Environmental Research 108 (2008) 168–176

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats ☆

Kembra L. Howdeshell [a,*], Cynthia V. Rider [b], Vickie S. Wilson [a], L. Earl Gray Jr.[a]

[a] Reproductive Toxicology Division, National Health and Environmental Effects Laboratory, Office of Research and Development, United States Environmental Protection Agency (US EPA), Research Triangle Park, NC 27711, USA
[b] North Carolina State University/US EPA Cooperative, Department of Molecular Biosciences, Raleigh, NC, USA

ARTICLE INFO

Article history:
Received 14 March 2008
Received in revised form
4 August 2008
Accepted 11 August 2008

Keywords:
Phthalate esters
Testosterone production
Leydig cell maturation
insl3
Human testicular dysgenesis syndrome
Phthalate mixtures

ABSTRACT

Phthalate esters are high production volume chemicals used to impart flexibility to polyvinyl chloride products as well as other applications. In the male laboratory rat, the period of sexual differentiation in utero is particularly sensitive to certain phthalate esters, which induce a suite of reproductive malformations, including epididymal and gubernacular agenesis. The fetal rat testes are a main target for phthalate esters as evidenced by a reduction in testosterone production and insulin-like hormone 3 (insl3) expression, a peptide hormone critical for testis descent. Histopathology of fetal and postnatal testes reveals that in utero exposure to phthalate esters disrupts Leydig and Sertoli cell maturation leading to a reduction in germ cells in the malformed seminiferous tubules in adulthood as well as an increased incidence of multinucleated germ cells. There are some strain-specific differences in the target organs in the male reproductive tract in rats affected by phthalate esters. Mixtures of phthalate esters with one another and with other anti-androgenic compounds exhibit cumulative, largely dose-additive effects on male reproductive tract development when administered during sexual differentiation in utero. Since phthalate ester metabolites are detected in maternal and fetal body fluids, and androgen-signaling and insl3 are highly conserved among mammals, phthalates may potentially affect human reproductive development.

Published by Elsevier Inc.

1. Introduction

Phthalate esters are high production volume chemicals used to impart flexibility to polyvinyl chloride plastics as well as many other applications. Phthalate esters are found in many commonly used products, including children's toys, health and beauty supplies (e.g. cosmetics and perfumes), medical equipment (e.g. dialysis tubing and intravenous bags), and the enteric coating of some pharmaceuticals. Phthalate esters readily migrate from such products, and their metabolites have been detected in several human bodily fluids, including maternal urine during pregnancy (Swan et al., 2005), breastmilk (Mortensen et al., 2005) and amniotic fluid (Latini et al., 2003; Silva et al., 2004b). Male laboratory rats exposed in utero to certain phthalate esters display malformations and alterations of reproductive tissues indicative of a suppression of fetal testicular testosterone and insulin-like 3 hormone (insl3) production (Foster, 2006; Gray et al., 2000; Wilson et al., 2004) and multinucleated germ cells (Ferrara et al., 2006; Foster, 2006; Parks et al., 2000; Scott et al., 2007). Environmental chemicals, such as the phthalate esters, have been suggested as potential causal agents of human testicular dysgenesis syndrome (TDS), which bears striking similarity to the reproductive malformations, decreased sperm abundance, and histopathological changes in rat testes following in utero exposure to certain phthalate esters (Skakkebaek et al., 2001; Virtanen et al., 2005). The potential for human developmental effects and the detection of phthalate ester metabolites in both the maternal and fetal compartments in humans have prompted the scientific community, health advocacy groups and legislators to call for the evaluation of mixtures of phthalates to better understand the health risks of human exposure to multiple sources of these chemicals (DiGangi et al., 2002; Howdeshell et al., 2007, 2008; Purvis and Gibson, 2005; Rider et al., 2008). In particular, the United States Environmental Protection Agency (US EPA) has funded a National Academy of Sciences panel to evaluate this issue and make recommendations to the US EPA on how to

☆ The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, US EPA, and has been approved for publication. Approval does not necessarily reflect the views and policies of the agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

* Corresponding author. Fax: +1 919 541 4017.
E-mail address: howdeshell.kembra@epa.gov (K.L. Howdeshell).

0013-9351/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.envres.2008.08.009

conduct cumulative risk assessments on mixture of phthalates; a report from this panel is scheduled to be published in late 2009 (http://www8.nationalacademies.org/cp/projectview.aspx?key=48860).

The effects of phthalate esters on reproductive parameters of the pubertal male have been studied in many mammalian species (i.e. rat, mouse, guinea pig and ferret) and have demonstrated the increased sensitivity of the immature versus the adult male; however, these studies will not be covered in this manuscript. The current review will focus on the effects of in utero exposure to phthalate esters on male rat reproductive tract development and function when administered during the period of sexual differentiation. We will review what is understood about the cellular and molecular mode of action behind phthalate action during reproductive tract development from studies on the well-characterized phthalate esters di(n)butyl phthalate (DBP, CAS RN 84-74-2) and diethylhexyl phthalate (DEHP, CAS RN 117-81-7), including evidence of strain differences in target organ responsiveness to DEHP. In particular, phthalate esters are known to induce gubernacular malformations due to suppression of insl3 production in the fetal rat testes. Although the effects of phthalate esters are most often studied in the male rat, we review evidence that female rat reproductive development is also affected by these chemicals. As humans are exposed to multiple phthalate esters on a daily basis, we summarize our research on the cumulative effects of binary and complex mixtures of phthalates with each other and/or with other androgen-disruptive chemicals on male reproductive development. Finally, we discuss how laboratory animal research regarding the effects of phthalate esters on reproductive development furthers our understanding of the potential health risks of such chemicals on the developing human.

2. Phthalate ester structure and activity

Phthalate esters are composed of paired ester groups on a benzene ring (Fig. 1). The ester linkages are hydrolyzed by esterases in the intestine and the resulting monoester metabolites are easily absorbed (Kluwe, 1982; Rock et al., 1986; Skibsted and Hansen, 1990). The monoester metabolites are generally assumed to be the bioactive component of the phthalate ester reproductive toxicants (Gray and Beamand, 1984; Sjoberg et al., 1986). The structure and position of the paired ester groups determines the reproductive toxicity of the phthalate ester. Using pubertal Sprague–Dawley (SD) rats, Foster et al. (1980, 1981) reported that phthalate esters possessing ester groups with 4–6 carbons, and in the ortho position, induced testicular atrophy and decreased testicular zinc content following 4-day oral administration of the chemicals. Conversely, phthalates with ester groups of shorter or longer than 4–6 carbons, or in the para position, were not active. The same structure–activity relationship was also observed for suppression of fetal testicular testosterone production following in utero exposure during sexual differentiation (Howdeshell et al., 2008; Wilson et al., 2004).

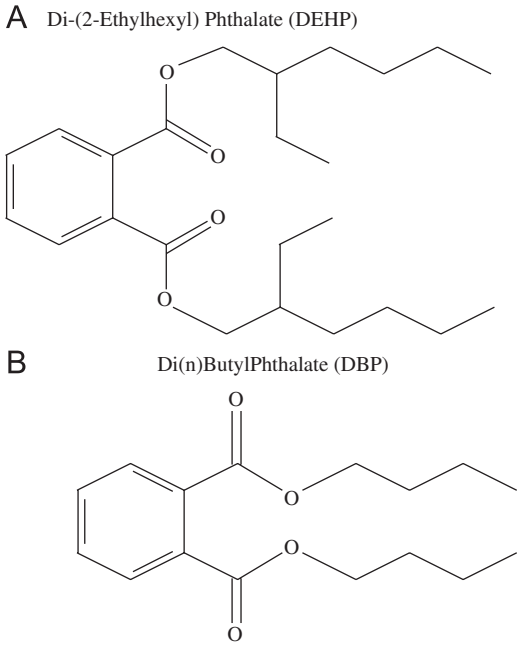

Fig. 1. Chemical structure of diethylhexyl phthalate (A) and di(n)butyl phthalate (B).

