Phthalate ester toxicity in Leydig cells: Developmental timing and dosage considerations

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Abstract
Humans have significant exposures to phthalates, as these chemical plasticizers are ubiquitously present in flexible plastics. Recent epidemiological evidence indicates that boys born to women exposed to phthalates during pregnancy have an increased incidence of congenital genital malformations and spermatogenic dysfunction, signs of a condition referred to as testicular dysgenesis syndrome (TDS). TDS is thought to develop as a result of environmental factors that cause a testicular disturbance at an early fetal stage with a resultant spectrum of clinical testicular dysfunction, ranging from impaired spermatogenesis and genital malformations to increased risk for development of testicular cancer. Proposed environmental factors in the etiology of TDS include endocrine disrupting compounds such as the phthalates. Leydig cells have been classified as one of the main targets for phthalate ester toxicity in the body based on studies in rodents. In support of this hypothesis, two Leydig cell products – insulin-like growth factor 3 (INSL3) and testosterone (T) – are both suppressed after phthalate exposures. Both fetal and adult generations of Leydig cells are affected by phthalate esters, although their sensitivities may differ. In rodent models, when pregnant dams are exposed to phthalate esters, fetal Leydig cells form enlarged clusters that are retained in the testis even after birth, in contrast to untreated controls. Despite the retention of fetal Leydig cells, however, their numbers and average cell volume of total in exposed males are reduced, as are INSL3 production and steroidogenic competence. These alterations are directly associated with clinical features of TDS, including cryptorchidism and impaired spermatogenesis.

Keywords: Phthalate Esters; Anti-androgens; Testosterone; Steroidogenesis; Puberty

1. Introduction
Reports of a higher incidence of urogenital anomalies in male newborns, including cryptorchidism (undescended testis) and hypospadias (failure of the urethra to tubularize completely, resulting in a urethral opening on the ventral aspect of the penis), and an increased incidence of testicular cancer and male infertility in adults are associated with early exposures to environmental chemicals with reproductive toxicity \cite{1,2}. For example, cryptorchidism affects 2–4\% of more of boys at birth, and is the most common congenital abnormality in male children \cite{3,4}. Although disorders linked to reproductive toxicants may become evident at different life stages, with cryptorchidism and hypospa-
commonly used phthalate is di(2-ethylhexyl) phthalate (DEHP), and the annual production volume of DEHP alone has been estimated at 2 million tons [7]. The Agency for Toxic Substances and Disease Registry (ATSDR) estimates that the maximum daily exposure to DEHP for the general population is about 2 mg/day. However, occupational and medical exposures can reach much higher levels [8]. For instance, exposure to DEHP from blood transfusions can be as high as 250–300 mg, equivalent to a dose of 3.5–4.3 mg/kg for an adult weighing 70 kg. Exposure may be much higher in infants and children due to lack of metabolic clearance [7].

Phthalates are diesters of phthalic acid with various side chain lengths. For example, DEHP has an eight-carbon hydrogen chain (Fig. 1). The diester forms of phthalate are rapidly hydrolyzed by esterases in the gut, liver, and blood and are present in the body in monoester forms, which are considered the bioactive toxicants. Accordingly, the monoester form of DEHP, mono(2-ethylhexyl) phthalate (MEHP) (Fig. 2), is thought to be 10-fold more potent in its toxicity to Leydig cells and Sertoli cells compared to DEHP. There is no direct effect of DEHP on Leydig cell function suggesting that DEHP is the pre-toxin which acts via metabolizing into MEHP [8]. A recent study by the Centers for Disease Control and Prevention (CDC) measured phthalate monoesters in human urine and reported that detectable levels of metabolites monoethyl phthalate (MEP), monobutyl phthalate (MBP), monobenzyl phthalate (MBzP), and MEHP are present in more than 75% of the population in the United States [6,9,10]. Exposure may be much higher in infants and children due to lack of metabolic clearance [7].

