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Effects of phthalate esters on the developing reproductive tract of male rats

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Phthalate esters are a large group of chemical agents used predominantly as plasticizers and solvents. Certain members of this chemical class have been shown to cause reproductive and developmental toxicity. Recent attention has focused on the potential of these agents to interfere with male reproductive development through a postulated antiandrogenic mechanism. Observations have focused on di-n-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP) and butyl benzylphthalate, with most information relating to dose–response relationships obtained for DBP. Neither DBP, DEHP nor their major metabolites interacted with human or rodent androgen receptors (AR) in transcriptional activation assays. DBP was administered during the critical window of development of the male reproductive system, after which the resulting offspring were examined until adulthood. DBP elicited marked effects on the developing male reproductive tract, including malformations of the epididymis and vas deferens, and hypospadias. Retention of thoracic nipples/areolae and reductions in anogenital distance were also noted. Surprisingly, Leydig cell adenomas were induced in some male offspring at 100 days of age. All these events occurred in the absence of any toxicity in the pregnant dam. Examination of testes from fetal rats indicated markedly reduced testosterone levels and increased Leydig cell numbers after DBP administration to the dams. Leydig cells were positive for AR and 3-betahydroxysteroid dehydrogenase.

Key words: antiandrogenic/endocrine disruption/phthalate, di-n-butyl/reproductive development/testis, fetal

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Introduction

Considerable concern has been focused on the potential of certain phthalate esters [and in particular di-n-butyl (DBP), di-2-ethylhexyl (DEHP) and butylbenzyl phthalate] to interfere with reproduction and development by an endocrine-mediated process. In general, phthalate esters are found in many different environmental media, but the concentrations of individual esters are low. Regulatory action on the presence of certain phthalate esters in toys for infants was prompted at least in part by concern over potential endocrine activity (European Commission, 1999). In general, standard assays of prenatal development in rats (where rat dams are treated from gestation days (GD) 6–15; i.e. not the major period for the development of the reproductive system) have only shown a teratogenic response at high dose levels (~ 1 g/kg per day) characterized by an increase in cleft palate in the presence of marked maternal toxicity (Ema *et al.*, 1993). DBP has been reported as showing oestrogenic activity in some in-vitro assay systems (Jobling *et al.*, 1995; Zacharewski *et al.*, 1998), but not others (reviewed by Andersen *et al.*, 1999). The major focus of this review will use DBP as a representative example of those esters that do exhibit adverse effects on reproductive development. DBP is used predominantly as a plasticizer in PVC products, as a solvent in inks used in food packaging, and in certain personal care products (e.g. nail polish). A recent review (Blount *et al.*, 2000) showed DBP to be the phthalate ester associated with reproductive effects most commonly found in the urine of women of childbearing age in this sample of the general US population. Further analysis of these data (Kohn *et al.*, 2000) estimated the maximal human exposure in this group to be 113 μg/kg bodyweight per day.

Table I. Summary of effects noted on reproduction and development following dietary di-n-butyl phthalate (DBP) administration (66 to 650 mg/kg per day) using a reproductive assessment by continuous breeding protocol[a]

Effect noted	F_0 generation	F_1 generation
Decrease in fertility	–	+
Decrease in litter size (in fertile animals)	+	+
Decrease in testes weight (+ histopathology)	–	+
Decrease in pup weight	+	+
Decrease in sperm count	–	+
Cryptorchidism	–	+
Male reproductive tract malformations (epididymis, external genitalia)	–	+
Female reproductive tract weight (and histopathology)	–	–
Oestrous cyclicity	–	–

[a]Data adapted from NTP (1991) and Wine *et al*. (1997).

Table II. Summary of effects and dose–response relationships for male reproductive development following in-utero exposure (gestation days 12–21) to di-n-butyl phthalate (DBP)[a]

Dose level (mg/kg per day)	Major effects noted
⩾500	Leydig cell adenoma and hyperplasia; reproductive tract malformations; testicular injury
⩾250	Reproductive tract malformations; Leydig cell hyperplasia; decreases in anogenital distance; delays in preputial separation; retained thoracic nipples; testicular injury
100	Retained thoracic nipples; small delay in preputial separation
<50	No significant effects

[a]Data from Mylchreest *et al*. (1998, 1999a, 2000).

