultz et al. (2001) showed the presence of histologic lesions in the fetal testis on several gestational days. However, the time course of lesion development, from fetal testicular lesions to gross malformations in the adult, was unknown. How the testicular lesions progressed with time to the ultimate adult phenotypes was also unclear. In the current study, males were exposed *in utero* to 500 mg/kg/day of DBP on GD 12 to 21. Testes and epididymides were examined on GD 17 to 21 to more fully understand the progression of fetal lesions. Postnatally, males were examined on PND 3, 7, 16, 21, 45, and 70 to follow the course of lesion development. Both the gross manifestations and the histologic changes in the testes and epididymides were described. This was the first study to describe DBP-induced male reproductive tract lesions over a continuum from the fetus to the adult. The manuscript from these data is currently in press in *Toxicologic Pathology* (Chapter 3).

Hypothesis 2: Fetal DBP exposure leads to altered gene expression in fetal testes that has consequences for fetal testosterone levels and long-term reproductive development.

Studies by Mylchreest et al. (2002) and Shultz et al. (2001) showed decreased levels of fetal intratesticular T following *in utero* DBP exposure, while a study by Lambright et al. (2003) showed decreased fetal testicular T production, also following gestational DBP exposure. The mechanism of this decreased T synthesis is not completely understood. Gene expression data from the study by Shultz, et al. (2001) indicated decreased gene expression for several of the steroidogenic enzymes in the T biosynthetic pathway. However, the data were generated using a small number of animals, one fetus per dam from three dams per group, for a total of three animals per group. They also did not analyze all the genes in the biosynthetic pathway. Therefore to confirm and extend the findings of Shultz et al., a study was conducted using a robust study design that included additional dams, a total of five per group, and additional fetuses, three per dam, for a total of 15 animals per treatment group. All the genes in the T biosynthetic pathway, from SRB1 (responsible for HDL uptake into the LCs) to 17β-HSD (responsible for the final enzymatic conversion of androstenedione to T) were analyzed. Additional genes related to androgen signaling or testicular development were also examined to help elucidate the molecular pathogenesis of gestational DBP exposure. The manuscript from these data has been submitted to *Toxicological Sciences* (Chapter 4).

Hypothesis 3: Altered testosterone levels and fetal Leydig cell lesions produced by DBP lead to Leydig cell adenomas in adult animals.

Mylchreest et al. found LC adenomas in two separate studies following *in utero* DBP exposure. The incidence of these adenomas was approximately 3%, which falls within the lifetime incidence range for LC adenomas in the Sprague-Dawley rat. However, these adenomas were observed at 90 days of age, while the incidence in control populations was based on two-year bioassays. Also, the animals were exposed to DBP only over a short gestational window, implicating DBP to be a transplacental carcinogen. Whether the incidence of LC adenomas was increased due to *in utero* DBP exposure or whether the lifetime incidence was the same as control animals but the adenomas were expressed earlier in life was unknown. Additionally, although AGD and areolae retention were known to be altered by gestational DBP exposure, it was not known whether these changes were permanent or whether these external, sexually dimorphic end points would be predictive of internal reproductive malformations. A study was designed to test the hypothesis that *in utero* DBP exposure leads to increased numbers of LC adenomas and also to determine whether alterations in AGD and areolae retention were permanent. Pregnant Sprague-Dawley rats were exposed to 100 and 500 mg/kg/day of DBP on GD 12 to 21, and male offspring were allowed to mature to 6, 12, and 18 months of age. The testes were examined histologically to determine the incidence of LC adenomas. Additionally, the AGD was measured on PND 1 and 180 to determine permanence of the effect and whether an association with gross or histologic lesions of the male reproductive tract existed. The same analysis was performed for areolae retention on PND 13 and 180. The manuscript generated from these data, intended for submission to *Toxicologic Pathology*, is currently under review and revision by the authors (Chapter 5).

The overall objectives of my thesis research were to study the pathogenesis of male reproductive tract lesions resulting from *in utero* DBP exposure, from the fetus to the adult, to examine the molecular changes induced by DBP on genes in the steroidogenic pathway and other genes involved in testicular differentiation, and to investigate the potential transplacental carcinogenic effects of DBP. All the studies used similar study designs of gestational DBP exposure on GD 12 to 21, the critical developmental window for the male reproductive tract. Specific methods, results, and discussions relevant to each major area of study are covered in the individual chapters of this thesis. Each chapter represents a manuscript being prepared and submitted for publication in peer-reviewed journals. In addition to the specific research on DBP, I was able to participate in the investigation of rodent reproductive development following *in utero* exposure to other antiandrogens, including linuron, flutamide, fenitrothion, and finasteride. Taking part in the these studies and performing all the pathological examinations allowed me to make unique evaluations on the phenotypes induced by each compound and compare them with those induced by DBP. These findings will be covered in the General Discussion of this dissertation (chapter 6).

CHAPTER THREE

PATHOGENESIS OF MALE REPRODUCTIVE TRACT LESIONS FROM GESTATION THROUGH ADULTHOOD FOLLOWING *IN UTERO* EXPOSURE TO DI(*N*-BUTYL) PHTHALATE

Norman J. Barlow[1,2] and Paul M. D. Foster[3]

[1]CIIT Centers for Health Research

Research Triangle Park, North Carolina

[2]North Carolina State University

Raleigh, North Carolina

[3] National Institute of Environmental Health Sciences

Research Triangle Park, North Carolina

ABSTRACT

Di(*n*-butyl) phthalate (DBP) acts as an antiandrogen by decreasing fetal testicular testosterone synthesis when male rats are exposed *in utero*. DBP-exposed male rats develop malformations of the reproductive tract secondary to the reduced fetal androgen levels. However, these malformations and the associated histologic lesions have only been described in adult rats. The objective of this study was to describe the male reproductive tract lesions in fetal, early postnatal, and young adult male rats following DBP exposure *in utero*. Pregnant Sprague-Dawley rats were exposed to 500 mg/kg/day DBP by gavage on gestation days (GD) 12 to 21. Male reproductive tracts were examined on GD 16 to 21 and on postnatal days (PND) 3, 7, 16, 21, 45, and 70. In the fetal testes, large aggregates of Leydig cells, multinucleated gonocytes, and increased numbers of gonocytes were first detected on GD 17 and increased in incidence to 100% by GD 20 and 21. These lesions resolved during the early postnatal period, while decreased numbers of spermatocytes were noted on PND 16 and 21. On PND 45, there was mild degeneration of the seminiferous epithelium, which progressed to severe seminiferous epithelial degeneration on PND 70. On PND 70, the degeneration was concurrent with ipsilateral malformed epididymides, which caused obstruction of testicular fluid flow and secondary pressure atrophy in the seminiferous tubules. In the fetus, the epididymal lesion was observed as decreased coiling of the epididymal duct. The decreased coiling progressed into the early postnatal period and adulthood, at which time malformed epididymides were apparent. As the animals were only dosed *in utero*, these findings indicate that DBP can initiate fetal testicular and epididymal changes that may not manifest as clear malformations until adulthood. The

pathogenesis of lesion development from the fetus to the adult is important for comparison of antiandrogens with differing modes of action.

INTRODUCTION

Antiandrogens have the potential to perturb male reproductive development and function. They can act via a variety of mechanisms, including decreased androgen synthesis, disturbance of the pituitary-gonadal axis, and blockade of the androgen receptor (AR) (2, 10, 16, 28). Environmental antiandrogens have recently gained much attention in the lay and scientific literature for their ability to disrupt reproductive development when male offspring are exposed during gestation. Endocrine-active agents such as di(*n*-butyl) phthalate (DBP), linuron, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p*,*p*′-DDE), and vinclozolin are readily found in the environment and have been shown to disrupt the androgen-dependent development of male rat pups born to dams exposed to these agents during pregnancy (11). Different phenotypes of reproductive lesions result from antiandrogens that share a common mode of action as well as those that act via different mechanisms (11, 17, 19, 20).

DBP is an intermediate-length phthalic acid ester that has recently been shown to have antiandrogenic properties when male rats are exposed *in utero* (11, 20, 23). Due to its widespread use, there is a potential for human exposure via direct contact, ingestion, or inhalation. Estimates of daily human DBP exposure from all sources were 0.03 to 15.9 μg/kg body weight per day (26). Based on measurements of the DBP metabolite monobutyl phthalate in the urine, another group estimated the average daily DBP intake to be 6.87 μg/kg body weight per day (4, 5, 15). While similar to estimated exposures, the level of exposure to DBP reported in the study by the Centers for Disease Control and Prevention (4) is not in proportion to the overall production volume of DBP. The authors proposed that

exposure was due to the use of DBP in personal care products. They also noted that women of childbearing age, 20 to 40 years old, had significantly higher estimated exposures (up to 117 μg/kg body weight per day) than the general population (4).

In a multigenerational study, F_1 male rats exposed to 256 and 509 mg/kg/day of DBP throughout gestation, lactation, and following weaning displayed seminiferous epithelial degeneration and Leydig cell hyperplasia in the testes, malformed epididymides, and hypospadias (25, 32). In contrast, no effects were present in the F_0 generation, indicating that gestational and early postnatal exposure were required for in the development of reproductive tract lesions in the male rat. Mylchreest et al found that when pregnant female rats were exposed to 100, 250, and 500 mg/kg/day of DBP during the last 10 days of gestation, the androgen-dependent tissues in the adult male offspring had various lesions, including hypoplastic or malformed epididymides, *vasa deferentia*, seminal vesicles, and prostates as well as atrophied testes and hypospadias (21). These pathological changes occurred in the absence of maternal toxicity and were induced by concentrations of DBP as low as 250 mg/kg/day when gavaged on gestation days 12 to 21. The findings in this gestational exposure study concurred with a National Toxicology Program multigenerational study of DBP in the diet (26, 32), but were in contrast to the findings of a standard teratology study that dosed on GD 7 to 15 with doses of up to 1000 mg/kg/day (6). In fact, DBP exposure on GD 16 to 19 alone, which is outside the dosing time used by Ema (6), was sufficient to cause male reproductive tract lesions (11). In addition to morphologic changes in the male reproductive tract, there were alterations in anogenital distance (AGD) and areolae retention in the male offspring of DBP exposed dams (11, 23).

In contrast to other chemicals that demasculinize male fetuses by acting as androgen receptor antagonists or estrogen receptor agonists, DBP does not bind to the androgen receptor; rather, its antiandrogenicity is due to decreased production of testosterone (T) by the fetal testes (8, 9, 22, 29). Though DBP has been reported to weakly bind to the estrogen receptor in several *in vitro* assays, no estrogenic activity has been noted following *in vivo* exposure (12, 14, 33). Though the lesions have been described in the adult, the pathogenesis of morphologic alterations due to *in utero* DBP exposure in the male reproductive tract has not been fully described. Determining the pathogenesis of DBP-induced alterations in the male reproductive tract from the fetus to the adult following *in utero* exposure will provide an indication of primary lesions expressed in the fetus and those changes that resolve or further develop as the animal ages. This information could aid in the identification of mechanisms of action important for risk extrapolation.

We examined the ontogeny of the reproductive tract lesions from DBP-exposed fetuses at different life stages from GD 16 to adulthood, specifically focusing on the progression of lesions in the testes and epididymides at both the gross and histologic levels. The chosen life stages (fetal, early postnatal and adult) were all compared to determine how the lesions differ at various times and how those lesions may have developed or resolved from earlier morphological alterations.

METHODS

Animals

This study was conducted in accordance with Federal guidelines for the care and use of laboratory animals (24). Animal use was approved by the Institutional Animal Care and Use Committee at the CIIT Centers for Health Research (CIIT). Animals were housed in CIIT's animal care facility, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Pregnant, time-mated, 8- to 10-week-old nulliparous Crl:CD(SD)Br rats were obtained from Charles River Laboratories, Inc. (Raleigh, NC) on GD 0. GD 0 is defined as the day sperm were identified in the vagina. Pregnant dams were assigned to dose groups by body weight randomization on the day of arrival and were housed in a HEPA-filtered, mass-air-displacement room with a standard 12-hour light-dark cycle at 18-26°C and a relative humidity of 30–70%. Individual dams and offspring were housed in polycarbonate cages on ALPHA-dri bedding (Shepherd Specialty Papers, Kalamazoo, MI) until weaning. Male pups were then housed 3 animals per cage with corresponding littermates. Animals had access *ad libitum* to NIH-07 rodent chow (Zeigler Brothers, Gardener, PA) and deionized water. Data were collected from all male fetuses and pups pre- and postweaning, and all data were included in the analyses performed at each time point.

Time course study

An initial study was performed utilizing one control dam dosed with corn oil and 3 to 4 dams exposed to 500 mg/kg/day DBP per group. Dams were gavaged daily on GD 12

to 21 at 8:00–9:00 am with either corn oil (Sigma Chemical Company, St. Louis, MO) or DBP (Aldrich Chemical Company, Milwaukee, WI) in corn oil at a concentration of 500 mg/ml. The dose level was chosen based on previous studies in which 500 mg/kg/day DBP on GD 12 to 21 induced a high incidence of reproductive tract malformations in male rats with no other discernable toxicity to the dams and no effects on litter size or pup survival (23). Dams were examined at dosing and several hours postdosing for clinical signs of toxicity. Male fetuses or pups were necropsied on GD 16, 17, 18, 19, 20, and 21 as well as PND 3, 7, 16, 21, 45, and 70. Examination of the DBP-exposed males revealed gross or histologic lesions at all the time points chosen. Based on the lesions observed, additional animals (4 control dams dosed with corn oil and 5 to 6 DBP-exposed dams per time point) were exposed (GD 12 to 21) and studied on GD 17, 19, and 21 and PND 7, 16, 45, and 70 to increase the overall numbers of animals for statistical analyses. A total of 4 to 5 dams per group (1 control, 3 to 4 dosed) were utilized on GD 16, 18, and 20 and PND 3 and 21. A total of 13 to 14 dams per group (5 control, 8 to 9 dosed) were utilized on GD 17, 19, and 21 and PND 7, 16, 45, and 70.

Fetal necropsies

Pregnant dams were anesthetized by an intraperitoneal injection of 1 ml/kg ketamine-acepromazine solution (100/0.6 mg/kg, wt/wt) and euthanized by aortic transection. Fetuses were removed from the uterine horns starting at the right ovary and progressing to the left ovary. Fetuses were weighed and then euthanized by decapitation and exsanguination prior to dissection. Fetuses were examined for external malformations and were sexed by inspection of the internal reproductive organs. The reproductive tracts of

all male fetuses were then removed using a dissecting microscope, and the testes and epididymides were examined both under direct light and with transillumination. Transillumination was found to be essential for evaluating the degree of ductular coiling in the developing epididymides. The epididymides and testes were kept as a unit when dissected from GD 18 and younger fetuses and were separated when removed from GD 19 and older fetuses. The right testes and epididymides were fixed in 10% neutral-buffered formalin for approximately 24 hours before being transferred to 70% ethanol. Processing, embedding, sectioning at 5 μm, and staining with hematoxylin and eosin followed the fixation process. The left testes and epididymides were frozen in Tissue-Tek Optimal Cutting Temperature Compound (OCT) (Sakura, Torrance, CA) and stored at -80°C.

Male pup necropsies

Male pups were euthanized by decapitation on PND 3, 7, 16, 21, 45 and 70. The testes, epididymides, and *vasa deferentia* were removed on PND 3, 7, 16, and 21. On PND 16 and 21, these tissues were removed and weighed along with the kidneys and liver. On PND 45 and 70, the testes, epididymides, *vasa deferentia*, prostates (dorsolateral and ventral lobes), seminal vesicles with coagulating gland and fluid, *levator ani* and *bulbocavernosus* muscles (LABC), liver, and kidneys were dissected and weighed. All organs were fixed in 10% neutral-buffered formalin except the testes and epididymides. The right testes and epididymides were fixed in Bouin's solution for 48 hours following capsular nicking approximately 4 to 6 hours into the fixation period. All tissues were trimmed, processed, embedded, sectioned at 5 μm, and stained with hematoxylin and eosin.

The left testes and epididymides were frozen in OCT embedding media and stored at -80°C.

Other measurements

Dam body weights were recorded daily during dosing. Male fetal and pup weights were collected at the time of necropsy. Weekly body weights were also recorded for male pups necropsied on PND 45 and 70. AGD was measured and areolae were counted on PND 1 and 13 respectively by a single investigator blinded to treatment. AGDs were measured with a dissecting microscope fitted with an eyepiece reticle (19). Dams were euthanized on the same day as male pups or on PND 21, the day of weaning. All female pups were euthanized on PND 21 without evaluation.

Testicular histopathology

Seminiferous epithelial degeneration on PND 45 and 70 was graded using the following system: grade 0, normal background tubular degeneration, i.e., 3 or fewer abnormal tubules per testis; grade I, less than 25% of the tubules affected; grade II, 25 to 50% of the tubules affected; grade III, 50 to 75% of the tubules affected; grade IV, 75 to 100% of the tubules were affected with spermatogenesis still occurring in some tubules; and grade V, 100% of the tubules affected with little to no spermatogenesis occurring (i.e., predominantly Sertoli cells with few spermatogonia and spermatocytes). The grade was based on evaluation of all the tubules in two cross sections. Examination of the histologic sections of the testes and epididymides was performed with knowledge of dose and age. In

the case of subtle lesions, DBP-exposed and control tissues were randomized and reexamined blinded to these parameters.

Statistics

The litter was the experimental unit for all of the age groups. Statistical analyses were conducted using JMP (version 4.0.0, SAS Institute, Cary, NC). Quantitative pup data were tested for homogeneity and analyzed by a repeated measure ANOVA (nested design) with the dams treated as the experimental unit to yield litter means. An ANOVA or ANCOVA was performed to test for significance of a treatment effect with covariates listed in the figure legends. Results are expressed as the least squares means ± standard error (SE). Pathology data were analyzed using the Fisher's Exact Test. Significance for all treatment effects was $p < 0.05$. Data from the two studies were analyzed separately for statistical differences. No differences were found, and all of the data were therefore combined before the final statistical analyses were performed.

RESULTS

General toxicity, dams and male offspring

DBP at 500 mg/kg/day on GD 12 to 21 did not cause any overt maternal toxicity or reduce dam body weight (Figure 1). Additionally, no decrease in litter size, pup survival, alteration of the sex ratio (data not shown), or body weights of male fetuses or pups compared to controls on any day examined was found(Figure 1).

Anogenital distance and areolae retention

DBP exposure *in utero* induced a statistically significant decrease of 14% in the male AGD on PND (Figure 2). This decrease was comparable to that measured in previous studies with *in utero* DBP exposure (11, 20). In addition, there was a significant increase in the number of retained areolae in DBP exposed male pups on PND 13 (Figure 2). The average number of areolae per male rat and the number of male rats and litters with males displaying ≥ 1 areola were significantly increased above control (data not shown).

Fetal gross and histopathology

Since the fetal epididymal ducts were just starting to coil in control animals on GD 19, discerning lesions in this tissue on GD 19 or earlier was difficult. There were subtle morphological differences between control and DBP-exposed fetal epididymides in some animals on GD 19, including slightly smaller epididymides and a gelatinous appearance to the adipose tissue surrounding the developing epididymal duct. On GD 20, the difference in the epididymal morphology between DBP-exposed males and controls was more

obvious, with decreased coiling of the epididymal duct in both the developing head and tail. This gross lesion in epididymides was most prevalent on GD 21, where there was distinctly less ductal coiling and the overall size of the organ was diminished (Figure 3A and 3B). A slight to mild decrease in the size of some testes was also noted. Enlarged seminiferous cords were observed at the gross level with transillumination on PND 19 to 21, which corresponded to the histologic findings. The *vasa deferentia* were smaller in DBP-exposed animals on GD 20 and 21.

The histologic lesions in the epididymides of DBP-exposed fetuses supported the gross findings with decreased coiling evidenced by reduction in the number of ductular cross sections in the tissue (Figure 4B). The histologic lesions were most severe on GD 21. The incidence of histologic lesions in this organ was not quantified since the fetal epididymides were extremely small and reliable sections of the tissue could not be obtained. However, there was a good correlation of the histologic and gross lesions in tissues where an adequate histologic section was obtained.

Fetal testes had three characteristic histologic lesions. The interstitium of the exposed fetal testes contained variably sized aggregates of fetal Leydig cells (LC). In control fetal testes, small clusters of LCs were observed, but they generally contained no more than four to six LCs (Figure 5A). The LC aggregates induced by DBP exposure generally contained large numbers of LCs and were often much larger than the diameter of the developing seminiferous cords (Figure 5B). These LCs were tightly packed with decreased amounts of cytoplasm and small, angular nuclei. The second prominent lesion observed in the fetal testes exposed to DBP was multinucleated gonocytes within the seminiferous cords (Figure 5B). These gonocytes were markedly enlarged with distinct cell

borders and abundant pale cytoplasm. They usually contained two to four nuclei, although some gonocytes were seen with up to 13 nuclei. The third lesion was an increased diameter of the seminiferous cords with markedly increased numbers of gonocytes (Figure 5B). Most seminiferous cords in control animals contained up to five or six gonocytes in a cross section (Figure 5A), whereas cords in DBP-exposed testes were often enlarged two to three times and were packed with smaller gonocytes containing less cytoplasm. These three morphologic features varied in incidence, from 0 to ~50%, on GD 16 to 18 while nearly 100% of the DBP-exposed fetuses examined had testes with two or three of the lesions on GD 19, 20, and 21 (Table I). Additionally, severity of the lesions increased in older fetuses with larger aggregates of LCs and increased numbers of multinucleated gonocytes.

Early postnatal gross pathology and histopathology

Gross and histologic lesions in the male reproductive tract of DBP-exposed animals continued to progress from those identified in the fetus, although the pups were no longer being exposed to DBP. Testicular lesions on PND 3, 7, 16, and 21 included testes that were smaller and not fully descended with lack of attachment to the *pars vaginalis gubernaculi* (gubernaculum) (Table II). The undescended testes were in variable locations in the abdomen and generally lacked attachment to the gubernaculum or the attachment was extremely loose, allowing free movement of the testes and twisting of the spermatic cord. Epididymal lesions similar to those seen in the fetus were also variably present (Table II). The epididymides from pups exposed *in utero* were smaller with decreased coiling of the epididymal duct. In some cases, the coiling was not continuous from the head to the tail

(Figure 3D). There was a variable incidence of absent *vasa deferentia*, both unilateral and bilateral (Table II).

A change in histologic lesions in the testes and epididymides was observed in the early postnatal period. Several of the same lesions, including LC aggregates and multinucleated gonocytes, were seen in the testes during the early postnatal period (PND 3, 7, 16), while decreased numbers of spermatocytes were observed later in the period from PND 16 to 21 (Table III, Figure 5D). No histologic lesions were identified in control animals on PND 3, 7, 16 or 21. Abnormal gonocytes that were multinucleated, increased in number, or contained bizarre mitotic figures were observed in seminiferous tubules of DBP-exposed males on PND 3 and 7, while these features were not observed on PND 16 or 21. Additionally, the number of gonocytes was normal or increased on PND 3 and 7, but the number of spermatocytes was decreased on PND 16 and 21 (Figure 5D). LC aggregates were readily apparent on PND 3 and 7 but were less significant on PND 16 and rare on PND 21 (Figure 5D). Histologically, epididymides from DBP-exposed rats had decreased ductular cross sections across all ages, PND 3 to 21 (Figure 4D). In addition, there was progressive mild dilation of ducts with flattening of the lining epithelium (Figure 4D). No changes were noted in the liver and kidney weights on PND 16 and 21.

Adult gross and histopathology

The incidence of gross lesions in the testes of young adult rats exposed to DBP *in utero* differed between PND 45 and 70 (Table IV). PND 45 testes did not show decreased weight, but there was a marked decreased in testicular size by PND 70 (Figures 3F and 3H, Table V). Testes in the DBP group were atrophied and flaccid, cryptorchid or ectopic.

Cryptorchid testes remained in the abdominal cavity, while ectopic testes descended through the inguinal ring but did not descend into the scrotum; rather, they remained in the subcutis of the inguinal region. Cryptorchid and ectopic testes were morphologically different from the atrophied testes in that they had a turgid capsule and normal color. In addition to atrophied testes, mildly to moderately enlarged testes were observed.

The gross lesions noted in the epididymides on PND 45 and 70 were similar and included intact, hypoplastic epididymides and malformed epididymides with variable degrees of agenesis (Figure 3F and 3H, Table IV). Hypoplastic epididymides were smaller and translucent, having relatively more fibrous connective tissue due to the decreased coiling of the duct and decreased amount of sperm in the duct, but they retained their normal shape. Epididymides of cryptorchid or ectopic testes were also small but had discernable coiling of the epididymal duct. Malformed epididymides had variable absence of different portions of the organs with the body being the most common absent part (Figure 3F and 3H). Absence of the tail was often associated with absence of the *vas deferens*. Lesions in testes and epididymides were unilateral with no side predilection, as well as bilateral. Epididymal weights correlated with the gross lesions and significantly decreased on both PND 45 and 70 (malformed epididymides were omitted from the analyses).

Young DBP-exposed adult males also had lesions in the accessory sex glands and other androgen-dependent tissues on PND 45 and 70, and approximately 25% had hypospadias with incomplete closure of the urethral folds on their ventral surfaces of the penises (Table IV). The most severe cases had a cleft along the entire length of the penis with exposure of the *os* penis (Figure 6B). There was a similar incidence of prostatic

lesions on PND 45 and 70, including small or completely absent ventral prostate lobes (VP) and small, misshapen dorsolateral prostate lobes (DLP) (Figure 6D). The seminal vesicles (SV) were also variably affected with small, misshapen or absent lobes (Figure 6D). The lesions occurred unilaterally and bilaterally. The *levator ani* and *bulbocavernosus* muscle (LABC) was markedly decreased in size and misshapen in DBP-exposed animals (Figure 6F). The weights of the DLP and SV, but not VP, were significantly decreased on PND 70, while only the DLP was decreased on PND 45 (Table V). The LABC weights were significantly decreased at both ages. There was a slight, statistically significant decrease in paired kidney weight on PND 45. This was judged to lack biologic significance since gross and histologic lesions were not seen in this organ and kidney weight differences were not seen at other ages.

The histologic lesions in the testes on PND 45 and 70 differed markedly in severity (Table VI). On PND 45, small numbers of lesioned tubules with degeneration of spermatocytes within the germinal epithelium were observed. However, the severity at this time was predominately diagnosed as grade I with approximately 10 to 15 tubules per cross section affected. The affected tubules had decreased numbers of spermatocytes with necrotic cell debris within the lumina (Figure 5F). This lesion was in contrast to the severe testicular lesion observed on PND 70 (Figure 5H). At this age, most of the testes had grade IV or V degeneration with 100% of the tubules affected. An additional lesion seen on PND 45 that was not observed on PND 70 was tubular luminal dilation in approximately 30% of the animals. Small numbers of animals on PND 70 had focal areas of LC hyperplasia that were smaller than the diameter of a normal seminiferous tubule. Seminiferous tubular

lesions were not seen in control animals at either age except for one animal with tubular luminal dilation on PND 45.

The histologic lesions in the epididymides on PND 45 and 70 were similar following *in utero* exposure to DBP. In hypoplastic epididymides, there was a relative increase in the amount of interstitial fibrous connective tissue due to the decreased size of the ductular lumen and the decreased number of ductular cross sections. Ducts smaller in diameter were lined by thickened epithelium with a cribiform pattern. There were small numbers of lymphocytes and macrophages scattered throughout the interstitium of some affected animals. The lesions in malformed epididymides were similar regardless of whether the portions were remnants of the head or tail. The number of cross sections was dramatically decreased with marked dilation of the remaining duct. The epithelium lining the duct was flattened compared to controls. In sections of the head (confirmed from the gross examination), sperm were never present in the lumen. Agenesis of grossly absent portions of the epididymides was confirmed histologically on multiple animals by serially sectioning the epididymal fat pad, which developed normally, in an attempt to find remnant ducts; however, none were observed.

DISCUSSION

This is the first study to document the progression of testicular and epididymal lesions from the start of sexual differentiation in the fetus through to adulthood in male offspring following exposure to DBP *in utero*. Lesions in the male reproductive tract noted on several days in the fetus and in the adult were similar to those previously reported (11, 23, 25, 32). This study examined additional fetal, early postnatal, and adult time points to reveal the morphologic ontogeny of the lesions in the male reproductive organs, focusing on the testes and epididymides. Understanding the ontogeny of the testicular and epididymal lesions is important for elucidating the cellular and molecular pathogenesis and determining whether this mechanism is applicable to humans. By knowing how the lesions progress from the fetus to the adult, it will be possible to compare and contrast the effects of other antiandrogens on the developing male reproductive tract and examine differences in mechanisms of antiandrogenicity leading to similar (and different) reproductive phenotypes.

The dose of DBP used in this study (500 mg/kg/day) was based on results in previous studies that showed a high incidence of lesions in the developing male reproductive tract with little to no generalized maternal or fetal toxicity (11, 23). While this dose of DBP may not be environmentally relevant, lower doses of DBP produced similar testicular and epididymal lesions although the incidences were much lower (20, 21). This dose of phthalate was chosen to give a high incidence of male reproductive tract lesions so the pathogenesis over time could be more easily studied with fewer DBP-exposed litters since most fetuses and pups were affected.

DBP effects on the testes

In the fetal rat, the characteristic lesions of aggregates of LCs, increased numbers of gonocytes, and multinucleated gonocytes became evident on GD 17 to 18 and increased in severity to ~100% by GD 21. During the early postnatal period (PND 3 and 7) the testicular lesions remained similar to those seen in the fetus. However, the incidence and severity of these lesions decreased from PND 3 to PND 16. On PND 16 and 21, seminiferous tubules contained decreased numbers of spermatocytes. The reason for the loss of spermatocytes may be due to degeneration of the altered gonocytes with lack of movement to the basal lamina to become spermatogonia. The spermatocyte loss and degeneration continued to PND 45, at which time small numbers of tubules with abnormal spermatogenesis were observed. The testicular lesions progressed in severity from PND 45 to 70. The transition from slightly dilated tubules with mild degeneration of the seminiferous epithelium to marked degeneration of the epithelium on PND 70 was most likely due to the malformed epididymides and obstruction of fluid flow from the testes. This finding was in concordance with the findings of McIntyre et al who showed secondary testicular lesions caused by malformed epididymides induced by *in utero* exposure to linuron (18). The seminiferous tubular degeneration noted in the present study was also consistent with the seminiferous epithelial lesions that occurred when epididymides were surgically obstructed (7). The progression in severity of testicular lesions as animals mature illustrates the ability of DBP to induce irreversible changes in the testes following a short gestational exposure that may not be fully manifest until adulthood.

DBP effects on the epididymides

Whereas the suite of morphological lesions in the fetal testes became less apparent over time in the postnatal period with development of a different adult phenotype, the DBP-induced epididymal lesions continued to progress into adulthood. Rather, they progressed in severity from the fetus through to adulthood. Gross lesions in the epididymides were initially difficult to diagnose since the epididymal fat pad developed normally, even with decreased epididymal duct development. Transillumination microscopy was found to be essential for detection of fetal epididymal duct lesions. Decreased coiling first became evident on GD 19 to 20 with continued development of the lesion to GD 21. The decreased size of the epididymides and the reduced coiling of the duct continued into the early postnatal period, although the males were no longer being exposed to DBP. By PND 16 and 21, there was ductular dilation and flattening of the epithelium in addition to decreased coiling. Another interesting change seen by PND 16 was degeneration of portions of the epididymides, as evidenced by animals detected in which the body of the epididymis did not contain a patent duct. The lack of an epididymal body in the early postnatal period progressed to the characteristic lesions seen in the adult. On PND 45 and 70, there were wholly absent epididymides or epididymides with missing structural portions, most frequently the body. These lesions were unilateral or bilateral, and there was no predilection for which part of the epididymis would be absent.

Development of the epididymal lesions lagged slightly behind that of the testicular lesions in the fetus. Epididymal lesions were not apparent until GD 19, whereas changes were first observed histologically in the testes on GD 17. This observation implicates a direct effect of DBP on the fetal testes, while the epididymal lesions were likely secondary

to decreased testicular T synthesis. T is predominantly responsible for differentiation and development of the tissues derived from the Wolffian ducts, i.e., the epididymides, *vasa deferentia*, and seminal vesicles (3). Fetal testicular T synthesis reaches its peak on approximately GD 19 (13, 31). DBP has been shown to decrease fetal testicular T levels to 25 to 30% of controls at this time (9, 22, 29). Therefore enough T may have been present to begin the early differentiation of the Wolffian ducts, but by GD 19 the amount of T present was no longer sufficient for complete development of the organ. Since DBP has a short half-life, internal exposure ended shortly following birth. However, the epididymal lesions did not resolve; in fact, they continued to become more severe to adulthood (27, 30). In the face of discontinued DBP exposure, this finding supports the concept that fetal epididymides are irreversibly altered by the decreased amounts of fetal T and that this lack of T at a critical time prevents or further alters their development later in life. Though a relatively high dose was given, DBP has a short half-life with most of the compound being excreted in the urine in 24 hours (1, 4, 27, 30). Saillenfait et al. noted that no DBP or its metabolites accumulated in the dams or embryonic tissues although DBP was rapidly transferred across the placentas to the embryos (27). Though the parent compound and its metabolites rapidly reach the developing embryos, the amounts were very low, accounting for only 0.12 to 0.15% of the administered dose.

DBP has a different mechanism of action from the antiandrogens linuron and flutamide, which are competitive AR antagonists of different potencies. Their differing mechanisms of action and potencies lead to different phenotypes in the developing male reproductive tract and to differences in the magnitude of the AGD reduction and areolae retention. DBP produces primarily epididymal and vasa deferentia lesions with increased

numbers of LCs secondary to decreased fetal testicular T synthesis. Though DBP has its effects predominantly on T-dependent tissues, it also causes lesions in dihydrotestosterone (DHT)-dependent tissues. In contrast, linuron affects testes and epididymides but has no morphological effects on fetal LCs or DHT-dependent tissues. Though it is also an AR antagonist, flutamide has no epididymal effects except at high dose levels, has no effect on fetal testicular T, and predominantly affects DHT-dependent tissues. These few examples illustrate that it is difficult to compare antiandrogens and predict their *in vivo* effects based solely on their pharmacological mechanisms of action.