Phthalate esters exert permanent reproductive tract alterations when administered during sexual differentiation in utero. Standard developmental toxicity studies evaluate the ability of a chemical to induce embryo or fetal death, alterations in body weights, and changes in size and appearance of the external and visceral organs, and skeletal system. Because the standard development toxicity studies involve dosing the dam on gestation day (GD) 6–15 and collecting the fetuses on GD 15, the developmental reproductive toxicity of the phthalates was initially overlooked. It has since been established that the administration of phthalate esters to the rat dam during a limited period of sexual differentiation cause reproductive malformations, and associated adverse effects on epididymides and testes of male offspring (GD 16–19, Long Evans (LE) strain (Gray et al., 1999); GD 16–18, SD strain (Carruthers and Foster, 2005) and Wistar strain (Ema et al., 1998)). This is the critical window of vulnerability for androgen receptor (AR) antagonists as well (vinclozolin, LE strain (Wolf et al., 2000) and Wistar strain (Welsh et al., 2008); and flutamide, SD strain (Foster and Harris, 2005)). Phthalate exposure during these periods results in adverse effects on the male rat reproductive tract that are indicative of a suppression of the androgen pathway, thus we classify the phthalates as anti-androgenic chemicals (Gray et al., 2006b). Although many anti-androgenic compounds antagonize the AR, phthalate esters do not bind the human AR in vitro at physiological concentrations (Parks et al., 2000); the absence of AR binding has been observed with DEHP and the DEHP metabolite monoethylhexyl phthalate (MEHP; Parks et al., 2000) as well as DBP and the DBP metabolite monobutyl phthalate (MBP) (Foster et al., 2001). While some phthalates have occasionally been reported to bind the estrogen receptor in vitro (Jobling et al., 1995), phthalate esters do not appear to exert estrogenic effects in vivo in rats, such as accelerated vaginal opening or induction of constant estrus (Grande et al., 2007; Gray et al., 1999; Mylchreest et al., 1998, 2000). Furthermore, DBP failed to induce estrogen-dependent uterotropic response or lordosis behavior in adult ovariectomized SD rats (Gray et al., 1999).

3. Mechanisms of phthalate action

In utero exposure of male SD rat offspring to high doses of DEHP, DBP, benzyl butyl phthalate (BBP) and diisononyl phthalate (DINP) induces a suite of reproductive tract malformations in adulthood, including agenesis of the epididymis, hypospadias, testicular malformations (including cryptorchid testes) and abnormal penile development (Foster, 2006; Gray et al., 2006b). Similar effects are seen in Wistar rats exposed in utero to comparable doses of DBP or DEHP, although there are lesser effects on the epididymis and a higher incidence of cryptorchidism in this strain (Fisher et al., 2003; Mahood et al., 2005; Wilson

et al., 2007). In addition, developmental exposure to high doses of DEHP and BBP decreased testicular and epididymal sperm counts in adult SD rats (Gray et al., 2006b). The effects of in utero exposure to phthalate esters observable in multiple rat strains early in postnatal life are decreased anogenital distance (AGD) on postnatal day 2 (PND2) and areolar/nipple retention in juveniles (PND14) (Gray et al., 2000). In normal development, androgens secreted by the fetal testes (1) act to lengthen the AGD in males relative to females and (2) cause regression of nipple anlagen in males. The magnitude of the decreases in AGD and increases in nipple retention seen in male rats following prenatal phthalate ester exposure were predictive of reproductive tract malformations in adulthood (Barlow et al., 2004; Hotchkiss et al., 2004).

The main target of prenatally administered phthalate esters appears to be the fetal testes. A high dose of DEHP (750 mg/kg/day) on GD14bPND3 suppressed the normal increase of testicular testosterone synthesis, which led to a reduction in testicular and whole-body level of testosterone in fetal SD rats (Parks et al., 2000). Short-term exposure to DEHP, DBP or BBP (1 g/kg/day on GD 14–18) also significantly suppresses fetal testicular testosterone synthesis and insl3 mRNA levels on GD 18 (Wilson et al., 2004). Histopathological examination of testes from DEHP-exposed male SD rat neonates revealed multiple areas of apparent Leydig cell hyperplasia (Parks et al., 2000). The Leydig cells in the areas of hyperplasia were smaller in size with less cytoplasm relative to controls and were grouped in large aggregates versus smaller clusters of cells in the control testes (Parks et al., 2000). Prenatal DEHP exposure also induced multinucleated gonocytes in the seminiferous tubules of male rats on GD 20 and PND3 (Parks et al., 2000). Foster and colleagues (2006) have reported similar fetal endocrine and histopathological changes in male offspring of SD rat dams treated with DBP (100–500 mg/kg/day) from GD 12 to 21. Interstitial cell hyperplasia has been reported in adult male offspring of SD rat dams treated with DBP (Mylchreest et al., 1999, 2000), and seminiferous tubule degeneration and atrophy have been reported for BBP (Tyl et al., 2004). The phthalate syndrome, which is induced by DBP, DEHP, BBP and other reproductive toxicant phthalates, includes a variety of gross testicular lesions (such as fluid-filled testes, hypoplastic testes and, in some cases, agenesis of the testis). These lesions arise from direct effects of phthalates on the testis as well as indirect effects due to nondescent of the testes (due to lack of a gubernacular cord; described below).

Sharpe and colleagues have further studied phthalate ester-induced testes histopathology using Wistar male rats exposed in utero (GD 13.5–21.5) to 500 mg/kg/day DBP (Fisher et al., 2003; Mahood et al., 2005). They suggest that the apparent Leydig cell hyperplasia observed in the fetal testes following DBP exposure is not due to increased cell proliferation, but rather a shift in the distribution of Leydig cells from numerous, small-sized clusters in control males to less abundant, medium- to large-sized clusters in DBP-treated male rats (Mahood et al., 2005). This hypothesis is corroborated by another laboratory (Lin et al., 2008), which reported that DEHP treatment (10, 100 or 750 mg/kg/day via the dam on GD 2–20) induced similar changes in Leydig cell aggregate distribution in GD 21 fetal LE rats. The abnormal clustering of Leydig cells in the testes of the DBP-exposed males appears to be due to abnormal cell migration, which also trapped Sertoli cells, and peritubular myoid cells among the Leydig cell aggregates (Fisher et al., 2003; Mahood et al., 2005, 2006). Shortly after birth, malformed seminiferous tubules formed around the Leydig cell aggregates and entrapped Sertoli cells. Germ cells were present in the malformed tubules in early puberty; however, the germ cells were absent by adulthood leaving Sertoli cell only-tubules (Mahood et al., 2005). In utero DBP exposure led to a delay in normal germ cell development (Ferrara et al., 2006) and reduced the abundance of Sertoli cells relative to controls (Scott et al., 2007). Germ cell number was significantly reduced in the testes of DBP males through PND25, but returned to normal numbers in adulthood (PND90). In addition, DBP treatment (500 mg/kg/day from GD 19.5 to 20.5) increased the incidence of multinucleated gonocytes in male rat fetuses similar to the induction seen with long-term treatment (GD 13.5–20.5); however, the short-term treatment during late gestation did not affect postnatal germ cell number. Ferrara et al. (2006) suggested that the phthalate-induced multinucleated gonocytes may be caused by disrupted interactions of the gonocytes and Sertoli cells (Kleymenova et al., 2005), a mechanism separate from DBP effects on early gonocyte development. In addition, two recent studies in mice and rats (Gaido et al., 2007; Scott et al., 2007) suggest that phthalates may induce multinucleated gonocytes via a mechanism separate from inhibition of testosterone synthesis.

Microarray and PCR analysis of gene expression in fetal rat testes following in utero exposure to phthalate esters have identified a number of potential mechanisms for phthalate action during sexual differentiation. The commonalities between the gene expression studies are the phthalate ester-induced alterations to cholesterol transport (e.g. steroidogenic acute regulatory gene, StAR), the steroidogenesis pathway (e.g. P450scc, cyp17a), and insl3 expression (Lehmann et al., 2004; Shultz et al., 2001). Prenatal phthalate esters exposure reduced testicular expression of genes involved in cell to cell interactions (e.g. gap-junction protein connexin 43, fibroblast growth factor and c-KIT (Barlow et al., 2003; Liu et al., 2005)), which further supports the hypothesis that phthalate exposure reduces Sertoli-germ cell connection thus leading to Sertoli cell only tubules in adulthood (Ferrara et al., 2006). Phthalate exposure inhibited testicular expression of alpha inhibin in the fetal SD rat (Liu et al., 2005); alpha inhibin is necessary for Sertoli cell development and oxidative stress pathway genes. However, another study reported that DBP-induced reduction of alpha inhibin expression and several other steroidogenic factor 1 (SF-1) related genes was restricted to the Leydig cells, while expression in the Sertoli cells was not affected by treatment on GD17.5 and GD 19.5 (500 mg/kg/day DBP administered to the Wistar rat dam on GD 12.5–19.5) (Plummer et al., 2007). Exposure to DEHP during gestation was also reported to increase mRNA expression of leukemia inhibitory factor (LIF) in fetal LE rat testes; there is some evidence that LIF may play a role in altering normal Leydig cell aggregate formation (Lin et al., 2008). Thus, phthalates may be acting on genes responsible for Leydig and Sertoli Cell development and cell to cell communication, but also indirectly and/or directly on genes involved in cholesterol transport and hormone production.