Effects on rodent male reproduction

Phthalate esters exhibit marked structure–activity relationships for the induction of testicular toxicity in pubertal rats (Foster *et al.*, 1980), with only esters of a specific chain being able to induce toxicity. Even within structural isomers, butyl phthalates also have specific structure–function relationships (Foster *et al*., 1981, 1982b). Phthalate-induced testicular toxicity appears preferentially targeted to the Sertoli cell (Foster *et al*., 1982a), and occurs rapidly (6–12 h) after high-dose, acute exposure. In-vitro studies have shown that phthalate monoesters (the major metabolites), but not the diesters, elicit similar morphological testicular responses to those observed *in vivo* (Gray and Beamand, 1984). Other studies have provided evidence that an interference with FSH action in the pubertal testis is one component of the mechanism of toxicity (Lloyd and Foster, 1988; Heindel and Chapin, 1989; Heindel and Powell, 1992). There is an age-dependency in response to testicular toxicity, with adults generally being less sensitive to the effects of phthalate esters than the young pubertal counterparts (Sjoberg *et al*., 1986). In general, conventional multigeneration reproduction studies in rodents are lacking for the active phthalate esters, but one study using DBP indicated a number of important features [National Toxicology Program (NTP), 1991; Wine *et al*., 1997]. Sprague-Dawley rats were administered DBP in the diet (dose levels up to ~650 mg/kg per day) in a continuous breeding protocol which indicated no adverse responses on fertility or litter size in the F_0 generation. However, F_1 animals, when exposed to the same dose levels, showed marked decreases in fertility at the highest dose level tested (only one out of 20 pairings resulted in a litter), and increases in the incidence of reproductive tract malformations (with single incidences observed at the lowest dose level) especially of the epididymis and penis. These results are summarized in Table I.

Effects on male reproductive development

A series of studies (Mylchreest *et al*., 1998, 1999a, 2000) was conducted to examine further the effects noted in the NTP study. These studies also employed Sprague-Dawley rats, but used gavage rather than dietary administration over either a gestational and lactational exposure, or just late gestation [gestation days (GD) 12–21, the period of major development for the male reproductive system in the rat]. Pregnant animals were allowed to litter their offspring, and observation on the animals' androgen status were made at birth (anogenital distance), pre-weaning (retention of thoracic nipples), puberty (preputial separation) and adulthood (~100 days of age; gross morphology of reproductive tract and histopathology). Dose levels selected were based on the NTP study and ranged from 100 to 750 mg/kg per day. The effects observed, both in incidence and severity, were very similar to those observed in the NTP study, despite differences in the dosing regimen. The

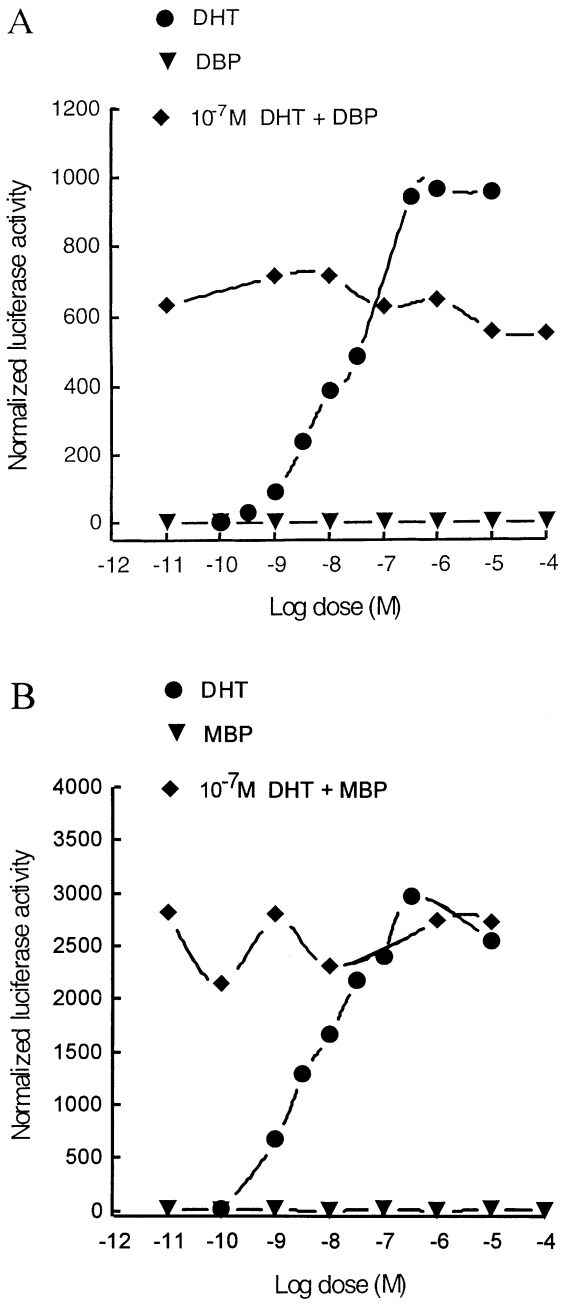

Figure 1. Effect of (**A**) di-n-butyl phthalate (DBP) or (**B**) mono-n-butyl phthalate (MBP) on luciferase transcriptional activation in a HepG2 human androgen receptor reporter gene assay. Values represent mean values from three separate experiments at each concentration employed. Standard deviations were <10% of the mean.