The male fetus is uniquely sensitive to the effects of altered androgen status. Early developmental events in the fetus, when disturbed by a shift in androgen levels or signaling *in utero*, lead to consequences later in life, long after the presence of the compound has ceased. Initial lesions in the fetal testes following DBP exposure resolved while a different set of morphological testicular alterations became obvious as the animals matured. The means by which decreased androgen levels program male reproductive tissues during gestation and cause consequences later in life are not clear. DBP decreases fetal T synthesis but the precise target is not known. However, several of the genes in the steroidogenic pathway are downregulated in rat fetal testes after DBP exposure (29). In addition, which downstream pathways are affected by decreased androgen levels, and therefore the complete molecular pathways of lesion development remain to be identified. During development some genes are expressed transiently or are only expressed in certain tissues. Thus, understanding the pathogenesis of developmental lesions and the timing of lesion occurrence is critical so that tissues can be analyzed at appropriate times when attempting to elucidate changes in gene expression

This study examined a single dose level of DBP given on GD 12 to 21 with examination of male fetuses on GD 16 to 21 and examination of male pups on PND 3, 7, 16, 21, 45, and 70. DBP alters the course of development of the male reproductive tract during this *in utero* exposure, which subsequently leads to a phenotype in the fetus that alters and develops as the animal matures even though there is no longer exposure to the compound. *In utero* exposure of the fetal gonocytes leads to impairment of spermatogenesis. This disruption in spermatogenesis is exacerbated by malformations in the epididymides that cause obstruction of testicular fluid outflow and secondary pressure atrophy of the seminiferous epithelium. While the characteristic morphologic changes of multinucleated gonocytes, increased numbers of gonocytes, and LC aggregates in the fetal testes were apparently resolved in the early postnatal period, the lesions in the developing epididymides progressed in severity from the fetus to adulthood. The lack of ductular coiling during fetal and early postnatal life ultimately leads to malformed epididymides that are nonpatent. The data from this study illustrate not only the characteristic lesions that develop but also the timing and progression of these events. This information is critical for comparing antiandrogens of the same and different mechanisms and for determining target tissues and the critical timing of collection of these tissues for further molecular analyses.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Barry S. McIntyre, Katie J. Turner, Christopher J. Bowman, and Katrina M. Waters, Mr. Duncan G. Wallace, and Mrs. Kim P. Lehmann for their assistance with the dissections and manuscript preparation. We would also like to thank all the staff in the Division of Biological Sciences at the CIIT Centers for Health Research for their help with the painstaking necropsies. This study would not have been possible without the assistance of the CIIT animal care staff and the necropsy and histology staff. This study was funded in part by the American Chemistry Council and by a National Research Service Award from the Department of Health and Human Services, National Institutes of Health, National Institute of Environmental Health Sciences (awarded to NJB). This manuscript does not necessarily reflect the views or policies of either of these organizations.

REFERENCES

1. Anderson WA, Castle L, Scotter MJ, Massey RC, Springall C (2001). A biomarker approach to measuring human dietary exposure to certain phthalate diesters. *Food Addit Contam* 18: 1068-1074.
2. Baskin LS, Himes K, Colborn T (2001). Hypospadias and endocrine disruption: Is there a connection? *Environ Health Perspect* 109: 1175-1183.
3. Berman DM, Tian H, Russell DW (1995). Expression and regulation of steroid 5 α-reductase in the urogenital tract of the fetal rat. *Mol Endocrinol* 9: 1561-1570.
4. Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Lucier GW, Jackson RJ, Brock JW (2000). Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 108: 979-982.
5. David RM (2000). Exposure to phthalate esters. *Environ Health Perspect* 108: A440-A440.
6. Ema M, Amano H, Itami T, Kawasaki H (1993). Teratogenic evaluation of di-*n*-butyl phthalate in rats. *Toxicol Lett* 69: 197-203.
7. Flickinger CJ, Baran ML, Howards SS, Herr JC (1999). Degeneration of the seminiferous epithelium following epididymal obstruction in prepubertal rats. *Anat Rec* 254: 76-86.
8. Foster PMD, Mylchreest E, Gaido KW, Sar M (2001). Effects of phthalate esters on the developing reproductive tract of male rats. *Human Reproduction Update* 7: 231-235.
9. Gray LE, Jr. (2002). Personal communication.

10. Gray LE, Jr., Ostby JS, Kelce WR (1994). Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol* 129: 46-52.

11. Gray LE, Jr., Wolf C, Lambright C, Mann P, Price M, Cooper RL, Ostby J (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* 15: 94-118.

12. Harris CA, Henttu P, Parker MG, Sumpter JP (1997). The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* 105: 802-811.

13. Huhtaniemi I, Pelliniemi LJ (1992). Fetal Leydig cells: cellular origin, morphology, life span, and special functional features. *Proc Soc Exp Biol Med* 201: 125-140.

14. Jobling S, Reynolds T, White R, Parker MG, Sumpter JP (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 103: 582-587.

15. Kohn MC, Parham F, Masten SA, Portier CJ, Shelby MD, Brock JW, Needham LL (2000). Human exposure estimates for phthalates. *Environ Health Perspect* 108: A440-A442.

16. LeBlanc GA, Bain LJ, Wilson VS (1997). Pesticides: multiple mechanisms of demasculinization. *Mol Cell Endocrinol* 126: 1-5.

17. McIntyre BS, Barlow NJ, Foster PMD (2001). Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of

early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci* 62: 236-249.

18. McIntyre BS, Barlow NJ, Sar M, Wallace DG, Foster PMD (2002). Effects of *in utero* linuron exposure on rat Wolffian duct development. *Reprod Toxicol* 16: 131-139.

19. McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PMD (2000). Effects of *in utero* exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat. *Toxicol Appl Pharmacol* 167: 87-99.

20. Mylchreest E, Cattley RC, Foster PMD (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to di(*n*-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci* 43: 47-60.

21. Mylchreest E, Sar M, Cattley RC, Foster PMD (1999). Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156: 81-95.

22. Mylchreest E, Sar M, Wallace DG, Foster PMD (2002). Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(*n*-butyl) phthalate. *Reprod Toxicol* 16: 19-28.

23. Mylchreest E, Wallace DG, Cattley RC, Foster PMD (2000). Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(*n*-butyl) phthalate during late gestation. *Toxicol Sci* 55: 143-151.

24. National Research Council (1996). *Guide for the Care and Use of Laboratory Animals*. National Academy Press, Washington, D.C.

25. National Toxicology Program (1991). *Di(n-butyl) Phthalate Reproduction and Fertility Assessment in CD Sprague-Dawley Rats When Administered Via Feed.* National Institute of Environmental Health Sciences, Research Triangle Park, NC.

26. National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (2000). *NTP-CERHR Expert Panel Report on Di(n-butyl) Phthalate.* National Institute of Environmental Health Sciences, Research Triangle Park.

27. Saillenfait AM, Payan JP, Fabry JP, Beydon D, Langonne I, Gallissot F, Sabate JP (1998). Assessment of the developmental toxicity, metabolism, and placental transfer of Di-*n*-butyl phthalate administered to pregnant rats. *Toxicol Sci* 45: 212-224.

28. Sharpe RM (2001). Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicol Lett* 120: 221-232.

29. Shultz VD, Phillips S, Sar M, Foster PMD, Gaido KW (2001). Altered gene profiles in fetal rat testes after in utero exposure to di(*n*-butyl) phthalate. *Toxicol Sci* 64: 233-242.

30. Tanaka A, Matsumoto A, Yamaha T (1978). Biochemical studies on phthalic esters. III. Metabolism of dibutyl phthalate (DBP) in animals. *Toxicology* 9: 109-123.

31. Tapanainen J, Kuopio T, Pelliniemi LJ, Huhtaniemi I (1984). Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biol Reprod* 31: 1027-1035.

32. Wine RN, Li LH, Barnes LH, Gulati DK, Chapin RE (1997). Reproductive toxicity of di-*n*-butylphthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ Health Perspect* 105: 102-107.

33. Zacharewski TR, Meek MD, Clemons JH, Wu ZF, Fielden MR, Matthews JB (1998). Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters. *Toxicol Sci* 46: 282-293.

Table I. Histopathology in the Testes of Fetuses Exposed to DBP *In Utero*

	Gestation Day					
	16	**17**	**18**	**19**	**20**	**21**
Number of fetuses (litters)	14 (3)	41 (8)	15 (3)	59 (8)	20 (4)	47 (9)
Leydig cell aggregates	0 (0)[a]	59* (88*)	47 (100*)	93* (100*)	95* (100)	89* (100*)
Multinucleated gonocytes	0 (0)	0 (0)	20 (67)	56* (100*)	90* (100)	98* (100*)
Increased numbers of gonocytes	0 (0)	7 (25)	20 (67)	90* (100*)	100* (100)	100* (100*)

[a] Percentage of fetuses in the 500 mg/kg/day group with lesions (% of litters).
* $p < 0.05$.

Table II. Early Postnatal Gross Lesions in the Testes, Epididymides, and *Vasa Deferentia* of Male Pups Exposed to DBP *In Utero*

	PND 7		PND 16		PND 21	
	DBP (mg/kg/day)					
	0	500	0	500	0	500
Number of animals (litters)	20 (4)	28 (5)	28 (5)	60 (8)	7 (1)	13 (2)
Unilateral testis[a]	0 (0)[b]	4 (20)	0 (0)	7 (38)	0 (0)	8 (50)
Bilateral testes[a]	0 (0)	4 (20)	0 (0)	20* (50)	0 (0)	31 (100)
Unilateral epididymis[c]	0 (0)	7* (80)	0 (0)	33* (100*)	0 (0)	23 (100)
Bilateral epididymides[c]	0 (0)	50* (80)	0 (0)	27* (88*)	0 (0)	8 (50)
Unilateral *vas deferens*[d]	0 (0)	11 (40)	0 (0)	27* (88*)	0 (0)	8 (50)
Bilateral *vasa deferentia*[d]	0 (0)	21* (40)	0 (0)	13* (75*)	0 (0)	15 (50)
Percentage of males with any reproductive lesion	0 (0)	75* (100*)	0 (0)	60* (100*)	0 (0)	62* (100)

[a] Lesioned testes include those that were not descended as far as corresponding controls, were not attached to the gubernaculum, and had hemorrhage or necrosis of the testes. Lesions occurred unilaterally and bilaterally with no side predilection.
[b] Percentage of animals with lesions (% of litters).
[c] Gross epididymal lesions included small epididymides with decreased coiling of the epididymal duct and epididymides with variable agenesis. Lesions occurred unilaterally and bilaterally with no side predilection.
[d] The gross lesion noted in the *vasa deferentia* was absence of the organ. This lesion was unilateral and bilateral with no side predilection.
* $p < 0.05$.

Table III. Early Postnatal Histopathology in the Testes and Epididymides of Pups Exposed to DBP *In Utero*

	Postnatal Day			
	3[a]	7	16	21
Number of animals (litters)	15 (3)	46 (8)	57 (8)	13 (2)
Testes[b]				
Leydig cell aggregates	100* (100)[c]	80* (100*)	93* (100*)	0 (0)
Abnormal gonocytes[d]	93* (100)	72* (100*)	0 (0)	0 (0)
Decreased numbers of spermatocytes	0 (0)	0 (0)	47* (75*)	62* (100)
Epididymides[e]				
Hypoplastic with decreased coiling of the epididymal duct	54* (100)	47* (88*)	43* (100*)	46* (100)
Dilated epididymal duct	8 (33)	16* (38)	36* (100*)	8 (50)
Percentage of males with any reproductive lesion	100* (100)	96* (100*)	95 (100*)	85* (100)

[a] Number of control animals (litters) examined on PND 3 = 7 (1), PND 7 = 26 (5), PND 16 = 30 (5), and PND 21 = 7 (1).
[b] Incidences are for the right testes only.
[c] Percentage of animals with lesions (% of litters).
[d] Abnormal germ cells include those that are multinucleated, are increased in number, or have bizarre mitotic figures.
[e] Incidences are for the right epididymides only.
* $p < 0.05$.

Table IV. Gross Lesions in the Reproductive Organs of Young Adult Male Rats Exposed to DBP *In Utero*

	PND 45		PND 70	
	DBP (mg/kg/day)			
	0	500	0	500
Number of animals (litters)	31 (5)	44 (8)	32 (5)	48 (8)
Unilateral testis[a, b]	0 (0)[b]	23* (63)	0 (0)	23* (75*)
Bilateral testes[a,b]	0 (0)	7 (38)	0 (0)	50* (88*)
Unilateral epididymis [b,c]	0 (0)	20* (63)	0 (0)	13* (50)
Bilateral epididymides[b,c]	0 (0)	34* (88*)	0 (0)	56* (88*)
Unilateral *vas deferens*[b,d]	0 (0)	18* (63*)	0 (0)	17* (63*)
Bilateral vasa deferentia[b,d]	0 (0)	2 (13)	0 (0)	35* (50)
Ventral prostate[e]	0 (0)	14* (38)	0 (0)	33* (75*)
Dorsolateral prostate[e]	0 (0)	14* (38)	0 (0)	31* (63*)
Seminal vesicles and coagulating glands[f]	0 (0)	21* (50)	0 (0)	52* (88*)
Penis[g]	0 (0)	32* (88*)	0 (0)	25* (38)
Percentage of animals with any reproductive lesion	0 (0)	75* (100*)	0 (0)	86* (100*)

[a] Lesioned testes include those that were enlarged or atrophied.
[b] Lesions occurred unilaterally and bilaterally. There was no side predilection.
[c] Gross epididymal lesions include small epididymides and those with variable agenesis.
[d] The gross lesion noted in the *vasa deferentia* was absence of the organ.
[e] Ventral and dorsolateral prostates were either grossly small, malformed, or absent.
[f] Gross lesions in the seminal vesicles and coagulating glands include small lobes with less fluid and malformed or absent lobes.
[g] The penises of affected animals were slightly smaller and had hypospadias of varying severity.

Table V. Organs Weights of Male Pups Exposed to DBP *In Utero*

	PND 21		PND 45		PND 70	
	DBP (mg/kg/day)					
	0	500	0	500	0	500
Right and left testes[a,e]	0.181 ± 0.005	0.174 ± 0.004	2.069 ± 0.069	2.082 ± 0.055	3.395 ± 0.245	2.775 ± 0.196
Right and left epididymides[a,b,e]	0.034 ± 0.008	0.033 ± 0.006	0.272 ± 0.005	0.244 ± 0.005*	0.904 ± 0.054	0.644 ± 0.053*
Ventral prostate[a,b,c]			0.157 ± 0.008	0.128 ± 0.007	0.411 ± 0.042	0.341 ± 0.034
Dorsolateral prostate[a,c]			0.182 ± 0.005	0.141 ± 0.004*	0.545 ± 0.036	0.375 ± 0.029*
Seminal vesicles and coagulating glands[a,c,d]			0.242 ± 0.030	0.257 ± 0.024	1.172 ± 0.038	1.029 ± 0.039*
Levator ani bulbocavernosus[a,c]			0.352 ± 0.012	0.240 ± 0.009*	0.893 ± 0.041	0.662 ± 0.673*
Liver[a]	1.637 ± 0.073	1.608 ± 0.053	11.967 ± 0.222	11.728 ± 0.178	18.501 ± 0.448	18.334 ± 0.358
Kidneys[a,e]	0.518 ± 0.023	0.481 ± 0.016	2.288 ± 0.042	2.111 ± 0.035*	3.355 ± 0.085	3.152 ± 0.068

[a] Nested litter means ± SE with body weight as a covariate. There were 1 control litter and 2 DBP–exposed litters on PND 21, 5 control and 8 DBP–exposed litters on PND 45, and 5 control and 8 DBP–exposed litters on PND 70.
[b] Malformed tissues were not included in the analyses.
[c] Organ weights for this tissue were not collected on PND 21.
[d] Seminal vesicle weights include the seminal fluid.
[e] Paired organ weights.
* Significantly different from control, $p < 0.05$.

Table VI. Testicular Histopathology Observed in Rats Exposed to DBP *In Utero*

	PND 45[a]	PND 70[a]
Number of animals (litters)	44 (8)	48 (8)
Degeneration of spermatogonia and spermatocytes		
Grade 0[b]	50* (100)[c]	35* (75)
Grade I[d]	41* (88*)	0 (0)
Grade II[e]	2 (13)	0 (0)
Grade III[d]	0 (0)	6 (38)
Grade IV[g]	7 (25)	35* (75*)
Grade V[h]	0 (0)	23* (100*)
Tubular luminal dilation	30* (63)	0 (0)
Leydig cell hyperplasia	0 (0)	10 (38)

[a] Histologic lesions were not seen in control animals on PND 45 or 70, except one animal with tubular luminal dilation on PND 45. Right side only examined.
[b] Grade 0, tubular dilation in less than 3 tubules (normal background)
[c] Percentage of animals with lesions (% of litters).
[d] Grade I, less than 25% of the tubules affected.
[e] Grade II, 25 – 50% of the tubules affected.
[f] Grade III, 50 – 75% of the tubules affected.
[g] Grade IV, 75 – 100% of the tubules affected with spermatogenesis still occurring in some tubules.
[h] Grade V, 100% of the tubules affected with no spermatogenesis occurring (Sertoli cells only).

FIGURE LEGENDS

Figure 1. Body weights for dams, male fetuses, and male pups, respectively. Dams were dosed by gavage with corn oil or 500 mg/kg/day DBP in corn oil on GD 12 to 21. There were no statistical differences between control and treated-exposed animals in any of the groups. Values for dams are group means ± SE; values for fetuses and pups are nested litter means ± SE.

Figure 2. Mean anogenital distance and areolae retention in DBP-exposed male rats from dams dosed by gavage with corn oil or 500 mg/kg/day DBP in corn oil on GD 12 to 21. Values are nested litter means ± SE with body weight as a covariate for AGD. *Significantly different from control ($p < 0.05$).

Figure 3. Gross photographs of GD 21 and PND 16, 45, and 70 testes and epididymides from fetuses or pups of dams dosed by gavage with corn oil or 500 mg/kg/day DBP on GD 12 to 21. (A) Control testis and epididymis on GD 21. Note the prominent coiling in the head and tail regions of the epididymis. Bar = 0.5 mm. (B) DBP-exposed testis and epididymis on GD 21. There is decreased coiling of the epididymal duct in the head and tail regions. The testis is slightly decreased in size. Bar = 0.5 mm. (C) Control epididymis on PND 16. There is marked coiling of the epididymal duct in the head with continuation through the body (arrow). Bar = 0.5 mm. (D) DBP-exposed epididymis on PND 16. Higher magnification of the epididymal head with an abrupt end of the coiled epididymal duct and lack of continuation through the epididymal body (arrow). Bar = 0.25 mm. (E) Control

testis and epididymis on PND 45. Bar = 0.5 cm. (F) DBP-exposed testis and epididymis on PND 45. The epididymis is malformed with a missing body and misshapen head. There is little difference in the size of the testis at this age. Bar = 0.5 cm. (G) Control testis and epididymis on PND 70. Bar = 0.5 cm. (H) DBP-exposed testis and epididymis on PND 70. The epididymis is malformed with a missing body and an extremely small head and tail. There is marked atrophy of the testis. Bar = 0.5 cm. H = epididymal head. T = epididymal tail.

Figure 4. Photomicrographs of GD 21 and PND 16, 45 and 70 epididymides from fetuses or pups of dams dosed by gavage with corn oil or 500 mg/kg/day DBP in corn oil on GD 12 to. (A) Control epididymal tail on GD 21. Multiple curved cross sections (coiling) of the epididymal duct are surrounded by mesenchymal tissue. H&E, 250×. (B) DBP-exposed epididymal tail on GD 21. There is a single section through a slightly curved duct (decreased coiling). The mesenchymal tissue is present in similar amounts to the control epididymis. H&E, 250×. (C) Control epididymal tail on PND 16. There are numerous ductular cross sections lined by low columnar to cuboidal epithelium. H&E, 125×. (D) DBP-exposed epididymal tail on PND 16. There are decreased numbers of ductular cross sections that are dilated and lined by flattened epithelium. H&E, 125×. (E) Control epididymal head on PND 45. The ductular cross sections are lined by cuboidal epithelium and contain a small amount of cellular debris but no sperm (immature). H&E, 125×. (F) DBP-exposed epididymal head on PND 45. Decreased numbers of cross sections are moderately dilated and lined by mildly flattened epithelium. H&E, 125×. (G) Control epididymal head on PND 70. The ductular cross sections are lined by cuboidal epithelial

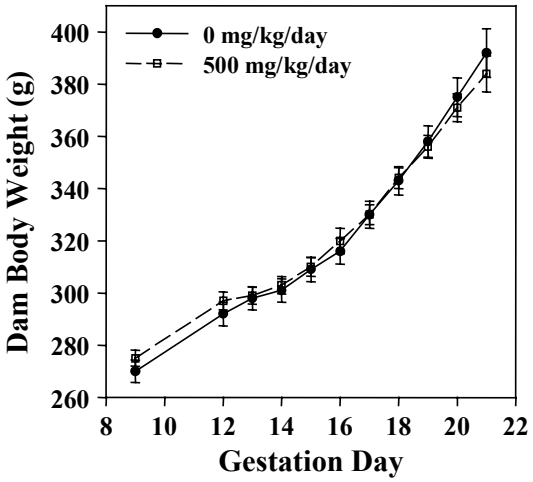
and contain a large number of sperm. H&E, 125×. (H) DBP-exposed epididymal head on PND 70. The number of cross sections is significantly reduced. The ducts that are present are markedly dilated and contain only a small amount of amorphous proteinaceous material. H&E, 125×.

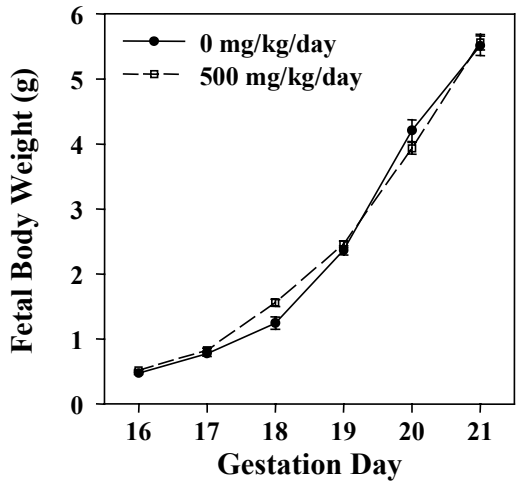
Figure 5. Photomicrographs of GD 21 and PND 16, 45, and 70 testes from fetuses or pups of dams dosed by gavage with corn oil or 500 mg/kg/day DBP on GD 12 to 21. (A) Control testis on GD 21. Cross sections of seminiferous cords contain gonocytes. Small clusters of fetal Leydig cells are seen scattered throughout the interstitium (arrow). H&E, 500×. (B) DBP-exposed testis on GD 21. Seminiferous cords contain increased numbers of gonocytes with several multinucleated gonocytes (arrowheads). Note the large aggregate of Leydig cells in the interstitium (arrow). H&E, 500×. (C) Control testis on PND 16. The gonocytes have migrated to the periphery of the tubules, becoming the spermatogonia. The cells containing the dark nuclei within the central portion of the tubules are spermatocytes. Note the tubular lumina have not formed. H&E, 250×. (D) DBP-exposed testis on PND 16. Seminiferous tubules contain subjectively decreased numbers of spermatocytes. There is a large remaining aggregate of Leydig cells in the interstitium (arrow). H&E, 250×. (E) Control testis on PND 45. H&E, 125×. (F) DBP-exposed testis on PND 45. Several tubules have degeneration of the seminiferous epithelium with cell debris and necrotic spermatocytes within the lumina, Grade I (arrows). H&E, 125×. (G) Control testis on PND 70. H&E, 125×. (H) DBP-exposed testis on PND 70. All of the tubules have marked degeneration of the seminiferous epithelium with Sertoli cells only lining most of the

tubules and a minimal amount of ineffective spermatogenesis occurring in several of the tubules, Grade V. H&E, 125×.

Figure 6. Gross photographs of penises, prostates, seminal vesicles, and the *levator ani* and *bulbocavernosus* muscles (LABC) on PND 45 and 70 from male pups of dams dosed by gavage with corn oil or 500 mg/kg/day DBP in corn oil on GD 12 to 21. (A) Control penis on PND 45. Ventral surface with the urethral opening at the tip, near the forceps. Bar = 0.5 cm. (B) DBP-exposed penis on PND 45. Hypospadias with incomplete closure of the urethral folds on the ventral surface of the penis exposing the os penis (OP). The urethral opening is at the base of the penis. Bar = 0.5 cm. (C) Control ventral (VP) and dorsolateral (DLP) prostates and seminal vesicle (SV) on PND 45. Bar = 1.0 cm. (D) DBP-exposed DLP and SV on PND 45. The DLP and SV are both decreased in size with a malformation of the left lobe of the SV. The VP is absent from the tissue. Bar = 0.5 cm. (E) Control LABC on PND 70. There are two portions of the muscle, the *levator ani* portion (LA) and the *bulbocavernosus* portion (BC). Bar = 1.0 cm. (F) DBP-exposed LABC on PND 70. Overall, the LABC is markedly decreased in size, especially the BC. Bar = 1.0 cm.

Figure 1


0 mg/kg/day
500 mg/kg/day
Dam Body Weight (g)
400
380
360
340
320
300
280
260
8
10
12
14
16
18
20
22
Gestation Day


0 mg/kg/day
500 mg/kg/day
Fetal Body Weight (g)
6
5
4
3
2
1
0
16
17
18
19
20
21
Gestation Day

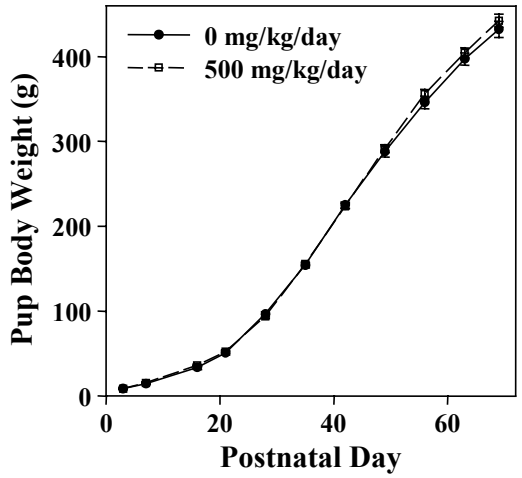

0 mg/kg/day
500 mg/kg/day
Pup Body Weight (g)
400
300
200
100
0
0
20
40
60
Postnatal Day

Figure 2

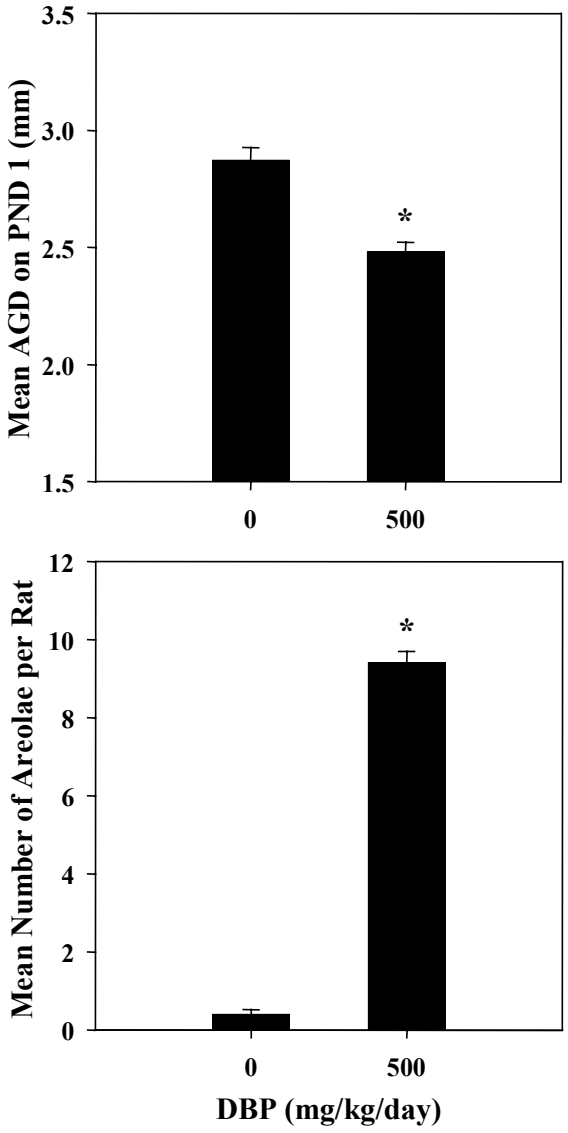

Mean AGD on PND 1 (mm)
3.5
3.0
2.5
2.0
1.5
*
0
500
Mean Number of Areolae per Rat
12
10
8
6
4
2
0
*
0
500
DBP (mg/kg/day)