Limited studies in human populations indicate a correlation between exposures of phthalates and adverse reproductive health outcomes. For example, phthalate monoester contamination of human breast milk had an adverse influence on the postnatal surge of reproductive hormones in newborn boys [11]. An association between adverse male genital development and phthalate exposure has also been reported [7,12]. Furthermore, chronic occupational exposures to high levels of phthalates have been linked to decreased pregnancy rates and higher miscarriage rates in female factory workers [13]. During pregnancy and delivery, both the mother and fetus may be exposed to DEHP through medical devices. Exposure of the fetus in utero is a concern because some phthalates, including DEHP and dibutyl phthalate (DBP), are now classified as potential developmental toxicants [14,15]. The Food and Drug Administration (FDA) recently issued a report acknowledging that the use of PVC-containing medical devices in the treatment of infants who are critically ill may be of concern [16]. These studies are indicative of human male health effects, possibly through interference with Leydig cell and Sertoli cell development and function. Additional epidemiological data are needed on human population exposures, along with a better mechanistic understanding of the reproductive health effects of phthalates. We and others have studied the effects of DEHP on Leydig cell development using rodent models. Phthalate exposures have profound effects on other testicular cell types such as Sertoli cells and gonocytes, which have been described elsewhere [16,17]. Herein, we review the current state of knowledge of the effects of phthalate on the Leydig cells as well as phthalate’s mechanism of action. Finally, we propose a hypothesis for phthalate-mediated activity in the male and its implications for human health.

2. Leydig cells

Leydig cells are the primary source of testosterone in the male, and differentiation of Leydig cells in the testes is one of the primary events in the development of the male body and fertility. In rodents, two separate generations of Leydig cells develop successively in the testis between embryogenesis and puberty. The first, designated fetal Leydig cells, differentiate from stem cells during gestation. The testis differentiates from the gonadal ridge primordium on day 10 (E10), and stem cells of the fetal Leydig cells commit and differentiate into mature fetal Leydig cells by E12, which are fully competent in steroidogenesis [18]. Fetal Leydig cells attain their highest steroidogenic activity 1 or 2 days prior to birth on day 19 of gestation [19], and the testosterone secreted is critical for male secondary sexual differentiation (i.e., development of the penis and sex accessory glands) [18]. Fetal Leydig cells also produce the hormone insulin-like growth factor 3 (INSL3), which binds to the leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8). The LGR8 specifically binds to INSL3 in the gubernaculum [20] and, together with testosterone, induces scrotal descent of the testis [21]. It now appears that INSL3 is the critical hormone responsible for early stage descent of the testis from the abdominal to inguinal position, as shown by the fact that loss of function mutations in humans and INSL3 knockout mice pre-
vents this process [22,23]. For this reason, interference with the development of fetal Leydig cells may be a precipitating cause of cryptorchidism.

Fetal Leydig cells remain in the testicular interstitium after birth, but rapidly involute [24]. The fate of fetal Leydig cells after birth remains a subject of debate in the literature [25]. However, recent studies of the luteinizing hormone receptor null (LHRKO) mice support the hypothesis that the cells are lost through attrition postnatally. This is based on the fact that the numbers of fetal Leydig cells and their capacity to produce testosterone are equivalent in null and wild-type mice just after birth, whereas there is a progressive failure of steroidogenesis with increasing age [26–28]. Therefore, the contribution of fetal Leydig cells to postnatal androgen secretion is negligible.

The cells that give rise to adult Leydig cells first become apparent by postnatal day 11 as spindle-shaped 3β-hydroxysteroid dehydrogenase (3β-HSD) expressing cells in the interstitium [29]. Postnatal development of the adult Leydig cell population involves self-renewal of stem Leydig cells, their commitment to the adult lineage, and further cytologic and biochemical differentiation into terminally differentiated adult cells that produce testosterone [30]. We propose that exposures of fetal and adult Leydig cells to phthalate may elicit divergent clinical consequences.