4. Gubernacular cord development and phthalates

Gubernacular cord underdevelopment and agenesis are reproductive malformations that are induced by prenatal phthalate ester exposure, and can result in cryptorchid (undescended) testes. Cryptorchidism has been reported by our laboratory and others with high dose exposure to predicted reproductive toxicant phthalate esters: DEHP (Gray et al., 1999, 2000), DBP (Fisher et al., 2003; Gray et al., 1999, 2000; McKinnell et al., 2005; Mylchreest et al., 1998, 1999) and di-isohepyl phthalate (DiHP; in SD rats (McKee et al., 2006)). While many laboratories evaluate the position of the testes, very few laboratories have directly measured the presence/absence or length of the gubernacular cord. We observed that male rats exposed to high doses of phthalates in utero had a significant increase in the incidence of underdeveloped (elongated), or absent gubernacular cords versus control males; interestingly, not all phthalate-treated males with

impaired gubernacular cord development had cryptorchid testes (Howdeshell et al., 2007).

Insulin like hormone 3 (insl3) is a peptide hormone produced by the Leydig cells of the testes, which is responsible for normal gubernacular cord development. Specifically, insl3 induces the gubernacular cord to differentiate and mature, thus facilitating the first phase of testes descent from the kidney area to the inguinal region during fetal life (Figs. 2A and C) (Ivell and Bathgate, 2002; Zimmermann et al., 1999). Mice without a functional insl3 gene display cryptorchid testes and normal androgen levels (Fig. 2D; Zimmermann et al., 1999). Androgen also plays a role in testis descent by acting to regress the cranial suspensory ligament during the first phase of testis descent (Figs. 2A, C). In the untreated (control) female rodent fetus, the gubernacular cord involutes in the absence of insl3 and the cranial suspensory ligament develops in the absence of testosterone to maintain the position of the ovaries near the kidneys (Fig. 2B).

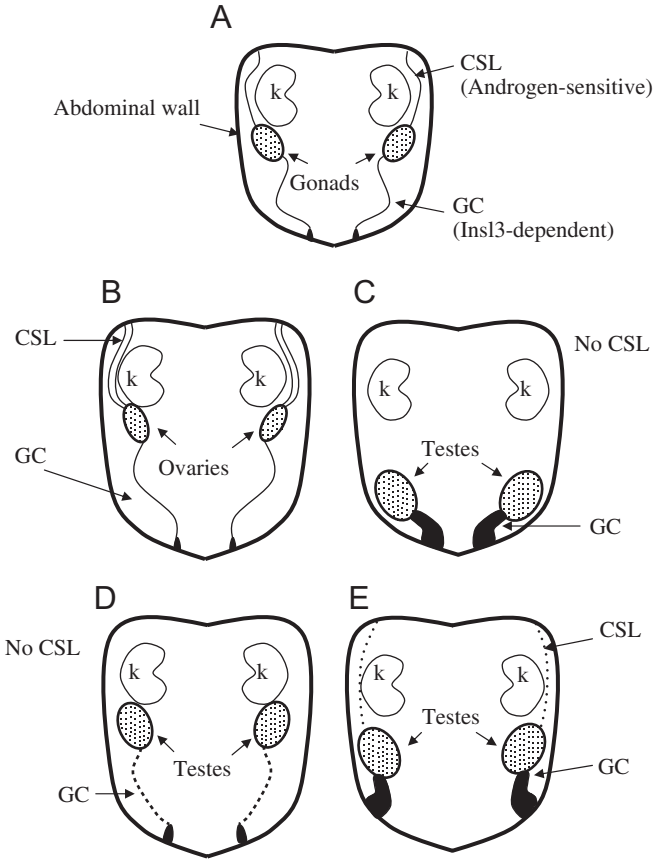

Fig. 2. Transabdominal descent of the testes during rat sexual differentiation is affected differently by phthalate esters than by androgen-receptor (AR) antagonists. (A) In the undifferentiated fetus, the gonad is positioned near the kidney (k; in both males and females) and is attached to the abdominal wall by the cranial suspensory ligament (CSL) mesentary and the gubernacular cord (GC). (B) In the control (or untreated) female fetus, the CSL further matures due to the lack of androgen, and the GC regresses due to the lack of insulin-like hormone 3 (insl3), thus maintaining the location of the ovaries near the kidneys. (C) In the control (or untreated) male fetus, androgen causes regression of the CSL and insl3 induces maturation of the GC, thus pulling the fetal testes into the inguinal region. (D) Anti-androgens can disrupt normal transabdominal testicular descent (D–E). (D) The inhibition of fetal testicular insl3 expression/production by phthalate esters can lead to underdevelopment or agenesis of the GC. (E) High doses of AR antagonists can result in the inappropriate placement of gubernacular cords in the abdominal wall (e.g. vinclozolin (Gray et al., 1994)) and/or persistence of the CSL (e.g. flutamide; van der Schoot and Emmen, 1996); however, fetal gubernacular cord development is not altered by exposure to AR antagonists.

Our laboratory was the first to identify that phthalate esters administered during sexual differentiation inhibit the expression of insl3 in the rat fetal testes (Fig. 2D) (Wilson et al., 2004). Fetal testicular insl3 expression on GD 18 was significantly suppressed by phthalate esters DBP, DEHP or BBP (750 mg/kg/day from GD 14–18), while AR antagonists and/or inhibitors of fetal testicular testosterone production (vinclozolin, prochloraz and linuron) did not influence insl3 mRNA levels (Wilson et al., 2004). MBP provided in the diet to pregnant SD rats significantly decreased fetal testicular insl3 expression and increased the incidence of undescended testes on GD 19 as well as PND60 SD male offspring versus controls (Shono et al., 2005). Fetal testicular insl3 mRNA is inhibited in a dose-dependent fashion by DBP or DEHP (Lehmann et al., 2004; Wilson et al., 2006). In addition, prenatal exposure to DBP reduced insl3 protein levels in the testes of GD 19 and adult Wistar rats relative to controls (McKinnell et al., 2005). Androgens are essential for the final stage of testicular descent from the inguinal region to the scrotal sac, which is not complete until postnatal week 3 in the rat (Ivell and Hartung, 2003). Exposure to AR antagonists can also lead to cryptorchid testes; however, this effect is not due to agenesis of the gubernacular cord. In contrast, morphologically normal gubernacular cords are often inappropriately attached to the abdominal wall in AR antagonist-treated male rats (Fig. 2E).

5. Strain differences in response to phthalates

A review of the literature reporting in utero effects of DBP indicates that male SD rat offspring display a different postnatal reproductive phenotype than Wistar male rat offspring (Fisher et al., 2003; reviewed in Wilson et al., 2007). When DEHP is administered to the rat dam at 750 mg/kg/day from GD 14 to 18, we observed dramatic differences in the occurrence of epididymal agenesis (SD, 67% versus Wistar, 8%) and gubernacular lesions (SD, 0% versus Wistar, 64%) among the male offspring of the two strains (Wilson et al., 2007). Control SD males had lower fetal testicular levels of testosterone production and higher insl3 mRNA expression than control Wistar males; however, treatment with DEHP lowered testosterone production and increased insl3 mRNA levels to similar levels in both strains. Effects seen in SD and Wistar rats with DBP are consistent with the strain-specific effects of DEHP. In Wistar rats, DBP treatment at 500 mg/kg/day during GD 13–21 induced cryptorchidism in 100% of the male offspring, but epididymal agenesis was absent or occurred in only 12% of the males (Fisher et al., 2003; Mahood et al., 2005). In SD male offspring, DBP at 500 mg/kg/day on GD 12–21 induced cryptorchidism in less than 7% of the male offspring, while epididymal agenesis was observed in 82% of the male offspring (Mylchreest et al., 2000).