most marked effect was on epididymal development, where a significant number of animals had either a major malformation of the epididymis, or it was absent. Other effects on male reproductive development included an increased incidence of hypospadias, cryptorchidism, a decrease in anogenital distance, delayed preputial separation, retention of thoracic nipples, and testicular lesions. The latter were typified by seminiferous tubular atrophy, Leydig cell hyperplasia and, at the highest dose level used in the late gestational study (500 mg/kg per day), a low incidence of Leydig cell adenoma. Adverse responses were noted at even the lowest dose level employed (100 mg/kg per day), which prompted the initiation of a further dose–response study (Mylchreest *et al.*, 2000) in which DBP was given during late gestation at levels ranging from 0.5 to 500 mg/kg per day. This study used a larger number of litters and essentially reproduced the initial studies with the establishment of a no-observed-adverse-effect-level (NOAEL) at 50 mg/kg per day. The dose–response relationships for the various end points evaluated in these studies are indicated in Table II. Similar effects to those noted above for DBP have been confirmed and also noted for DEHP (Gray *et al.*, 1999).

DBP as an antiandrogen

The pattern of response obtained with DBP and DEHP (Gray *et al.*, 1999; Mylchreest *et al.*, 1999a) was indicative of an antiandrogenic mode of action (since significant effects were noted on androgen-dependent sexual differentiation of the reproductive tract, particularly Wolffian duct differentiation). No adverse findings indicative of an adverse oestrogenic response have been noted for any phthalate ester *in vivo* (Wine *et al.*, 1997; Mylchreest *et al.*, 1998; Zacharewski *et al.*, 1998; Gray *et al.*, 1999). The studies with DBP and DEHP not only indicated effects on testosterone-mediated reproductive development, but also showed that dihydrotestosterone-dependent end points were also perturbed (external genitalia, anogenital distance, retention of thoracic nipples). However, whilst this pattern was consistent with these agents being antiandrogens, there was a marked difference in response between these agents and the classical androgen receptor (AR) antagonist, flutamide. In particular, flutamide targets at low dose levels the development of the prostate, but is much less active on the developing epididymis (Imperato-McGinley *et al.*, 1992), whereas the reverse is true for the phthalate esters. This response prompted studies of the actions of DBP and DEHP and their major metabolites [the corresponding monoesters mono-n-butyl phthalate (MBP) and mono-2-ethylhexyl phthalate (MEHP)] in a human AR transcriptional activation assay described previously (Maness *et al.*, 1998). These assays showed that, unlike flutamide, neither DBP (Figure 1) nor DEHP nor their metabolites were able to interact directly with the human androgen receptor *in vitro*.

A number of the members of the phthalate ester class have been shown to interact with the peroxisome proliferator activated receptor α (PPARα), and significant interest has been focused on whether the effects noted above for DBP and DEHP can be attributed to this potential mechanism. If such a mechanism could be sustained, it would have important implications for human risk assessment, since this receptor is not believed to be of importance in inducing phthalate-mediated hepatic toxicity in humans. A number of studies have indicated that, for at least one member of the class (DEHP), PPARα is essential for the induction of hepatic changes in rodents, since no effects were observed in the PPARα-knockout mouse. However, in PPARα-knockout mice exposed to DEHP, both testicular toxicity (Ward *et al.*, 1998) and developmental effects (Peters *et al.*, 1997) typical of DEHP were observed, demonstrating that interaction with PPARα was not involved in the mechanism for effects on reproductive development.