3. Effects of phthalates on fetal Leydig cells during prenatal exposure

Toxic effects of phthalates in the pregnant rodent and fetus have been observed, and reduced numbers of implantations, increased fetal resorption, decreased body weight, and increased malformations are reported as major effects [31]. However, there have been no studies investigating whether fetal Leydig cells are subject to a direct action of phthalates until recently. In utero exposure to phthalate esters has been shown to induce cryptorchidism, hypospadias, impaired spermatogenesis, and reduced male fertility in rats [32–36]. These postnatal changes are apparently preceded by a decrease in fetal Leydig cell function, including lower testicular levels of testosterone [32,36–38] and INSL3 mRNA [39]. The dysfunction of fetal Leydig cells is postulated to have a direct association with decreased anogenital distance, an androgen-dependent parameter of male sexual development. These effects were clearly demonstrated in experiments using phthalate esters that are structurally related to DEHP, including DBP. Decreased anogenital distance in male fetuses has been observed following exposure of pregnant dams to doses of DBP at 500 mg/kg or above [33]. Mylchreest et al. observed that increased frequencies of underdeveloped epididymis, testicular atrophy, hypospadias and ectopic or absent testes characterize phthalate exposures of doses of 250 mg/kg/day or higher [35]. For a 10-day prenatal (embryonic and fetal) exposure to DBP, the non-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) of these adverse consequences were 50 and 100 mg/kg/day, respectively [36].

We [40] and others [32,41] have studied postnatal effects of phthalates after prenatal exposures to phthalates. In the case of prenatal exposure, focal dysgenetic areas arise within the testes and the seminiferous tubules are malformed, often containing intratubular Leydig cells. Prenatal exposure to phthalates produces abnormal aggregations of fetal Leydig cells, and it has been thought that this represents Leydig cell hyperplasia or neoplasia [36,41]. These aggregations of Leydig cells are evident when rats are exposed to 500 mg/kg of DBP [36,41]. Stereological analysis, however, does not support the concept that Leydig cell numbers are increased in the aggregations [41]. The LOAEL for the formation of Leydig cell aggregations following 20-day prenatal DEHP exposure was 10 mg/kg/day, and at this dose fetal Leydig cells showed a clear tendency to group together in clusters of two cells or more [40]. This clustering of fetal Leydig cells with two to six cells per aggregate was associated with increased testicular testosterone levels at that dose of DEHP. When the doses of DEHP increased to 100 mg/kg/day or above, a sharp increase in cell clustering occurred. However, the absolute numbers of Leydig cells per testis and their cell volumes were decreased, resulting in a significant decrease in testicular testosterone levels [40]. We may infer that the decreased Leydig cell numbers and cell volumes explain the lowered testosterone levels in treated animals, and may also be associated with cryptorchidism [39]. The associations between adverse male genital development and phthalate exposure have also been reported in human male newborns [12]. A reduction of the anogenital index is seen in infant boys with increasing levels of MBP, MEP, monobenzyl- and mono-isobutyl phthalate in maternal urine samples during late-pregnancy. Boys with a short anogenital index also showed a high prevalence of cryptorchidism and small genital size.

The mechanism of phthalate toxicity on fetal Leydig cell steroidogenesis has not been well characterized. Liu et al. performed gene expression profiling following in utero exposure to phthalate esters and demonstrated a global decline in levels of gene expression associated with Leydig cell signaling (Lhcr), lipid transport (Scarb1 and Star), and the steroidogenic pathway (Cyp11a1, Hsd3b1, and Cyp17a1) [42], which confirmed the trends for genes that had been observed earlier, such as Scarb1, Star, Cyp11a1, Hsd3b1, and Cyp17a1 [43]. Prenatal phthalate exposure is also known to inhibit INSL3 gene expression [39]. The lower expression levels of these genes apparently leads in turn to decreases in their protein levels [42,43]. Whether these changes of Leydig cell gene expression levels are a direct action of phthalates or are secondary to the decreased Leydig cell numbers and size induced by phthalates is unknown. However, the induction of both angiotensin and vasopressin receptor (Nalp6), together with aminopeptidase A, an enzyme responsible for converting angiotensin II to angiotensin III, following phthalate exposure, suggests a role for either abnormal angiotensin or vasopressin activity in the suppression of testosterone synthesis in fetal Leydig cells [42]. Both angiotensin and vasopressin have been shown to inhibit testosterone production by fetal Leydig cells [44–46].
4. Effects of phthalates on adult Leydig cell development

4.1. Prenatal exposure

The postnatal lineage giving rise to adult Leydig cells begins to differentiate on postnatal day (PND) 11 and mid-puberty is reached at PND 35. Prenatal exposure to phthalates changes Leydig cell development during this period. Pups born to pregnant dams exposed to DEHP at 100 mg/kg/day have a reduction in serum testosterone and LH levels at PNDs 21 and 35, although a recovery occurs by PND 90 [2]. Similar effects were observed in rats exposed to DBP at 100 mg/kg/day or higher [43]. Male reproductive tract abnormalities, as well as depressed testosterone levels, were also observed at 6, 12, and 18 months of age following in utero exposure to DBP [41], suggesting that the disturbance of fetal Leydig cells described earlier is associated with a sustained disturbance of steroidogenesis in adult Leydig cells.