6. Effects in females

Although less studied, phthalate esters also act as reproductive toxicants on the developing female rat. A combination of in utero and lactational exposure to DBP (250 and 500 mg/kg/day) caused reduced fecundity and induced a low incidence of uterine malformations (partial agenesis of the uterus or absence of implants in one horn) in female rats, while their male siblings exhibited a low incidence of hypospadias, undescended testes and lowered epididymal sperm counts (Gray et al., 1999). A low frequency of delayed vaginal opening and partial to complete agenesis of the uterus have also been reported for DBP at 500 and 750 mg/kg/day (Mylchreest et al., 1998). Thus, reproductive tract malformations are occurring in both males and females at about

the same phthalate ester doses; however, they occur less frequently in the female at these dosage levels. Although not a reproductive malformation, the number of tertiary atretic follicles was significantly increased in the ovaries of Wistar rats was observed following in utero and lactational exposure to 405 mg DEHP/kg/day (via oral dosing of the dam) relative to controls (Grande et al., 2007).

Gestational phthalate exposure is also known to induce fetal mortality. Female LE rats orally dosed with DBP (250–1000 mg/kg/day from weaning through pregnancy/lactation) cycled and mated normally (with untreated males); however, several females exposed to 500 mg DBP/kg/day aborted their litters around mid-pregnancy, and treatment with 1000 mg/kg/day DBP resulted in complete infertility (Gray et al., 2006a). Increased postimplantation embryo loss has also been reported in Wistar rats treated with DBP (750–1500 mg/kg/day from GD 0 to 8)(Ema et al., 2000) as well as in SD dams treated with BBP, di-isobutyl phthalate (DiBP) or dipentyl phthalate (DPP) at comparable high doses from GD 8 to 18 (DPP causes significant fetal mortality as low as 300 mg/kg/day; Howdeshell et al., 2008). These effects may be due to direct effects of phthalate esters on fetal development and/or maternal endocrinology. Female LE rats treated with DBP (500 and 1000 mg/kg/day from weaning through pregnancy/lactation through several pregnancies) had significantly decreased litter size associated with reduced progesterone serum levels and ovarian progesterone production on GD 13 (Gray et al., 2006a). Interestingly, a reduction in serum levels of progesterone and estradiol was observed in prepubertal female SD rats orally dosed with 500 mg DEHP/kg/day for 10 days (Svechnikova et al., 2007). In a non-human primate, chronic DEHP exposure (500 and 2500 mg/kg/day) from weaning to adulthood led to increased ovarian weights in juvenile common marmoset females (Tomonari et al., 2006). The enlarged ovaries had large corpus luteum, which is a common characteristic of older common marmoset females and is suggestive of accelerated aging with DEHP treatment (Tomonari et al., 2006). Further research is needed to understand the mechanism by which phthalate esters induce fetal mortality.

7. Phthalate ester and anti-androgenic mixtures

Risk assessments have typically been conducted on an individual chemical basis. However in 1996, the US Congress passed the Food Quality Protection Act (FQPA), which mandates that the US EPA evaluate the cumulative risk of exposure to chemicals that share a common mechanism of toxicity (Congress, 1996). The FQPA legislation was originally written for the evaluation of the food use pesticides; however, our laboratory's focus has evolved more broadly to address the cumulative risk of environmental chemicals impacting testosterone availability. We have investigated the cumulative effects of prenatal exposure to binary mixtures of phthalates as well as mixtures of phthalates and anti-androgenic agricultural chemicals for their effects on the male rat reproductive system using a 5-day exposure during the period of sexual differentiation (GD 14–18).

In one study, we hypothesized that the two phthalates DBP and DEHP, which act via a common mechanism of action but have different active metabolites (MBP and MEHP, respectively), would act in a cumulative fashion to induce reproductive malformations, suppress fetal testosterone production and inhibit the expression of insl3 and genes involved in steroidogenesis (Howdeshell et al., 2007). Pregnant SD rat dams were gavaged on GD 14–18 with vehicle control, 500 mg/kg DBP, 500 mg/kg DEHP, or a combination of DBP and DEHP (500 mg/kg each chemical; DBP+DEHP). The DBP+DEHP mixture was predicted to induce epididymal agenesis in 50% of the males, while the individual doses were predicted to have minimal or no effect on male rat offspring (Howdeshell et al., 2007). The observed DBP+DEHP effects were subsequently compared to responses predicted from models of dose addition (appropriate for chemicals acting via the same mechanism of action) and response addition (describing the independent action of chemicals acting via different mechanisms of action) modeled responses (Rider and LeBlanc, 2005) that used dose–response data from preliminary studies of the individual chemicals. As hypothesized, the DBP+DEHP mixture acted in a cumulative, largely dose-additive fashion to induce malformations and organ weight reductions in androgen-dependent (including hypospadias and epididymal agenesis) and insl3-dependent reproductive tissues (i.e. gubernacular cord agenesis) in prenatally exposed adult males (see subset of data—Fig. 3A; Howdeshell et al., 2007). Androgen-sensitive developmental endpoints of decreased neonatal AGD and increased areolar retention at PND13 were also dose-additively affected in the DBP+DEHP males. Fetal testicular testosterone production as well as insl3 and cyp11a gene expression were cumulatively decreased in GD 18 male fetuses exposed to DBP+DEHP compared to DBP or DEHP alone, which provides further evidence that phthalate ester alterations in the fetal endocrine environment lead to reproductive tract malformations in adulthood.

In another binary mixture study, we administered BBP (500 mg/kg/day) and the herbicide linuron (50 mg/kg/day; an AR antagonist and testosterone synthesis inhibitor) individually and in combination from GD 14 to 18 (Hotchkiss et al., 2004). As predicted for the BBP plus Linuron mixture, AGD at PND2 was decreased and areolae/nipple retention at PND13 was increased in a cumulative fashion relative to controls, and these two endpoints remained similarly affected in adulthood. Prenatal exposure to the BBP plus Linuron mixture resulted in nearly 60% incidence of hypospadias, while such malformations were nearly absent with the individual chemical treatments (Fig. 3B). The incidence of internal malformations and organ weight reductions of the androgen-dependent reproductive tissues also demonstrated cumulative effects for the mixture dose (see subset of data—Fig. 3B; Hotchkiss et al., 2004). As expected, the BBP plus Linuron treatment did not induce gubernacular agenesis as only the phthalates produce this malformation via inhibition of insl3 mRNA. Similar to the DBP+DEHP data, the alterations in developmental and adult androgen-responsive tissues were associated with a cumulative decrease in fetal testicular testosterone production and testosterone levels as well as a decrease in testicular progesterone production. We also observed cumulative effects on external and internal reproductive tract malformations with binary combinations of two phthalate esters with a common metabolite (DBP and BBP, 500 mg/kg/day each chemical individually or in combination) as well as a mixture of DBP (500 mg/kg/day) and procymidone (50 mg/kg/day), a pesticide with AR-antagonist activity when administered during in utero sexual differentiation (Figs. 3C–D; Gray et al., 2006b; L. E. Gray Jr., unpublished observation; A. K. Hotchkiss, J. Furr and L. E. Gray Jr., manuscript in preparation).