Figure 3

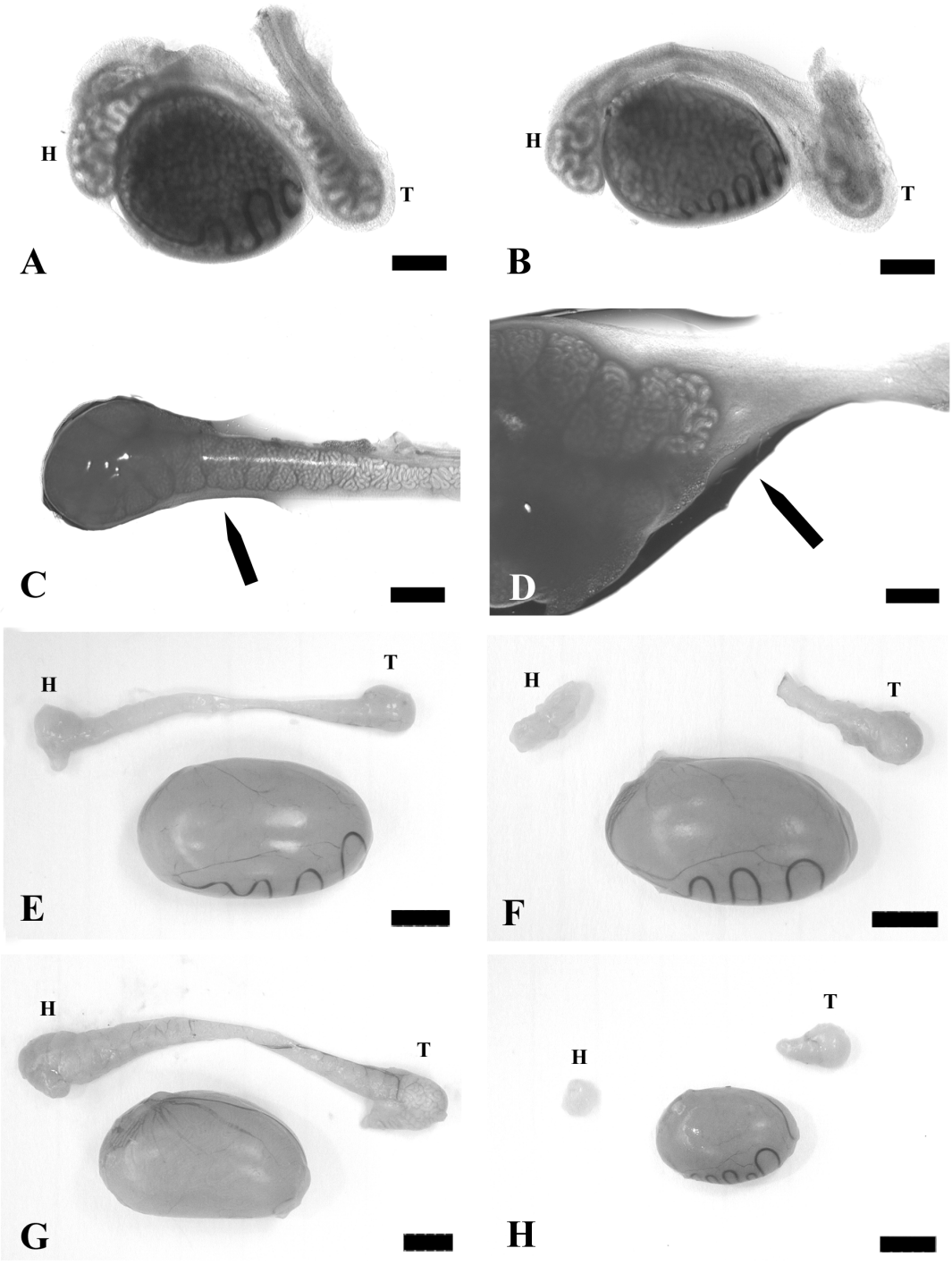


Figure 4

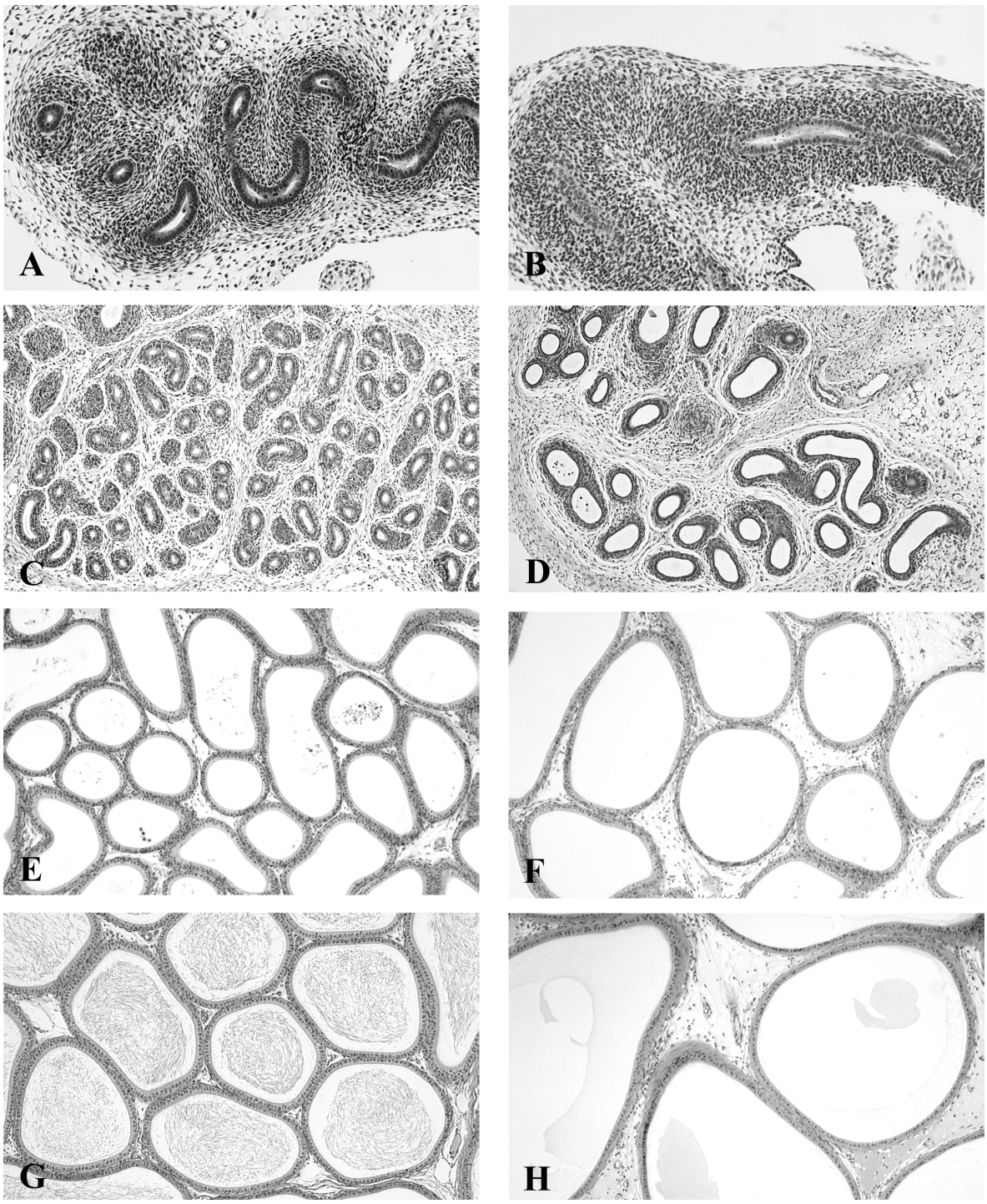


Figure 5

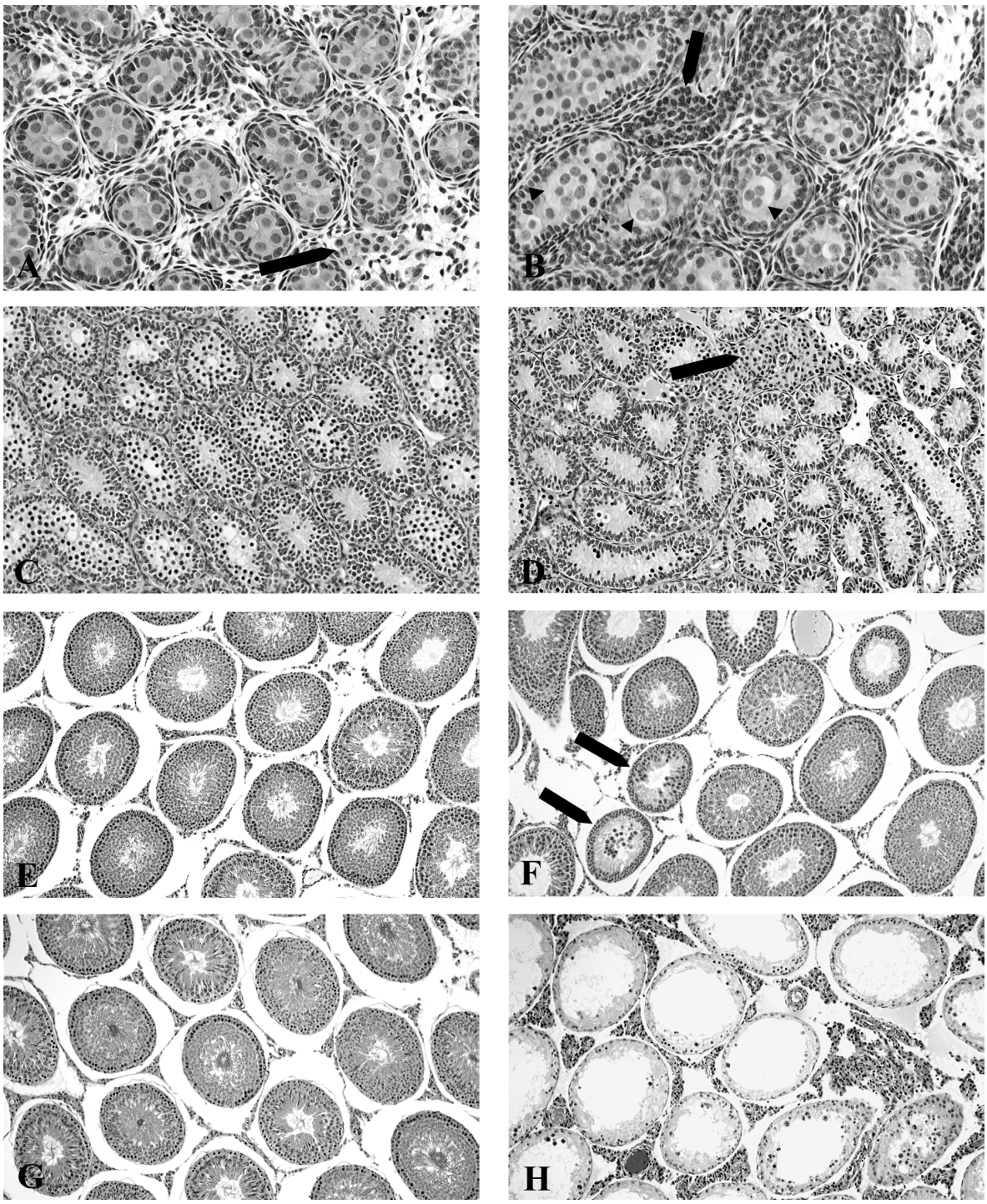
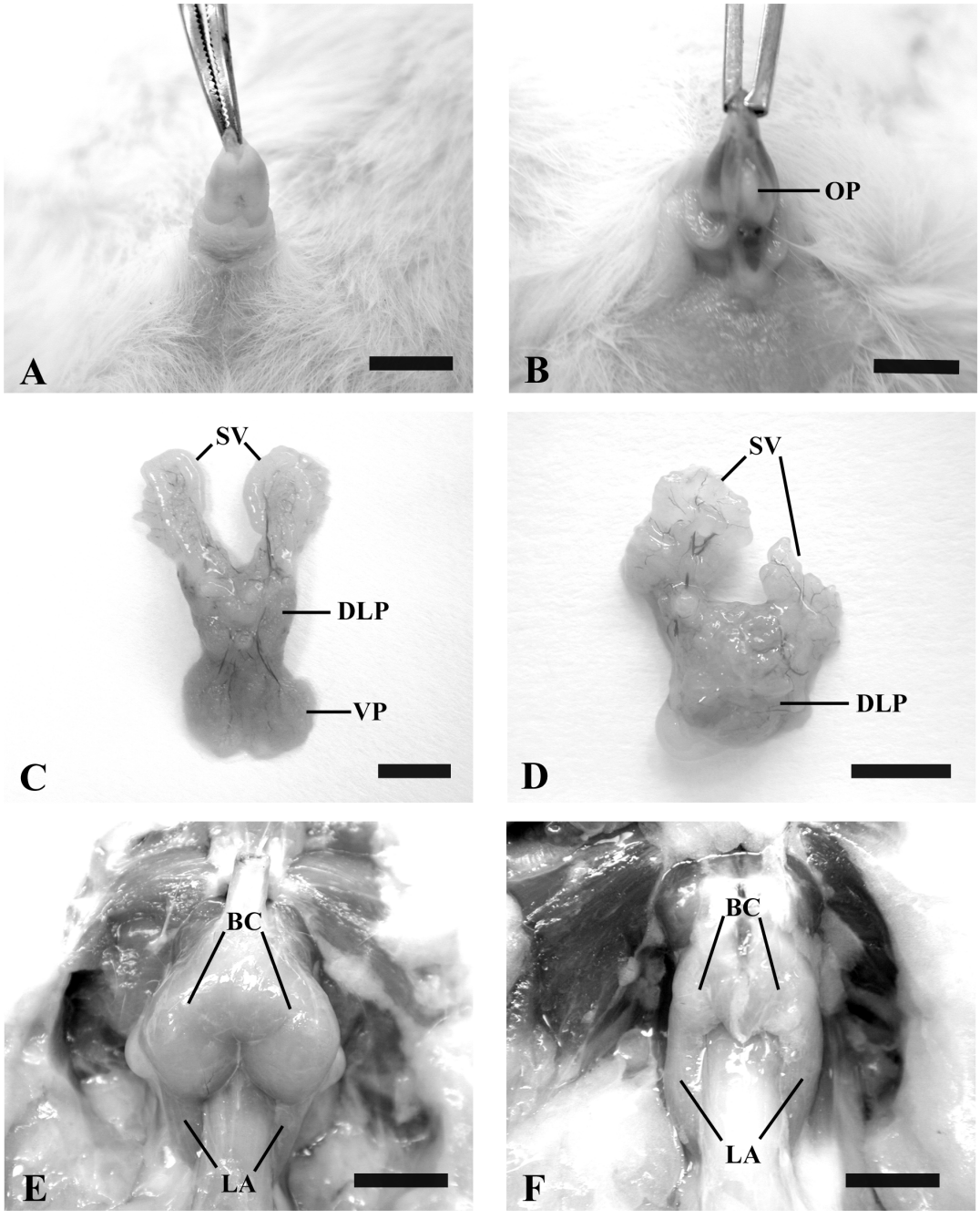


Figure 6



CHAPTER 4

QUANTITATIVE CHANGES IN GENE EXPRESSION IN FETAL RAT TESTES FOLLOWING EXPOSURE TO DI(*N*-BUTYL) PHTHALATE

Norman J. Barlow[1,2], Suzanne L. Phillips[1], Duncan G. Wallace[1], Madhabananda Sar[1], Kevin W. Gaido[1], and Paul M. D. Foster[3]

[1]CIIT Centers for Health Research

Research Triangle Park, North Carolina

[2]North Carolina State University

Raleigh, North Carolina

[3] National Institute of Environmental Health Sciences

Research Triangle Park, North Carolina

ABSTRACT

Di(*n*-butyl) phthalate (DBP) alters male reproductive development by decreasing testicular testosterone (T) production when fetuses are exposed on gestation days (GD) 12 to 21. Previous studies have shown altered gene expression for enzymes in the T biosynthetic pathway following exposure to DBP. The objectives of this study were to develop a more detailed understanding of the effect of DBP on steroidogenesis using a robust study design with increased numbers of dams and fetuses compared to previous studies and to explore mRNA expression for other critical genes involved in androgen biosynthesis and signaling. Additionally, immunohistochemical localization of protein expression for several key genes was performed to further confirm mRNA changes. Fetal Leydig cell lipid levels were also examined histochemically using oil red O. Six to seven pregnant Crl:CD(SD)BR rats per group were gavaged with corn oil or DBP at 500 mg/kg/day on GD 12 to 19. Testicular RNA isolated from three randomly selected GD 19 fetuses per litter was used for real-time RT-PCR for the following genes: scavenger receptor class B-1 (SRB1), steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), P450c17, 17β-hydroxysteroid dehydrogenase (17β-HSD), androgen receptor (AR), luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (FSHR), stem cell factor tyrosine kinase receptor (*c-kit*), stem cell factor (SCF), proliferating cell nuclear antigen (PCNA), and testosterone-repressed prostate message-2 (TRPM-2). mRNA expression was downregulated for SRB1, StAR, P450scc, 3β-HSD, P450c17, and *c-kit* following DBP exposure, while TRPM-2 was upregulated. 17β-HSD, AR, LHR, FSHR, and PCNA were

not significantly changed. Immunohistochemical staining for *c-kit* was seen in fetal Leydig cells, which has not been previously reported. Downregulation of most of the genes in the T biosynthetic pathway confirms and extends previous findings. Diminished Leydig cell lipid content and alteration of cholesterol transport genes also support altered cholesterol metabolism and transport as a potential mechanism for decreased T synthesis following exposure to DBP.

INTRODUCTION

Phthalate esters are used in the plastics, coatings, and cosmetics industries and are widely distributed environmental contaminants (IPCS 1992, 1997). Examining urinary phthalate metabolites, Blount et al. (2000) found that the general population appears to be exposed to disproportionately higher amounts of di(*n*-butyl) phthalate (DBP) compared with other phthalates. In addition, women of childbearing age, 20 to 40 years old, which is the target population for the teratogenic effects of DBP, had estimated exposures higher than other groups and had six of the eight highest levels found in the study (Blount *et al.*, 2000; Kohn *et al.*, 2000). A DBP multigenerational study performed by the National Toxicology Program showed DBP to be a male reproductive toxicant that had effects on animals exposed pre- and postnatally, while similar reproductive effects were not seen in the parental generation (NTP, 1991; Wine *et al.*, 1997). Furthermore, studies by Mylchreest et al. (1999, 2000) showed that DBP exposure during gestation had the ability to profoundly affect the developing male reproductive tract in the absence of maternal toxicity. Dams gavaged on gestation days (GD) 12 to 21 produced male offspring that had multiple malformations of epididymides, *vasa deferentia*, seminal vesicles, and dorsolateral and ventral prostate lobes that persisted into adulthood. *In utero* exposure also led to hypospadias, cryptorchidism, decreased anogenital distance (AGD) on postnatal day (PND) 1, and increased areolae retention on PND 13 (Barlow and Foster, 2003; Gray *et al.*, 1999; Mylchreest *et al.*, 1999; Mylchreest *et al.*, 2000).

Although DBP and its major metabolite do not bind the androgen receptor (Foster *et al.*, 2001), DBP has been characterized as an antiandrogen because it caused a 66-88%

decrease in fetal intratesticular testosterone (T) levels in the rat on GD 18, 19, and 21 (Mylchreest *et al.*, 2002; Shultz *et al.*, 2001) and had profound effects on the developing male reproductive tract (Barlow and Foster, 2003; Gray *et al.*, 1999; Mylchreest *et al.*, 1999; Mylchreest *et al.*, 2000). The mechanism by which DBP caused reduced T levels was through decreased production of androgen by the fetal Leydig cells (LC) (Lambright *et al.*, 2003). Decreased T in the testes may have led to altered differentiation of the Wolffian ducts and induced malformations in those tissues that were then detected in adult offspring (Mylchreest *et al.*, 2002). In addition to effects on T-dependent tissues, the epididymides, *vasa deferentia*, and seminal vesicles, effects were also seen in dihydrotestosterone (DHT)-dependent tissues, the prostate and external genitalia, although those alterations were less prevalent (Barlow and Foster, 2003).

Three characteristic histologic lesions diagnosed in fetal testes exposed to DBP were large aggregates of LCs, multinucleated gonocytes, and seminiferous cords that contained increased numbers of gonocytes. Although histologic changes were first observed on GD 17, gross lesions were not detected until GD 19 to 20, at which time the developing epididymides appeared smaller with decreased coiling of the epididymal duct (Barlow and Foster, 2003). GD 19 was chosen for the current study because nearly 100% of the animals exhibited three characteristic DBP-induced lesions at this age, especially increased numbers of LCs (Barlow and Foster, 2003). In addition to morphologic lesions on GD 19, this age was chosen because T synthesis is at or near its zenith (Huhtaniemi and Pelliniemi, 1992; Tapanainen *et al.*, 1984).

Utilizing microarray analyses and real-time quantitative RT-PCR , Shultz et al. (2001) identified genes in fetal testes whose expression was altered by DBP exposure.

They found decreased gene expression on GD 19 for structure-specific recognition protein, prothymosin-alpha, heart fatty acid binding protein, P450 side-chain cleavage enzyme (P450scc), scavenger receptor class B-1 (SRB1), and eukaryotic translation initiation factor. The results were based on total RNA from both testes of one fetus per dam and three dams per treatment group. Changes in gene expression seen with microarrays on GD 19 were supported by RT-PCR data for P450scc and SRB1. RT-PCR was also performed on other genes that were not on the microarrays, steroidogenic acute regulatory protein (StAR), P450c17 (17α-hydroxylase/17,20-lyase), myristoylated alanine-rich C-kinase substrate (MARCKS), testosterone-repressed prostate message-2 (TRPM-2), proliferating cell nuclear antigen (PCNA), and stem cell factor tyrosine kinase receptor (*c-kit*). Testicular RNA from a single fetus for each of three dams per group was also used for these analyses (Shultz *et al.*, 2001).

One objective of the present study was to confirm DBP-induced alterations in fetal testicular mRNA expression found by Shultz et al. (2001), utilizing increased numbers of dams per treatment group and more fetuses per dam. Additional genes in the steroidogenic pathway not previously examined, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD), were added to the analyses to give a more complete picture of the changes in gene expression in the entire steroid biosynthetic pathway. Androgen receptor (AR), luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (FSHR), and stem cell factor (SCF) were also examined for changes in mRNA expression. Immunolocalization of the proteins for StAR, SRB1, TRPM-2, *c-kit*, and SCF was performed to assess cell localization within the testis and to determine whether protein expression corresponded to changes in gene expression. Data

from this study indicated that there were a multitude of changes in gene expression for most of the enzymes of the T biosynthetic pathway, for several associated with cholesterol transport, and for other androgen-related genes. These changes in mRNA expression were supported by immunohistochemical localization of selected proteins and by staining for lipids.

MATERIALS AND METHODS

Animals. Animals were housed in the animal facility of the CIIT Centers for Health Research (CIIT), which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. This study followed Federal guidelines for the care and use of laboratory animals (National Research Council, 1996) and was approved by the Institutional Animal Care and Use Committee at CIIT. Thirteen pregnant CRL:CD(SD)BR rats were time-mated at Charles River Breeding Laboratories, Inc. (Raleigh, NC) and shipped to CIIT on GD 0, the day sperm was detected in the vaginal smear. Dams were assigned to a treatment group by body weight randomization using Provantis (Instem LSS, Stone, UK), 6 animals in the control group and 7 animals in the DBP-dosed group. Animals were identified by ear tags and cage cards and housed individually in polycarbonate cages with Alpha-dri cellulose bedding (Shepherd Specialty Papers, Kalamazoo, MI). Rodent diet NIH-07 (Zeigler Brothers, Gardener, PA) and reverse-osmosis water were provided *ad libitum*. Animals were kept in a humidity- and temperature-controlled, HEPA-filtered, mass air-displacement room. The room was maintained on a 12-hr light-dark cycle at approximately 22 ± 4ºC with a relative humidity of approximately 30-70%.

Study design. Dams were gavaged daily from GD 12 to 19 with corn oil vehicle (Sigma Chemical Co., St. Louis, MO) or DBP (Aldrich Chemical Company, Milwaukee, WI) in corn oil at 500 mg/kg/day. This dose of DBP was selected because a previous morphologic study (Barlow and Foster, 2003) had shown that nearly 100% of male fetuses had testicular lesions, especially of LCs, on GD 19 and because this was the dose of DBP

used by Shultz et al. (2001) to examine gene expression. 500 mg/kg/day of DBP during gestation is not a likely environmental exposure for humans. However, the lesion incidence of nearly 100% provides a robust model for studying altered tissue morphologies and associated changes in mRNA expression.

Dam body weights were recorded on GD 9 and daily during the dosing period. The dams from each dose group were euthanized on GD 19 by CO_2 asphyxiation and exsanguination via aortic transection. Fetuses were immediately removed from the uterus, weighed, euthanized by decapitation, and sexed by internal examination of the reproductive organs. The right and left testes and epididymides were removed from male fetuses using a dissecting microscope. The epididymides were separated from the testes using a dissecting microscope with transillumination. Both testes were snap-frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

Real-Time Quantitative RT-PCR. Total RNA was isolated from both testes using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer's suggested protocol. Total testicular RNA (1 µg) was treated with DNase I (Amersham Pharmacia Biotech, Newark, NJ) at 37°C for 30 min in the presence of RNasin (Applied Biosystems, Foster City, CA). DNase I was heat-inactivated at 75°C for 5 min, and cDNA was synthesized using random hexamers and TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer's suggested protocol. Total RNA from each tissue was separated into four aliquots for reverse transcription (RT) with one aliquot receiving no enzyme and designated to serve as a negative control. Quality of RT reactions was confirmed by comparison of triplicate RT versus no enzyme control for each RNA sample using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set. Rat-

specific primers and probes were designed for the genes of interest (Tables 1 and 2) using Primer Express software (Applied Biosystems) with the following parameters: low T_m = 60ºC, high T_m = 64ºC, optimum T_m = 62ºC, amplicon length = 80 to 150 base pairs, and primer length = 20 to 24 base pairs, with an optimum of 22. Production of a single PCR product was confirmed using agarose gel electrophoresis, and primer and probe efficiencies were determined according to manufacturer's recommended protocol (Applied Biosystems). RT-PCR was performed on an ABI PRISM 7700 Sequence Detection System and on the ABI PRISM 7900HT Sequence Detection System using SYBR Green PCR and TaqMan Universal PCR Master Mix reagent kits according to the manufacturer's instructions for quantification of gene expression (Applied Biosystems). GAPDH was used as an internal calibrator for all RT-PCR reactions. Five dams from the control group were used since the sixth dam had only two male fetuses. Five dams were randomly selected from the seven DBP-exposed dams to maintain the same number of animals between control and DBP-exposed groups. RT-PCR was performed in triplicate on three randomly selected fetuses from each dam for a total of 15 fetuses per group.

Immunohistochemistry. GD 19 fetal testes for immunohistochemistry were randomly selected from a prior DBP study that used the same dosing schedule utilized in this study. Testes were immersion-fixed in 10% neutral-buffered formalin for 24 h, transferred to 70% ethanol, processed into paraffin, sectioned at 5 μm, placed on charged slides, and stored at room temperature until processed for immunostaining. The sections were deparaffinized and then treated with 3% H_2O_2 in methanol for 10 min to suppress endogenous peroxidase activity. Antigen retrieval was performed by heating the sections for 3 min in citrate buffer (1:10 dilution in deionized water, pH 5.5–5.7) (BioGenex, San

Ramon, CA). The avidin-biotin peroxidase method was used for immunostaining as described previously (Sar and Welsch, 1999). The sections were incubated with 10% Carnation powdered nonfat milk (Nestlé, Solon, OH) for 10 min followed by 2% normal serum (rabbit or goat) in PBS for 20 min each to reduce nonspecific staining. Tissues were also treated for 15 min each with avidin and biotin to suppress endogenous biotin activities. Incubation occurred overnight at 4ºC with the following primary antibodies: StAR (rabbit polyclonal IgG, 1 μg/ml), c-kit (rabbit polyclonal IgG, 2 μg/ml), SRB1 (goat polyclonal IgG, 1 μg/ml), SCF (goat polyclonal IgG, 2 μg/ml), and TRPM-2 (goat polyclonal IgG, 4 μg/ml).

All primary antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA except StAR, which was purchased from the Affinity BioReagents, Golden, CO. The optimal working dilution of each antibody was determined by incubating sections with various concentrations of antibody ranging from 1 to 5 μg/ml. Following incubation with the primary antibody, the slides were washed in PBS for 5 min followed by incubation with a biotinylated secondary antibody antirabbit IgG or antigoat IgG (1:200) and then with avidin-biotin peroxidase (1:200) (Vector Labs, Burlingame, CA) for 30 min at room temperature. The sections were exposed to liquid diaminobenzidine (BioGenex, San Ramon, CA) after a 5-min PBS wash. The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted with Permount. The specificity of immunostaining was determined by incubating adjacent sections with the preabsorbed antibody, which was prepared by incubating each antibody with 4- to 5-fold excess of the synthetic peptide used as the immunogen.

Oil red O histochemistry. Frozen sections from GD 19 testes were cut and placed on slides. Oil red O staining was performed according to Pearse (1996). Skin was used as the positive control with dark red staining seen in subcuticular adipose tissue.

Statistical Analyses. Statistical analyses were conducted using JMP (version 4.0.0, SAS Institute, Cary, NC). Gene expression data were analyzed by a repeated measure ANOVA (nested design) with the dams treated as the experimental unit. Relative expression ratios were calculated using the equation set forth by Pfaffl (2001) in which efficiencies for both the gene of interest and the calibrator, GAPDH, were used. Actual efficiencies were calculated from standard curves for TaqMan data, while efficiencies were assumed to be optimal and similar between the gene of interest and GAPDH for SYBR Green data. Therefore, 2 was used as the efficiency, as recommended by the manufacturer (Applied Biosystems). An average threshold cycle (CT) for all of the control fetuses was generated for both the gene of interest and GAPDH. Each individual CT was then subtracted from the average CT for both the control and DBP-exposed fetuses yielding ΔCTs. These data were entered into the Pfaffl equation,

$$\text{Expression ratio} = E_{\text{gene}}^{\Delta\text{CT gene (control-exposed)}} / E_{\text{GAPDH}}^{\Delta\text{CT GAPDH (control-exposed)}}$$

to generate relative expression ratios for each fetus. Since a group control average was used for the gene of interest and GAPDH, the expected expression ratio for each animal in the control group was 1 (or 100% of control). Statistical analyses were performed on the expression ratios of individual fetus, nested by dam. For data generated using SYBR Green fluorescence, the Pfaffl equation is equal to the equation put forth in Applied Biosystem's User Bulletin No. 2, $2^{-\Delta\Delta CT}$. Analyses of relative expression ratios were considered to be statistically significant at $p < 0.05$.

As three fetuses per dam and five dams per group were used in this study, we examined the intralitter versus interlitter variability to determine whether there was more variability between fetuses in a litter or whether there was more variability between dams (litter means). The analysis was achieved by comparing the dam and fetus variance components.

RESULTS

Oil red O histochemistry. Oil red O stains lipid deep red within histologic sections. Fetal LCs randomly dispersed throughout the interstitium of control testes contained large numbers of variably sized lipid vacuoles (Figure 1A). The interstitium of DBP-exposed testes contained large aggregates of LCs with red staining lipid vacuoles. However, the size and number of the lipid vacuoles in the LCs of these large clusters were decreased (Figure 1B). Additionally, there were decreased numbers of oil red O positive LCs throughout the rest of the interstitium.

Cholesterol transport genes and TRPM-2. Gene expression for the high-density lipoprotein (HDL) receptor on the cell surface, SRB1, was significantly decreased, ~41% of control (Figure 2). Immunostaining for SRB1 in control testes was localized to the cytoplasm of LCs with low-intensity staining of the cytoplasm of some Sertoli cells (Figure 1C). The staining for this protein was decreased in LCs in DBP-exposed fetuses (Figure 1D). In addition to decreased staining in the LCs, there was marked increase in the cytoplasmic staining of Sertoli cells (Figure 1D). Similar to SRB1, gene expression for StAR, the protein responsible for cholesterol transport to the inner mitochondrial membrane, was also significantly reduced to ~34% of control (Figure 2). Immunostaining for StAR was intense and localized to the cytoplasm of LCs in control and DBP-exposed testes (Figures 1E and 1F). However, the staining intensity of LCs in DBP-exposed testes was markedly decreased (Figure 1F). mRNA expression for the androgen-related gene TRPM-2 was significantly increased to approximately 276% of control (Figure 2), which corresponded to immunostaining for this gene. Staining for TRPM-2 protein was observed

in the cytoplasm of small numbers of Sertoli cells in control animals (Figure 3A), while staining was markedly increased in Sertoli cells of DBP-exposed testes (Figure 3B).

Steroidogenic enzymes. Gene expressions for all the enzymes involved in testosterone biosynthesis from the conversion of cholesterol to pregnenolone (P450scc) to the formation of T from androstenedione (17β-HSD) were examined. The initial three enzymes that catalyze the first four reactions in T biosynthesis all had significantly decreased expression ratios. P450scc was most severely decreased with a gene expression of ~5% of control (Figure 4). 3β-HSD and P450c17 were similarly decreased, with gene expressions of ~52 and 59% of control (Figure 4). Whereas the initial three enzymes were significantly decreased, gene expression for 17β-HSD was increased 42% relative to control (Figure 4). However, there was a large component of variability in the DBP-exposed fetal testes for 17β-HSD and the increased gene expression was not a statistically significant different from control.

Androgen and gonadotropin receptors. There were no significant differences from control for AR, LHR, or FSHR (Figure 5).

Gonocyte and Sertoli cell surface proteins and PCNA. Relative expression ratios for two cell surface proteins were significantly decreased. C-kit is the tyrosine kinase receptor for SCF and is located on the cell surface of gonocytes. Gene expression for this protein was decreased to ~9% of control (Figure 6). Intense c-kit immunostaining was noted in the cytoplasm of gonocytes and LCs in control testes (Figure 3C), while the staining was appreciably decreased in both cell types of DBP-exposed testes (Figure 3D). The ligand for c-kit is the Sertoli cell surface protein SCF. Its expression was also significantly decreased to ~10% of control (Figure 6). SCF protein was observed in the

cytoplasm of Sertoli cells, LCs, and some gonocytes in control testes (Figure 3E). Staining intensity in Sertoli cells was dramatically increased following DBP exposure, while the cytoplasm of most of the LCs in the large aggregates did not appear to stain (Figure 3F). There was no statistically significant change in gene expression for the general cell proliferation protein PCNA (Figure 6).

Intra- versus interlitter variability. The variance component for individual fetuses was significantly increased over the dam variance component for 11 of the 13 genes examined, indicating that the variability between fetuses within a litter was greater than the variability between dams. The difference in variability was statistically significant for SRB1 ($p < 0.001$), P450scc ($p < 0.024$), P450c17 ($p < 0.039$), 17β-HSD ($p < 0.001$), LHR ($p < 0.001$), *c-kit* ($p < 0.001$), and TRPM-2 ($p < 0.001$). While not significant, the dam variance component for 3β-HSD and FSHR was greater than the fetal variance component.

DISCUSSION

Histologic lesions seen with *in utero* DBP exposure include large aggregates of abnormal LCs within the interstitium and multinucleated gonocytes and increased numbers of gonocytes in seminiferous cords (Barlow and Foster, 2003; Mylchreest *et al.*, 2002). A fetal LC and the T biosynthetic pathway within that cell are illustrated in Figure 7. Genes examined in this study, the cellular location of their proteins, and their mRNA expression levels (percentage of control) are shown. In addition, photomicrographs of control and DBP-exposed fetal testes and a graph of fetal intratesticular T levels found by Shultz et al. (2001) are included to highlight all the changes that occur in fetal testes following *in utero* DBP exposure. Though intratesticular T levels were not measured in this study, the dosing regimen used in the current study has been shown to significantly reduce fetal intratesticular T and decrease production of T by DBP-exposed testes (Figure 7) (Lambright *et al.*, 2003; Mylchreest *et al.*, 2002; Shultz *et al.*, 2001). Decreased androgen synthesis was supported by increased mRNA and protein expression for TRPM-2, an androgen-responsive gene found to be increased during prostatic regression following castration and by the use of antiandrogens (Lakins *et al.*, 1998; Wong *et al.*, 1993). As the name of the gene states, normal androgen levels repress TRPM-2 expression in the prostate (Leger *et al.*, 1987). If androgen status is disrupted and androgen levels drop, there is increased expression of this gene and the corresponding protein (Miyake *et al.*, 2000a; Miyake *et al.*, 2000b).

Proteins directly responsible for T synthesis in fetal rat testes include StAR, P450scc, 3β-HSD, 17α-hydroxylase/17,20-lyase, and 17β-HSD. Additionally, SRB1 is

responsible for transport of high-density lipoprotein (HDL) cholesteryl esters into the cell (Cao *et al.*, 1999; Trigatti *et al.*, 2000). Although cholesterol for steroidogenesis in the fetal testis may be obtained from the conversion of intracellular acetate, the preferred source of cholesterol is uptake of cholesteryl esters from HDL by SRB1 (Andersen and Dietschy, 1978; Cao *et al.*, 1999). Even so, phthalates have been shown to interfere with cholesterologenesis in the testes and decrease plasma cholesterol levels (Bell, 1982). While conversion of acetate to cholesterol and cholesterol uptake was not measured in the current study, decreased cholesterol uptake into fetal LCs following DBP exposure has been noted (Thompson *et al.*, 2003). SRB1 gene expression was significantly decreased in the current study (Figure 7), and DBP likely altered intracellular cholesterol synthesis, both of which may have contributed to decreased intracellular cholesterol levels. There were decreased numbers of LCs staining for lipid and the LCs of the large aggregates (Figure 7) had decreased lipid staining. Transmission electron microscopy was performed in a separate morphological study on testes exposed to DBP by the same dosing scheme and also showed decreased lipid vacuoles in LCs within large aggregates (N.J. Barlow, unpublished data). Taken together, these data support alterations in cholesterol synthesis, transport, and storage that likely play a role in decreased testosterone production by fetal LCs.

StAR is necessary for delivery of cholesterol to the inner mitochondrial membrane and is thought to be the overall rate-limiting step in steroid production (Hasegawa *et al.*, 2000; Manna *et al.*, 2001). Gene expression and protein levels of this cholesterol transport molecule were significantly decreased relative to control (Figure 7). Studies by Thompson et al. (2003) have shown that, in addition to decreased uptake of cholesterol by LCs, there

is also decreased uptake of cholesterol into DBP-exposed mitochondria, further supporting altered cholesterol handling in the pathogenesis of decreased T synthesis (Figure 7).

P450scc conversion of cholesterol to pregnenolone is the limiting enzymatic step in T biosynthesis (Miller, 1988; Omura and Morohashi, 1995). Although alteration of cholesterol transport and metabolism appear to contribute to decreased T synthesis, the significantly decreased level of mRNA expression for P450scc indicates another possible contributor (Figure 7). Decreased expression of P450scc may be partially due to reduced delivery of cholesterol, and therefore gene expression for the protein responsible for conversion to the next intermediate may have been downregulated. However, GD 19 DBP-exposed testes still produce small amounts of T, indicating that T biosynthesis was not completely inhibited. Whether the significantly decreased gene expression of P450scc was due to direct effects of DBP on gene expression for this enzyme or whether there was secondary downregulation following decreased cholesterol delivery is currently unknown. Gene expressions for 3β-HSD and P450c17 were both significantly decreased (Figure 7). However, Thompson et al. (2003) showed that when DBP-exposed testes were incubated with pregnenolone, progesterone, or 17α-hydroxyprogesterone that T production increased, though it never attained the same level as control testes. Collectively these data may indicate that decreased delivery of intermediates leads to decreased gene expression for these two proteins or that proteins levels were still high enough to support steroidogenesis even though gene expressions were decreased.

Gene expression changes for three receptors responsible for androgen signaling and male reproductive development and function were not significantly different from control. Unlike LHR and FSHR, testicular AR showed a trend for an increase above control.

Mylchreest et al. (2002) found similar protein expression for AR in DBP-exposed fetal testes. AR protein expression throughout the testis, especially in the large areas of LCs, was increased and double staining with 3β-HSD found that many of the AR-positive cells were 3β-HSD negative, which correlates with the decreased gene expression for 3β-HSD observed in the current study. Steroidogenesis in fetal LCs is initially independent of luteinizing hormone (LH) (El-Gehani *et al.*, 1998; Noumura *et al.*, 1966; O'Shaughnessy *et al.*, 1998). Although the LHR is first detectable in the fetal rat testis on GD 16.5, significant amounts of LH are not seen until T levels begin to decrease near the end of gestation (El-Gehani *et al.*, 1998; Zhang *et al.*, 1994). In addition, the male reproductive tract of LHR knockout mice is similar to control animals at birth, further supporting LH-independent production of T (Zhang *et al.*, 2001). Given that LH does not play a major role in steroidogenesis before GD 19, it is not surprising that we did not see alterations of gene expression for LHR in the current study.