4.2. Neonatal exposure

Neonatal exposure to phthalates via the mother’s milk has been examined, and a correlation exists between exposure levels and adverse reproductive health outcomes. In particular, it has been reported that phthalate monoester contamination of human breast milk has an adverse influence on the postnatal surge of reproductive hormones in newborn boys, which is seen as a sign of TDS [11]. In rats, nursing dams exposed orally to DEHP at 100 mg/kg/day from PND 1 to 21 have lower serum testosterone concentrations on PND 21, with a progressive recovery from PNDs 35 to 90 [2]. Neonatal exposure to DBP subcutaneously at 20 mg/animal (about 200 mg/kg) reduced the weights of testes and accessory sex organs at PND 42 [15]. These data indicate that neonatal exposures to phthalates could affect Leydig cell function at later stages.

4.3. Pubertal exposure

Pubertal exposures to phthalates have produced complex results, which are dependent on both duration and dose. Oral doses of 10, 100, or 200 mg/kg/day DEHP for two different 14-day periods (PND 21–34 or 35–48) do not alter serum LH and T levels [2,47]. However, rats exposed orally to DEHP at 10, 100, or 200 mg/kg/day for a 28-day period (PND 21–48) have increased serum LH and T levels [2]. These increases can be maintained for up to 100 days after exposure [2].

In contrast, low-doses of DEHP have been shown to increase testosterone levels. The NOAEL and LOAEL for stimulation of testosterone can be achieved for DEHP at 1 and 10 mg/kg/day, respectively [2,48]. This low-dose stimulation by DEHP is also seen after inhalation exposures to DEHP at doses comparable to oral administration of 1–5 mg/kg/day [49]. This increase in testosterone production is sufficient to advance the timing of puberty as judged by an earlier onset of prepubital separation.

The mechanism of DEHP-induced testosterone production in adults is not well understood. One of the causes of increased testosterone production may be DEHP-mediated Leydig cell hyperplasia induced by increased estrogen action [48]. Aromatase expression and activity are increased in Leydig cells after phthalate exposure [48]. This, in turn, could lead to abnormal estrogen action at the testicular level, which is a known cause of Leydig cell hyperplasia and Leydig cell tumors in rodents [50,51]. Steroidogenic capacity normalized to Leydig cell numbers decreases after 28-day DEHP exposures to low doses [2,48], and we infer that the observed increases in serum T levels may be explainable by increased cell numbers, which is consistent with the elevated serum LH levels seen after treatment [2,48]. Low dose DEHP exposures are within the maximum daily exposure range as evaluated by the U.S. Department of Health and Human Services in 1985 [52]. It remains true that most studies of DEHP have focused on high doses (500 mg/kg/day and above). These exposures have shown varying degrees of reproductive anomalies, including testicular atrophy [37,53–56]. When rats were exposed to up to 200 mg/kg/day DEHP for up to 100 days, no cellular or morphological changes were detected in the testis as evaluated by light microscopy, and the weights of the seminal vesicles (with coagulating glands) were likewise unaffected [2,48]. Suppressive effects of DEHP were only seen in rats exposed to 750 mg/kg/day [57]. Since the body weight was also reduced at the high dose, systemic toxicity could not be ruled out [57]. From these studies, we can conclude that high-dose phthalate exposures have an inhibitory effect on Leydig cell function, with the LOAEL in the range of 600–800 mg/kg and NOAEL at 10–200 mg/kg [2,48].