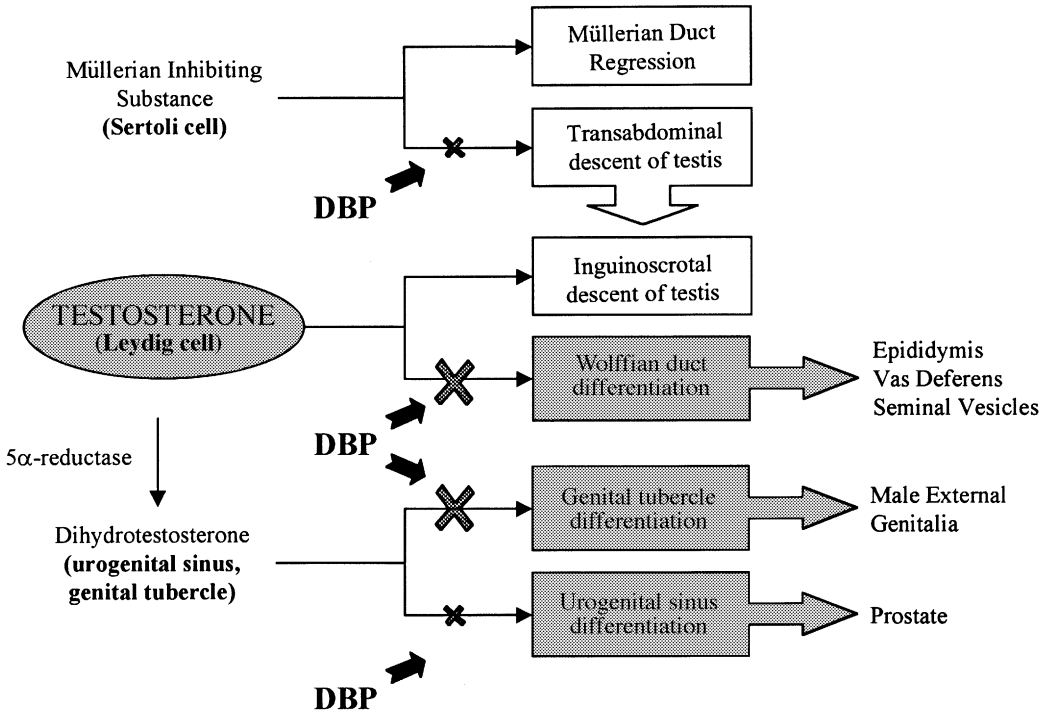

Figure 2. Schematic representation of the effects of di-n-butyl phthalate (DBP) on male reproductive development in the rat.

Early changes in the fetal testis

Studies on early fetal effects of DBP have indicated that on GD 18, morphological changes can be observed in the testis, characterized by an apparent proliferation of Leydig cells, with multinuclear gonocytes found in the seminiferous cords (Mylchreest *et al.*, 1999b). This study also indicated that fetal testicular testosterone was also significantly reduced on GD 18 and 21, and that the Leydig cells undergoing hyperplasia were both AR- and 3β-hydroxysteroid dehydrogenase-positive by immunocytochemistry. AR localization appeared decreased in the ducts of the developing epididymis. Taken together, these changes would suggest that a primary androgen insufficiency coupled with a decreased expression of the AR is responsible for the failure of the epididymis to develop in DBP-treated fetuses. Interestingly, immunostaining for proliferating cell nuclear antigen (PCNA) indicated normal presence in the fetal Sertoli cells, but Leydig cells were not immunopositive. Moreover, the gonocytes in control fetuses exhibited very limited staining, whereas those from DBP-treated animals were markedly immunopositive. These data would suggest that the observation of multinuclear gonocytes in DBP treated fetal testes was due to an inappropriate initiation of cell division, whilst the observed increase in Leydig cell number may be due to a compensatory mechanism as a result of lowered fetal testicular testosterone levels. We also postulate that the lack of a PCNA response may be due to a failure of normal apoptosis, rather than a major proliferative event.

Conclusions

DBP produces marked effects on the development of the rat male reproductive tract, with the epididymis being the most sensitive to the induction of malformations. The initial event underlying the induction of malformations appears to be a decrease in fetal testicular testosterone concentrations. Associated with this is an apparent proliferation of fetal testicular Leydig cells that may be associated with the Leydig cell hyperplasia and tumours found in adult animals that have been exposed *in utero*. Studies in older animals exposed to phthalate esters have indicated that the Sertoli cell is the likely testicular target (Foster *et al.*, 1982a). Although the effects on gonocytes may be due to an indirect Sertoli cell effect, there were no indications of retained Müllerian structures in the male fetuses exposed *in utero* to DBP, i.e. the secretion of Müllerian inhibiting substance (MIS) by the fetal Sertoli cells did not appear affected. However, testicular descent is also believe to be under the control MIS (Hutson *et al.*, 1994) and a small number of animals have exhibited cryptorchidism following DBP and other phthalate treatments (Imajima *et al.*, 1997) during the critical windows for male reproductive development. The major structures targeted by in-utero exposure to DBP which are consistent with an antiandrogenic mode of action, not mediated directly via the AR, are summarized in Figure 2. A NOAEL for reproductive development was established for DBP at 50 mg/kg per day. This is significantly higher than our current estimates for the general population (maximum of 113 μg/kg per day; Kohn *et al.*, 2000). However, similar types of data on potential worker exposure or estimates of risk from aggregate phthalate exposure are not available.

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