Lesions in DBP-exposed seminiferous cords include the formation of large, multinucleated gonocytes and an increased number of gonocytes within the developing seminiferous cords (Figure 7). C-kit is the receptor for SCF, both of which are located on gonocytes and Sertoli cells respectively (Zsebo *et al.*, 1990). Mutations in either of these two genes have been shown to disrupt the interaction between the proteins, which leads to a lack of spermatogenesis and infertility in sexually mature animals (Loveland and Schlatt, 1997). Gene expressions for both *c-kit* and SCF were significantly decreased to approximately 10% of control, indicating that altered gene expression for these two proteins may play a part in the formation of multinucleated gonocytes or the increased numbers of gonocytes, although a clear mechanistic link between the reduced expression

for these two genes and the histologic lesions has not been definitively made (Figure 7). Immunostaining for c-kit was seen in both gonocytes and fetal LCs in control animals with decreased protein expression in both cell populations in DBP-exposed testes. Although observed in LCs in early postnatal and adult males, c-kit protein expression has not been previously described in fetal LCs (Fox *et al.,* 2000; Loveland and Schlatt, 1997; Manova *et al.*, 1990; Orth *et al.*, 1996). The purpose of *c-kit* in adult LCs may be related to soluble SCF and regulation of testosterone biosynthesis (Fox *et al.*, 2000; Loveland and Schlatt, 1997). Whether the same relationship is present in the fetus is not clear at this time. Though it is not known if *c-kit* and SCF expression remains low following birth and discontinued exposure to DBP, the early postnatal testicular lesion of decreased germ cells observed in SCF mutant mice is similar to the testicular lesion seen early postnatally following *in utero* DBP exposure (Barlow and Foster, 2003; Brannan *et al.*, 1992). Immunostaining for SCF in Sertoli cells of DBP-exposed testes was increased, which was opposite the observed SCF mRNA expression. The reason for the disparity between gene expression data and protein expression is not clear at this time.

A generally assumed biological phenomenon is that fetuses or pups within a litter are more closely related to each other than offspring from other litters (Elswick *et al.*, 2000; Haseman and Hogan, 1975; Shirley and Hickling, 1981; Williams, 1975). Under this principle, it may be assumed that one fetus or pup from a litter is representative of the entire litter. In this study, testes from three male fetuses were used to calculate a litter mean. When statistically analyzed, there was more variability among the three fetuses in the litter for most of the genes than there was between dam litter means. Since we have seen differences in animal sensitivity to DBP following *in utero* exposure, the intralitter

variability is not surprising (Elswick *et al.*, 2000; Mylchreest *et al.*, 1999; Mylchreest *et al.*, 2000). Although the reason for this variability is not known, it may be due to a variety of factors including uterine blood flow, which may alter the amount of compound being delivered to each fetus (Buelke-Sam *et al.*, 1982; Even *et al.*, 1994), and the location of each fetus within the uterus, which may lead to increased or decreased exposure to testosterone from neighboring fetuses (Clark *et al.*, 1993; Even *et al.*, 1992; Nonneman *et al.*, 1992). Given the intralitter variability seen in this study, selection of one fetus from a litter may be an inappropriate representation of the entire litter and may lead to erroneous conclusions. Therefore increased numbers of fetuses or pups from each litter (the entire litter complement if possible) should be used for statistical analyses.

This study, using a more robust design that included increased numbers of fetuses and dams, confirmed the gene expression changes found by Shultz et al. (2001). Additional genes (including 3β-HSD, 17β-HSD, AR, LHR, FSHR, SCF, and PCNA) were examined for changes in gene expression. Taken together the data correlate with decreased T synthesis by fetal LCs. Whether the changes in gene expression were the primary molecular cause of decreased steroidogenesis or whether the decreased expression of the steroidogenic enzymes was simply a physiological response to decreased amounts of intermediates is not known. Decreased amounts of lipid within LCs and the decreased gene expression for SRB1 and StAR, in conjunction with data from Thompson et al. (2003) and Bell (1982), favor the latter mechanism, although other mechanisms such as reduced intracellular signaling or lack of appropriate LC differentiation cannot be completely ruled out. Gene expression for the rate-limiting enzyme in T biosynthesis, P450scc, is decreased to approximately 5% of control; therefore this decrease may also play a significant part in

the mode of action of DBP on the fetal testis. While gene expressions for many of enzymes in the T biosynthetic pathway were downregulated, gene expression for 17β-HSD was not statistically different from control, arguing against a wholesale downregulation of all cellular genes in response to exposure to a toxicant. The finding of increased intralitter variability compared with interlitter variability was unexpected and warrants further investigation to determine the number of animals appropriate for gene expression analysis when using a nested study design.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Katie J. Turner and Mrs. Kim P. Lehmann for their assistance with dissections and statistical analyses of the gene expression data. We would also like to thank Drs. Christopher J. Thompson and L. Earl Gray, Jr. for their discussion of data in relation to the current study. The assistance of Mrs. Renee Thacker and Mr. Otis Lyght of the CIIT histology laboratory was greatly appreciated. Mr. E. Stan Piestrak and Mr. James Mangum were instrumental in the capturing of images and design of figures and illustrations. This study would not have been possible without the assistance of the CIIT animal care staff. Funding was provided in part by the American Chemistry Council and by a National Research Service Award from the National Institute of Environmental Health Sciences (awarded to NJB). This manuscript does not necessarily reflect the views or policies of either of these organizations.

REFERENCES

Andersen, J. M., and Dietschy, J. M. (1978). Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. *J. Biol. Chem.* **253**, 9024-9032.

Barlow, N. J., and Foster, P. M. D. (2003). Pathogenesis of male reproductive tract lesions from gestation through adulthood following *in utero* exposure to di(*n*-butyl) phthalate. *Toxicol. Pathol.* In press.

Bell, F. P. (1982). Effects of phthalate esters on lipid metabolism in various tissues, cells and organelles in mammals. *Environ. Health Perspect.* **45**, 41-50.

Blount, B. C., Silva, M. J., Caudill, S. P., Needham, L. L., Pirkle, J. L., Sampson, E. J., Lucier, G. W., Jackson, R. J., and Brock, J. W. (2000). Levels of seven urinary phthalate metabolites in a human reference population. *Environ. Health Perspect.* **108**, 979-982.

Brannan, C. I., Bedell, M. A., Resnick, J. L., Eppig, J. J., Handel, M. A., Williams, D. E., Lyman, S. D., Donovan, P. J., Jenkins, N. A., and Copeland, N. G. (1992). Developmental abnormalities in Steel17H mice result from a splicing defect in the steel factor cytoplasmic tail. *Genes Dev.* **6**, 1832-1842.

Buelke-Sam, J., Holson, J. F., and Nelson, C. J. (1982). Blood flow during pregnancy in the rat: II. Dynamics of and litter variability in uterine flow. *Teratology* **26**, 279-88.

Cao, G., Zhao, L., Strangle, H., Hasegawa, T., Richardson, J. A., Parker, K. L., and Hobbs, H. H. (1999). Developmental and hormonal regulation of murine scavenger receptor, class B, type 1. *Mol. Endocrinol.* **13**, 1460-73.

Clark, M. M., Bishop, A. M., vom Saal, F. S., and Galef, B. G., Jr. (1993). Responsiveness to testosterone of male gerbils from known intrauterine positions. *Physiol. Behav.* **53**, 1183-7.

El-Gehani, F., Zhang, F. P., Pakarinen, P., Rannikko, A., and Huhtaniemi, I. (1998). Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. *Biol. Reprod.* **58**, 116-23.

Elswick, B. A., Welsch, F., and Janszen, D. B. (2000). Effect of different sampling designs on outcome of endocrine disrupter studies. *Repro. Tox.* **14**, 359-367.

Even, M. D., Dhar, M. G., and vom Saal, F. S. (1992). Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. *J. Reprod. Fertil.* **96**, 709-16.

Even, M. D., Laughlin, M. H., Krause, G. F., and vom Saal, F. S. (1994). Differences in blood flow to uterine segments and placentae in relation to sex, intrauterine location and side in pregnant rats. *J. Reprod. Fertil.* **102**, 245-52.

Foster, P. M. D., Mylchreest, E., Gaido, K. W., and Sar, M. (2001). Effects of phthalate esters on the developing reproductive tract of male rats. *Hum. Reprod. Update* **7**, 231-235.

Fox, R. A., Sigman, M., and Boekelheide, K. (2000). Transmembrane versus soluble stem cell factor expression in human testis. *J. Androl.* **21**, 579-85.

Gray, L. E., Jr., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R. L., and Ostby, J. (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual

differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health* **15**, 94-118.

Hasegawa, T., Zhao, L., Caron, K. M., Majdic, G., Suzuki, T., Shizawa, S., Sasano, H., and Parker, K. L. (2000). Developmental roles of the steroidogenic acute regulatory protein (StAR) as revealed by StAR knockout mice. *Mol. Endocrinol.* **14**, 1462-71.

Haseman, J. K., and Hogan, M. D. (1975). Selection of the experimental unit in teratology studies. *Teratology* **12**, 165-71.

Huhtaniemi, I., and Pelliniemi, L. J. (1992). Fetal Leydig cells: cellular origin, morphology, life span, and special functional features. *Proc. Soc. Exp. Biol. Med.* **201**, 125-140.

International Programme on Chemical Safety (IPCS) (1992). Environmental Health Criteria 131. Diethylhexyl Phthalate. World Health Organization, Geneva.

International Programme on Chemical Safety (IPCS) (1997). Environmental Health Criteria 189. Di-*n*-butyl Phthalate. World Health Organization, Geneva.

Kohn, M. C., Parham, F., Masten, S. A., Portier, C. J., Shelby, M. D., Brock, J. W., and Needham, L. L. (2000). Human exposure estimates for phthalates. *Environ. Health Perspect.* **108**, A440-A442.

Lakins, J., Bennett, S. A., Chen, J. H., Arnold, J. M., Morrissey, C., Wong, P., O'Sullivan, J., and Tenniswood, M. (1998). Clusterin biogenesis is altered during apoptosis in the regressing rat ventral prostate. *J. Biol. Chem.* **273**, 27887-95.

Lambright, C. S., Wilson, V. S., Furr, J. R., Wolf, C. J., Noriega, N., and Gray, L. E., Jr. (2003). Effects of endocrine disrupting chemicals on fetal testes hormone production. *The Toxicologist* **62**. In press.

Leger, J. G., Montpetit, M. L., and Tenniswood, M. P. (1987). Characterization and cloning of androgen-repressed mRNAs from rat ventral prostate. *Biochem. Biophys. Res. Commun.* **147**, 196-203.

Loveland, K. L., and Schlatt, S. (1997). Stem cell factor and *c-kit* in the mammalian testis: lessons originating from Mother Nature's gene knockouts. *J. Endocrinol.* **153**, 337-44.

Manna, P. R., Roy, P., Clark, B. J., Stocco, D. M., and Huhtaniemi, I. T. (2001). Interaction of thyroid hormone and steroidogenic acute regulatory (StAR) protein in the regulation of murine Leydig cell steroidogenesis. *J. Steroid Biochem. Mol. Biol.* **76**, 167-77.

Manova, K., Nocka, K., Besmer, P., and Bachvarova, R. F. (1990). Gonadal expression of *c-kit* encoded at the W locus of the mouse. *Development* **110**, 1057-69.

Miller, W. L. (1988). Molecular biology of steroid hormone synthesis. *Endocr. Rev.* **9**, 295-318.

Miyake, H., Chi, K. N., and Gleave, M. E. (2000a). Antisense TRPM-2 oligodeoxynucleotides chemosensitize human androgen- independent PC-3 prostate cancer cells both in vitro and in vivo. *Clin. Cancer Res.* **6**, 1655-63.

Miyake, H., Nelson, C., Rennie, P. S., and Gleave, M. E. (2000b). Testosterone-repressed prostate message-2 is an antiapoptotic gene involved in progression to androgen independence in prostate cancer. *Cancer Res.* **60**, 170-6.

Mylchreest, E., Sar, M., Cattley, R. C., and Foster, P. M. D. (1999). Disruption of androgen-regulated male reproductive development by di(n- butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol.* **156**, 81-95.

Mylchreest, E., Sar, M., Wallace, D. G., and Foster, P. M. D. (2002). Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. *Reprod. Toxicol.* **16**, 19-28.

Mylchreest, E., Wallace, D. G., Cattley, R. C., and Foster, P. M. D. (2000). Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicol. Sci.* **55**, 143-151.

National Research Council (1996). Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.

National Toxicology Program (NTP) (1991). Di(*n*-butyl) phthalate reproduction and fertility assessment in CD Sprague-Dawley rats when administered via feed. National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Nonneman, D. J., Ganjam, V. K., Welshons, W. V., and Vom Saal, F. S. (1992). Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. *Biol. Reprod.* **47**, 723-9.

Noumura, T., Weisz, J., and Lloyd, C. W. (1966). In vitro conversion of 7-3-H-progesterone to androgens by the rat testis during the second half of fetal life. *Endocrinology* **78**, 245-53.

Omura, T., and Morohashi, K. (1995). Gene regulation of steroidogenesis. *J. Steroid Biochem. Mol. Biol.* **53**, 19-25.

Orth, J. M., Jester, W. F., Jr., and Qiu, J. (1996). Gonocytes in testes of neonatal rats express the *c-kit* gene. *Mol. Reprod. Dev.* **45**, 123-31.

O'Shaughnessy, P. J., Baker, P., Sohnius, U., Haavisto, A. M., Charlton, H. M., and Huhtaniemi, I. (1998). Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function. *Endocrinology* **139**, 1141-6.

Pearse, A. G. E. (1996). *Histotechnology: a self-instructional text*. ASCP Press, Chicago, IL.

Sar, M., and Welsch, F. (1999). Differential expression of estrogen receptor-beta and estrogen receptor- alpha in the rat ovary. *Endocrinology* **140**, 963-71.

Shirley, E. A., and Hickling, R. (1981). An evaluation of some statistical methods for analysing numbers of abnormalities found amongst litters in teratology studies. *Biometrics* **37**, 819-29.

Shultz, V. D., Phillips, S., Sar, M., Foster, P. M. D., and Gaido, K. W. (2001). Altered gene profiles in fetal rat testes after *in utero* exposure to di(n-butyl) phthalate. *Toxicol. Sci.* **64**, 233-42.

Tapanainen, J., Kuopio, T., Pelliniemi, L. J., and Huhtaniemi, I. (1984). Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biol. Reprod.* **31**, 1027-1035.

Thompson, C. T., Ross, S. M., and Gaido, K. W. (2003). Di(*n*-butyl) phthalate interferes with fetal testicular steroidogenesis at the level of cholesterol transport and cleavage. *The Toxicologist* **62**. In press.

Trigatti, B., Rigotti, A., and Krieger, M. (2000). The role of the high-density lipoprotein receptor SR-BI in cholesterol metabolism. *Curr. Opin. Lipidol.* **11**, 123-31.

Williams, D. A. (1975). The analysis of binary responses from toxicological experiments involving reproduction and teratogenicity. *Biometrics* **31**, 949-52.

Wine, R. N., Li, L. H., Barnes, L. H., Gulati, D. K., and Chapin, R. E. (1997). Reproductive toxicity of di-*n*-butylphthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ. Health Perspect.* **105**, 102-7.

Wong, P., Pineault, J., Lakins, J., Taillefer, D., Leger, J., Wang, C., and Tenniswood, M. (1993). Genomic organization and expression of the rat TRPM-2 (clusterin) gene, a gene implicated in apoptosis. *J. Biol. Chem.* **268**, 5021-31.

Zhang, F. P., Hamalainen, T., Kaipia, A., Pakarinen, P., and Huhtaniemi, I. (1994). Ontogeny of luteinizing hormone receptor gene expression in the rat testis. *Endocrinology* **134**, 2206-13.

Zhang, F. P., Poutanen, M., Wilbertz, J., and Huhtaniemi, I. (2001). Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol. Endocrinol.* **15**, 172-83.

Zsebo, K. M., Williams, D. A., Geissler, E. N., Broudy, V. C., Martin, F. H., Atkins, H. L., Hsu, R. Y., Birkett, N. C., Okino, K. H., Murdock, D. C., and et al. (1990). Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* **63**, 213-24.

Table 1. Primer Sets for Real-Time Quantitative RT-PCR Analyses

Gene	Forward Primer	Reverse Primer
GAPDH	AATGTATCCGTTGTGGATCTGA	GCCTGCTTCACCACCTTCT
SRB1	ATGGTACTGCCGGGCAGAT	CGAACACCCTTGATTCCTGGTA
StAR	CTGCTAGACCAGCCCATGGAC	TGATTTCCTTGACATTTGGGTTCC
P450scc	GGAGGAGATCGTGGACCCTGA	TGGAGGCATGTTGAGCATGG
3β-HSD	AGCAAAAAGATGGCCGAGAA	GGCACAAGTATGCAATGTGCC
P450c17	TGGCTTTCCTGGTGCACAATC	TGAAAGTTGGTGTTCGGCTGAAG
17β-HSD	AATGTGCTTTCCATTTGCAAGGT	ATGCCACTGGCAGAGGAGATG
AR	CTAGCGCGTGCCTTCCTTTACA	CCCACCTGCGGGAAGCT
LHR	CTGCGCTGTCCTGGCC	CGACCTCATTAAGTCCCCTGAA
FSHR	TTTACTTGCCTGGAAGCGACTAA	CCCAGGCTCCTCCACACA
C-kit	ATCCAGCCCCACACCCTGTT	TGTAGGCAAGAACCATCACAATGA
SCF	TTCGCTTGTAATTGGCTTTGC	TTCAACTGCCCTTGTAAGACTTGA
PCNA	CAACTTGGAATCCCAGAACAGGAG	TAAGGTCCCGGCATATACGTGC
TRPM-2	GCTTTCCCGGAAGTGTGTAACG	CGTGCGTAGAACTTCATGCAGG

Note. Rat-specific primers were designed using Primer Express software with the following parameters: low T_m = 60ºC, high T_m = 64ºC, optimum T_m = 62ºC, amplicon length = 80 to 150 base pairs, and primer length = 20 to 24 base pairs, with an optimum of 22.

Table 2. Probe Sequences for Real-Time Quantitative RT-PCR Analyses

Gene	Probe Sequence
GAPDH	CCGCCTGGAGAAACCTGCCAAGTATG
StAR	ACCGCATGGAGGCCATGGGAGA
P450scc	CCAGCGGTTCATCGACACGCCGT
3β-HSD	TGGCAGCCAATGGGAGCATCCT
P450c17	CCTACGTACTGGTCAATCTCCTTTTGGATCTTCTTCTT

Note. Rat-specific probes were designed using Primer Express software with the following parameters: low T_m = 60ºC, high T_m = 64ºC, optimum T_m = 62ºC, amplicon length = 80 to 150 base pairs, and primer length = 20 to 24 base pairs, with an optimum of 22.

FIGURE LEGENDS

FIG. 1. Histochemical and immunohistochemical staining of gestation day (GD) 19 fetuses from control males (A, C, and E) and males exposed to di(*n*-butyl) phthalate (DBP) (500 mg/kg/day) (B, D, and F) on GD 12 to 19. Control testis (A) stained with oil red O for lipids have Leydig cells (LC) randomly scattered throughout the interstitium that contain large numbers of variably sized, red-staining lipid vacuoles. DBP-exposed testis (B) has several large aggregates of numerous LCs that individually contained fewer lipid vacuoles. Control testis (C) with Sertoli cells (arrowheads) lining the seminiferous cords and LCs scattered throughout the interstitium. DBP-exposed testis (D) immunostained for scavenger receptor B-1 shows increased staining of Sertoli cells (arrowheads) lining the cords compared to control (C) and decreased staining of LCs in a large interstitial aggregate (*). Steroid acute regulatory protein expression is confined to LCs in both the control (E) and DBP-exposed testes (F). There is decreased staining intensity in the DBP-exposed LCs. Original magnification = ×500 (A and B) and ×250 (C, D, E, and F).

FIG. 2. Real-time quantitative RT-PCR analyses of testicular mRNA on gestation day 19 for scavenger receptor B-1 (SRB1), steroid acute regulatory protein (StAR), and testosterone repressed prostate message-2 (TRPM-2) from control and di(*n*-butyl) phthalate-exposed (500 mg/kg/day) fetuses. Gene expression levels are graphed as relative mRNA expressions (% of control). Gene expressions were analyzed for 15 fetuses per group, nested by dam. Values are nested litter means ± SEM. *Significantly different from control ($p < 0.05$).

FIG. 3. Immunohistochemical staining of gestation day (GD) 19 fetuses from control males (A, C, and E) and males exposed to di(*n*-butyl) phthalate (DBP) (500 mg/kg/day) (B, D, and F) on GD 12 to 19. TRPM-2 staining was markedly increased in Sertoli cells (arrowheads) of the DBP-exposed testis (B) while minimal staining for clusterin was seen in small numbers of Sertoli cells (arrowheads) and Leydig cells (LC) in the control testis (A). Immunostaining for c-kit was prominent in gonocytes (arrows) and LCs of the control testis (C). While these cells stained for c-kit in the DBP-exposed testis (D), staining intensity was diminished (Gonocytes = arrows, LC aggregate = *). Small numbers of LCs, Sertoli cells, and gonocytes were lightly stained for stem cell factor (SCF) in control animals (E), while staining intensity was markedly increased in Sertoli cells (arrowheads) in the DBP-exposed testis. LCs within a large aggregate (*) have little staining for SCF. Original magnification = 500 × (A and B) and 250 × (C, D, E, and F).

FIG. 4. Real-time quantitative RT-PCR analyses of testicular mRNA on gestation day 19 for P450scc, 3β-hydroxysteroid dehydrogenase, P450c17, and 17β- hydroxysteroid dehydrogenase from control and di(*n*-butyl) phthalate-exposed (500 mg/kg/day) fetuses. Gene expression levels are graphed as relative mRNA expressions (% of control). Gene expressions were analyzed for 15 fetuses per group, nested by dam. Values are nested litter means ± SEM. *Significantly different from control ($p < 0.05$).

FIG. 5. Real-time quantitative RT-PCR analyses of testicular mRNA on gestation day 19 for androgen receptor (AR), luteinizing hormone receptor (LHR), and follicle stimulating hormone receptor (FSHR) from control and di(*n*-butyl) phthalate-exposed (500 mg/kg/day) fetuses. Gene expression levels are graphed as relative mRNA expressions (%

of control). Gene expressions were analyzed for 15 fetuses per group, nested by dam. Values are nested litter means ± SEM. *Significantly different from control ($p < 0.05$).

FIG. 6. Real-time quantitative RT-PCR analyses of testicular mRNA on gestation day 19 for stem cell factor tyrosine kinase receptor (*c-kit*), stem cell factor (SCF), and proliferating cell nuclear antigen (PCNA) from control and di(*n*-butyl) phthalate-exposed (500 mg/kg/day) fetuses. Gene expression levels are graphed as relative mRNA expressions (% of control). Gene expressions were analyzed for 15 fetuses per group, nested by dam. Values are nested litter means ± SEM. *Significantly different from control ($p < 0.05$).

FIG. 7. Testosterone (T) biosynthetic pathway in a fetal Leydig cell and the effects of DBP on gene expression. Photomicrographs of control and DBP-exposed testes and a graph of the intratesticular T levels on gestation day 19. Cholesterol is synthesized from extracellular high-density lipoprotein (HDL) cholesteryl esters or from intracellular acetate. Cholesterol is then carried from the outer mitochondrial membrane by steroid acute regulatory protein (StAR) where it is converted to pregnenolone by P450side-chain cleavage enzyme (P450scc). Pregnenolone is then transported to the smooth endoplasmic reticulum (SER), where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone is converted to 17-hydroxyprogesterone and androstenedione by P450c17. Androstenedione is converted to the end product, T, by 17β-hydroxysteroid dehydrogenase (173β-HSD). The percentages in the boxes are relative gene expression ratios (% of control) for mRNA expression of each protein. *Significantly different from control ($p < 0.05$). Intratesticular T levels were significantly decreased on GD 19 following *in utero* DBP exposure (Shultz *et al.,* 2001). DBP-testes (B) have large

areas of abnormal Leydig cells (*), increased numbers of Leydig cells within the seminiferous cords (arrows), and multinucleated gonocytes (B inset) compared to control testes (A). Original magnification = ×250 (A and B) and ×500 (inset).

Figure 1

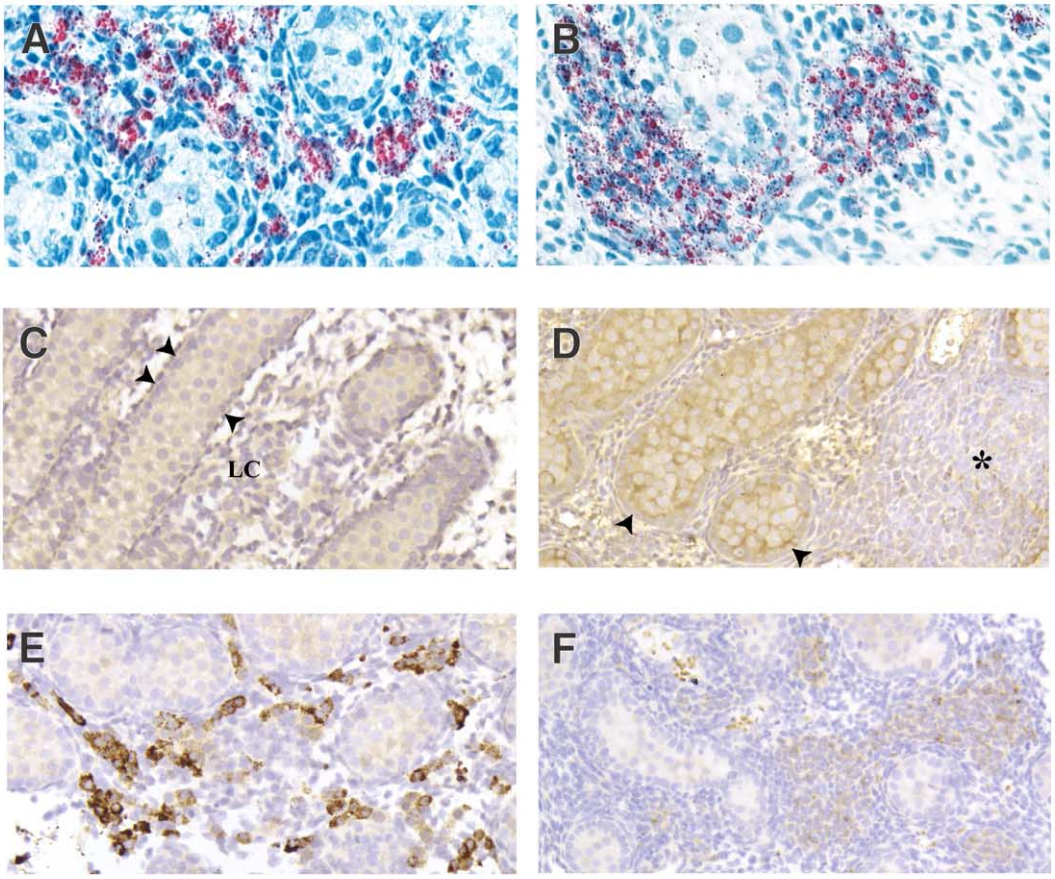


Figure 2

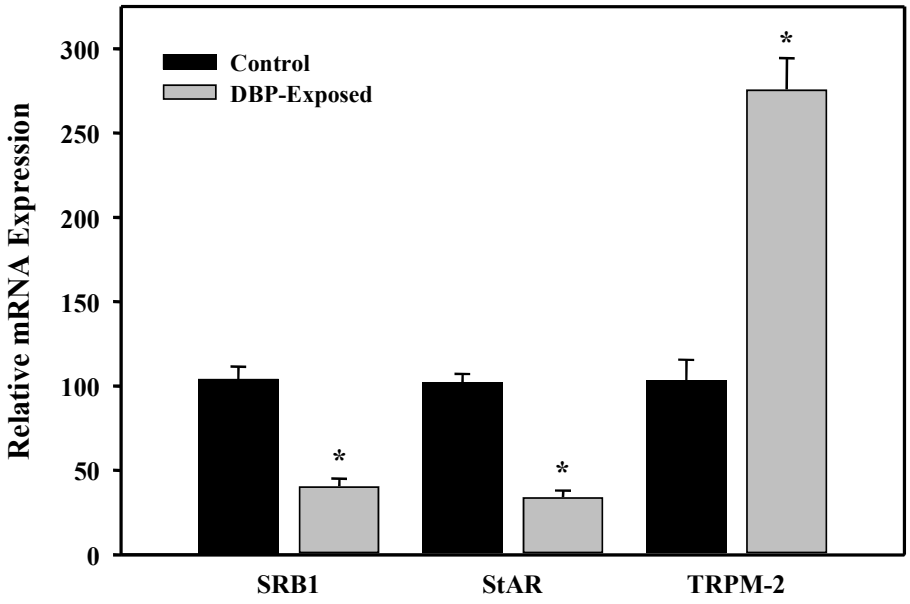

Relative mRNA Expression
300
250
200
150
100
50
0
Control
DBP-Exposed
*
*
*
SRB1
StAR
TRPM-2

Figure 3

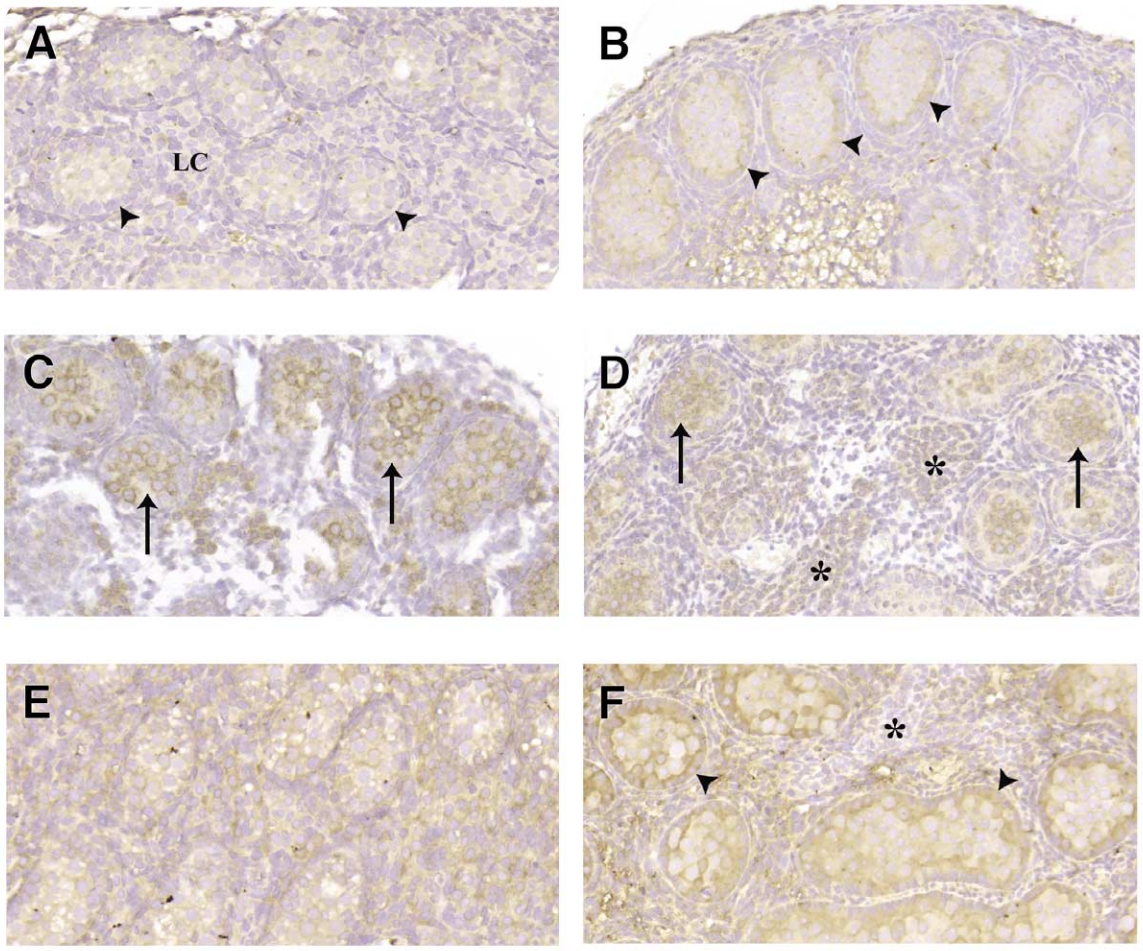


Figure 4

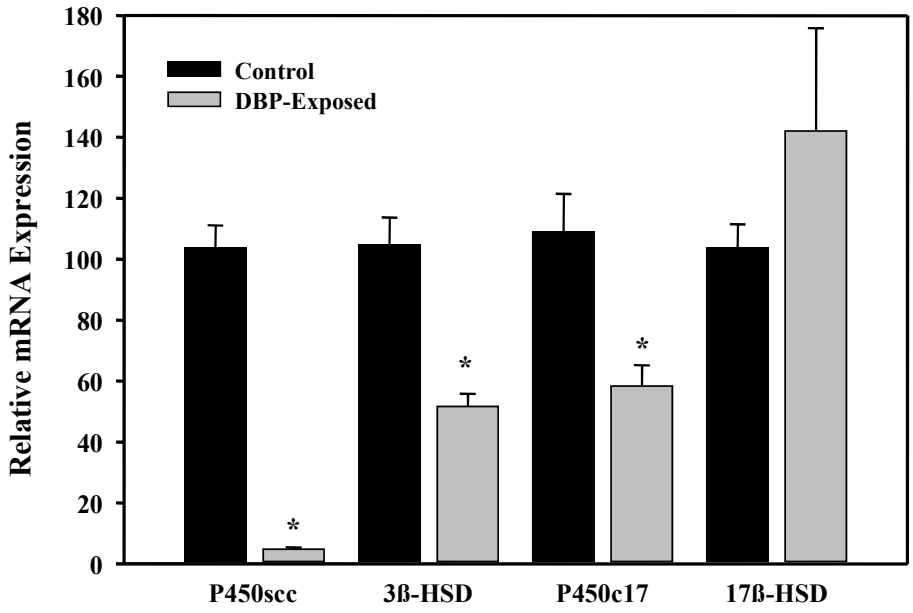

Relative mRNA Expression
180
160
140
120
100
80
60
40
20
0
Control
DBP-Exposed
*
*
*
P450scc
3ß-HSD
P450c17
17ß-HSD