Based on the dose-additive effects on postnatal endpoints in the binary phthalate studies, we hypothesized that phthalate esters administered during the period of sexual differentiation would work together to suppress fetal testosterone production in a dose-additive fashion (Howdeshell et al., 2008). In the first series of experiments, we determined the sigmoidal dose–response curve of six individual phthalates (BBP, DBP, DEHP, DEP, DiBP and DPP) on GD 18 testicular testosterone production following exposure to SD rat dams on GD 8–18. As predicted by their structure–activity relationship, BBP, DBP, DEHP and DiBP were equipotent, DPP was approximately three times more

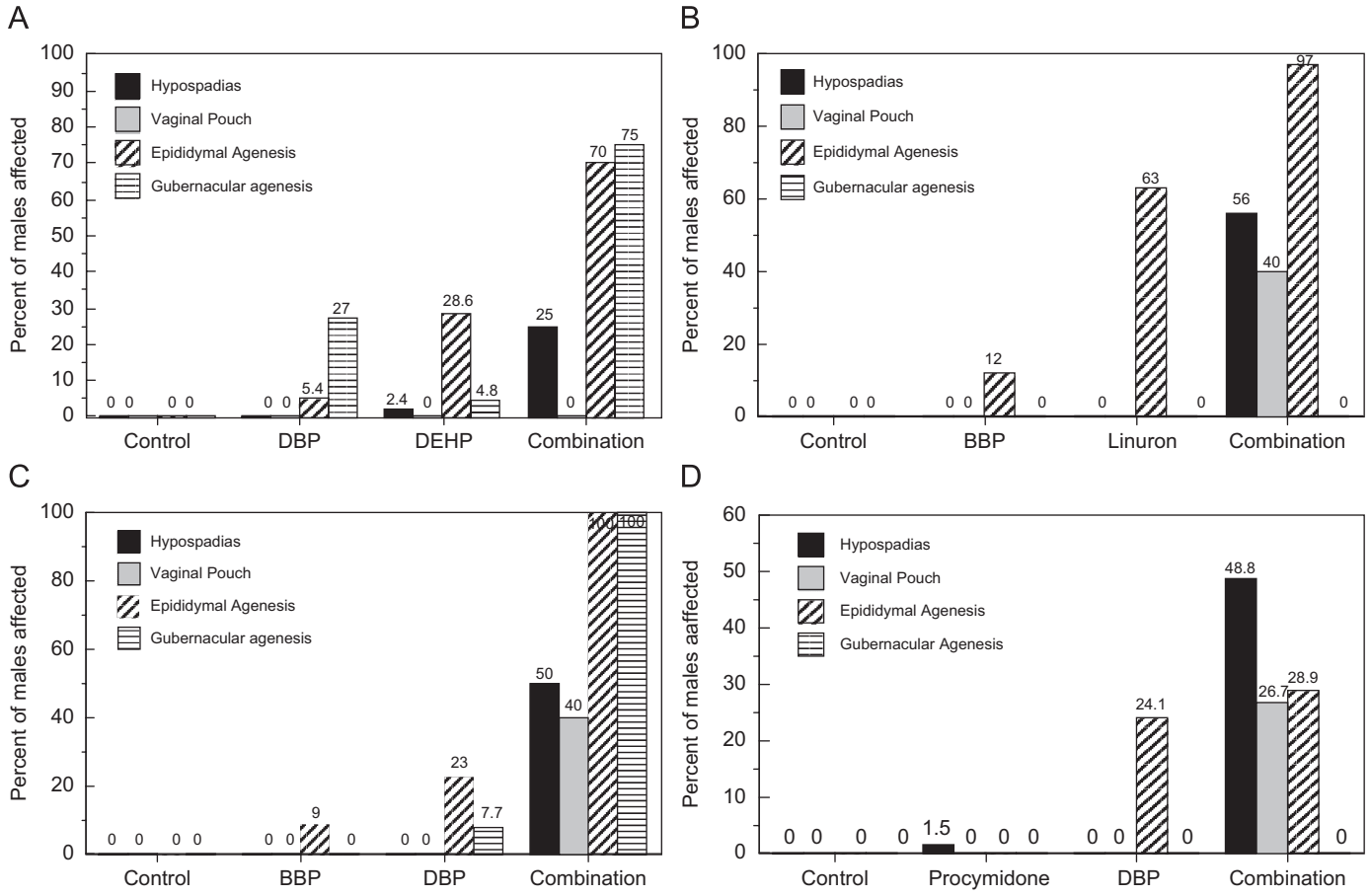

Fig. 3. Androgen-dependent reproductive malformations of androgen and insl3-dependent tissues induced by binary combinations of phthalate esters and food-use pesticide, AR-antagonists administered orally in corn oil to Sprague–Dawley rat dams on gestation day 14–18. Mixtures studies included (A) dibutyl phthalate (DBP; 500 mg/kg/day individual dose) and diethylhexyl phthalate (DEHP; 500 mg/kg/day individual dose) (Howdeshell et al., 2007), (B) BBP and linuron (50 mg/kg/day)(Hotchkiss et al., 2004), (C) benzylbutyl phthalate (BBP; 500 mg/kg/day individual dose) and DBP (500 mg/kg/day) (Gray et al., 2006b; A. K. Hotchkiss, J. Furr, and L. E. Gray Jr., manuscript in preparation), and (D) procymidone (50 mg/kg/day) and DBP (500 mg/kg/day)(Gray et al., 2006b; A. K. Hotchkiss, J. Furr and L. E. Gray Jr., manuscript in preparation). The dose level of each of the individual chemicals was one-half of the effective dose needed to induce reproductive malformations in 50% of the males (1/2 of the ED50). The combination dose was predicted to be the ED50 dose if the two individual chemical doses together acted in a cumulative, dose-additive fashion.

potent, while DEP had no effect on fetal testosterone production. In the second experiment, SD dams were dosed at 100%, 80%, 60%, 40%, 20%, 10%, 5% or 0% of a mixture of BBP, DBP, DEHP, DiBP (300 mg/kg/day per chemical) and 100 mg DPP/kg/day; the individual chemical doses in the mixture were selected such that each phthalate would contribute equally to the reduction in testosterone. As hypothesized, fetal testosterone production was inhibited in a dose-additive manner. In addition, several of the individual phthalates and the mixture induced fetal mortality in a manner predicted by dose addition. These data demonstrate that individual phthalates with a similar mechanism of action can elicit dose-additive effects on fetal testosterone production and fetal mortality when administered as a mixture (Howdeshell et al., 2008).

We recently published a complex mixture study assessing the joint action of seven antiandrogenic chemicals that target male reproductive tract development. The mixture consisted of three phthalates (BBP, DBP and DEHP), vinclozolin, procymidone, prochloraz and linuron (Gray et al., 2006b). Collectively, the selected compounds exhibit two distinct mechanisms of action for impairing androgen-dependent development: (1) inhibition of testosterone synthesis and (2) AR antagonism. Our hypothesis was that chemicals that target androgen signaling via different mechanisms of action act in a dose-additive manner to disrupt male rat reproductive tract development. Our hypothesis was contrary to the more commonly held hypothesis that multiple chemicals of differing mechanisms of action would be best fit by integrated or response-addition models (Teuschler et al., 2004). The integrated addition model estimates the dose-additive responses among chemicals with a similar mechanism, then combines the mechanism-based groups using a response-addition model; for example, the responses to phthalates or the AR-antagonists would be calculated separately using a dose-addition model, then the responses to the two groups would be combined using a response-addition model. Pregnant SD rat dams were gavaged on GD 14–18 with a dose range of the complex mixture containing each anti-androgenic chemical at 1/7th of its potency for inducing reproductive tract malformations (BBP, DBP and DEHP at 150 mg/kg/day, vinclozolin 15 mg/kg/day, procymidone 15 mg/kg/day, prochloraz 35 mg/kg/day and linuron 20 mg/kg/day). Prenatal exposure to the seven chemical mixture elicited dose-dependent cumulative effects to decrease AGD in male rat offspring, retain nipples, induce reproductive malformations and suppress androgen-dependent organ weights, all of which were predicted by the dose-addition model (Gray et al., 2006b).

The results of the binary studies, the five phthalate mixture study, and the seven chemical antiandrogen mixture study support our hypothesis that chemicals that target androgen signaling can act together to induce dose-additive adverse effects

on male reproductive tract development, even if their individual mechanisms of action are different. As demonstrated in the fetal component of the binary studies, fetal testicular testosterone action is cumulatively impacted regardless of whether the mixture is comprised of two phthalate esters (testosterone synthesis inhibitors) or a phthalate ester (testosterone synthesis inhibitor) and an AR-antagonist (blocking the ability of testosterone to bind to its receptor). The decreased testosterone activity in the fetus, regardless of the mechanism of action of the individual chemicals, results in the induction of alterations in androgen-dependent tissues. If these results hold true for lower dosage levels of these endocrine-disruptive chemicals, then these findings would have important implications for risk assessments; this would indicate that phthalates and other anti-androgenic chemicals present below their no observed adverse effect levels (NOAELs) for inducing reproductive malformations can contribute to the overall developmental reproductive toxicity effects of chemical mixtures.