4.4. Adult exposures

It is well-documented that oral exposure to DEHP in adult rats and mice causes: decreases in the weights of the testes, prostate, seminal vesicles, and epididymis; atrophy and degeneration of the seminiferous tubules; and/or altered sperm parameters and reduced fertility [53,54,58–63]. The lowest doses for reproductive effects in these studies are an NOAEL and LOAEL for testicular histopathology of 3.7 and 38 mg/kg/day, respectively, in rats exposed for 90 days [64], and 5.8 and 29 mg/kg/day, respectively, in rats exposed for 104 weeks [58]. The 14 mg/kg/day NOAEL in the critical study [65] is higher than the NOAELs of 3.7 and 5.9 mg/kg/day [58,64], but has the advantage of being based on an assessment of fertility rather than histological examination alone.

In summary, phthalates have biphasic effects on both fetal Leydig cells and adult Leydig cells. Phthalates cause fetal Leydig cell aggregation, and, at low doses, increase testicular testosterone production. High doses, however, decrease testosterone production thereby reducing anogenital distance and inducing cryptorchidism. Low doses of phthalates increase the number of adult Leydig cells and increase testosterone production thus advancing puberty, while high doses decrease testosterone synthesis in adult Leydig cells, delaying puberty (Table 1).

5. Mechanism of phthalates on Leydig cells

It is clear that actions of phthalates on Leydig cell function have not been fully studied. A building consensus views
phthalate-mediated toxicity as arising from antiandrogenic or proestrogenic activity. However, neither DEHP nor its metabolite, MEHP, binds to the androgen receptor (AR) \textit{in vitro} at concentrations of up to 10 \textmu M [37]. This indicates that phthalates are not AR antagonists. DEHP and MEHP do not have estrogenic activity \textit{in vitro} although some phthalates do show limited estrogenic activity [66]. The mode of action of phthalates on Leydig cells is likely to depend on developmental timing and dosing. Over a wide range of doses, phthalates promote the aggregation of fetal Leydig cells [36,40,41]. However, this phenomenon has not been observed in adult Leydig cells after postnatal exposures [37,53–56]. A biphasic effect on adult Leydig cell populations appears to be a characteristic of phthalate exposures. At lower doses, phthalates increase testosterone production either by increasing Leydig cell numbers or by directly stimulating testosterone production. Phthalates inhibit testosterone production in both fetal and adult Leydig cells when rats are exposed to higher doses. The mechanisms of phthalate-mediated toxicity on Leydig cells at the higher doses are far from clear. One possibility is that MEHP is a peroxisome proliferator and that this activity is responsible for its toxic effects in the Leydig cell [56,67]. Peroxisome proliferators stimulate the peroxisome proliferator-activated receptors (PPARs), which are members of a nuclear receptor superfamily. PPAR acts as a heterodimer with the retinoid X receptor (RXR) to regulate transcription, and once activated, PPAR induces the transcription of genes containing peroxisome proliferator response elements [68].

The PPAR family contains three subtypes, PPAR\(\alpha\), PPAR\(\beta\), and PPAR\(\gamma\), encoded by different genes [69]. The activation of PPARs alters the expression levels of their target genes, which are generally involved in metabolism, cell growth, and stress responses [69].

Leydig cells and other testicular cells express different PPAR subtypes depending on the developmental stage. Prior to birth, both PPAR\(\alpha\) and PPAR\(\gamma\) are strongly expressed in fetal Leydig cells. In the postnatal period, PPAR\(\alpha\) is expressed in both Leydig and Sertoli cells of the adult rat [70]. PPAR\(\beta\) is also expressed in Leydig and Sertoli cells [71]. PPAR\(\gamma\), however, is either weakly expressed or not expressed at all in these cell types [71]. Unlike fetal Leydig cells, the spectrum of PPAR subtype expression in adult rat Leydig cells is similar to that in a mouse MA-10 Leydig cell line, which contains both PPAR\(\alpha\) and PPAR\(\beta\) but not PPAR\(\gamma\) [56]. Both PPAR\(\alpha\) and PPAR\(\beta\) were identified in human testis homogenates [72]. PPAR\(\alpha\) is expressed in human Leydig cells and spermatocytes but not in the Sertoli cells [70].