Figure 5

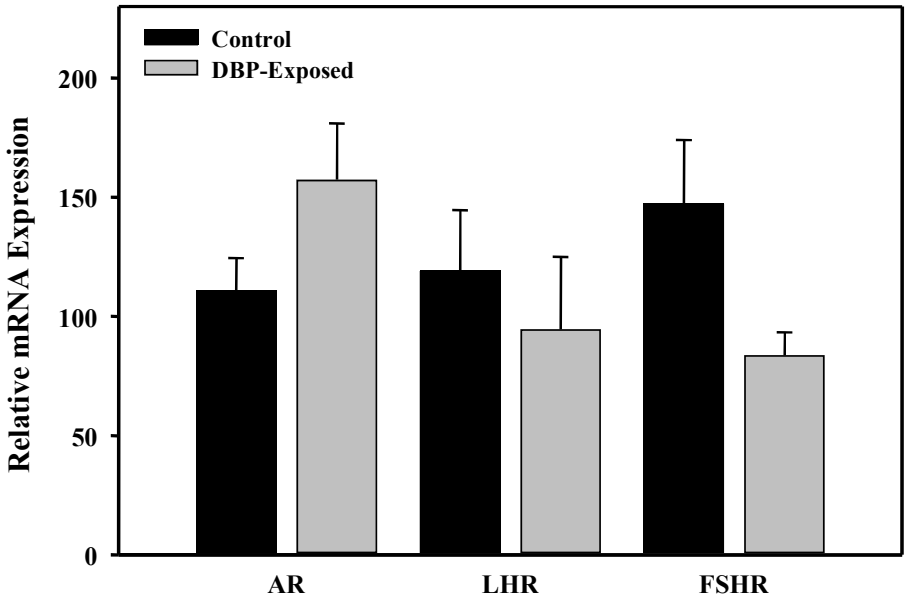

Control
DBP-Exposed
Relative mRNA Expression
200
150
100
50
0
AR
LHR
FSHR

Figure 6

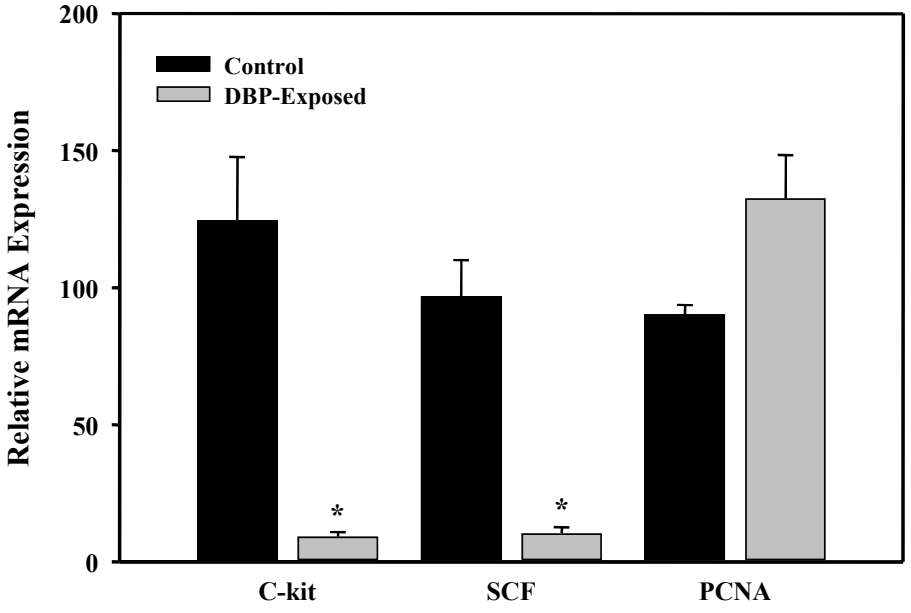
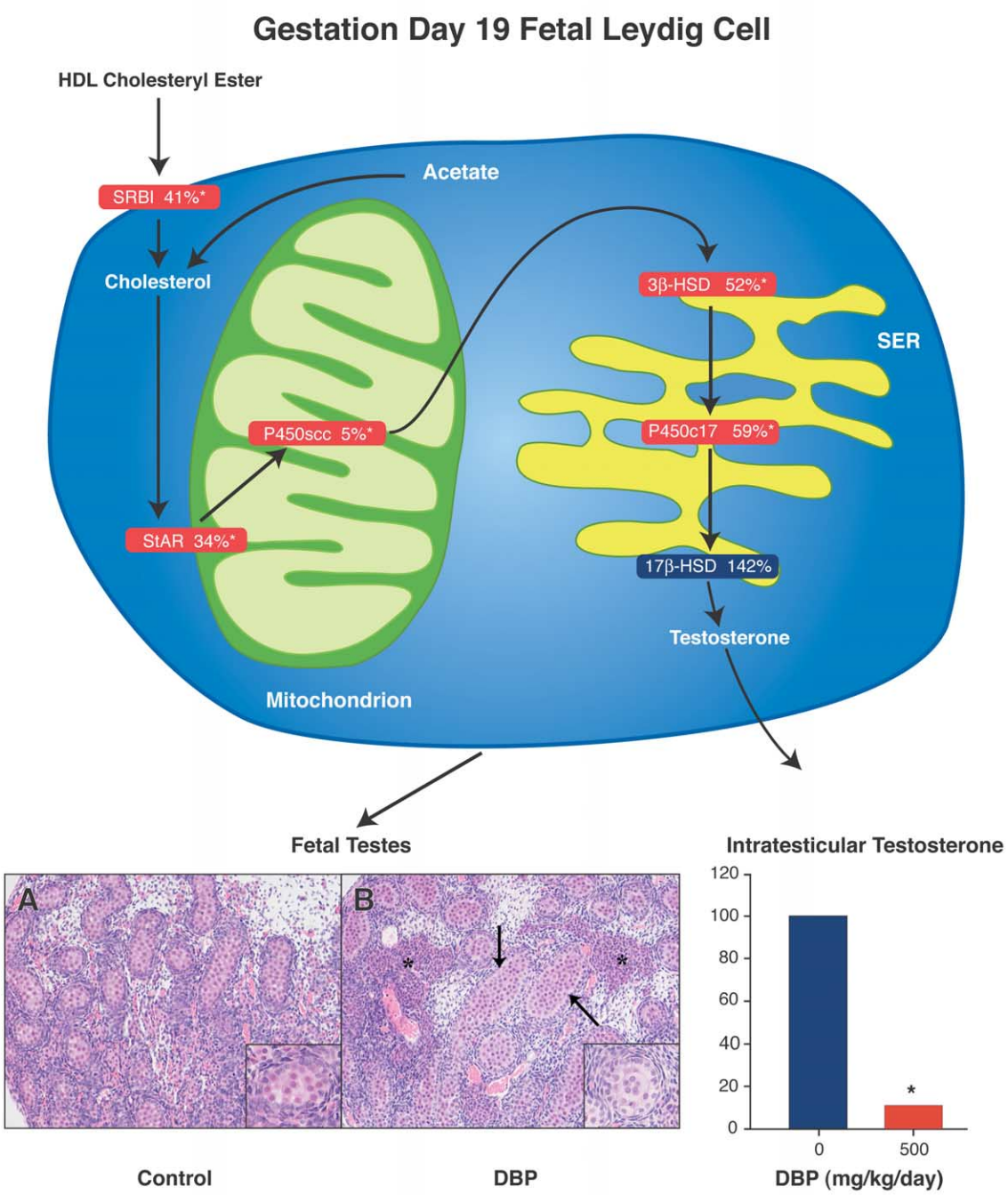

200
150
100
50
0
Relative mRNA Expression
Control
DBP-Exposed
*
*
C-kit
SCF
PCNA

Figure 7


Gestation Day 19 Fetal Leydig Cell
HDL Cholesteryl Ester
SRBI 41%*
Acetate
Cholesterol
3β-HSD 52%*
SER
P450scc 5%*
P450c17 59%*
StAR 34%*
17β-HSD 142%
Testosterone
Mitochondrion
Fetal Testes
A
B
Control
DBP
Intratesticular Testosterone
120
100
80
60
40
20
0
0
500
DBP (mg/kg/day)

CHAPTER FIVE

MALE REPRODUCTIVE TRACT LESIONS AT SIX, TWELVE, AND EIGHTEEN MONTHS OF AGE FOLLOWING *IN UTERO* EXPOSURE TO DI(*N*-BUTYL) PHTHALATE

Norman J. Barlow[1,2], Barry S. McIntyre[3], and Paul M. D. Foster[4]

[1]CIIT Centers for Health Research

Research Triangle Park, North Carolina

[2]North Carolina State University

Raleigh, North Carolina

[3]Schering-Plough Research Institute

Lafayette, New Jersey

[4]National Institute of Environmental Health Sciences

Research Triangle Park, North Carolina

ABSTRACT

In utero DBP exposure leads to malformations in the adult male reproductive tract and histologic testicular lesions including marked seminiferous epithelial degeneration and a low incidence of Leydig cell (LC) adenomas on postnatal day (PND) 90. Additionally, this antiandrogen causes decreased anogenital distance (AGD) on PND 1 and increased areolae retention on PND 13. The objective of this study was to examine animals at later time points to determine if there was an increased incidence of LC adenomas over time or if the incidence noted in previous studies was similar regardless of the time examined. A second objective was to examine AGD and areolae retention during the early postnatal period and during adulthood to determine if the changes early in life are permanent and if they are associated with lesions in the male reproductive tract. Pregnant Crl:CD(SD)BR rats were gavaged with corn oil or DBP at 100 and 500 mg/kg/day, ten dams per group. Three replicates of animals (n=30 animals per replicate) were exposed from gestation days 12 to 21 and the male offspring allowed to mature to 6, 12, and 18 months of age. Gross malformations in the male reproductive tract and histologic lesions in the testes were similar to those previously described. However, classical LC adenomas were not increased in the DBP-groups at any age. Rather, a lesion of proliferating LCs and aberrant tubules, defined as testicular dysgenesis, was diagnosed. The incidence of this lesion was approximately 20% unilateral and 7-18% bilateral in the high dose group and was similar between all ages examined implicating a developmental alteration rather than an age-related change. AGD and areolae retention were found to be permanent changes following *in utero* exposure to 500 mg/kg/day of DBP. Deceased AGD was a sensitive predictor of

lesions in the male reproductive tract with only small changes necessary to see a significant incidence of male reproductive malformations. While *in utero* DBP did not induce traditional LC adenomas, it did induce a proliferative developmental lesion composed of LCs and aberrant seminiferous tubules. The pathogenesis of this DBP-induced testicular lesion is unclear and warrants further investigation.

INTRODUCTION

Di(*n*-butyl) phthalate (DBP) acts as an antiandrogen when male rats are exposed *in utero* by decreasing fetal testicular testosterone (T) production (8, 19, 28). Due to its use in soft plastics and personal care products such as shampoo, deodorant, and hair spray, there is potential for human exposure (7). When male rats were exposed to 500 mg/kg/day of DBP during the latter half of gestation and examined on GD 17 to 21 they exhibited a characteristic suite of testicular lesions including the formation of large aggregates of Leydig cells (LCs), increased numbers of gonocytes within seminiferous cords, and multinucleated gonocytes (1). Nearly 100% of males exposed to DBP during the last ten days of gestation had at least two of these three morphologic characteristics. During the early postnatal period these lesions resolved, though the adult testis developed seminiferous epithelial degeneration, which became more severe following puberty (1). Studies in our laboratory and others have demonstrated that male rats exposed *in utero* to DBP exhibit malformations of epididymides, seminal vesicles, prostates, and penises (3, 18, 20).

AGD and areolae retention are sexually dimorphic endpoints that are androgen-dependent in the male rat. T is locally converted to dihydrotestosterone (DHT) causing lengthening of the perineum, the male AGD being approximately two to three times longer than the female AGD on postnatal day (PND) 1 (3, 14, 22). Antiandrogens may cause decreases in AGD through a variety of mechanisms including disruption of androgen synthesis or signaling (3, 22). Permanent decreases in AGD have been found for several antiandrogens including the androgen receptor antagonists flutamide and linuron (14, 15). In addition to being permanently decreased by flutamide, AGD on PND 1 was predictive of

subsequent lesions in DHT-dependent tissues of adult offspring, while flutamide-induced changes in AGD were not sensitive indicators of altered T-mediated development (14). Although AGD was permanently decreased following *in utero* exposure to linuron, this decrease was not associated with subsequent morphologic changes in the adult male reproductive tract (15). Male offspring exposed *in utero* to the organophosphate insecticide fenitrothion, another androgen receptor antagonist, exhibited a slight decrease in AGD on PND 1, but this change was transient and was not observed in adulthood (30). Gestational DBP exposure has been shown to decrease AGD on PND 1 (1, 18, 20). It was unknown if the decreased AGD following gestational exposure to DBP, an androgen biosynthesis inhibitor, was permanent and if the reduction was associated with morphological alterations of the male reproductive tract in the adult offspring (3, 18, 20).

DHT causes apoptotic regression of the nipple anlagen in male rats (6, 22). Therefore, alteration of androgen levels in male fetuses or blockade of the androgen receptor may lead to increased retention of areolae that would otherwise have undergone DHT-dependent regression. Similar to antiandrogen-mediated decreases in AGD, DBP, linuron, flutamide, and fenitrothion induced areolae retention on PND 13 (3, 14, 15, 18, 20, 30). Permanent nipples observed during adulthood have been noted for linuron, flutamide, and DBP, whereas this was a transient effect for fenitrothion. The association between the number of DBP-induced areolae on PND 13 and morphologic lesions in the adult male reproductive tract has not been established.

In two separate studies by Mylchreest et al. (1999 and 2000) it was found that *in utero* DBP exposure led to a low incidence of LC adenomas in male offspring at 3 months of age. This was an unprecedented finding as the animals in the Mylchreest studies were

exposed to DBP *in utero* only and were 90 days of age at necropsy The combined incidence in the two Mylchreest studies was ~3%, which is within the range of incidence (1.4–13.3%) for control animals from nine separate two-year carcinogenicity studies using Sprague-Dawley rats (16). The cumulative incidence from these studies was 6.5%, while Charles River Laboratories in Raleigh, NC, the supplier of animals for this and the Mylchreest studies, reported an incidence in old rats of approximately 5% (9).

Endocrine active compounds have been shown to induce neoplasia following prenatal or early postnatal exposure. Diethylstilbestrol (DES), a synthetic estrogen, is the most widely studied endocrine active agent that has been shown to lead to formation of uterine and vaginal neoplasia in offspring of females, humans and rodents, exposed to DES during gestation (5, 23, 26). DES has also been shown to cause an increased incidence of testicular neoplasia in male mice exposed over a short gestational window (25). The phytoestrogen genistein, which is found in high levels in many soy-based formulas, results in a potentially high postnatal exposure in human infants. When rats were dosed with equivalent estrogenic doses on PND 1 to 5 and examined at 18 months of age it was found that genistein induced a rate of uterine adenocarcinoma, approximately 35%, which was similar to DES when exposed on the same days (24). Taken together, these compounds illustrate the unique sensitivity of the developing reproductive tract to perturbation by endocrine active agents.

The potential for DBP to act as a transplacental carcinogen was investigated using a novel study design. Fetuses were exposed to DBP on GD 12 to 21, an exposure of only 10 days, and the male offspring were examined at 6, 12, and 18 months of age. Comparison with the Mylchreest studies (1999 and 2000) was made to determine if the incidence of LC

adenomas following *in utero* DBP exposure increased over time or if the incidence was the same as that seen in 90-day old rats regardless of the age of the animal at the time of examination. In addition, animals in the 6-month group were utilized to determine if the changes in AGD and areolae retention observed on PND 1 and 13 were permanent. Males in this cohort of animals were uniquely identified on PND 1 and AGD and areolae retention during the early postnatal period were compared to these parameters collected at necropsy on PND 180. As animals were uniquely identified, association of decreased AGD and increased areolae retention with male reproductive tract lesions was also investigated.

METHODS

Animals

This study was conducted in concordance with Federal guidelines for the care and use of laboratory animals and was approved by the Institutional Animal Care and Use Committee at the CIIT Centers for Health Research (21). The CIIT animal care facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Ninety pregnant CRL:CD(SD)BR rats were time mated at Charles River Breeding Laboratories (Raleigh, NC) and shipped to CIIT on gestation day (GD) 0, the day sperm was detected in the vaginal smear. Dams were assigned to replicates and treatment groups by body weight randomization using Provantis (Instem LSS, Stone, UK). Ten dams were assigned to each treatment group, thirty dams per male necropsy time point. Treatments included a control (0 mg/kg/day DBP), low-dose (100 mg/kg/day DBP), and a high-dose group (500 mg/kg/day DBP). The dose levels of DBP were chosen based on studies by Mylchreest et al. (1999 and 2000) that indicated LC adenomas developed when male pups were exposed to 500 mg/kg/day on GD 12 to 21 and on the statistically significant retention of areolae at 100 mg/kg/day. When more than 30 dams for each replicate were received from the breeding facility the additional dams were assigned to the high-dose group. Dams were identified by ear tags and cage cards and male pups were identified by subcutaneous transponders and cage cards. Dams were housed individually in polycarbonate cages with Alpha-dri cellulose bedding (Shepherd Specialty Papers, Kalamazoo, MI). Males were group housed by litter following weaning, three per cage, until size necessitated housing two per cage and ultimately one per cage for the duration of the study. Rodent diet NIH-07

(Zeigler Brothers, Gardener, PA) and reverse-osmosis water were provided *ad libitum*. Animals were kept in a humidity- and temperature-controlled, HEPA-filtered, mass air-displacement room. The room was maintained on a 12-hr light-dark cycle at approximately 18º–26ºF with a relative humidity of approximately 30–70%.

Study design

Dams were dosed by gavage daily from GD 12 to 21 with corn oil vehicle (Sigma Chemical Co., St. Louis, MO) or DBP in corn oil at 100 and 500 mg/kg/day (Aldrich Chemical Company, Milwaukee, WI). Dam body weights were recorded on GD 9 and daily during the dosing period. Male pups were weaned on PND 21, while the dams and female pups were euthanized by CO_2 asphyxiation and aortic transection without examination. Male pups were necropsied at 6, 12, and 18 months of age.

Male necropsies and other antiandrogenic endpoints

The males in the 6-month replicate were utilized for additional measurements including AGD and areolae or nipple retention. A single investigator, blinded to treatment, performed all measurements. Male pups were uniquely identified on PND 1 using footpad tattoos to allow for data collection and comparison of data collected from individual animals over their lifetime. AGD, the distance from the anus to the genital papilla, was measured on PND 1 using a dissecting microscope with an eyepiece reticle. AGD was measured at necropsy (PND 180) with a manual caliper. Areolae were counted on PND 13. There was no discrimination between areolae with or without a nipple bud. The ventral surface of the thorax and abdomen of all animals necropsied on PND 180 was shaved for

the counting of nipples. All morphologic structures in appropriate areas, from small blemishes to florid nipples, were counted.

Necropsies were performed on PND 180, 370 and 540. Males were anesthetized with isoflurane (Fort Dodge, Overland Park) by nose cone exposure and euthanized by cardiac puncture. Necropsies included full evaluation of the external genitalia and the internal reproductive organs by a single veterinary pathologist. Epididymides and all organs with gross lesions were fixed in 10% neutral-buffered formalin. Testes were fixed in modified Davidson's fixative for 48 hours and then transferred to 10% neutral-buffered formalin (10, 12). All moribund animals and animals found dead prior to their scheduled sacrifice were necropsied with collection of study protocol-specified tissues and all gross lesions. The entire testes were cross sectioned with all sections being examined microscopically. The testes, epididymides, and all tissues from unscheduled deaths were processed, embedded in paraffin, sectioned at five microns, and stained with hematoxylin and eosin. Tissues from moribund or dead animals were examined histologically to confirm the cause of death or significant gross findings. Data from testes or epididymides of dead or moribund animals were not included in the data from the animals necropsied at the scheduled sacrifices.

Statistical Analyses

Statistical analyses were conducted using JMP (version 4.0.0, SAS Institute, Cary, NC). The litter was the experimental unit for all of the age groups and data were analyzed nested by dam to yield litter means. Significance for all treatment effects was $p < 0.05$. Pathology data were analyzed using a Chi Square test followed by the Fisher's Exact Test.

Quantitative pup data were analyzed by a repeated measure ANOVA (nested design) or ANCOVA with the covariates listed in the figure legends. Contrasts of least square means were used to test for significance of treatment effects. Results are expressed as the least squares means ± standard error of the mean. Linear regression was used to determine the association of AGD on PND 1 to AGD on PND 180. To determine the association of decreased AGD on PND 1 to lesions within the male reproductive tract logistic regression was employed. Following logistic regression the receiver operator characteristic (ROC) and inverse prediction functions were performed. The ROC values approaching 1 were predictive of an association between decreased AGD and lesions in the reproductive tract, while values nearing 0.5 were not predictive (4). The inverse prediction function determines the AGD at which 50 and 10% of the animals will have a lesion in a particular tissue of the reproductive tract.

RESULTS

Gross pathology

Malformations observed in the aged male reproductive tracts were similar to those previously described in young adult males and did not vary significantly by age (1, 3, 18, 20). Lesions in the testes included atrophy with flaccid capsules, occasional enlargement with abundant amounts of interstitial fluid, and rarely absence of the testis . These alterations occurred unilaterally and bilaterally, though the bilateral manifestation predominated for the atrophied and enlarged testes (Table I). Enlarged testes were only observed in the 500 mg/kg/day dose group and were often accompanied by malformed epididymides. Epididymal lesions occurred predominantly in the high dose group (Table I). While small numbers of epididymal changes were present in the control and low dose groups, these lesions were hypoplastic with the head, body, and tail present. In the high dose group the majority of epididymides were malformed with absence of various portions or absence of the entire epididymides. The morphologic alteration observed in the *vasa deferentia* was absence of tissue. Absent *vasa deferentia* were frequently associated with malformed or absent epididymides and occurred unilaterally and bilaterally, though the numbers tended to be more evenly distributed (Table I). The other organ derived from the Wolffian ducts, the seminal vesicles, also developed DBP-induced lesions, which occurred only in the 500 mg/kg/day dose group (Table I). Seminal vesicles were small or malformed with decreased intraluminal secretions or were completely absent. On PND 540, age-induced lesions of the seminal vesicles were observed in the control and low-dose DBP animals (Table I). The alteration noted in these animals, a slight decrease in size, was

markedly different from those in the high-dose DBP group, which had marked decreases in seminal vesicle size with malformed or absent lobes.

Changes noted in DHT-dependent tissues were also most frequently observed in the high dose group (Table I). Prostates were frequently small to absent with both the ventral and dorsolateral lobes affected. Small numbers of lesions were noted in control and low-dose animals on PND 370 with markedly increased numbers seen in these groups on PND 540 (Table I). However, prostates in these animals were only slightly decreased in the size. Hypospadias, a malformation of the penis with ectopic location of the urethral opening on the ventral surface, was observed only in the high dose group (Table I). Pituitary gland masses were present in all dose groups of the PND 370 and 540 replicates, but were not observed in the PND 180 animals. There were no statistically significant differences in the occurrence of pituitary adenomas between control and dosed animals.

Histopathology

Proliferative LC lesions were observed at all time points examined (Table II). Classical LC adenomas, masses of well differentiated LCs larger than the diameter of three seminiferous tubules (Figure 1B), were only observed in small numbers on PND 540 in the control and low dose groups, except for a single tumor in a control animal on PND 370. LC hyperplasia, areas of well differentiated LCs smaller than three seminiferous tubules, was noted in the high, low and high, and all three doses on PND 180, 370, and 540, respectively. The incidence LC hyperplasia was not significantly different for any of the dose groups.

Testicular dysgenesis, defined as aberrant seminiferous tubules associated with proliferative LCs, was seen in the high dose group at all three time points, except for a single animal in the control group on PND 370. These dysgenetic areas varied markedly in morphology but were always located in the same region of the testis, the central area of sections containing the rete testis. They were most often observed in a single section but were occasionally seen in the same area of multiple sections indicating a single lesion that extended through the testis. The size of the dysgenetic areas varied, with some areas greater than the diameter of three seminiferous tubules. Testicular dysgenesis occurred with approximately the same incidence across all age groups and was much more prevalent as a unilateral finding (Table II). Dysgenetic areas contained variable numbers of seminiferous tubules and LCs. The tubules were often convoluted and lined by immature-appearing Sertoli cells (Figures 1C and 1D). In many lesions the tubules had small lumina while in some they were mildly dilated (Figure 1D); and in others they were markedly dilated with only flattened Sertoli cells (Figure 1E). LCs within dysgenetic areas varied in number and differentiation and surrounded the immature tubules, often forming mass lesions (Figures 1C and 1D). These LCs were spindloid with decreased amounts of cytoplasm. In other areas, especially around the markedly dilated tubules, there were very few LCs (Figure 1E). In several testes there were proliferative LCs around small blood vessels that were not associated with seminiferous tubules (Figure 1F). While these areas did not appear to be dysgenetic, i.e., they did not contain seminiferous tubules, they were located in the same central region as the other dysgenetic lesions.

The incidence of seminiferous epithelial degeneration was similar to that previously reported in adult animals exposed *in utero* to 500 mg/kg/day of DBP (Table II) (1). The

degeneration in most testes affected nearly 100% of the tubules with complete lose of germ cells, leaving only Sertoli cells. Many of the testes contained tubules or areas of granulomatous inflammation with inflammation frequently surrounding areas of mineralization. The rete testis was often expanded by the presence of sperm and surrounded by granulomatous inflammation. The inflammatory lesion of the rete was most prevalent on PND 180 and 370. In the PND 370 group, inflammation often appeared to be resolving with increased amounts of fibrous connective tissue surrounding the rete. Rete fibrosis with minimal to mild granulomatous inflammation was the predominant lesion on PND 540 (Figure 1H).

Anogenital distance

The AGD of uniquely identified male pups was measured on PND 1 and 180. Male pups in the 500 mg/kg/day group exhibited a statistically significantly decreased of 14 and 10%, respectively, (Figure 2). Linear regression on the AGD of individual animals on PND 1 versus the same animal's AGD on PND 180 was performed with a coefficient of determination (r^2) of 0.80. indicating that the decreased AGDs on PND 1 and 180 were significantly correlated (Figure 3). While the litter means for AGD of animals in the 100 mg/kg/day group were not significantly different from control (Figure 2), analysis of individual males did show separation from controls by a subset of animals in this dose group (Figure 3).

The ability of decreased AGD on PND 1 to predict male reproductive tract alterations was tested using logistic regression. All of the values for reproductive tissues examined in this study were significant, including any changes in the reproductive tract,

regardless of the organ. Decreased AGD on PND 1 was a strong predictor of a lesion in each particular tissue, as indicated by the ROC values near 1.0 (Table III). The M_{50} and M_{10} values (AGD in mm), and their corresponding confidence intervals, are the AGDs at which 50 and 10% of the animals will have a lesion, gross or histologic, in a particular tissue. While some tissues have a ROC value close to 1.0 (e.g., penises ROC=0.96) indicating that decreased AGD is strongly predictive of a lesion in this tissue, it may take a marked reduction in AGD to achieve either 50 or 10% incidence (Table III). Other organs that may not be as predictive (e.g., testes ROC=0.9) may only need slight reductions in AGD to achieve the same incidences.

Areolae and nipple retention

Areolae and nipples were counted on uniquely identified pups on PND 13 and 180. There was no distinction between areolae with or without a nipple bud on PND 13. Nipples on PND 180 ranged from small, slightly raised areas on the skin in the appropriate position of a nipple to fully developed nipples. Areolae retention was significantly increased in both the low and high dose DBP groups relative to control (Figure 4). On PND 180 retained nipples were significantly increased above control in the 500 mg/kg/day dose group (Figure 4). Though significantly increased, there were fewer nipples per animal in the 500 mg/kg/day group on PND 180 than there were on PND 13, ~4.5 nipples versus ~10 areolae.

To determine if areolae detected on PND 13 in the DBP-exposed animals were associated with gross or histologic lesions in the male reproductive tract , the number of areolae were plotted against the proportion of the total number of animals responding (Figure 5). The number of areolae were segregated into five blocks, 0, 1–3, 4–6, 7–9, and

10–12 areolae. A minimal response was seen in the testes in both the 0 and 1–3 areolae group (Figure 5). Approximately 7–12% of the animals with morphologic alterations in all of the tissues besides the penis were in the 4–6 areolae response group. All of the tissues behaved similarly in the 7–9 and 10–12 areolae groups with approximately 20% of the animals in the former and 65–75% of the lesioned animals in the latter group. All of the responders for hypospadias were in the 10–12 areolae group.

Survival

Adverse effects on survival were only observed in the 500 mg/kg/day dose group of the 540-day replicate (Table I, Figure 6). There were minimal to no losses of animals in all dose groups for the 180-day and the 370-day replicates. In the control and 100 mg/kg/day dose group of the 540-replicate no animals died prior to PND 350, which is similar to that seen for the other two replicates. However, there were significant early deaths of animals in the high dose group. The rate of these losses eventually declined and the high dose survival from 350 to 540 days was similar to the control and low dose groups for that replicate (Figure 6).

Animals in the high dose group that died early, i.e., prior to PND 350, exhibited urinary tract obstructions, often associated with cysto- and nephrolithiasis (Table IV). The urinary bladders of these animals were often markedly enlarged and filled with red urine (Figure 7A). Dilated ureters and hydronephrosis were also observed. The point of obstruction was frequently at the cranial flexure of the urethra as it exited the pelvic canal. This area frequently contained uroliths or large, gritty, proteinaceous plugs (Figure 7B). Urinary bladders were also completely filled and dilated by large uroliths (Figure 7C).

When the urinary bladders were opened they contained variably sized uroliths that often had a rough surfaces (Figure 7D). The stones from a single animal were cultured and analyzed (data not shown). *Proteus mirabilis* was cultured from the urine of this animal. The stones were found to be magnesium ammonium phosphate (struvite). As the clinical syndrome was similar for all animals and the uroliths had similar appearances, analyses on the stones or urine of other animals were not performed. In addition to hydronephrosis kidneys with ascending pyelonephritis were also observed (Figure 7E). Animals for which a cause of death was not determined were not included in Table IV.

DISCUSSION

The main objective of this study was to further investigate the potential transplacental carcinogenicity of DBP, specifically the induction of LC adenomas in male Sprague-Dawley rats exposed to DBP on GD 12 to 21. Animals were examined at 6, 12, and 18 months of age and there was no observed increase in classical LC adenomas, histologically defined as nodular proliferations of generally well-differentiated LCs (2, 17, 27). LC adenomas frequently encompass seminiferous tubules and compress tubules adjacent to the mass. The Society of Toxicologic Pathology (STP) recommends the diagnosis of adenoma for proliferations of LCs greater than three seminiferous tubules in diameter, while those less than that are areas classified as? hyperplasia (13). Only small numbers of classically defined hyperplasia and adenomas were observed in this study with no differences in incidence between control, low, and high doses of DBP.

While no differences were observed in LC hyperplasia or adenomas, there were proliferative areas, defined as testicular dysgenesis, observed in the high dose group. Testicular dysgenesis was always found in a similar area of the testis, the central region of sections containing the rete. These lesions varied in size and, by the STP criteria, would have been diagnosed as areas of LC hyperplasia and adenomas. However, the morphology of the dysgenetic areas differed from traditional LC proliferations. The dysgenetic areas were observed in the high dose group only, save for one lesion in a control animal, and the incidence was similar between the 6, 12, and 18-month replicates. This implies that testicular dysgenesis did not appear over time, rather it formed *in utero*, while the differentiating testes were being exposed to DBP, and continued to develop as animals

aged. While occasionally found in both testes this was predominantly a unilateral lesion. The lesions from Mylchreest et al. (1999 and 2000) were reviewed and were found similar to the areas diagnosed as testicular dysgenesis in the current study. However, the overall incidence of this lesion in the two Mylchreest studies was only 3%, versus the approximate 20% incidence in this study. Knowing that only a small number of LC adenomas were expected, the current study examined cross sections of the entire testis, three to five sections, which increased the ability to find histologic lesions and was a potential explanation for the difference in the incidence level between the three studies. The pathogenesis of testicular dysgenesis is not completely understood.

Similar to flutamide and linuron, AGD was permanently decreased following *in utero* exposure to DBP (14, 15). The DBP-induced decrease was significant at the high dose on both PND 1 and 180. While AGD was not significantly decreased at 100 mg/kg/day, it was slightly decreased on both PND 1 and 180. The animals in this dose group with slightly decreased AGD on PND 1 were the same animals with decreased AGD on PND 180, emphasized by the subset of animals separated from the control animals and other low dose group animals in Figure 3. In addition to being a permanent change induced by *in utero* exposure to 500 mg/kg/day of DBP, the decreased AGD was also predictive of gross or histologic changes in the male reproductive tract as illustrated by the ROC values in Table III. While AGD seemed to be more predictive of lesions in tissues such as the seminal vesicles and prostate (ROC = 0.97) versus the testes and epididymides (ROC = 0.90 and 0.92), it took a greater magnitude of change in AGD to observe either a 50 or 10% incidence of animals with lesions. In the case of the seminal vesicles or prostate, a decrease in AGD of approximately 12% was necessary to observe a 50% incidence of animals with

an alteration in those organs, while only an 8% decrease was necessary to see the same incidence in the testes. To note a 10% incidence of a lesion in the testes or epididymides, a decrease in AGD of only 2–2.5% was necessary. Though the decreased AGD at 100 mg/kg/day was not statistically significant it is possible that this decrease is biologically significant as this magnitude of change was all that was necessary to predict lesions in the testes and epididymides.

While areolae observed on PND 13 and 14 have been shown to be permanent following *in utero* DBP exposure, it was of interest to determine if nipples induced by exposure to 100 mg/kg/day, a relatively low dose of DBP, would be permanent (3). Areolae were significantly increased at the low dose on PND 13 but were not elevated on PND 180, indicating this to be a transient effect at 100 mg/kg/day. Additionally, in the 500-mg/kg/day dose group, approximately ten areolae per animal were counted on PND 13 whereas only four nipples per animal were counted on PND 180. Therefore, even at the high dose, many of the areolae counted on PND 13 did not persist as nipples. While decreased AGD on PND 1 was predictive of lesions in the male reproductive tact, increased areolae retention on PND 13 does not appear to behave in the same fashion, in fact, a full complement of areolae, i.e., greater than nine areolae, were necessary before markedly increased numbers of malformations were present. While nipples were permanent following *in utero* linuron exposure, which is similar to DBP, they were not predictive of reproductive tract lesions in adult male offspring (15).