8. Relevance of animal studies to humans

As phthalates inhibit testosterone production and insl3 expression in rat studies, it is important to note that the androgen-signaling and insl3 pathways are highly conserved in mammals, including humans (Baker, 2004; Ivell et al., 2005). Disruption of these pathways by genetic mutation or drugs can produce common adverse effects in humans and rodents (Hughes, 2001; Ivell and Bathgate, 2002; Scott et al., 2007). In utero exposure to phthalate esters during sexual differentiation is reported to disrupt androgen-signaling and/or androgen-dependent reproductive development in wide range of mammals. In Dutch-belted rabbits exposed in utero via the doe to DBP (400 mg/kg/day), phthalate treatment decreased the weights of the testes and accessory sex glands, reduced the sperm concentration and ejaculate volume, and doubled the number of abnormal sperm relative to controls (Higuchi et al., 2003). Treatment with DBP-exposed rabbits increased frequency of testicular germ cell loss; cryptorchidism and hypospadia were observed in one of 17 male rabbits exposed in utero to DBP (Higuchi et al., 2003). Serum testosterone levels in neonatal common marmosets were decreased 5 h after a single dose of DBP or MBP (500 mg/kg/day)(Hallmark et al., 2007); these data suggest that the developing primate, and thus possibly humans, may respond to phthalate exposure similarly to the rat.

Multinucleated gonocytes have been observed in fetal mouse testes following oral DBP exposure to the dam (C57B16 mouse strain; Gaido et al., 2007). The DBP-induction of multinucleated gonocytes in mice was observed in the absence of DBP effects on testicular testosterone levels (in C56B1/6J and C3H/HeJ mouse strains). These results lead the authors to hypothesize that the phthalates may affect gonocytes via a pathway separate from effects on androgen production and that the mouse model may be less sensitive to the anti-androgenic effects of phthalates (Gaido et al., 2007).

Phthalate esters also have been shown to cause reproductive tract malformations in lower vertebrates. Exposure to DBP affected the reproductive development of African clawed frogs, when exposed via their aquaria water as tadpoles during sexual maturation to DBP at 0.1–10 ppm (Lee and Veeramachaneni, 2005). As adults, the DBP-treated male frogs had reduced absolute testes-kidney weight, reduced seminiferous tubule diameter, reduced germ cell nests per tubule and impaired spermatogenesis (Lee and Veeramachaneni, 2005). In addition, the percent of Sertoli cell-only seminiferous tubules were increased in DBP-treated male frogs relative to controls (Lee and Veeramachaneni, 2005).

9. Phthalate exposures in humans

Phthalate ester metabolites have been detected in humans of all ages and some humans are exposed to high doses. The potential for human exposure during fetal development is evidenced by detection of phthalates in urine of women of reproductive age (Blount et al., 2000; Silva et al., 2004a), and in maternal urine and amniotic fluid (Silva et al., 2004b). In a study of 54 pregnant women, the DBP monoester metabolite MBP was detected in 93% of the urine samples (Silva et al., 2004b). While the majority of the corresponding amniotic fluid samples had low median levels of monoester metabolites, 2% of the samples had levels of MBP within five-fold of the levels detected in rat amniotic fluid from SD dams administered of DBP at doses near the respective low adverse effect levels (Calafat et al., 2006; Mylchreest et al., 2002). In one case, the ingestion of pharmaceuticals coated with DBP led to high levels (16,868 ng/ml) of MBP in human urine, indicating the potential for oral high dose exposure in humans (Hauser et al., 2004); this level was 100 × greater than the 95th percentile of the men in the 1999–2000 National Health and Nutrition Examination Survey.

Some neonates are also exposed to high levels of phthalates. Infants maintained in neonatal intensive care units are a subpopulation at greatest risk to the effects of direct exposure to phthalates. A positive association has been reported between exposure to phthalate-containing medical equipment (e.g. medical tubing, intravenous bags) and urinary MEHP levels in neonates maintained within an intensive care unit for a minimum of three days (Green et al., 2005). In addition to MEHP, urine samples from these infants also had detectable levels of the oxidative metabolites of DEHP as well as the monoester metabolites of DBP and BBP (Weuve et al., 2006), which indicated that these infants were exposed to multiple phthalates; these three phthalates are known reproductive toxicants in rats.

Finally, researchers working in cooperation with the German Federal Environmental Agency evaluated the phthalate metabolite load of urine samples of 239 healthy children aged 2 to 14 years old in Germany (reviewed in Wittasek and Angerer, 2008). Approximately one-fifth of children exceeded a cumulative Tolerable Daily Intake (TDI) for DBP and DEHP, based on the metabolites of DBP and DEHP (Wittasek and Angerer, 2008). These studies emphasize the need for more research on the human health risks to mixtures of phthalates.

10. TDS in humans and phthalates: A hypothesis

TDS is a syndrome in human males that is characterized by cryptorchidism, hypospadias, decreased sperm counts and testicular cancer (Fisher et al., 2003; Virtanen et al., 2005); the etiology of this syndrome is unknown, but endocrine-disruptive chemicals (such as phthalates esters) have been suggested as one of many potential causal agents (Sharpe and Skakkebaek, 2008). Phthalate exposure during sexual differentiation in male rats induces many of the same effects (cryptorchidism, hypospadias and decreased sperm counts) as seen in men with TDS, except testicular cancer. Although prenatal phthalate exposure does not induce testis cancer (i.e. seminonas) in rats, developmental exposure to phthalates can induce the expression of some genes that are markers of carcinoma in situ (CIS) of the testis in humans. A recent study of testis histopathology in adult Wistar rats developmentally exposed to DBP observed delayed expression of the transcription factor Oct-4 in the gonocytes; Oct-4 is a transcription factor that is used as a marker for carcinoma in situ cells in TDS males (Ferrara et al., 2006; Fisher et al., 2003).

Finally, Swan et al. (2005) reported a negative association between pregnant mothers' urinary phthalate levels and the anogenital distance index (AGD/bw) of their infant boys. In rats and other mammals (including humans), the lengthening of the AGD in males is dependent on androgen and in utero phthalate exposure is known to significantly decrease AGD in rats (Foster, 2006; Gray et al., 2006b). The Swan et al. (2005) study suggests that, similar to effects in male rat offspring, exposure to phthalate esters in the womb can impact human androgen-dependent male reproductive development (Swan, 2008).

11. Conclusion

In summary, exposure of the fetal rat testis to reproductive toxicant phthalates during the period of sexual differentiation alters the testicular paracrine and/or autocrine growth factors or hormones involved in Leydig cell migration and maturation. As a result, the fetal Leydig cells of male rats prenatally exposed to phthalate esters produce less testosterone and insl3, which are required for differentiation of the male phenotype from an indifferent reproductive tract. Lower hormone levels in utero induce postnatal reproductive malformations in the phthalate-exposed male rats, such as epididymal agenesis, cryptorchidism and hypospadias. Phthalates can also induce reproductive malformations in the female offspring, and increase fetal mortality when administered in high doses or for a long duration.

Continued research efforts are needed to assess the cumulative risk of chemical mixtures on human reproductive health. Our research in laboratory rats has demonstrated that mixtures of phthalate esters with one another, and with other anti-androgenic chemicals, can alter reproductive development and hormone production in a largely dose-additive fashion. Finally, as testosterone and insl3 expression are critical for the proper differentiation of the male reproductive tract in all mammals, disruption of these signaling pathways by antiandrogenic chemicals or mixtures of these chemicals could lead to adverse reproductive effects in humans.

References

Baker, M.E., 2004. Co-evolution of steroidogenesis and steroid-inactivating enzymes and adrenal and sex steroid receptors. Mol. Cell Endocrinol. 215, 55–62.

Barlow, N.J., Phillips, S.L., Wallace, D.G., Sar, M., Gaido, K.W., Foster, P.M., 2003. Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate. Toxicol. Sci. 73, 431–441.

Barlow, N.J., McIntyre, B.S., Foster, P.M., 2004. Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(n-butyl) phthalate. Toxicol. Pathol. 32, 79–90.

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

Calafat, A.M., Brock, J.W., Silva, M.J., Gray Jr., L.E., Reidy, J.A., Barr, D.B., Needham, L.L., 2006. Urinary and amniotic fluid levels of phthalate monoesters in rats after the oral administration of di(2-ethylhexyl) phthalate and di-n-butyl phthalate. Toxicology 217, 22–30.