Leydig cell PPAR\(\alpha\) targeted genes are responsive to peroxisome proliferators, and this is seen, for example, when the peroxisome proliferator cipiroflibrate is administered to adult rats for 2 weeks. The result is increased expression of two PPAR\(\alpha\) targeted genes acyl-CoA oxidase and multifunctional protein-1 in Leydig cells [73]. Using transactivation assays at micromolar concentrations, many phthalate monoesters have been shown to induce PPARs \textit{in vitro} [74–77]. For example, MEHP activates mouse and human PPAR\(\alpha\) and PPAR\(\gamma\) in COS-1 cells [78]. One potential mechanism of PPAR activation may involve the peripheral-type benzodiazepine receptor (PBR). The PBR has been implicated in the rate-determining step of steroidogenesis consisting of the transfer of cholesterol from intracellular stores into mitochondria [79]. Treatment of mice with DEHP \textit{in vivo} reduces both testicular PBR mRNA and circulating testosterone levels, in agreement with the proposed role of PBR in steroidogenesis. DEHP did not inhibit testosterone production and testicular PBR expression in PPAR\(\alpha\)-null mice. These results suggest that the antiandrogenic effect of DEHP is mediated by a PPAR\(\alpha\)-dependent inhibition of Leydig cell PBR gene expression [56]. Other mechanisms of PPAR\(\alpha\)-mediated toxicity include activation of metabolizing enzymes leading to free radical production and oxidative stress [80]. In this regard, Maloney and Waxman [78] showed that MEHP activates mouse and human PPAR\(\alpha\) and PPAR\(\gamma\) in COS-1 cells. Although this response is generally thought to contribute to the carcinogenic effect of DEHP in the liver, it may be causing more generalized toxicity in cells such as the Leydig cell.

Another signaling pathway in Leydig cells that may be affected by phthalate is controlled by the aryl hydrocarbon receptor. Fetal testes of animals treated with DBP \textit{in vivo} have an altered pattern of gene expression, increased expression levels of aryl hydrocarbon receptor, CYP1B1, and epoxide hydrolase as assayed by microarray, that is suggestive of an oxidative stress response. Activation of metabolizing enzymes by PPAR\(\alpha\) also raises the question of whether exposure to DEHP may increase susceptibility to other toxins requiring metabolic activation.

The reproductive toxicity of phthalates cannot be entirely explained by the action of a PPAR\(\alpha\)-mediated pathway, because PPAR\(\alpha\)-null mice remain sensitive to DEHP-mediated repro-
ductive toxicity. Since TDS induced by phthalates involves disturbances in Sertoli cell cytology, it may be that testosterone production is altered as a result of a dysfunctional interaction between Leydig and Sertoli cells [81]. In addition, the structurally related phthalates present a challenge to toxicologists faced with identifying endocrine disruptors in the environment. While DEHP acts through a PPAR-mediated pathway in Leydig cells, other phthalates such as DBP may activate liver enzymes to alter steroid metabolism. It is clear that steroid hormone metabolism can be altered by several different mechanisms, including both receptor-mediated and non-receptor-mediated events.

In recent years, a growing interest in environmental endocrine disruptors has led to the development of screening assays to identify such compounds. One such assay has been designed to detect interference with estrogen receptor binding activity. This assay would not identify compounds such as MEHP that may be an option. None of the phthalates tested elicited classic in vitro estrogenic responses. Among them, DBP and DHP have been tested in vivo and have toxicological effects in rats that are similar to DEHP [39]. It is becoming apparent that phthalates are endocrine disruptors of the non-classic type [39,48,83], i.e., not through modulation of binding activity of a steroid hormone receptor. Whereas classical endocrine disruptors interfere with endocrine processes at the receptor level, DEHP alters reproductive function by affecting hormone synthesis. For this reason, DEHP will be inconspicuous in receptor-based screening assays, and new assay methods will be needed to do toxicity assessment [52].

In summary, phthalates have profound effects on Leydig cells in utero and after birth. A biphasic effect of phthalates on Leydig cell numbers and function has been observed. High doses of phthalates on Leydig cells in utero may be associated with TDS postnatally. The existing screens for antiandrogenic and estrogenic activity are inappropriate for phthalates, given that they do not appear to act on steroid hormone receptor binding activity. As an alternative test, screening for direct phthalate-mediated effects on testosterone and estradiol production by Leydig cells in vitro may be an option.

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