Prior to this study DBP had not been shown to cause decreased survival of the male offspring at doses up to 500 mg/kg/day (3, 18, 20). Decreased survival was seen in one group in this study, the high dose group of the 18-month replicate. Urinary obstruction with

ascending pyelonephritis was the common finding in most animals that died prior to PND 350. The definitive cause of the urolithiasis and urinary obstruction in these animals was not identified. These animals did have malformations of their genitourinary tracts that may have predisposed them to urinary tract infections, urolith formation, and urinary obstruction. *Proteus mirabilis* is a common crystal forming bacteria often cultured from the urinary tract of animals with uroliths (11, 29). *Proteus* is a urease producer, which leads to breakdown of urea to ammonium ions and the formation of alkaline urine, a prerequisite for struvite crystal formation (11). Additionally, in a controlled study of multiple urease-producing bacteria, *Proteus mirabilis* produced the most severe kidney lesions (11). All of the animals were housed in the same room with the same bedding, food, and water and animals in the high-dose group had similar reproductive tract lesions regardless of replicate so it is unclear why one particular group of the 18-month replicate was affected.

In summary, the current study demonstrated that while *in utero* exposure to DBP did not increase the incidence of classically defined LC adenomas; rather there was a previously undescribed lesion in the testis that was similar in incidence regardless of the age examined, implicating formation during development of the testes. The pathogenesis of this lesion is not completely understood but, given its similar morphology and similar location in the testes, it implies that possibly there is a group of cells or a structural area in the fetal testis that is particularly susceptible to DBP exposure. This is supported by the presence of dysgenetic areas in DBP-exposed testes with no other lesions. Further studies to elucidate the pathogenesis and significance of this lesion are necessary. It was also shown that alterations in AGD and areolae retention are permanent at 500 mg/kg/day and that decreased AGD is predictive of DBP-induced male reproductive tract lesions. This

information on these endpoints supplements that which is known about other endocrine disrupting compounds such as flutamide, linuron, and fenitrothion, which act as antiandrogens via a mechanism different from DBP.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Katie J. Turner and Christopher J. Bowman, Mr. Duncan G. Wallace, Mrs. Kim P. Lehmann, and the necropsy and histology staff for their assistance with dissections. We would also like to acknowledge the technical assistance of Drs. Robert Chapin and Diane Creasy in interpreting the histopathology in the testes. This study would not have been possible without the assistance of the CIIT animal care staff. Funding was provided in part by the American Chemistry Council and by a National Research Service Award from the Department of Health and Human Services, National Institutes of Health, National Institute of Environmental Health Sciences (awarded to NJB). This manuscript does not necessarily reflect the views or policies of either of these organizations.

REFERENCES

1. Barlow NJ and Foster PMD (2003). Pathogenesis of male reproductive tract lesions from gestation through adulthood following *in utero* exposure to di(*n*-butyl) phthalate. *Toxicol Pathol*
2. Boorman GA, Chapin RE, and Mitsumori K, *Testis and Epididymis*, in *Pathology of the Fischer Rat*, G.A. Boorman, *et al.*, Editors. 1990, Academic Press, Inc.: San Diego.
3. Gray LE, Jr., Wolf C, Lambright C, Mann P, Price M, Cooper RL, and Ostby J (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p*,*p*'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* 15:94-118.
4. Hanley JA and McNeil BJ (1982). The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 143:29-36.
5. Herbst AL, Kurman RJ, Scully RE, and Poskanzer DC (1972). Clear-cell adenocarcinoma of the genital tract in young females. Registry report. *N Engl J Med* 287:1259-64.
6. Imperato-McGinley J, Binienda Z, Gedney J, and Vaughan ED, Jr. (1986). Nipple differentiation in fetal male rats treated with an inhibitor of the enzyme 5 α-reductase: definition of a selective role for dihydrotestosterone. *Endocrinology* 118:132-7.

7. International Programme on Chemical Safety (1997), Environmental Health Criteria 189. Di-*n*-butyl Phthalate, 1997, World Health Organization: Geneva.

8. Lambright CS, Wilson VS, Furr JR, Wolf CJ, Noriega N, and Gray LE, Jr. (2003). Effects of endocrine disrupting chemicals on fetal testes hormone production. *The Toxicologist* 61:

9. Lang PL (1992), Spontaneous neoplastic lesions and selected non-neoplastic lesions in the Crl:CDBr rat, 1992, Charles River Laboratory: Raleigh.

10. Lanning LL, Creasy DM, Chapin RE, Mann PC, Barlow NJ, Regan KS, and Goodman DG (2002). Recommended approaches for the evaluation of testicular and epididymal toxicity. *Toxicol Pathol* 30:507-20.

11. Larsson PA, Cano M, Grenabo L, Brorson JE, Hedelin H, Pettersson S, and Johansson SL (1989). Morphological lesions of the rat urinary tract induced by inoculation of mycoplasmas and other urinary tract pathogens. *Urol Int* 44:210-7.

12. Latendresse JR, Warbrittion AR, Jonassen H, and Creasy DM (2002). Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. *Toxicol Pathol* 30:524-33.

13. McConnell RF, Westen HH, Ulland BM, Bosland MC, and Ward JM, *Proliferative Lesions of the Testes in Rats with Selected Examples from Mice*, in *Standardized System of Nomenclature and Diagnostic Criteria*, Society of Toxicologic Pathology, 1992, STP/ARP/AFIP: Washington.

14. McIntyre BS, Barlow NJ, and Foster PMD (2001). Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and

correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci* 62:236-49.

15. McIntyre BS, Barlow NJ, and Foster PMD (2002). Male rats exposed to linuron *in utero* exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. *Toxicol Sci* 65:62-70.

16. McMartin DN, Sahota PS, Gunson DE, Hsu HH, and Spaet RH (1992). Neoplasms and related proliferative lesions in control Sprague-Dawley rats from carcinogenicity studies. Historical data and diagnostic considerations. *Toxicol Pathol* 20:212-25.

17. Mitsumori K and Elwell MR (1988). Proliferative lesions in the male reproductive system of F344 rats and B6C3F1 mice: incidence and classification. *Environ Health Perspect* 77:11-21.

18. Mylchreest E, Sar M, Cattley RC, and Foster PMD (1999). Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156:81-95.

19. Mylchreest E, Sar M, Wallace DG, and Foster PMD (2002). Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(*n*-butyl) phthalate. *Reprod Toxicol* 16:19-28.

20. Mylchreest E, Wallace DG, Cattley RC, and Foster PMD (2000). Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(*n*-butyl) phthalate during late gestation. *Toxicol Sci* 55:143-151.

21. National Research Council (1996), Guide for the Care and Use of Laboratory Animals, 1996, National Academy Press: Washington, D.C. p. 125.

22. Neumann F, Berswordt-Wallrabe RV, Elger W, Steinbeck H, Hahn JD, and Kramer M (1970). Aspects of androgen-dependent events as studied by antiandrogens. *Recent Prog Horm Res* 26:337-410.

23. Newbold R (1995). Cellular and molecular effects of developmental exposure to diethylstilbestrol: implications for other environmental estrogens. *Environ Health Perspect* 103 Suppl 7:83-7.

24. Newbold RR, Banks EP, Bullock B, and Jefferson WN (2001). Uterine adenocarcinoma in mice treated neonatally with genistein. *Cancer Res* 61:4325-8.

25. Newbold RR, Hanson RB, Jefferson WN, Bullock BC, Haseman J, and McLachlan JA (2000). Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol. *Carcinogenesis* 21:1355-63.

26. Newbold RR, Moore AB, and Dixon D (2002). Characterization of uterine leiomyomas in CD-1 mice following developmental exposure to diethylstilbestrol (DES). *Toxicol Pathol* 30:611-6.

27. Qureshi SR, Perentes E, Ettlin RA, Kolopp M, Prentice DE, and Frankfurter A (1991). Morphologic and immunohistochemical characterization of Leydig cell tumor variants in Wistar rats. *Toxicol Pathol* 19:280-6.

28. Shultz VD, Phillips S, Sar M, Foster PMD, and Gaido KW (2001). Altered gene profiles in fetal rat testes after *in utero* exposure to di(*n*-butyl) phthalate. *Toxicol Sci* 64:233-42.

29. Takeuchi H, Ueda M, Satoh M, and Yoshida O (1991). Effects of dietary calcium, magnesium and phosphorus on the formation of struvite stones in the urinary tract of rats. *Urol Res* 19:305-8.

30. Turner KJ, Barlow NJ, Struve MF, Wallace DG, Gaido KW, Dorman DC, and Foster PM (2002). Effects of *in utero* exposure to the organophosphate insecticide fenitrothion on androgen-dependent reproductive development in the Crl:CD(SD)BR rat. *Toxicol Sci* 68:174-83.

Table I. Gross Lesions in the Male Reproductive Tract and Pituitary Gland Following *In Utero* Exposure to DBP

	PND 180			PND 370			PND 540		
DBP (mg/kg/day)	0	100	500	0	100	500	0	100	500
Number of animals (litters)	60 (10)	65 (10)	45 (11)	61 (10)	61 (9)	74 (11)	45 (9)	49 (10)	35 (8)
Animals surviving to necropsy	97%	100%	92%	98%	100%	96%	77%	83%	57%*
Testes[a]									
Unilateral	3 (20)[b]	11 (60)	16 (36)	2 (10)	3 (22)	20* (82*)	2 (11)	6 (30)	23* (75*)
Bilateral	0 (0)	0 (0)	69* (82*)	0 (0)	3 (22)	61* (100*)	2 (11)	2 (10)	69* (88*)
Epididymides[c]									
Unilateral	2 (10)	8 (40)	27* (64*)	2 (10)	2 (11)	12* (55*)	2 (11)	6 (30)	23* (63)
Bilateral	0 (0)	0 (0)	62* (82*)	0 (0)	2 (11)	72* (100*)	2 (11)	2(10)	69* (88*)
Vasa deferentia[d]									
Unilateral	0 (0)	0 (0)	31* (82*)	0 (0)	0 (0)	24* (82*)	0 (0)	0 (0)	17* (50*)
Bilateral	0 (0)	0 (0)	22* (45*)	0 (0)	0 (0)	9* (45*)	0 (0)	0 (0)	23* (50*)
Seminal vesicles[e]	0 (0)	0 (0)	69* (91*)	0 (0)	0 (0)	38* (91*)	16 (56)	27 (70)	80* (100)
Prostates[f]	0 (0)	0 (0)	67* (82*)	3 (20)	3 (22)	58* (100*)	45 (89)	48 (70)	94* (100)
Penises[g]	0 (0)	0 (0)	16* (27)	0 (0)	0 (0)	22* (64*)	0 (0)	0 (0)	26* (50*)
Pituitary glands[h]	0 (0)	0 (0)	0 (0)	5 (30)	3 (22)	0 (0)	14 (67)	31 (80)	31 (63)

[a] Lesioned testes include those that were atrophied, enlarged, or absent.
[b] Percentage of animals with lesions (% of litters with affected animals).
[c] Gross epididymal lesions include small epididymides and those with variable agenesis.
[d] The gross lesion noted in the *vasa deferentia* was absence of the organ.
[e] Gross lesions in the seminal vesicles include small lobes with less fluid and malformed or absent lobes.
[f] Ventral and dorsolateral prostates were small or absent.
[g] Penises had hypospadias of varying severity.
[h] Lesioned pituitary glands contained variably sized tan to red masses (adenomas).
* $p < 0.05$

Table II. Histopathology of the Testes Following *In Utero* Exposure to DBP

	PND 180			PND 370			PND 540		
DBP (mg/kg/day)	0	100	500	0	100	500	0	100	500
Animals (litters)	60 (10)	65 (10)	45 (11)	61 (10)	61 (9)	74 (11)	45 (9)	49 (10)	35 (8)
Testis Unilateral									
LC adenoma[a]	0 (0)[b]	0 (0)	0 (0)	2 (10)	0 (0)	0 (0)	2 (11)	2 (10)	0 (03)
LC hyperplasia[c]	0 (0)	0 (0)	2 (9)	0 (0)	2 (11)	4 (27)	7 (11)	4 (10)	3 (13)
Testicular dysgenesis[d]	0 (0)	0 (0)	22* (64*)	2 (10)	0 (0)	20* (73*)	0 (0)	0 (0)	20* (38)
Germ cell degeneration[e]	3 (20)	11 (50)	18* (55)	2 (10)	5 (22)	22* (73*)	7 (22)	12 (60)	23 (63)
Rete testis[f]	2 (10)	6 (30)	38* (55)	2 (10)	0 (0)	24* (82*)	0 (0)	4 (20)	26* (50*)
Bilateral									
LC adenoma	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (10)	0 (0)
LC hyperplasia	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (20)	0 (0)
Testicular dysgenesis	0 (0)	0 (0)	7 (27)	0 (0)	0 (0)	18* (73*)	0 (0)	0 (0)	11* (38)
Germ cell degeneration	0 (0)	0 (0)	75* (73*)	2 (10)	3 (22)	67* (100*)	7 (22)	4 (20)	71* (88*)
Rete testis	0 (0)	0 (0)	7 (18)	0 (0)	3 (22)	7* (45*)	0 (0)	2 (10)	14* (38)

[a] Leydig cell adenomas were greater than three seminiferous tubules in diameter.
[b] Percentage of animals with lesions (% of litters with affected animals).
[c] Leydig cell hyperplasia was less than three seminiferous tubules in diameter.
[d] Testicular dysgenesis is defined as areas of aberrant or immature seminiferous tubules associated with proliferative Leydig cells. This lesion was always located in the central portion of the testis, near the rete testis.
[e] Seminiferous epithelial degeneration with Sertoli cell only tubules was observed in nearly 100% of the tubules of affected testes.
[f] The lesion of the rete testis was sperm stasis with granulomatous inflammation and fibrosis.
* $p < 0.05$

Table III. Predictive Ability of Anogenital Distance on PND 1 for Lesions in Tissues of the Male Reproductive Tract

Tissue	ROC[a]	M_{50}[b]	CL_{50} Lower[c]	CL_{50} Upper[c]	M_{10}[b]	CL_{10} Lower[c]	CL_{10} Upper[c]
Testes	0.90	2.49	2.44	2.53	2.64	2.61	2.71
Epididymides	0.92	2.49	2.41	2.5	2.63	2.58	2.69
Vasa deferentia	0.96	2.34	2.28	2.39	2.5	2.44	2.58
Seminal vesicles	0.97	2.38	2.33	2.42	2.51	2.47	2.59
Prostates	0.97	2.37	2.32	2.42	2.51	2.46	2.59
Penises	0.96	2.19	2.00	2.24	2.31	2.26	2.49
All reproductive organs	0.90	2.49	2.44	2.53	2.65	2.61	2.71

[a] Receiver operator characteristic (ROC) describes that ability of anogenital distance (AGD) on PND 1 to predict lesions in the tissues of the male reproductive tract. Gross and histologic lesions were included in the analyses. 1.0 is highly predictive while 0.5 is not predictive.
[b] Inverse prediction value at which 50 and 10% of the animals will exhibit lesions (gross or histologic) in a given tissue.
[c] 95% confidence limits of the inverse prediction value.

Table IV. Critical Findings in Moribund or Dead Animals

Age (PND)	Dose (mg/kg/day)	Critical Findings	Dam Number	% of Litter Unscheduled
60	500	Urinary obstruction, Renal tubular mineralization	22	55
64	500	Urinary obstruction	28	100
77	500	Urinary obstruction, Renal tubular mineralization	22	55
82	500	Urinary obstruction, Ascending pyelonephritis	26	20
177	500	Urinary obstruction, Ascending pyelonephritis	86	13
188	500	Cystolithiasis	28	100
189	500	Urinary obstruction, Renal tubular mineralization	22	55
217	500	Urinary obstruction	29	14
223	500	Urinary obstruction, Ascending pyelonephritis	85	20
273	500	Urinary obstruction	28	100
315	500	Urinary obstruction, Ascending pyelonephritis	24	43
329	500	Urinary obstruction, Ascending pyelonephritis	27	50
347	500	Nephro– and cystolithiasis, Ascending pyelonephritis	22	55
357	0	Chronic interstitial nephritis, Nephrolithiasis	7	14
375	0	Polyarteritis	6	71
392	100	Pituitary adenoma	12	38
409	100	Pituitary adenoma	20	33
421	100	Chronic interstitial nephritis, Pituitary adenoma	12	38
421	100	Pituitary adenoma	12	38
421	100	Chronic interstitial nephritis, Pituitary adenoma	17	33
422	500	Chronic interstitial nephritis	24	43
443	100	Chronic interstitial nephritis	20	33
455	500	Urinary obstruction, Nephro– and cystolithiasis	23	14
462	100	Basal cell adenoma	14	17
465	500	Pituitary adenoma	22	55
467	0	Chronic interstitial nephritis	6	71
476	0	Nephro– and cystolithiasis	2	33
483	0	Chronic interstitial nephritis, Pituitary adenoma	4	100
483	500	Sarcoma, NOS	22	55
483	500	Pituitary adenoma	91	57
487	500	Chronic interstitial nephritis, Cystolithiasis	25	40
490	0	Chronic interstitial nephritis	4	100
490	500	Chronic interstitial nephritis	27	50
511	0	Chronic interstitial nephritis	6	71
514	0	Pituitary adenoma	1	14
520	100	Chronic interstitial nephritis, Pituitary adenoma	13	33
522	0	Chronic interstitial nephritis	6	71
525	0	Chronic interstitial nephritis, Pituitary adenoma	10	9
526	0	Chronic interstitial nephritis	4	100
526	500	Chronic interstitial nephritis, Pituitary adenoma	91	57
527	0	Chronic interstitial nephritis	6	71
530	0	Pituitary adenoma	8	17
536	500	Chronic interstitial nephritis	91	57
538	100	Pituitary adenoma	17	33

FIGURE LEGENDS

Figure 1. Photomicrographs of testes from animals exposed to vehicle and DBP, 100 and 500 mg/kg/day, on gestation days 12 to 21. (A) Control testis on postnatal day (PND) 540. H&E. ×50. (B) DBP–exposed testis (100 mg/kg/day) on PND 540. A large Leydig cell (LC) adenoma (*) is present with multiple smaller areas of LC hyperplasia (arrowheads). There is no seminiferous epithelial degeneration in this testis. H&E. ×50. (C) DBP–exposed testis (500 mg/kg/day) on PND 370. Area of testicular dysgenesis including convoluted, immature seminiferous tubules and large numbers of LCs. The surrounding tubules have marked seminiferous epithelial degeneration and the interstitium is markedly expanded by interstitial fluid. H&E. ×50. (D) DBP–exposed testis (500 mg/kg/day) on PND 540. Higher magnification of dysgenetic area of the testis with immature appearing and dilated tubules and numerous spindloid LCs. H&E. ×125. (E) DBP–exposed testis (500 mg/kg/day) on PND 540. Area of testicular dysgenesis with markedly dilated tubules and relatively few LCs. H&E. ×50. (F) DBP–exposed testis (500 mg/kg/day) on PND 370. Focal area of LCs located in a similar area to other dysgenetic lesions but containing no aberrant tubules. The interstitium of this testis is markedly expanded by interstitial fluid. H&E. ×125. (G) Control testis on PND 540. The rete testis is the flattened duct near the capsule of the testis (arrowheads). A single tubule (tubuli recti) (arrow) that connects the seminiferous tubules and rete testis is present. H&E. ×125. (H) DBP–exposed testis (500 mg/kg/day) on PND 540. The area of the rete testis is markedly expanded by fibrous connective tissue (*). Sertoli cells with few germ cells line the surrounding seminiferous tubules. H&E. 125×.

Figure 2. Mean anogenital distance (AGD) from control and DBP–exposed male pups on postnatal days 1 and 180. Dams were exposed to vehicle or DBP, 100 and 500 mg/kg/day, on gestation days 12 to 21. Values are nested litter means ± SEM with body weight as a covariate for AGD. *Significantly different from control ($p < 0.05$).

Figure 3. Individual anogenital distances (AGD) on postnatal day (PND) 1 versus the same animal's AGD on PND 180. Male pups were exposed to exposed to DBP at 100 and 500 mg/kg/day on gestation days 12 to 21. Linear regression of the fit line yields a coefficient of determination of 0.80. The AGD for the 500 mg/kg/day dose group was significantly decreased from controls on PND 1 and 180. While the 100 mg/kg/day group was not significantly different on a litter mean basis, a subset of the animals did have a slightly lower AGD on both time points.

Figure 4. Areolae and nipple retention from control and DBP–exposed male pups on postnatal days 13 and 180. Dams were exposed to vehicle or DBP, 100 and 500 mg/kg/day, on gestation days 12 to 21. Values are nested litter means ± SEM. *Significantly different from control ($p < 0.05$).

Figure 5. Areolae retention on postnatal day 13 versus the percentage of animals with gross or histologic lesions in a particular tissue. Male pups were exposed to exposed to DBP at 100 and 500 mg/kg/day on gestation days 12 to 21. The number of areolae were segregated into blocks, 0, 1–3, 4–6, 7–9, and 10–12 areolae. Animals with lesions in each tissue were

plotted as a response against each areolae block. The response rate was the number of lesioned animals in that group divided by the total number of lesioned animals, totaling 100% in all 5 blocks. The same percentage response was calculated and graphed for the animals with no lesions.

Figure 6. Survival curves for male pups exposed to DBP at 0, 100, and 500 mg/kg/day on gestation days 12 to 21. Losses of animals were similar for all dose groups of the 180– and 370–day replicates. There were no differences in survival for the control and 100 mg/kg/day dose groups in the 540–day replicate, 77 and 83% survival, respectively. However, there was a significant difference in survival for the high dose group of this replicate, approximately 57%.

Figure 7. Gross lesions from animals exposed to 500 mg/kg/day DBP on gestation days 12 to 21. All lesions were from animals found moribund or dead prior to their scheduled necropsy times. (A) The urinary bladder (*) was markedly dilated and filled with dark red fluid. There was bilateral dilation of the ureters (arrows). (B) The obstruction of the animal in Figure 7A occurred at the flexure of the urethra as it exited the pelvic canal. A large, gritty, proteinaceous plug was found at the site (arrow). Once removed the urethra cranial and caudal to the plug was patent. (C) The urinary bladder was markedly dilated and filled with large uroliths (arrow). (D) Another example of a urinary bladder filled with variably sized, rough stones. (E) Kidney from an animal with urinary obstruction. There were multiple, coalescing, firm, raised inflammatory nodules throughout the parenchyma. (F)

Caudal view of a large pituitary gland adenoma that had caused compression of the overlying brain.

Figure 1

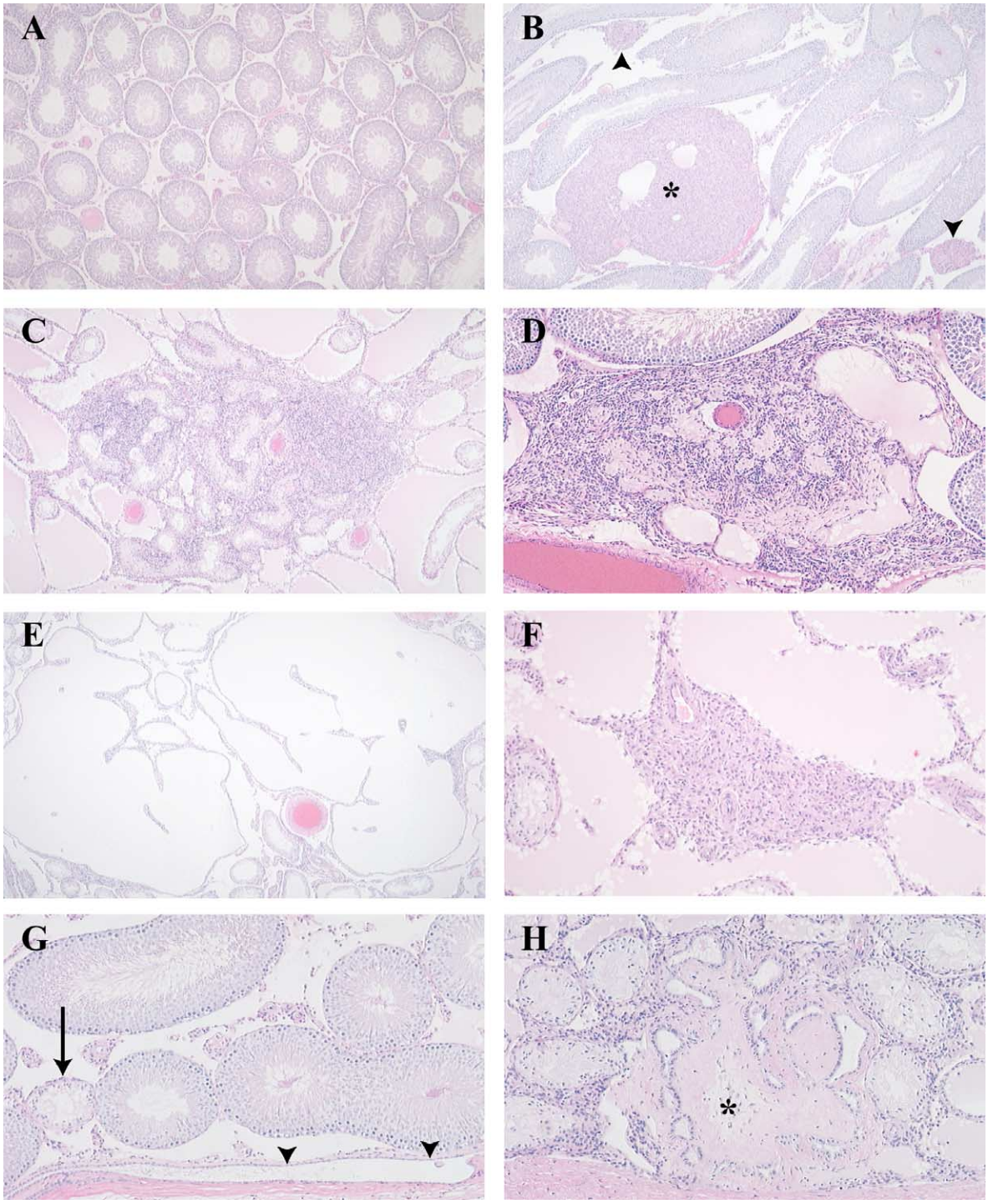


Figure 2

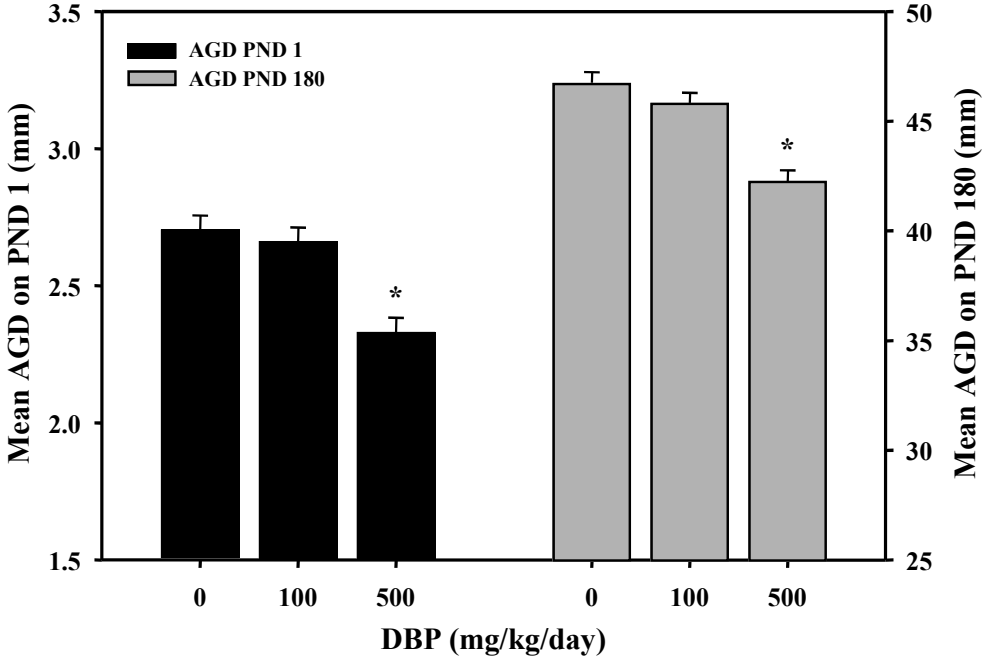

AGD PND 1
AGD PND 180
Mean AGD on PND 1 (mm)
Mean AGD on PND 180 (mm)
3.5
3.0
2.5
2.0
1.5
50
45
40
35
30
25
*
*
0
100
500
0
100
500
DBP (mg/kg/day)

Figure 3

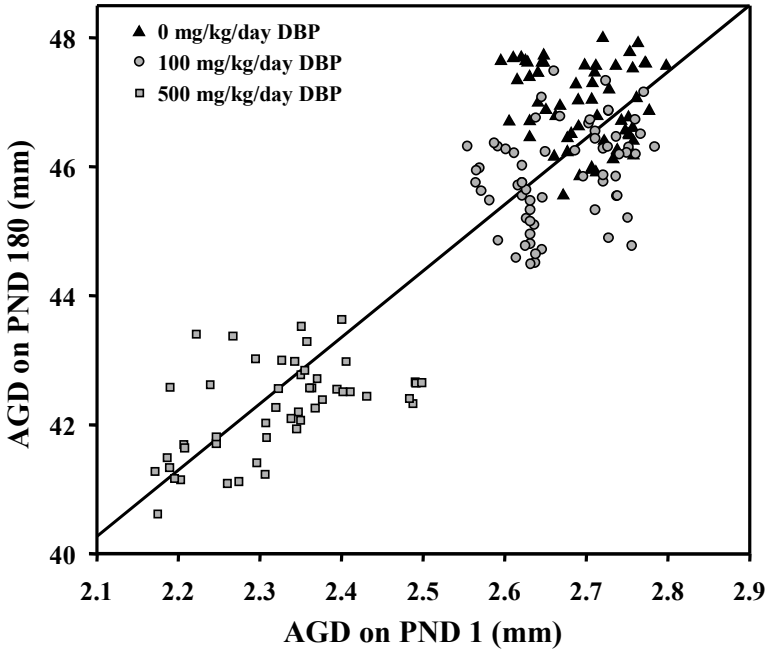

0 mg/kg/day DBP
100 mg/kg/day DBP
500 mg/kg/day DBP
AGD on PND 180 (mm)
AGD on PND 1 (mm)
40
42
44
46
48
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9

Figure 4

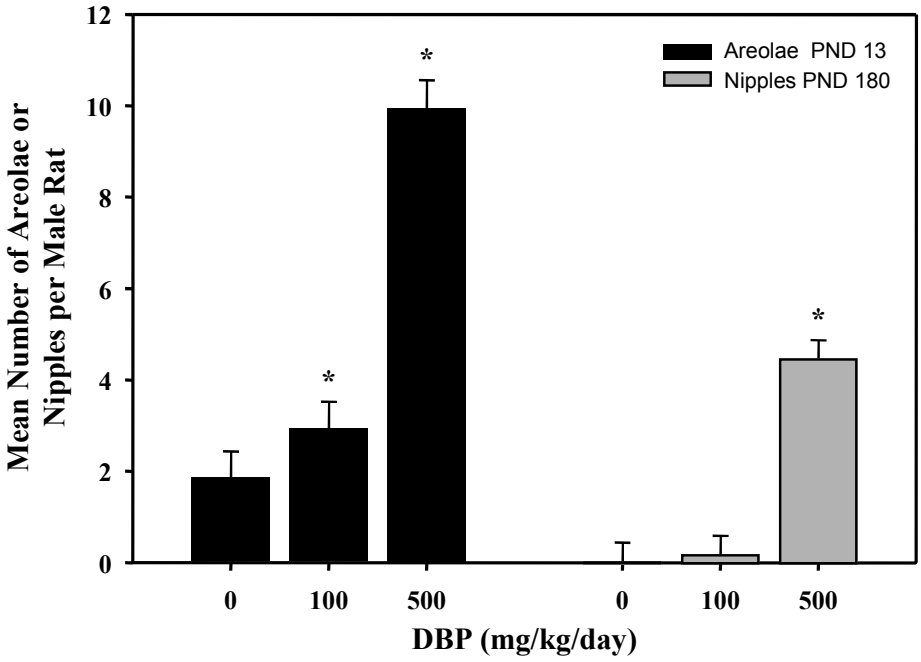

Areolae PND 13
Nipples PND 180
Mean Number of Areolae or Nipples per Male Rat
12
10
8
6
4
2
0
*
*
*
0
100
500
0
100
500
DBP (mg/kg/day)

Figure 5

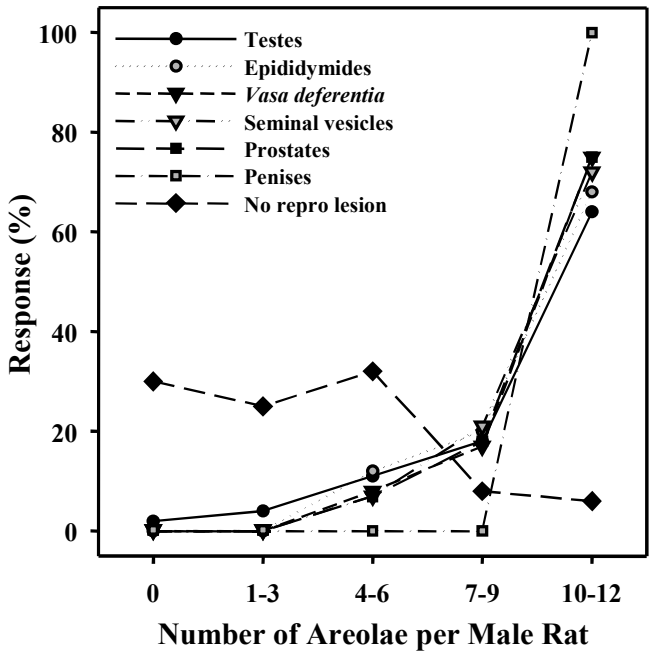

Testes
Epididymides
Vasa deferentia
Seminal vesicles
Prostates
Penises
No repro lesion
Response (%)
100
80
60
40
20
0
0
1-3
4-6
7-9
10-12
Number of Areolae per Male Rat