Carruthers, C.M., Foster, P.M., 2005. Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. Birth Defects Res. B Dev. Reprod. Toxicol. 74, 277–285.

Congress, 1996. US, Food Quality Protection Act of 1996. Vol. Public Law 104–107, pp. 1–50.

DiGangi, J., Schettler, T., Cobbing, M., Rossi, M., 2002. Aggregate Exposures to Phthalates in Humans. Health Care Without Harm, Washington, DC, pp. 1–49.

Ema, M., Miyawaki, E., 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.

Ema, M., Miyawaki, E., Kawashima, K., 2000. Effects of dibutyl phthalate on reproductive function in pregnant and pseudopregnant rats. Reprod. Toxicol. 14, 13–19.

Ferrara, D., Hallmark, N., Scott, H., Brown, R., McKinnell, C., Mahood, I.K., Sharpe, R.M., 2006. Acute and long-term effects of in utero exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. Endocrinology 147, 5352–5362.

Fisher, J.S., Macpherson, S., Marchetti, N., Sharpe, R.M., 2003. Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. Hum. Reprod. 18, 1383–1394.

Foster, P.M., 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int. J Androl. 29, 140–147 discussion 181-5.

Foster, P.M., Harris, M.W., 2005. Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages. Toxicol. Sci. 85, 1024–1032.

Foster, P.M., Thomas, L.V., Cook, M.W., Gangolli, S.D., 1980. Study of the testicular effects and changes in zinc excretion produced by some n-alkyl phthalates in the rat. Toxicol. Appl. Pharmacol. 54, 392–398.

Foster, P.M., Lake, B.G., Cook, M.W., Thomas, L.V., Gangolli, S.D., 1981. Structure–activity requirements for the induction of testicular atrophy by butyl phthalates in immature rats: effect on testicular zinc content. Adv. Exp. Med. Biol. 136 (part A), 445–452.

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

Gaido, K.W., Hensley, J.B., Liu, D., Wallace, D.G., Borghoff, S., Johnson, K.J., Hall, S.J., Boekelheide, K., 2007. Fetal mouse phthalate exposure shows that gonocyte multinucleation is not associated with decreased testicular testosterone. Toxicol. Sci. 97, 491–503.

Grande, S.W., Andrade, A.J., Talsness, C.E., Grote, K., Golombiewski, A., Sterner-Kock, A., Chahoud, I., 2007. A dose–response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): reproductive effects on adult female offspring rats. Toxicology 229, 114–122.

Gray, T.J., Beamand, J.A., 1984. Effect of some phthalate esters and other testicular toxins on primary cultures of testicular cells. Food Chem. Toxicol. 22, 123–131.

Gray Jr., L.E., Ostby, J.S., Kelce, W.R., 1994. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicol. Appl. Pharmacol. 129, 46–52.

Gray Jr., L.E., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R.L., 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.

Gray Jr., L.E., Ostby, J., Furr, J., Price, M., Veeramachaneni, D.N., Parks, L., 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol. Sci. 58, 350–365.

Gray Jr., L.E., Laskey, J., Ostby, J., 2006a. Chronic di-n-butyl phthalate exposure in rats reduces fertility and alters ovarian function during pregnancy in female Long Evans hooded rats. Toxicol. Sci. 93, 189–195.

Gray Jr., L.E., Wilson, V.S., Stoker, T., Lambright, C., Furr, J., Noriega, N., Howdeshell, K., Ankley, G.T., Guillette, L., 2006b. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int. J Androl. 29, 96–104.

Green, R., Hauser, R., Calafat, A.M., Weuve, J., Schettler, T., Ringer, S., Huttner, K., Hu, H., 2005. Use of di(2-ethylhexyl) phthalate-containing medical products and urinary levels of mono(2-ethylhexyl) phthalate in neonatal intensive care unit infants. Environ. Health Perspect. 113, 1222–1225.

Hallmark, N., Walker, M., McKinnell, C., Mahood, I.K., Scott, H., Bayne, R., Coutts, S., Anderson, R.A., Greig, I., Morris, K., Sharpe, R.M., 2007. Effects of monobutyl- and di(n-butyl)-phthalate in vitro on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects in vivo in the fetal rat and neonatal marmoset and in vitro in the human. Environ. Health Perspect. 115, 390–396.

Hauser, R., Duty, S., Godfrey-Bailey, L., Calafat, A.M., 2004. Medications as a source of human exposure to phthalates. Environ. Health Perspect. 112, 751–753.

Higuchi, T.T., Palmer, J.S., Gray Jr., L.E., Veeramachaneni, D.N., 2003. Effects of dibutyl phthalate in male rabbits following in utero, adolescent, or post-pubertal exposure. Toxicol. Sci. 72, 301–313.

Hotchkiss, A., Parks-Saldutti, L., Ostby, J., Lambright, C., Furr, J., Vandenbergh, J., Gray Jr., L.E., 2004. A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. Biol. Reprod. 71, 1852–1861.

Howdeshell, K.L., Furr, J., Lambright, C., Rider, C.V., Wilson, V.S., Gray Jr., L.E., 2007. Cumulative effects of diethyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. Toxicol. Sci. 99, 190–202.

Howdeshell, K.L., Wilson, V.S., Furr, J., Lambright, C.R., Rider, C.V., Blystone, C.R., Hotchkiss, A.K., Gray Jr., L.E., 2008. A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague Dawley rat in a cumulative, dose additive manner. Toxicol Sci 105, 153–165.

Hughes, I.A., 2001. Minireview: sex differentiation. Endocrinology 142, 3281–3287.

Ivell, R., Bathgate, R.A., 2002. Reproductive biology of the relaxin-like factor (RLF/INSL3). Biol. Reprod. 67, 699–705.

Ivell, R., Hartung, S., 2003. The molecular basis of cryptorchidism. Mol. Hum. Reprod. 9, 175–181.

Ivell, R., Hartung, S., Anand-Ivell, R., 2005. Insulin-like factor 3: where are we now? Ann. NY Acad. Sci. 1041, 486–496.

Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumpter, J.P., 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ. Health Perspect. 103, 582–587.

Kleymenova, E., Swanson, C., Boekelheide, K., Gaido, K.W., 2005. Exposure in utero to di(n-butyl) phthalate alters the vimentin cytoskeleton of fetal rat Sertoli cells and disrupts Sertoli cell–gonocyte contact. Biol. Reprod. 73, 482–490.

Kluwe, W.M., 1982. Overview of phthalate ester pharmacokinetics in mammalian species. Environ. Health Perspect. 45, 3–9.

Latini, G., De Felice, C., Presta, G., Del Vecchio, A., Paris, I., Ruggieri, F., Mazzeo, P., 2003. In utero exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. Environ. Health Perspect. 111, 1783–1785.

Lee, S.K., Veeramachaneni, D.N., 2005. Subchronic exposure to low concentrations of di-n-butyl phthalate disrupts spermatogenesis in *Xenopus laevis* frogs. Toxicol. Sci. 84, 394–407.

Lehmann, K.P., Phillips, S., Sar, M., Foster, P.M., Gaido, K.W., 2004. Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. Toxicol. Sci. 81, 60–68.

Lin, H., Ge, R.S., Chen, G.R., Hu, G.X., Dong, L., Lian, Q.Q., Hardy, D.O., Sottas, C.M., Li, X.K., Hardy, M.P., 2008. Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero. Proc. Natl. Acad. Sci. 150, 7218–7222.

Liu, K., Lehmann, K.P., Sar, M., Young, S.S., Gaido, K.W., 2005. Gene expression profiling following in utero exposure to phthalate esters reveals new gene targets in the etiology of testicular dysgenesis. Biol. Reprod. 73, 180–192.

Mahood, I.K., Hallmark, N., McKinnell, C., Walker, M., Fisher, J.S., Sharpe, R.M., 2005. Abnormal Leydig cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. Endocrinology 146, 613–623.

Mahood, I.K., McKinnell, C., Walker, M., Hallmark, N., Scott, H., Fisher, J.S., Rivas, A., Hartung, S., Ivell, R., Mason, J.I., Sharpe, R.M., 2006. Cellular origins of testicular dysgenesis in rats exposed in utero to di(n-butyl) phthalate. Int. J Androl. 29, 148–154 discussion 181–185.