Figure 6

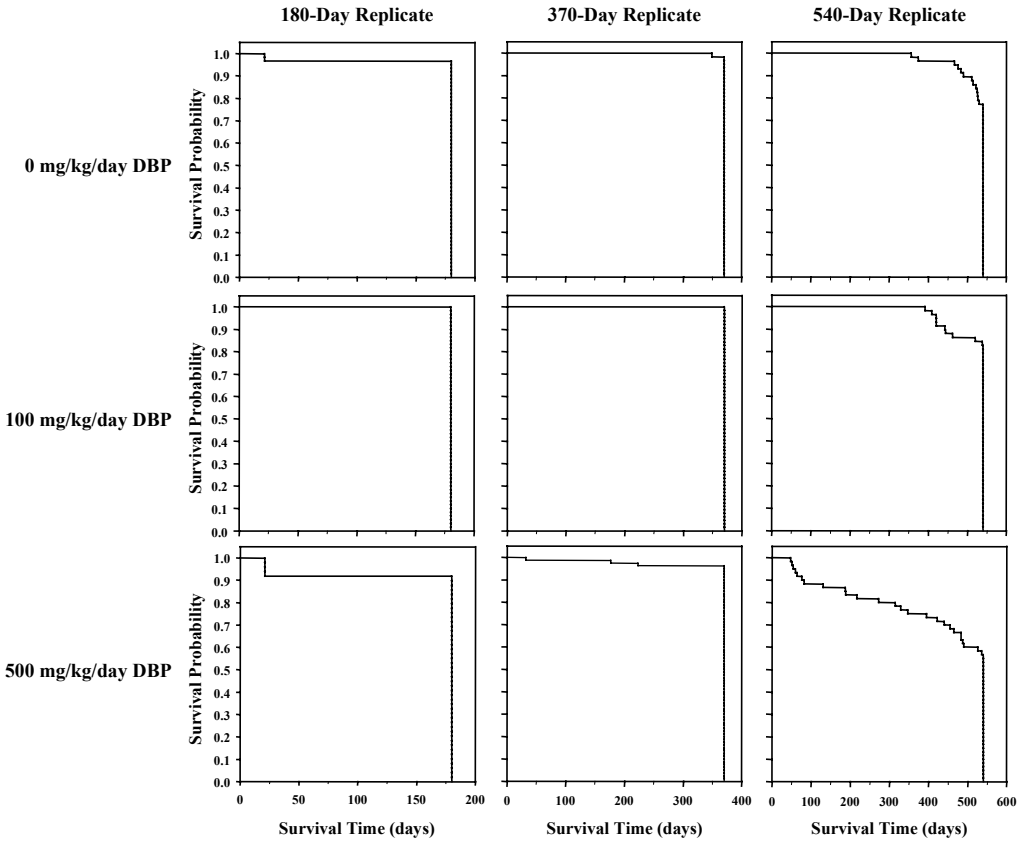
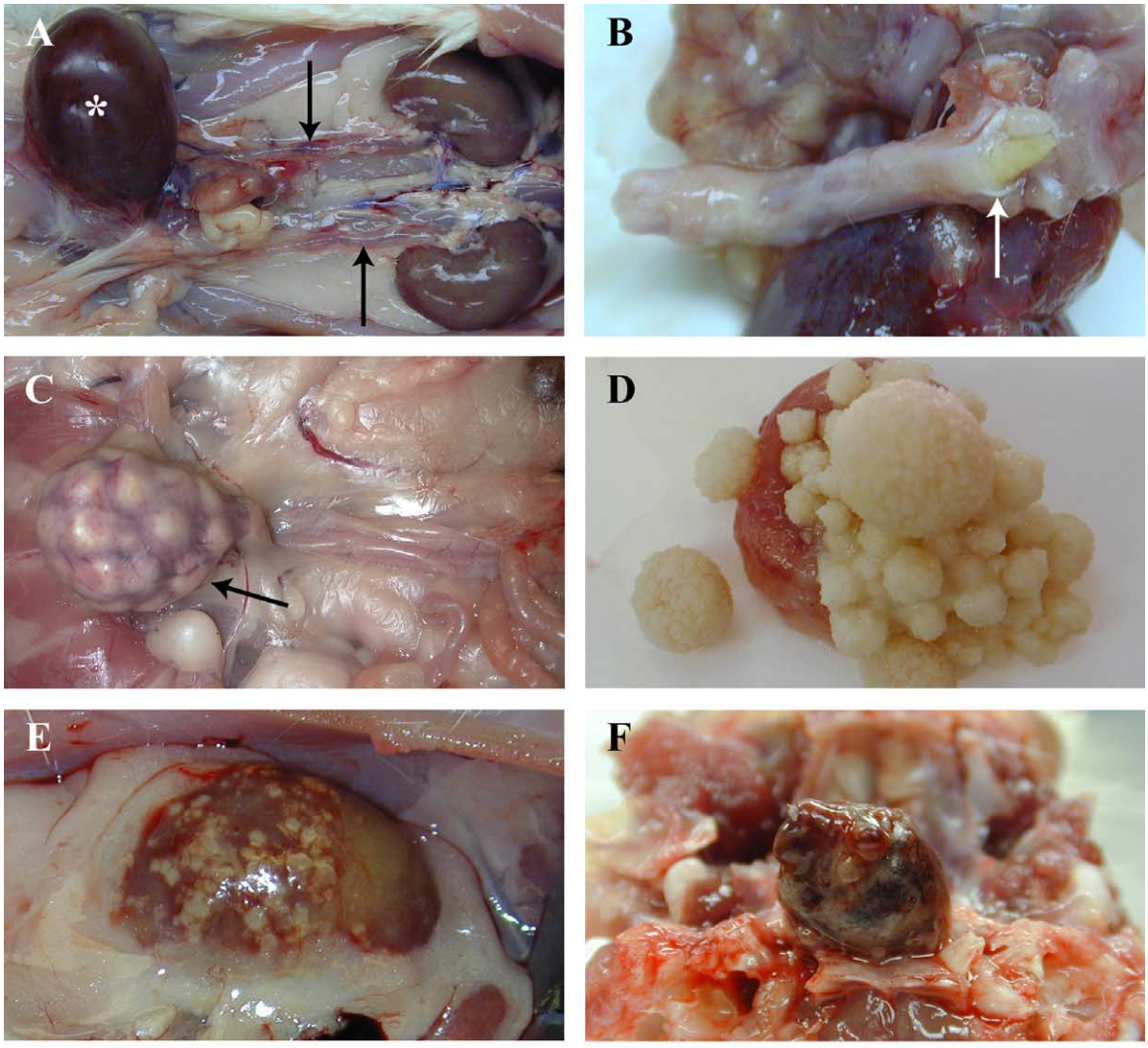

180-Day Replicate
370-Day Replicate
540-Day Replicate
0 mg/kg/day DBP
100 mg/kg/day DBP
500 mg/kg/day DBP
Survival Probability
Survival Time (days)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
50
100
150
200
300
400
500
600

Figure 7



CHAPTER SIX

General Discussion

Hypotheses and objectives

During my doctoral research I was given the opportunity to work within the broad area of reproductive toxicologic pathology, in a program specifically centered on antiandrogens. During the course of my investigations, I was afforded the opportunity to make scientific contributions to numerous studies utilizing gestational exposure to antiandrogens. The focus of my research was on the pathogenesis of DBP-induced male reproductive tract lesions. Mylchreest et al. were the first to show that gestational exposure to DBP led to altered male reproductive differentiation and development. The lesions in the male reproductive tract following *in utero* exposure were well characterized, but the pathogenesis of these lesions was not known. The first hypothesis of my doctoral research was, **Decreased fetal T induced by *in utero* DBP exposure leads to altered morphogenesis of the male reproductive tract that begins in the fetus and is ultimately manifest in the adult male reproductive tract.** The objective of this study was to examine animals at multiple times during gestation, in the early postnatal period, and as young adults to determine the morphogenesis of male reproductive tract lesions. The second hypothesis was, **Fetal DBP exposure leads to altered gene expression in fetal testes that has consequences for fetal testosterone levels and long-term reproductive development.** The objective of this study was to fully characterize gene expressions for the enzymes in the T biosynthetic pathway and other androgen-related genes in an attempt to

determine the degree to which *in utero* DBP exposure affects mRNA expression in this pathway. The final hypothesis was, **Altered testosterone levels and fetal Leydig cell lesions produced by DBP lead to Leydig cell adenomas in adult animals.** The objective of the study was to expose males to DBP for a 10-day gestational period and then allow the male offspring to mature to 6, 12, and 18 months of age to determine whether there was an increased incidence of LC adenomas.

Overall results

Although all three of my major studies were conducted separately, they were not conducted in isolation. They were designed to be continuations of previous work in our laboratory and to provide information that could be used across studies. The testes of the fetal rat exhibited characteristic lesions of DBP exposure, including large aggregates of LCs, multinucleated gonocytes, and increased proliferation of gonocytes (8). In addition, there was decreased coiling of the epididymal duct although the entire structure was present. From previous studies, we knew that DBP caused decreased production of T in fetal testes (107, 123, 154). The mechanism of this decreased T production is incompletely understood. Marked decreases in mRNA expression for most of the steroidogenic enzymes and for other genes that support steroidogenesis have been observed (7). However, these decreases may have been a direct effect of DBP on the fetal LCs, or the decreased gene expression may have been due to end-product inhibition of the steroidogenic pathway. The increased numbers of fetal LCs observed following DBP exposure appears to be a reactive response to decreased T levels, that is, decreased T leads to recruitment of additional LCs. The increased number of LCs may also be due to altered paracrine signaling with the Sertoli cells. Since phthalates are known Sertoli cell toxicants in the adult, they may also

target fetal Sertoli cells (33, 51). Whether there was altered communication between Sertoli cells and LCs was not definitively determined in our studies, but we did determine that there were altered gene expression levels for SCF and its receptor, *c-kit*, which implicates altered communication between Sertoli cells and gonocytes (7). The altered expression for these genes may have led to multinucleation and increased proliferation of gonocytes in the fetus and ultimately to the germinal epithelial degeneration observed in adult males exposed to DBP *in utero* (8).

The large aggregates of fetal LCs were noted in the testes of early postnatal animals, but they did not appear to be enlarging; rather, they became relatively smaller due to the marked growth of the testis (8). By PND 21, clusters of fetal LCs could no longer be identified. However, when sections of testis were examined from early postnatal animals, only two sections from the same transverse cut were examined, increasing the possibility that these lesions could have been overlooked. Whether these LCs remain within the interstitium of the immature and mature rat or whether they eventually degenerate is not known. One hypothesis is that the DBP-induced aggregates of fetal LCs are the cells that eventually become LC adenomas in the adult rats exposed to DBP *in utero*.

Whereas we did not see an increased incidence of classical LC adenomas following gestational exposure to DBP, we did identify a novel proliferative lesion in the adult testes composed of LCs and seminiferous cords or tubules (testicular dysgenesis) (6). In the areas of testicular dysgenesis, the LCs were poorly differentiated and morphologically dissimilar to those of a classic LC adenoma. Variable numbers of LCs were seen within the areas of testicular dysgenesis. While the lesion is being called testicular dysgenesis rather than a frank neoplasm, the size of some of the lesions was greater than three seminiferous tubules,

which would classify them as an LC adenomas by the standards for proliferative LC lesions of the Society of Toxicologic Pathology (114). The tubules associated with the proliferative LC lesions appeared immature and did not have spermatogenesis occurring (6). The lumina were small, and the Sertoli cells lining the tubules appeared similar to those in an early postnatal testis. The testicular dysgenesis lesions were always located in the same area of the adult testis, implying that they developed from a specific anatomic location. The lesions diagnosed as LC adenomas in the studies by Mylchreest et al. (1999 and 2000) were reviewed and found to have a similar morphology to the lesions we have diagnosed as testicular dysgenesis, further supporting the early development of these lesions with continued proliferation throughout the lifetime of the animal.

The small size of the fetal rat testis precludes definitive orientation for histopathology. Therefore we obtained cross, longitudinal, or oblique sections. We identified areas of abnormal LCs (the large aggregates) in the fetal rat testes but were not able to localize them to a particular area due to the inconsistent sectioning. However, an association between these large aggregates of LCs in the fetus and the testicular dysgenesis lesion in the adult is likely. We began dosing pregnant dams on GD 12, which coincides with the formation of the seminiferous cords (102, 147). DBP exposure starting on GD 12 may have caused altered seminiferous cord formation, especially if DBP directly affects fetal Sertoli cells, leading to an early precursor of testicular dysgenesis phenotype.

Gonocytes in the fetal testis are altered by DBP exposure. There are increased numbers of gonocytes within seminiferous cords and many of them are multinucleated, especially in testes from fetuses near the end of gestation (8). A breakdown in communication between the gonocytes and Sertoli cells may have led to these

morphological alterations. The gonocytes of control animals in the early postnatal testis migrate toward the basal lamina to become spermatogonia (55, 135). We believe that many of the gonocytes with altered morphology undergo degeneration and are not able to migrate and transform into spermatogonia. Altered spermatogonia would be one potential cause of germinal epithelial loss in the adult. The seminiferous epithelial degeneration in the adult also supports the notion that Sertoli cells exposed to DBP *in utero* are permanently altered and possibly cannot sustain normal spermatogenesis as adults. Whereas animals with marked seminiferous epithelial degeneration had malformed epididymides, there were also animals that had intact epididymides and yet exhibited marked testicular lesions (8). Sertoli cells may have been altered by *in utero* DBP treatment, which did not manifest until adulthood. Findings were similar in aged animals since rats with intact epididymides also had marked testicular degeneration at 6, 12, and 18 months of age. While the epididymides may have been intact, it is quite possible that they had functional lesions and could not reabsorb the testicular fluid being produced. In essence increased amounts of fluid due to improper fluid absorption would be a functional obstruction and could contribute to the observed testicular degeneration.

Epididymides exposed to DBP were complete when examined with transillumination during gestation. Although they were underdeveloped, a distinct duct could always be identified. The epididymal lesions appeared to progress slightly as the animals aged, since malformed epididymides with missing portions were identified in adults (8). Exposure to DBP during development may alter the ability of the epididymides to develop during the postnatal period. Absence of epididymides was observed in the studies of adult animals exposed *in utero* to DBP implying that complete regression of the

this structure can occur. Epididymal fat pads with no obvious epididymides were sectioned to look for remnant ducts, but none were found. This indicates that an epididymis may develop in the fetus, albeit abnormally, and that it can completely regress during the postnatal period.

The molecular studies carried out have confirmed the findings of Shultz, et al. and extended those findings to include genes involved with the rest of the steroidogenic pathway (7). There was decreased gene expression for all but one of the steroidogenic enzyme genes (17β-HSD) together with reductions in other genes supporting steroidogenesis such as SRB1 and StAR. Assuming the levels of functional enzymes follow the altered mRNA expression is supportive of the view that altered gene expression was the cause of decreased fetal testicular T synthesis. However, a specific target was not identified. Given other molecular lesions in cholesterol management genes, altered cholesterol metabolism may lead to decreased formation of T precursors and that this in turn leads to altered expression of steroidogenic enzyme genes. Decreased lipid vacuoles were seen in fetal Leydig cells with a fat stain and previous transmission electron microscopy (data not shown) identified decreased numbers of these vacuoles at the ultrastructural level. Taken together the gene expression data and the morphological data indicate that DBP has a broad effect on the fetal testis and does not have a specific cellular target. However, paracrine signaling occurring between Sertoli cells, gonocytes, and LCs that has not been completely elucidated makes it is possible that DBP may be affecting a particular cell such as the Sertoli cell, which would then have subsequent effects on gonocytes and LCs.

Data gaps filled by my studies

Multiple gaps in knowledge were filled by data generated in the three studies presented in this dissertation. The morphogenesis of the gross and histologic lesions from the fetus to the adult following *in utero* DBP exposure has now been described (8). This information is critical for comparison with other antiandrogens and for determining at which time point and in which tissues molecular analyses should be conducted. Since the exposure to DBP in our protocols utilized only an *in utero* exposure, the morphogenesis of the fetal lesions was important for determining the precise phenotype of the tissue during key molecular events. In addition to comparing the lesions of *in utero* DBP exposure to the altered phenotypes induced by other antiandrogens, the gene expression data generated will allow for comparisons at the molecular level among other endocrine disrupting compounds (7). This further extends our ability to correlate morphological events to molecular mechanisms. For example, decreased lipid in LCs, which was associated with the decreased levels of SRB1 gene expression, has been demonstrated (7). These two pieces of data suggest the possibility that decreased T synthesis may be related to altered cholesterol mobilization and metabolism.

In the molecular study presented in Chapter 3, we used a large numbers of fetuses for determination of gene expression levels. The data from the 15 fetuses allowed us to compare variability both within a litter and between litters. Contrary to what was expected, we found increased variability within litters compared with that between litters. There are plausible biological explanations for this phenomenon, including differential uterine blood flow and fetal uterine location (21, 45, 46, 130). What these data highlight is that one fetus

from a litter may not be representative of the total litter complement and that erroneous conclusions may be drawn based on the data from the examination of a single fetus.

While there was no increase in classical LC adenomas following *in utero* DBP exposure and aging of the male offspring to 6, 12, and 18 months, a novel testicular lesion was identified. These areas of testicular dysgenesis were composed of abnormal LCs and immature-appearing tubules. Given the similar location in all of the testes examined and the similar incidence between ages, this lesion may have initially developed in the fetus and continued to proliferate into adulthood.

Decreased AGD on PND 1 following *in utero* exposure to DBP is a sensitive marker of the antiandrogenic effects of DBP (121, 122, 124). However, whether this end point was permanently altered and whether there was any predictive value to this measurement was unknown. DBP-induced reduction in AGD was found to a permanent change that was highly predictive of lesions in the male reproductive tract (6). Similarly, retention of nipples in males following gestational exposure to DBP was permanent, although nipple retention was not closely associated with lesions in the male reproductive tract.

Alternative antiandrogens

In addition to my primary research with DBP, I was given the opportunity to take part in a number of other studies utilizing various antiandrogens (18, 115-118, 138, 174). This experience with compounds other than that used in my own experiments has allowed me to make comparisons among different antiandrogen-induced phenotypes following *in utero* exposures.

DBP induced seminiferous epithelial degeneration in the testes of exposed animals that increased in severity following puberty (8, 122, 124). This dramatic difference was predominantly attributed to malformed epididymides causing secondary pressure atrophy (116). However, significant numbers of animals with intact epididymides had severe seminiferous epithelial degeneration were observed. Therefore the testicular lesions could not be solely attributed to epididymal malformations. Epididymides were the most severely affected tissues following *in utero* DBP exposure (8, 63, 124). The predominant epididymal lesions were hypoplasia or partial agenesis. Malformed epididymides most often lacked the body of the organ, although the head and tail had a similar incidence of agenesis (data not shown). When the tail was absent the ipsilateral *vas deferens* was almost always absent as well. The seminal vesicles and prostates were significantly decreased in size, and a number of prostates were totally absent at the gross level. Hypospadias were also observed in up to approximately 25% of the animals. AGD was permanently decreased and nipple retention was permanently increased following *in utero* DBP exposure (6).

DBP caused severe lesions in T-dependent tissues, Wolffian duct derivatives, and DHT-dependent tissues. The lesions were more severe in the T-dependent tissues, but effects on the penis, AGD, and nipple retention highlight the breadth of end point sensitivity to the *in utero* antiandrogenic effects of DBP. This is not surprising since a significant decrease in fetal testosterone provides decreased substrate for 5α-reductase leading to decreased concentrations of DHT (13, 123, 154). In addition to working with DBP, I was also able to contribute to a DEHP study conducted by the U.S. EPA (138). Given that these phthalates appear to work via the same mechanism (decreased fetal T

production) the lesions in fetal and early postnatal animals were similar to those seen with animals of the same age exposed to DBP.

Flutamide, a potent AR antagonist, primarily affected DHT-dependent tissues, with effects on Wolffian duct derivatives at high doses (115). Marked prostatic hypoplasia and prostatic agenesis following *in utero* exposure to flutamide was present in adult animals. The incidence of hypospadias was markedly higher in flutamide exposed animals compared with DBP-exposed animals since this lesion was observed at all doses of flutamide tested. In addition to internal lesions, permanent decreases in AGD and increases in nipple retention was also noted. Unlike DBP, which was predictive of lesions in both T- and DHT-dependent tissues, decreased AGD following flutamide exposure was predictive only of lesions in DHT-dependent tissues of the male reproductive tract (115). Flutamide induced severe male reproductive tract lesions in DHT-dependent tissues at relatively low doses, while higher doses induced changes in T-dependent tissues as well. The reason for this dichotomy in tissues effects is not known. Possibly local concentrations of testosterone in the developing Wolffian ducts are enough to outcompete the AR antagonism of flutamide. Another possibility is that increased numbers of the androgen receptor in the Wolffian ducts, effectively allows testosterone to overcome the antagonistic effects of flutamide.

Linuron is also an AR antagonist but is not as potent as flutamide (118). Therefore, lesions similar to flutamide with a reduced incidence or severity were expected. However, the morphologic profile following *in utero* linuron exposure was more similar to DBP than to flutamide. Linuron induced epididymal malformations but did not appear to have direct effects on the testes of either fetal or adult animals. Testicular lesions of adult rats were

associated with malformed epididymides and obstruction and secondary pressure atrophy was the cause of the testicular lesions rather than a primary testicular event (116). While the dorsolateral prostate was slightly decreased in weight at the highest dose tested, no lesions were seen in this tissue or the seminal vesicles (118). AGD was permanently decreased and nipple retention was permanently increased, but neither of these alterations was predictive for lesions in the male reproductive tract. While *in utero* exposure to linuron induced epididymal lesions similar to DBP, there were no effects on other T- or DHT-dependent tissues except for one hypospadias (116). Although its mechanism of antiandrogenicity is speculated to be the same as flutamide based on competitive antagonism of the AR, the difference in potency appears to elicit an entirely different phenotypes for these two compounds.

Fenitrothion was shown to be an androgen receptor antagonist in both an *in vitro* receptor binding assay and the *in vivo* Hershberger pharmacological assay (168). Its potency *in vitro* was comparable to flutamide, and it was about 30 times more potent than linuron. Aside from mild and transient decreases in AGD and increases in areolae retention however, fenitrothion did not induce any male reproductive tract lesions (174). Although *in vivo* screens indicated fenitrothion was a competitive AR antagonist, its metabolism in pregnant dams and other toxicities precluded using higher dose levels, which likely lead to its decreased effectiveness as an antiandrogen (174).

The mechanism of action of finasteride is different from all the other antiandrogens discussed thus far, as it is a competitive inhibitor of 5α-reductase, the enzyme that converts T to DHT (29, 30, 82-84). *In utero* exposure to this compound yields lesions in DHT-dependent tissues, including the prostate and penis (18). Prostates were markedly decreased

in size and appeared completely absent for some of the animals, and there was a high incidence of hypospadias. Finasteride exposure also induced permanently decreased AGD and permanently increased nipple retention. This compound elicited the morphologic lesions expected of an inhibitor of 5α-reductase and, given the complete absence of lesions in the tissues derived from the Wolffian duct, was dissimilar to DBP.

Environmental relevance

The doses of DBP used in the studies reported in this thesis were fairly high compared with the levels encountered by the average human. These doses were purposely chosen to induce lesion formation in a large percentage of animals, thereby making it easier to study the morphological development of the lesions and providing a more robust experimental model. Although not of environmental relevance at these doses, the data are important for several reasons. (1) The interaction of compounds acting via the same pharmacological mechanism is not completely understood. Humans are exposed to multiple phthalates. While the exposure levels to one of them may not be harmful, the exposure to three or four, all working via the same mechanism, may be enough to induce gene changes and alter development of the male reproductive tract. (2) Interactions with other antiandrogens are not well characterized. Although compounds may work via different mechanisms, there is overlap between lesions seen with different chemicals, and therefore combinations of low doses, ineffectual at inducing lesions alone, may be enough to cause altered development. (3) Human risk assessment is not fully developed in terms of sensitive populations. Since we see variability in response within and between litters the concept of more sensitive individuals is highly plausible. It may take only very low exposure to induce developmental defects in a sensitive individual.

Potential future studies

Since the most acutely affected fetal testicular cell has yet to be identified, I think one direction of future research should be to give an acute dose on different gestation days and look at early time points using transmission electron microscopy to discern changes in cell structure. These analyses would help identify the testicular cell type that is first affected and might help identify critical gestation days and time points for further molecular analyses. Another interesting experiment would be to attempt to reverse the affects of decreased T synthesis by replacing T in the fetus via the dam. If the primary effect is on the fetal LC and all of the other lesions stem from that, then replacing T, if the appropriate dose can be delivered to the fetuses, should ameliorate all DBP-induced testicular lesions.

Proposed summary mechanism

The definitive mechanism for the antiandrogenicity of DBP remains to be elucidated. However, the studies performed and presented in this thesis give some clues as to likely events perturbed in the fetal testis. I propose that DBP directly affects multiple cell types in the fetal testis, altering paracrine signals between Sertoli cells, gonocytes, and LCs. While there is significant discussion about the paracrine regulation of these three cell types, specific factors and their definitive functions remain to be identified (26, 102, 127). One known interaction is between SCF and *c-kit* (119). SCF is produced by the Sertoli cell, while *c-kit* is a surface receptor for SCF on gonocytes and is found in LCs. Disruption of the interaction between SCF and *c-kit* on gonocytes may lead to the pathology observed, multinucleated gonocytes and increased numbers of gonocytes. Perturbation of the SCF-*c-kit* interaction between Sertoli cells and LCs could lead to the published suppression of T

biosynthesis (107, 123, 154). However, these two interactions alone do not explain all the observed morphological and molecular alterations. There are decreased numbers of lipid vacuoles in fetal LCs, and gene expression is decreased for SRB1 and StAR (7). Phthalates are known to alter lipid and cholesterol metabolism (10-12), and I propose that this plays a part in decreased steroidogenesis. Whether DBP directly affects gene expression for the enzymes of the steroidogenic pathway or whether changes in mRNA expression are secondary remains a mystery. Recent data from Earl Gray and Vickie Wilson at the U.S. EPA (personal communication) showed that in vitro culture of fetal testes with MEHP, the active metabolite of DEHP, did not alter steroidogenesis *in vitro*. This would argue that the effects we see on steroidogenic enzyme gene expression are secondary to some other mechanism. *Insl3* gene expression in fetal LCs is also known to be decreased following phthalate exposure (L.E. Gray and V. Wilson, personal communication). How this is related to decreased T synthesis is unknown.

Decreased T led to decreased differentiation of the Wolffian ducts and decreased levels of DHT. Altered stability and development of the Wolffian ducts leads to malformed epididymides, absent *vasa deferentia*, and malformed seminal vesicles. These tissues are imprinted during fetal life and have permanent malformations that do not resolve even if T is restored during puberty. Decreased DHT leads to decreased AGD, increased nipple retention, hypospadias, and malformed prostates. Ultimately the toxicity of DBP on the fetal testes may only involve one cell type. Given the diversity of changes seen at the molecular level and the morphological alterations identified histologically, the more likely explanation is that DBP is affecting more than one cell type in the fetal testis with a cumulative response ensuing.

GENERAL REFERENCES

1. Agarwal DK, Eustis S, Lamb JC, Reel JR, and Kluwe WM (1986). Effects of di(2-ethylhexyl) phthalate on the gonadal pathophysiology, sperm morphology, and reproductive performance of male rats. *Environ Health Perspect* 65:343-50.
2. Albro PW, Hass JR, Peck CC, Odam DG, Corbett JT, Bailey FJ, Blatt HE, and Barrett BB (1981). Identification of the metabolites of di(2-ethylhexyl) phthalate in urine from the African green monkey. *Drug Metab Dispos* 9:223-5.
3. Andersen JM and Dietschy JM (1978). Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. *J Biol Chem* 253:9024-32.
4. Anderson WA, Castle L, Scotter MJ, Massey RC, and Springall C (2001). A biomarker approach to measuring human dietary exposure to certain phthalate diesters. *Food Addit Contam* 18:1068-74.
5. Auger J, Kunstmann JM, Czyglik F, and Jouannet P (1995). Decline in semen quality among fertile men in Paris during the past 20 years. *N Engl J Med* 332:281-5.
6. Barlow N, McIntyre B, and Foster P (2003). Male reproductive tract lesions at six, twelve, and eighteen months following *in utero* exposure to di(*n*-butyl) phthalate (in preparation). *Toxicol Path*
7. Barlow N, Phillips S, Wallace D, Sar M, Gaido K, and Foster P (2003). Quantitative changes in gene expression in fetal rat testes following exposure to di(*n*-butyl) phthalate (submitted). *Toxicol Sci*

8. Barlow NJ and Foster PMD (2003). Pathogenesis of male reproductive tract lesions from gestation through adulthood following *in utero* exposure to di(*n*-butyl) phthalate (in press). *Toxicol. Pathol.*

9. Baskin LS, Himes K, and Colborn T (2001). Hypospadias and endocrine disruption: Is there a connection? *Environ Health Perspect* 109:1175-83.

10. Bell FP (1982). Effects of phthalate esters on lipid metabolism in various tissues, cells and organelles in mammals. *Environ Health Perspect* 45:41-50.

11. Bell FP, Makowske M, Schneider D, and Patt CS (1979). Inhibition of sterologenesis in brain and liver of fetal and suckling rats from dams fed di-2-ethylhexyl phthalate plasticizer. *Lipids* 14:372-7.

12. Bell FP, Patt CS, and Gillies PJ (1978). Effect of phthalate esters on serum cholesterol and lipid biosynthesis in liver, testes, and epididymal fat in the rat and rabbit. *Lipids* 13:673-8.

13. Berman DM, Tian H, and Russell DW (1995). Expression and regulation of steroid 5α-reductase in the urogenital tract of the fetal rat. *Mol Endocrinol* 9:1561-70.

14. Bigsby R, Chapin RE, Daston GP, Davis BJ, Gorski J, Gray LE, Howdeshell KL, Zoeller RT, and vom Saal FS (1999). Evaluating the effects of endocrine disruptors on endocrine function during development. *Environ Health Perspect* 107 Suppl 4:613-8.

15. Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Lucier GW, Jackson RJ, and Brock JW (2000). Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 108:979-82.

16 Boisen KA, Main KM, Rajpert-De Meyts E, and Skakkebaek NE (2001). Are male reproductive disorders a common entity? The testicular dysgenesis syndrome. *Ann N Y Acad Sci* 948:90-9.

17. Bowman C, Barlow N, Turner K, Wallace D, and Foster P (2003). Effects of *in utero* exposure to finasteride on androgen-dependent reproductive development in the male rat (in preparation). *Toxicol Sci*

18. Brock JW, Caudill SP, Silva MJ, Needham LL, and Hilborn ED (2002). Phthalate monoesters levels in the urine of young children. *Bulletin of Environmental Contamination and Toxicology* 68:309-314.

19. Brock JW, Silva MJ, Malek NA, Hodge CC, and Blount BC (2001). A new method to measure phthalate exposure in humans. *APMIS* 109:S330-S331.

20. Buelke-Sam J, Holson JF, and Nelson CJ (1982). Blood flow during pregnancy in the rat: II. Dynamics of and litter variability in uterine flow. *Teratology* 26:279-88.

21. Bureau of National Affairs (1988). Environmental group considering challenge to EPA position on cancer risk from linuron. *Chem Regul Rep* 12:774-800.

22. Byskov A and Hoyer P, *Embryology of mammalian gonads and ducts*, in *The Physiology of Reproduction*, e. Knobil and J. Neill, Editors. 1994, Raven Press: New York.

23. Caine M, Perlberg S, and Gordon R (1975). The treatment of benign prostatic hypertrophy with flutamide (SCH: 13521): a placebo-controlled study. *J Urol* 114:564-8.

24. Calley D, Autian J, and Guess W (1966). Toxicology of a series of phthalate esters. *J. Pharm. Sci.* 55:158-162.

25. Cao G, Zhao L, Stangl H, Hasegawa T, Richardson JA, Parker KL, and Hobbs HH (1999). Developmental and hormonal regulation of murine scavenger receptor, class B, type 1. *Mol Endocrinol* 13:1460-73.

26. Capel B (1998). Sex in the 90s: SRY and the switch to the male pathway. *Annu Rev Physiol* 60:497-523.

27. Carlsen E, Giwercman A, Keiding N, and Skakkebaek NE (1992). Evidence for decreasing quality of semen during past 50 years. *BMJ* 305:609-13.

28. Cater BR, Cook MW, Gangolli SD, and Grasso P (1977). Studies on dibutyl phthalate-induced testicular atrophy in the rat: effect on zinc metabolism. *Toxicol Appl Pharmacol* 41:609-18.

29. Clark RL, Anderson CA, Prahalada S, Robertson RT, Lochry EA, Leonard YM, Stevens JL, and Hoberman AM (1993). Critical developmental periods for effects on male rat genitalia induced by finasteride, a 5α-reductase inhibitor. *Toxicol Appl Pharmacol* 119:34-40.

30. Clark RL, Antonello JM, Grossman SJ, Wise LD, Anderson C, Bagdon WJ, Prahalada S, MacDonald JS, and Robertson RT (1990). External genitalia abnormalities in male rats exposed in utero to finasteride, a 5α-reductase inhibitor. *Teratology* 42:91-100.

31. Colborn T, vom Saal FS, and Soto AM (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 101:378-84.

32. Cook JC, Mullin LS, Frame SR, and Biegel LB (1993). Investigation of a mechanism for Leydig cell tumorigenesis by linuron in rats. *Toxicol Appl Pharmacol* 119:195-204.

33. Creasy DM, Foster JR, and Foster PMD (1983). The morphological development of di-*n*-pentyl phthalate induced testicular atrophy in the rat. *J Pathol* 139:309-21.

34. David RM (2000). Exposure to phthalate esters. *Environ Health Perspect* 108:A440-A440.

35. Dostal LA, Chapin RE, Stefanski SA, Harris MW, and Schwetz BA (1988). Testicular toxicity and reduced Sertoli cell numbers in neonatal rats by di(2-ethylhexyl)phthalate and the recovery of fertility as adults. *Toxicol Appl Pharmacol* 95:104-21.

36. Drews U (2000). Local mechanisms in sex specific morphogenesis. *Cytogenet Cell Genet* 91:72-80.

37. Eigenberg DA, Bozigian HP, Carter DE, and Sipes IG (1986). Distribution, excretion, and metabolism of butylbenzyl phthalate in the rat. *J Toxicol Environ Health* 17:445-56.

38. Ema M, Kurosaka R, Amano H, and Ogawa Y (1995). Developmental toxicity evaluation of mono-*n*-butyl phthalate in rats. *Toxicol Lett* 78:101-6.

39. Ema M and Miyawaki E (2001). Adverse effects on development of the reproductive system in male offspring of rats given monobutyl phthalate, a metabolite of dibutyl phthalate, during late pregnancy. *Reproductive Toxicology* 15:189-194.

40. Ema M, Miyawaki E, and Kawashima K (1998). Further evaluation of developmental toxicity of di-*n*-butyl phthalate following administration during late pregnancy in rats. *Toxicol Lett* 98:87-93.

41. Ema M, Miyawaki E, and Kawashima K (2000). Critical period for adverse effects on development of reproductive system in male offspring of rats given di-*n*-butyl phthalate during late pregnancy. *Toxicol Lett* 111:271-8.

42. Ema M, Miyawaki E, and Kawashima K (2000). Effects of dibutyl phthalate on reproductive function in pregnant and pseudopregnant rats. *Reprod Toxicol* 14:13-9.

43. Emmen JMA, McLuskey A, Adham IM, Engel W, Grootegoed JA, and Brinkmann AO (2000). Hormonal control of gubernaculum development during testis descent: Gubernaculum outgrowth in vitro requires both insulin-like factor and androgen. *Endocrinology* 141:4720-4727.

44. Even MD, Dhar MG, and vom Saal FS (1992). Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. *J Reprod Fertil* 96:709-16.

45. Even MD, Laughlin MH, Krause GF, and vom Saal FS (1994). Differences in blood flow to uterine segments and placentae in relation to sex, intrauterine location and side in pregnant rats. *J Reprod Fertil* 102:245-52.

46. Fisch H, Goluboff ET, Olson JH, Feldshuh J, Broder SJ, and Barad DH (1996). Semen analyses in 1,283 men from the United States over a 25-year period: no decline in quality. *Fertil Steril* 65:1009-14.

47. Fisch H, Ikeguchi EF, and Goluboff ET (1996). Worldwide variations in sperm counts. *Urology* 48:909-11.

48. Foster P, *Testicular organization and biochemical function*, in *Physiology and toxicology of male reproduction*, J. Lamb and P. Foster, Editors. 1988, Academic Press: San Diego.

49. Foster PMD, Cook MW, Thomas LV, G. WD, and Gangolli SD (1982). Differences in urinary metabolic profile from di-*n*-butyl phthalate-treated rats and hamsters: A possible explanation for species differences in susceptibility to testicular atrophy. *Drug Metabolism and Disposition* 11:59-61.

50. Foster PMD, Foster JR, Cook MW, Thomas LV, and Gangolli SD (1982). Changes in ultrastructure and cytochemical localization of zinc in rat testis following the administration of di-*n*-pentyl phthalate. *Toxicol Appl Pharmacol* 63:120-32.

51. Foster PMD, Lake BG, Thomas LV, Cook MW, and Gangolli SD (1981). Studies on the testicular effects and zinc excretion produced by various isomers of monobutyl-o-phthalate in the rat. *Chem Biol Interact* 34:233-8.

52. Foster PMD and McIntyre BS (2002). Endocrine active agents: implications of adverse and non-adverse changes. *Toxicol Pathol* 30:59-65.

53. Foster PMD, Thomas LV, Cook MW, and Gangolli SD (1980). Study of the testicular effects and changes in zinc excretion produced by some *n*-alkyl phthalates in the rat. *Toxicol Appl Pharmacol* 54:392-8.

54. Franchi L and Mandl A (1964). The ultrastructure of germ cells in foetal and neonatal rats. *J Embryol Exp Morphol* 12:289-308.

55. Fukuoka M, Tanimoto T, Zhou Y, Kawasaki N, Tanaka A, Ikemoto I, and Machida T (1989). Mechanism of testicular atrophy induced by di-*n*-butyl phthalate in rats. Part 1. *J Appl Toxicol* 9:277-83.

56. Gangolli SD (1982). Testicular effects of phthalate esters. *Environ Health Perspect* 45:77-84.

57. George FW and Peterson KG (1988). 5 α-dihydrotestosterone formation is necessary for embryogenesis of the rat prostate. *Endocrinology* 122:1159-64.

58. Gilbert S, *Developmental Biology*. 1994, Sunderland: Sinauer Associates, Inc.

59. Gray LE, Jr. (1998). Xenoendocrine disrupters: laboratory studies on male reproductive effects. *Toxicol Lett* 102-103:331-5.

60. Gray LE, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, Veeramachaneni DN, Wilson V, Price M, Hotchkiss A, Orlando E, and Guillette L (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *Human Reproduction Update* 7:248-264.

61. Gray LE, Jr., Ostby JS, and Kelce WR (1994). Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol* 129:46-52.

62. Gray LE, Jr., Wolf C, Lambright C, Mann P, Price M, Cooper RL, and Ostby J (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* 15:94-118.

63. Gray TJ and Butterworth KR (1980). Testicular atrophy produced by phthalate esters. *Arch Toxicol Suppl* 4:452-5.

64. Gray TJ, Butterworth KR, Gaunt IF, Grasso GP, and Gangolli SD (1977). Short-term toxicity study of di(2-ethylhexyl) phthalate in rats. *Food Cosmet Toxicol* 15:389-99.

65. Gray TJ and Gangolli SD (1986). Aspects of the testicular toxicity of phthalate esters. *Environ Health Perspect* 65:229-35.

66. Gray TJ, Rowland IR, Foster PMD, and Gangolli SD (1982). Species differences in the testicular toxicity of phthalate esters. *Toxicol Lett* 11:141-147.

67. Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, and Lovell-Badge R (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* 346:245-50.

68. Guillette LJ, Jr., Gross TS, Masson GR, Matter JM, Percival HF, and Woodward AR (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect* 102:680-8.

69. Hall P, *Testicular steroid synthesis: Organization and regulation*, in *The Physiology of Reproduction*, E. Knobil and J. Neill, Editors. 1994, Raven Press, Ltd.: New York.

70. Harris CA, Henttu P, Parker MG, and Sumpter JP (1997). The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* 105:802-11.

71. Hasegawa T, Zhao LP, Caron KM, Majdic G, Suzuki T, Shizawa S, Sasano H, and Parker KL (2000). Developmental roles of the steroidogenic acute regulatory protein (StAR) as revealed by StAR knockout mice. *Mol Endocrinol* 14:1462-1471.

72. Hasmall SC, James NH, Macdonald N, Soames AR, and Roberts RA (2000). Species differences in response to diethylhexylphthalate: suppression of apoptosis,

induction of DNA synthesis and peroxisome proliferator activated receptor alpha-mediated gene expression. *Archives of Toxicology* 74:85-91.

73. Heindel JJ, Gulati DK, Mounce RC, Russell SR, and Lamb JCt (1989). Reproductive toxicity of three phthalic acid esters in a continuous breeding protocol. *Fundam Appl Toxicol* 12:508-18.

74. Hites RA and Biemann K (1972). Water pollution: organic compounds in the Charles River, Boston. *Science* 178:158-60.

75. Hodge H (1943). Acute toxicity for rats and mice of 2-ethylhexanol and 2-ethylhexyl phthalate. *Proc. Soc. Exp. Biol. Med.* 53:20.

76. Hoppin JA, Brock JW, Davis BJ, and Baird DD (2002). Reproducibility of urinary phthalate metabolites in first morning urine samples. *Environ Health Perspect* 110:515-518.

77. Hughes IA (2001). Minireview: sex differentiation. *Endocrinology* 142:3281-7.

78. Hughes IA, Lim HN, Martin H, Mongan NP, Dovey L, Ahmed SF, and Hawkins JR (2001). Developmental aspects of androgen action. *Mol Cell Endocrinol* 185:33-41.

79. Huhtaniemi I and Pelliniemi LJ (1992). Fetal Leydig cells: cellular origin, morphology, life span, and special functional features. *Proc Soc Exp Biol Med* 201:125-140.

80. Hutson JM (1985). A biphasic model for the hormonal control of testicular descent. *Lancet* 2:419-21.

81. Imperato-McGinley J, Binienda Z, Arthur A, Mininberg DT, Vaughan ED, Jr., and Quimby FW (1985). The development of a male pseudohermaphroditic rat using an inhibitor of the enzyme 5 α-reductase. *Endocrinology* 116:807-12.

82. Imperato-McGinley J, Binienda Z, Gedney J, and Vaughan ED, Jr. (1986). Nipple differentiation in fetal male rats treated with an inhibitor of the enzyme 5α-reductase: definition of a selective role for dihydrotestosterone. *Endocrinology* 118:132-7.

83. Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B, and Vaughan ED (1992). Comparison of the effects of the 5α-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. *Endocrinology* 131:1149-56.

84. International Programme on Chemical Safety (1992), Environmental Health Criteria 131. Diethylhexyl Phthalate, World Health Organization: Geneva.

85. International Programme on Chemical Safety (1997), Environmental Health Criteria 189. Di-*n*-butyl Phthalate, World Health Organization: Geneva.

86. Itoh N, Kayama F, Tatsuki TJ, and Tsukamoto T (2001). Have sperm counts deteriorated over the past 20 years in healthy, young Japanese men? Results from the Sapporo area. *J Androl* 22:40-4.

87. Ivell R and Bathgate RA (2002). Reproductive biology of the relaxin-like factor (RLF/INSL3). *Biol Reprod* 67:699-705.

88. Jaeger RJ and Rubin RJ (1970). Plasticizers from plastic devices extraction, metabolism, and accumulation by biological systems. *Science* 170:460-2.

89. Jaeger RJ and Rubin RJ (1972). Migration of a phthalate ester plasticizer from polyvinyl chloride blood bags into stored human blood and its localization in human tissues. *N Engl J Med* 287:1114-8.

90. Jobling S, Reynolds T, White R, Parker MG, and Sumpter JP (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ Health Perspect* 103:582-7.

91. Josso N, *Anatomy and Endocrinology of Fetal Sex Differentiation*, in *Endocrinology*, L.J. DeGroot and J.L. Jameson, Editors. 2001, W.B. Saunders Company: Philadelphia.

92. Jost A (1947). Recherches sur la differenciation sexuelle de l'embryon de lapin. *Arch. Anat. Microscop. Morphol. Exp.* 36:

93. Jost A (1972). A new look at the mechanisms controlling sex differentiation in mammals. *Johns Hopkins Med J* 130:38-53.

94. Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, Golub M, Henderson R, Hinberg I, Little R, Seed J, Shea K, Tabacova S, Tyl R, Williams P, and Zacharewski T (2002). NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-*n*-butyl phthalate. *Reproductive Toxicology* 16:489-527.

95. Kelce WR, Gray LE, and Wilson EM (1998). Antiandrogens as environmental endocrine disruptors. *Reprod Fertil Dev* 10:105-11.

96. Kelce WR, Monosson E, Gamcsik MP, Laws SC, and Gray LE, Jr. (1994). Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol Appl Pharmacol* 126:276-85.

97. Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, and Wilson EM (1995). Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature* 375:581-5.

98. Kent J, Wheatley SC, Andrews JE, Sinclair AH, and Koopman P (1996). A male-specific role for SOX9 in vertebrate sex determination. *Development* 122:2813-22.

99. Kluwe WM (1982). Overview of phthalate ester pharmacokinetics in mammalian species. *Environ Health Perspect* 45:3-9.

100. Kohn MC, Parham F, Masten SA, Portier CJ, Shelby MD, Brock JW, and Needham LL (2000). Human exposure estimates for phthalates. *Environ Health Perspect* 108:A440-A442.

101. Koopman P (1999). Sry and Sox9: mammalian testis-determining genes. *Cell Mol Life Sci* 55:839-56.

102. Koopman P, Gubbay J, Vivian N, Goodfellow P, and Lovell-Badge R (1991). Male development of chromosomally female mice transgenic for Sry. *Nature* 351:117-21.

103. Lake BG, Brantom PG, Gangolli SD, Butterworth KR, and Grasso P (1976). Studies on the effects of orally administered di(2-ethylhexyl) phthalate in the ferret. *Toxicology* 6:341-56.

104. Lake BG, Phillips JC, Linnell JC, and Gangolli SD (1977). The in vitro hydrolysis of some phthalate diesters by hepatic and intestinal preparations from various species. *Toxicol Appl Pharmacol* 39:239-48.

105. Lambright C, Ostby J, Bobseine K, Wilson V, Hotchkiss AK, Mann PC, and Gray LE (2000). Cellular and molecular mechanisms of action of linuron: An

antiandrogenic herbicide that produces reproductive malformations in male rats. *Toxicol Sci* 56:389-399.

106. Lambright CS, Wilson VS, Furr JR, Wolf CJ, Noriega N, and Gray LE, Jr. (2003). Effects of endocrine disrupting chemicals on fetal testes hormone production (accepted). *The Toxicologist* 61:

107. LeBlanc GA (1995). Are environmental sentinels signaling? *Environ Health Perspect* 103:888-90.

108. LeBlanc GA, Bain LJ, and Wilson VS (1997). Pesticides: multiple mechanisms of demasculinization. *Mol Cell Endocrinol* 126:1-5.

109. Lovell-Badge R and Robertson E (1990). XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. *Development* 109:635-46.

110. Luo X, Ikeda Y, and Parker KL (1994). A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* 77:481-90.

111. Mackay S, *Gonadal Development in Mammals at the Cellular and Molecular Levels*, in *International Review of Cytology: A survey of cell biology*, K.W. Jeon, Editor. 2000, Academic Press: San Diego. p. 47-99.

112. Mayer FL, Stalling DL, and Johnson JL (1972). Phthalate esters as environmental contaminants. *Nature* 238:411-3.

113. McConnell RF, Westen HH, Ulland BM, Bosland MC, and Ward JM, *Proliferative Lesions of the Testes in Rats with Selected Examples from Mice*, in *Standardized System of Nomenclature and Diagnostic Criteria*, Society of Toxicologic Pathology, 1992, STP/ARP/AFIP: Washington.

114. McIntyre BS, Barlow NJ, and Foster PMD (2001). Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol Sci* 62:236-49.

115. McIntyre BS, Barlow NJ, and Foster PMD (2002). Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. *Toxicol Sci* 65:62-70.

116. McIntyre BS, Barlow NJ, Sar M, Wallace DG, and Foster PMD (2002). Effects of in utero linuron exposure on rat Wolffian duct development. *Reprod Toxicol* 16:131-9.

117. McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, and Foster PMD (2000). Effects of in utero exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat. *Toxicol Appl Pharmacol* 167:87-99.

118. Merchant-Larios H and Moreno-Mendoza N (2001). Onset of sex differentiation: dialog between genes and cells. *Arch Med Res* 32:553-8.

119. Miller WL (1988). Molecular biology of steroid hormone synthesis. *Endocr Rev* 9:295-318.

120. Mylchreest E, Cattley RC, and Foster PMD (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to di(*n*-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci* 43:47-60.

121. Mylchreest E, Sar M, Cattley RC, and Foster PMD (1999). Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156:81-95.

122. Mylchreest E, Sar M, Wallace DG, and Foster PMD (2002). Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(*n*-butyl) phthalate. *Reprod Toxicol* 16:19-28.

123. Mylchreest E, Wallace DG, Cattley RC, and Foster PMD (2000). Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(*n*-butyl) phthalate during late gestation. *Toxicol Sci* 55:143-151.

124. Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, and Ingraham HA (1998). Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. *Cell* 93:445-54.

125. Nazir DJ, Alcaraz AP, Bierl BA, Beroza M, and Nair PP (1971). Isolation, identification, and specific localization of di-2-ethylhexyl phthalate in bovine heart muscle mitochondria. *Biochemistry* 10:4228-32.

126. Nef S and Parada LF (2000). Hormones in male sexual development. *Genes Dev* 14:3075-86.

127. Nef S, Shipman T, and Parada LF (2000). A molecular basis for estrogen-induced cryptorchidism. *Dev Biol* 224:354-61.

128. Neubert D (2002). Reproductive toxicology: the science today. *Teratog Carcinog Mutagen* 22:159-74.

129. Nonneman DJ, Ganjam VK, Welshons WV, and Vom Saal FS (1992). Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. *Biol Reprod* 47:723-9.

130. National Toxicology Program (1991), Di(*n*-butyl) phthalate reproduction and fertility assessment in CD Sprague-Dawley rats when administered via feed, National Institute of Environmental Health Sciences: Research Triangle Park, NC.

131. Oishi S and Hiraga K (1980). Testicular atrophy induced by phthalic acid esters: effect on testosterone and zinc concentrations. *Toxicol Appl Pharmacol* 53:35-41.

132. Oishi S and Hiraga K (1980). Testicular atrophy induced by phthalic acid monoesters: effects of zinc and testosterone concentrations. *Toxicology* 15:197-202.

133. Omura T and Morohashi K (1995). Gene regulation of steroidogenesis. *J Steroid Biochem Mol Biol* 53:19-25.

134. Orth JM, Jester WF, Li LH, and Laslett AL, *Gonocyte-Sertoli Cell Interactions During Development of the Neonatal Rodent Testis*, in *Current Topics in Developmental Biology*, G.P. Schatten, Editor. 2000, Academic Press: San Diego. p. 103-124.

135. Cosmetic Ingredient Review Expert Panel (1985). Final report on the safety assessment of dibutyl phthalate, dimethyl phthalate, and diethyl phthalate. *J Am Col Tox* 4:267-303.

136. Parker KL and Schimmer BP (1997). Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr Rev* 18:361-77.

137. Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, and Gray LE (2000). The plasticizer diethylhexyl phthalate induces malformations by

decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicological Sciences* 58:339-349.

138. Payne A and O'Shaughnessy P, *Structure, function and regulation of steroidogenic enzymes in the leydig cell*, in *The Leydig Cell*, A. Payne, M. Hardy, and L. Russell, Editors. 1996, Cache River Press: Vienna, IL.

139. Peck C, Albro P, Hass J, Odom D, Barrett B, and Bailey F (1978). Metabolism and excretion of the plasticizer di-2-ethylhexyl-phthalate in man. *Clinical Research* 26:101A.

140. Peck C, Bailey F, Odom D, Blatt H, and Barett B (1976). Transfusion plasticizer kinetics in a subhuman primate species. *Transfusion* 16:526.

141. Peck C, Bailey F, Odom D, Blatt H, and Barrett B (1976). Plasticizer disposition in a conscious primate. *Pharmacologist* 18:195.

142. Peck CC, Odom DG, Friedman HI, Albro PW, Hass JR, Brady JT, and Jess DA (1979). Di-2-ethylhexyl phthalate (DEHP) and mono-2-ethylhexyl phthalate (MEHP) accumulation in whole blood and red cell concentrates. *Transfusion* 19:137-46.

143. Peets EA, Henson MF, and Neri R (1974). On the mechanism of the anti-androgenic action of flutamide (alpha- alpha-alpha-trifluoro-2-methyl-4'-nitro-m-propionotoluidide) in the rat. *Endocrinology* 94:532-40.

144. Pelliniemi L, Kuopio T, and Frojdman K, *The Cell Biology and Function of the Fetal Leydig Cell*, in *The Leydig Cell*, A. Payne, M. Hardy, and L. Russell, Editors. 1996, Cache River Press: Vienna, IL.

145. Prout GR, Jr., Irwin RJ, Jr., Kliman B, Daly JJ, MacLaughlin RA, and Griffin PP (1975). Prostatic cancer and SCH-13521: II. Histological alterations and the pituitary gonadal axis. *J Urol* 113:834-40.

146. Quigley C, *Genetic basis of sex determination and sex differentiation*, in *Endocrinology*, L. DeGroot and J. Jameson, Editors. 2001, W.B. Saunders Company: Philadelphia.

147. Rowland IR, Cottrell RC, and Phillips JC (1977). Hydrolysis of phthalate esters by the gastro-intestinal contents of the rat. *Food Cosmet Toxicol* 15:17-21.

148. Rubin RJ and Nair PP (1973). Plasticizers in human tissues. *N Engl J Med* 288:915-6.

149. Sadovsky Y and Dorn C (2000). Function of steroidogenic factor 1 during development and differentiation of the reproductive system. *Reviews of Reproduction* 5:136-142.

150. Shaffer C, Carpenter D, and Smyth H (1945). Acute and subacute toxicity of di(2-ethylhexyl) phthalate with note upon its metabolism. *Journal of Industrial Hygiene Toxicology* 27:130-135.

151. Sharpe RM (2001). Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicol Lett* 120:221-32.

152. Sharpe RM and Skakkebaek NE (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* 341:1392-5.

153. Shultz VD, Phillips S, Sar M, Foster PMD, and Gaido KW (2001). Altered gene profiles in fetal rat testes after in utero exposure to di(*n*-butyl) phthalate. *Toxicol Sci* 64:233-42.

154. Sjoberg P, Lindqvist NG, and Ploen L (1986). Age-dependent response of the rat testes to di(2-ethylhexyl) phthalate. *Environ Health Perspect* 65:237-42.

155. Skakkebaek NE, Rajpert-De Meyts E, and Main KM (2001). Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Human Reproduction* 16:972-978.

156. Sohoni P, Lefevre PA, Ashby J, and Sumpter JP (2001). Possible androgenic/anti-androgenic activity of the insecticide fenitrothion. *J Appl Toxicol* 21:173-8.

157. Sonnenschein C and Soto AM (1998). An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol* 65:143-50.

158. Srivastava SP, Srivastava S, Saxena DK, Chandra SV, and Seth PK (1990). Testicular effects of di-*n*-butyl phthalate (DBP): biochemical and histopathological alterations. *Arch Toxicol* 64:148-52.

159. Stocco D, *Acute regulation of leydig cell steroidogenesis*, in *The Leydig Cell*, A. Payne, M. Hardy, and L. Russell, Editors. 1996, Cache River Press: Vienna, IL.

160. Stocco DM and Clark BJ (1996). Regulation of the acute production of steroids in steroidogenic cells. *Endocr Rev* 17:221-44.

161. Sultan C, Balaguer P, Terouanne B, Georget V, Paris F, Jeandel C, Lumbroso S, and Nicolas J (2001). Environmental xenoestrogens, antiandrogens and disorders of male sexual differentiation. *Mol Cell Endocrinol* 178:99-105.

162. Sumpter JP (1995). Feminized responses in fish to environmental estrogens. *Toxicol Lett* 82-83:737-42.

163. Sunami O, Kunimatsu T, Yamada T, Yabushita S, Sukata T, Miyata K, Kamita Y, Okuno Y, Seki T, Nakatsuka I, and Matsuo M (2000). Evaluation of a 5-day Hershberger assay using young mature male rats: methyltestosterone and p,p'-DDE, but not fenitrothion, exhibited androgenic or antiandrogenic activity in vivo. *J Toxicol Sci* 25:403-15.

164. Sundaram K and Kumar N, *Metabolism of testosterone in Leydig cells and peripheral tissues*, in *The Leydig cell*, A. Payne, M. Hardy, and L. Russell, Editors. 1996, Cache River Press: Vienna, IL.

165. Swain A and Lovell-Badge R, *Sex determination and differentiation*, in *Mouse Development*, J. Rossant and P. Tam, Editors. 2002, Academic Press: San Diego.

166. Swan SH, Elkin EP, and Fenster L (1997). Have sperm densities declined? A reanalysis of global trend data. *Environ Health Perspect* 105:1228-32.

167. Tamura H, Maness SC, Reischmann K, Dorman DC, Gray LE, and Gaido KW (2001). Androgen receptor antagonism by the organophosphate insecticide fenitrothion. *Toxicol Sci* 60:56-62.

168. Tanaka A, Matsumoto A, and Yamaha T (1978). Biochemical studies on phthalic esters. III. Metabolism of dibutyl phthalate (DBP) in animals. *Toxicology* 9:109-23.

169. Tapanainen J, Kuopio T, Pelliniemi LJ, and Huhtaniemi I (1984). Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biol Reprod* 31:1027-1035.

170. Thomas JA, Darby TD, Wallin RF, Garvin PJ, and Martis L (1978). A review of the biological effects of di-(2-ethylhexyl) phthalate. *Toxicol Appl Pharmacol* 45:1-27.

171. Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jr., Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, and Skakkebaek NE (1996). Male reproductive health and environmental xenoestrogens. *Environ Health Perspect* 104 Suppl 4:741-803.

172. Trigatti B, Rigotti A, and Krieger M (2000). The role of the high-density lipoprotein receptor SR-BI in cholesterol metabolism. *Curr Opin Lipidol* 11:123-31.

173. Turner KJ, Barlow NJ, Struve MF, Wallace DG, Gaido KW, Dorman DC, and Foster PM (2002). Effects of *in utero* exposure to the organophosphate insecticide fenitrothion on androgen-dependent reproductive development in the Crl:CD(SD)BR rat. *Toxicol Sci* 68:174-83.

174. Tyler CR, Jobling S, and Sumpter JP (1998). Endocrine disruption in wildlife: a critical review of the evidence. *Crit Rev Toxicol* 28:319-61.

175. United States Environmental Protection Agency, *Environmental Protection Agency preliminary determination to conclude special review, Continue registration of linuron, 53 FR 31262, August 17, 1998*. 1988, Washington, D.C.

176. Van Thiel DH (1984). Ethyl alcohol and gonadal function. *Hosp Pract* 19:152-8.

177. Ward JM, Peters JM, Perella CM, and Gonzalez FJ (1998). Receptor and nonreceptor-mediated organ-specific toxicity of di(2-ethylhexyl)phthalate (DEHP) in peroxisome proliferator-activated receptor alpha-null mice. *Toxicol Pathol* 26:240-6.

178. Warren DW, Haltmeyer GC, and Eik-Nes KB (1973). Testosterone in the fetal rat testis. *Biol Reprod* 8:560-5.

179. White RD, Carter DE, Earnest D, and Mueller J (1980). Absorption and metabolism of three phthalate diesters by the rat small intestine. *Food Cosmet Toxicol* 18:383-6.

180. White RD, Earnest DL, and Carter DE (1983). The effect of intestinal esterase inhibition on the *in vivo* absorption and toxicity of di-*n*-butyl phthalate. *Food Chem Toxicol* 21:99-101.

181. Wine RN, Li LH, Barnes LH, Gulati DK, and Chapin RE (1997). Reproductive toxicity of di-*n*-butylphthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ Health Perspect* 105:102-7.

182. Witschi E (1951). Embryogenesis of the adrenal and the reproductive glands. *Rec Prog Horm Res* 6:1-23.

183. Yano K, Hirosawa N, Sakamoto Y, Katayama H, Moriguchi T, Joung KE, Sheen YY, and Asaoka K (2002). Phthalate levels in beverages in Japan and Korea. *Bull Environ Contam Toxicol* 68:463-9.

184. Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, Engel W, and Adham IM (1999). Targeted disruption of the *Insl3* gene causes bilateral cryptorchidism. *Mol Endocrinol* 13:681-91.

APPENDIX ONE

Extramural funding, Publications, and Presentations

Extramural Funding:

National Research Service Award of the National Institute of Environmental Health Sciences

Title: Pathogenesis of Phthalate-Induced Reproductive Lesions

Grant #: 1 F32 ES011061-01

Manuscripts:

McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PMD: Effects of *In Utero* Linuron Exposure on Androgen-Dependent Reproductive Development in the Male CRL:CD (SD) Br Rat. Toxicology and Applied Pharmacology 167:87-99 (2000).

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray, LE: The Plasticizer Diethylhexyl Phthalate Induces Malformations by Decreasing Fetal Testosterone Synthesis During Sexual Differentiation in the Male Rat. Toxicological Sciences 58:339-349 (2000).

McIntyre BS, Barlow NJ, Foster PMD: Androgen-Mediated Development in Male Rat Offspring Exposed to Flutamide *in Utero*: Permanence and Correlation of Early Postnatal Changes in Anogenital Distance and Nipple Retention with Malformations in Androgen-Dependent Tissues. Toxicological Sciences 62:236-249 (2001).

Manuscripts continued:

McIntyre BS, Barlow NJ, Foster PMD: Male Rats Exposed to Linuron *In Utero* Exhibit Permanent Changes in Anogenital Distance, Nipple Retention, and Epididymal Malfunctions that Result in Subsequent Testicular Atrophy. Toxicological Sciences 65:62-70 (2002).

McIntyre BS, Barlow NJ, Sar M, Wallace DGW, Foster PMD: Effects of *In Utero* Linuron Exposure on Rat Wolffian Duct Development. Reproductive Toxicology 16:131-139 (2002).

Turner KJ, Barlow NJ, Struve MJ, Wallace DG, Gaido KW, Dorman DC, Foster PMD: Effects of *In Utero* Exposure to the Organophosphate Insecticide Fenitrothion on Androgen-Dependent Reproductive Development in the Crl:CD(SD)BR Rat: Implications for Screening and Testing of Compounds with Potential Antiandrogenic Activity. Toxicological Sciences 68:174-183 (2002).

Lanning LL, Creasy DM, Chapin RE, Mann PC, Barlow NJ, Regan KS, Goodman DG: Recommended Approaches for the Evaluation of Testicular and Epididymal Toxicity. Toxicologic Pathology (Society of Toxicologic Pathology Position Paper) 30:507-520 (2002).

Barlow NJ, Foster PMD: Pathogenesis of Male Reproductive Tract Lesions from Gestation through Adulthood Following *in Utero* Exposure to Di(*n*-butyl) Phthalate. Toxicologic Pathology (in press).

Barlow NJ, Phillips SL, Wallace DG, Sar M, Gaido KW, Foster PMD: Quantitative Changes in Gene Expression in Fetal Rat Testes Following Exposure to Di(*n*-butyl) Phthalate. Toxicological Sciences (submitted).

Manuscripts continued:

Turner KJ, Turner KJ, McIntyre BS, Phillips SL, Barlow NJ, Bowman CJ, Foster PMD: Altered Gene expression during Rat Wolffian Duct Development in Response to *In Utero* Exposure to the Environmental Antiandrogen Linuron. Toxicological Sciences (submitted).

Barlow NJ, McIntyre BS, Foster PMD: Male Reproductive Tract Lesions at Six, Twelve, and Eighteen Months Following in Utero Exposure to DBP. Toxicologic Pathology (in preparation).

Bowman CJ, Barlow NJ, Turner KJ, Wallace DG, Foster PMD: Effects of *in utero* exposure to finasteride on androgen-dependent reproductive development in the male rat. Toxicological Sciences (in preparation).

Abstracts:

McIntyre BS, Barlow NJ, Mylchreest E, Maness SC, Gaido KW, Foster PMD: Effects of the Antiandrogen Linuron on Maternal Toxicity and Androgen-sensitive Reproductive Endpoints in the CD Rat. Society of Toxicology-North Carolina Chapter Annual Winter Meeting (1999).

McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PMD: Effects of Linuron on Androgen-Dependent Reproductive Development in the Male Sprague-Dawley Rat. The Toxicologist 54S:291 (2000).

Parks LG, Ostby JS Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LE: Malformations Induced by the Antiandrogenic Action of DEHP Result from Decreased Fetal Testosterone Synthesis, not AR-Antagonism. 5th Symposium of the National Health and Environmental Effects Research Laboratory (2000).

Abstracts continued:

Barlow NJ, Sar M, Mylchreest E, Foster PMD: Immunohistochemical Localization of the Androgen Receptor and 3β-Hydroxysteroid Dehydrogenase in Hyperplastic Leydig Cells of the Fetal Testis Following *In Utero* Exposure to Di(*n*-butyl) Phthalate. Toxicologic Pathology 28(6):855 (2000).

McIntyre BS, Barlow NJ, Foster PMD: Effects of Androgen Receptor Antagonists of Differing Potencies on Male Rat Reproductive Development. International Symposium, Hormones and Endocrine Disrupters in Food and Water: Possible Impact on Human Health, Copenhagen, Denmark (2000).

Barlow NJ: Reproductive Toxicology Protocol Design in the Study of Antiandrogens. International Journal of Toxicology 19(6):2 (2000).

Barlow NJ, Foster PMD: Chronology of Di(*n*-butyl) Phthalate-Induced Reproductive Tract Lesions in Male Rats Exposed *In Utero*. The Toxicologist 60S(1):216 (2001).

Foster PMD, Barlow NJ, Wallace DG, McIntyre BS: Effects of *In Utero* Flutamide Exposure on Androgen-Dependent Male Rat reproductive Development: A Dose Response Study. The Toxicologist 60S(1):219 (2001).

McIntyre BS, Barlow NJ, Foster PMD: Testicular Atrophy Resulting From *In Utero* Linuron Exposure is Secondary to Epididymal Malformations. The Toxicologist 60S(1):215 (2001).

Barlow NJ, McIntyre BS, Foster PMD: Permanent Alteration of Anogenital Distance and Nipple Retention in Male Rats Exposed to Di(*n*-butyl) Phthalate *In Utero*. The Toxicologist 66S(1):233 (2002).

Abstracts continued:

Foster PMD, Turner KJ, Barlow NJ: Antiandrogenic Effects of a Phthalate Combination on *In Utero* Male Reproductive Development in the Sprague-Dawley Rat: Additivity of response? The Toxicologist 66S(1):233 (2002).

Turner KJ, Barlow NJ, Struve MF, Wallace DG, Gaido KW, Dorman DC, Foster PMD: Effects of *In Utero* Fenitrothion Exposure on Androgen-Dependent Male Rat Reproductive Development: A Dose-Response Study. The Toxicologist 66S(1):234 (2002).

Presentations:

Invited Speaker

Reproductive Toxicology Protocol Design in the Study of Antiandrogens. The American College of Toxicology's Twenty-first Annual Meeting: San Diego, California, November 12 - 15, 2000.

Pathologic Endpoints in the Study of Antiandrogens. The North Carolina Society of Histopathology Technologists Annual Spring Meeting: Raleigh, North Carolina, April 19-20, 2002.

Seminars

Di(*n*-butyl) Phthalate: Reproductive Consequences of *In Utero* Exposure in the Male CD Rat. North Carolina State University Microbiology, Pathology and Parasitology Department Research In Progress Seminar: Raleigh, North Carolina, April 4, 2001.

Morphologic and Molecular Consequences of *In Utero* Exposure to the Environmental Antiandrogen Di(*n*-butyl) Phthalate. The United States Environmental Protection Agency's Endocrine Disrupting Compounds Forum: Research Triangle Park, North Carolina, March 13, 2002.

Presentations continued:

Platform Presentations

Reproductive Tract Malformations in Male CD Rats Following *In Utero* Exposure to the Antiandrogen Linuron. The American College of Veterinary Pathologists' Fiftieth Annual Meeting: Chicago, Illinois, November 15 - 19, 1999.

Di(*n*-butyl) Phthalate-Induced Testicular Lesions Following *In Utero* Exposure: Primary and Secondary Phenomena? The American College of Veterinary Pathologists' Fifty-first Annual Meeting: Amelia Island, Florida, December 3 - 6, 2000.