McKee, R.H., Pavkov, K.L., Trimmer, G.W., Keller, L.H., Stump, D.G., 2006. An assessment of the potential developmental and reproductive toxicity of di-isoheptyl phthalate in rodents. Reprod. Toxicol. 21, 241–252.

McKinnell, C., Sharpe, R.M., Mahood, K., Hallmark, N., Scott, H., Ivell, R., Staub, C., Jegou, B., Haag, F., Koch-Nolte, F., Hartung, S., 2005. Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by in utero exposure to di (n-butyl) phthalate. Endocrinology 146, 4536–4544.

Mortensen, G.K., Main, K.M., Andersson, A.M., Leffers, H., Skakkebaek, N.E., 2005. Determination of phthalate monoesters in human milk, consumer milk, and infant formula by tandem mass spectrometry (LC-MS-MS). Anal. Bioanal. Chem. 382, 1084–1092.

Mylchreest, E., Cattley, R.C., Foster, P.M., 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.

Mylchreest, E., Sar, M., Cattley, R.C., Foster, P.M., 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., Wallace, D.G., Cattley, R.C., Foster, P.M., 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.

Mylchreest, E., Sar, M., Wallace, D.G., Foster, P.M., 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.

Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J., Gray Jr., L.E., 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol. Sci. 58, 339–349.

Plummer, S., Sharpe, R.M., Hallmark, N., Mahood, I.M., Elcombe, C., 2007. Time-dependent and compartment-specific effects of *in utero* exposure to di(n-butyl) phthalate on gene/protein profiling and laser capture microdissection. Toxicol. Sci. 97, 520–532.

Purvis, M., Gibson, R., 2005. The Right Start: The Need to Eliminate Toxic Chemicals From Baby Products. US Public Interest Research Group Education Fund, Washington, DC, pp. 1–32.

Rider, C.V., LeBlanc, G.A., 2005. An integrated addition and interaction model for assessing toxicity of chemical mixtures. Toxicol. Sci. 87, 520–528.

Rider, C.V., Furr, J., Wilson, V.S., Gray Jr., L.E., 2008. A mixture of seven antiandrogens induces reproductive malformations in rats. Int. J Androl. 31, 249–262.

Rock, G., Labow, R.S., Tocchi, M., 1986. Distribution of di(2-ethylhexyl) phthalate and products in blood and blood components. Environ. Health Perspect. 65, 309–316.

Scott, H.M., Hutchinson, G.R., Mahood, I.M., Hallmark, N., Welsh, M., De Gendt, K., Verhoeven, G., O'Shaughnessy, P., Sharpe, R.M., 2007. Role of androgens in fetal testis development and dysgenesis. Endocrinology 148, 2027–2036.

Sharpe, R.M., Skakkebaek, N.E., 2008. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. Fertil. Steril. 89, 33–38.

Shono, T., Shima, Y., Kondo, T., Suita, S., 2005. In utero exposure to mono-n-butyl phthalate impairs insulin-like factor 3 gene expression and the transabdominal phase of testicular descent in fetal rats. J Pediatr. Surg. 40, 1861–1864.

Shultz, V.D., Phillips, S., Sar, M., Foster, P.M., Gaido, K.W., 2001. Altered gene profiles in fetal rat testes after in utero exposure to di(n-butyl) phthalate. Toxicol. Sci. 64, 233–242.

Silva, M.J., Barr, D.B., Reidy, J.A., Malek, N.A., Hodge, C.C., Caudill, S.P., Brock, J.W., Needham, L.L., Calafat, A.M., 2004a. Urinary levels of seven phthalate metabolites in the US population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. Environ. Health Perspect. 112, 331–338.

Silva, M.J., Reidy, J.A., Herbert, A.R., Preau Jr., J.L., Needham, L.L., Calafat, A.M., 2004b. Detection of phthalate metabolites in human amniotic fluid. Bull Environ. Contam. Toxicol. 72, 1226–1231.

Sjoberg, P., Bondesson, U., Gray, T.J., Ploen, L., 1986. Effects of di-(2-ethylhexyl) phthalate and five of its metabolites on rat testis in vivo and in in vitro. Acta Pharmacol. Toxicol. (Copenh) 58, 225–233.

Skakkebaek, N.E., Rajpert-De Meyts, E., Main, K.M., 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum. Reprod. 16, 972–978.

Skibsted, U., Hansen, P.E., 1990. 1H NMR spin-echo spectroscopy of human erythrocytes. Transformation of exogenous compounds. NMR Biomed. 3, 248–258.

Svechnikova, I., Svechnikov, K., Söder, O., 2007. The influence of di-(2-ethylhexyl) phthalate on steroidogenesis by the ovarian granulosa cells of immature female rats. J Endocrinol. 194, 603–609.

Swan, S.H., 2008. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ. Res., this issue, doi:10.1016/j.envres.200.

Swan, S.H., Main, K.M., Liu, F., Stewart, S.L., Kruse, R.L., Calafat, A.M., Mao, C.S., Redmon, J.B., Ternand, C.L., Sullivan, S., Teague, J.L., 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ. Health Perspect. 113, 1056–1061.

Teuschler, L.K., Rice, G.E., Wilkes, C.R., Lipscomb, J.C., Power, F.W., 2004. A feasibility study of cumulative risk assessment methods for drinking water disinfection by-products mixtures. J Toxicol. Environ. Health A 67, 755–777.

Tomonari, Y., Kurata, Y., David, R.M., Gans, G., Kawasuso, T., Katoh, M., 2006. Effect of di(2-ethylhexyl) phthalate (DEHP) on genital organs from juvenile common marmosets: I. Morphological and biochemical investigation in 65-week toxicity study. J Toxicol. Environ. Health A 69, 1651–1672.

Tyl, R.W., Myers, C.B., Marr, M.C., Fail, P.A., Seely, J.C., Brine, D.R., Barter, R.A., Butala, J.H., 2004. Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. Reprod. Toxicol. 18, 241–264.

van der Schoot, P., Emmen, J.M., 1996. Development, structure and function of the cranial suspensory ligaments of the mammalian gonads in a cross-species perspective; their possible role in effecting disturbed testicular descent. Hum. Reprod. Update 2, 399–418.

Virtanen, H.E., Rajpert-De Meyts, E., Main, K.M., Skakkebaek, N.E., Toppari, J., 2005. Testicular dysgenesis syndrome and the development and occurrence of male reproductive disorders. Toxicol. Appl. Pharmacol. 207, 501–505.

Welsh, M., Saunders, P.T.K., Fisken, M., Scott, H.M., Hutchison, G.R., Smith, L.B., Sharpe, R.M., 2008. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. J Clin. Invest. 118, 1479–1490.

Weuve, J., Sánchez, B.N., Calafat, A.M., Schettler, T., Green, R.A., Hu, H., Hauser, R., 2006. Exposure to phthalates in neonatal intensive care unit infants: urinary concentrations of monoesters and oxidative metabolites. Environ. Health Perspect. 114, 1424–1431.

Wilson, V.S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., Gray Jr., L.E., 2004. Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. Toxicol. Lett. 146, 207–215.

Wilson, V.S., Lambright, C., Furr, J., Bobseine, K., Wood, C., Howdeshell, K.L., Gray Jr., L.E., 2006. Ontogeny of changes in fetal testis gene expression induced in male offspring after maternal treatment with DEHP (diethylhexyl phthalate). Toxicologist 90, 238.

Wilson, V.S., Howdeshell, K.L., Lambright, C., Furr, J., Gray Jr., L.E., 2007. Differential expression of the phthalate syndrome in male Sprague Dawley and Wistar rats after in utero DEHP exposure. Toxicol. Lett. 170, 177–184.

Wittasek, M., Angerer, J., 2008. Phthalates: metabolism and exposure. Int. J Androl. 31, 131–138.

Wolf, C.J., LeBlanc, G.A., Ostby, J.S., Gray Jr., L.E., 2000. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol. Sci. 55, 152–161.

Zimmermann, S., Steding, G., Emmen, J.M., Brinkmann, A.O., Nayernia, K., Holstein, A.F., Engel, W., Adham, I.M., 1999. Targeted disruption of the insl3 gene causes bilateral cryptorchidism. Mol. Endocrinol. 13, 681–691.