In utero exposure to di(n-butyl) phthalate and testicular dysgenesis

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Disorders of male reproductive health are common and perhaps increasing in the Western world (Sharpe and Skakkebak 2003; Toppoli et al. 1996). These include cryptorchidism and hypospadias, which present at birth, and low sperm counts and testicular germ cell cancer, which manifest in young adulthood. Evidence suggests that these disorders may have a common origin in fetal life (Sharpe and Skakkebak 2003; Skakkebak et al. 2001). Based on this evidence, these disorders have been hypothesized to constitute a testicular dysgenesis syndrome (TDS), which results from abnormal development of the testis and consequent malfunction of the Leydig cells (LC) and/or Sertoli cells (SC) during male sexual differentiation (Sharpe and Skakkebak 2003; Skakkebak et al. 2001).

Certain phthalate esters such as di(n-butyl) phthalate (DBP) have been shown in rats to interfere with normal development of the testis and reproductive tract when exposure occurs during gestation (Barlow and Foster 2003; Ema et al. 1998, 2000; Fisher et al. 2003; Gray et al. 1999; Mylchreest et al. 1998, 1999, 2000), resulting in postnatal downstream disorders that are similar to those reported in human TDS, that is, cryptorchidism, hypospadias, impaired spermatogenesis, and reduced male fertility. Fetal exposure of male rats to DBP therefore has potential as an animal model in which to explore the events in the fetal testis that give rise to TDS (Fisher et al. 2003). In searching for such mechanisms, various phthalate-induced changes in the fetal testis have been reported, namely the occurrence of multinucleated gonocytes (MNGs), abnormal LC aggregation, decreased testicular testosterone levels, and decreased insulin-like factor 3 mRNA/protein expression (Fisher et al. 2003; Mahood et al. 2005; McKinnell et al. 2005; Parks et al. 2000; Shultz et al. 2000; Wilson et al. 2004). We have also shown that in utero exposure of rats to 500 mg/kg DBP causes postnatal development of focal areas of dysgenesis, comprising malformed seminiferous cords/tubules with intracordal/intratubular LC and immature SC, within otherwise normal testes that exhibit complete spermatogenesis (Fisher et al. 2003; Mahood et al. 2005, 2006), similar changes have been reported in TDS (testicular cancer) patients (Hoei-Hansen et al. 2003; Sharpe 2006).

The similarity between DBP-induced TDS-like disorders in rats and the relatively high prevalence of TDS disorders in humans has raised the question of whether DBP, or other phthalates, might contribute causally to human TDS. This possibility arises because of the widespread exposure of humans to DBP and other phthalates (Hauser et al. 2004; Rudel et al. 2003; Schettler 2006), although there is only one piece of published evidence to indicate that phthalate exposure during pregnancy might cause changes in the male offspring that are analogous to those reported in rats (Swan et al. 2005). An important issue in this debate is that the lowest doses (50–100 mg/kg/day) of DBP, or other phthalates, that have been shown to cause abnormal changes in rats, namely reduced anogenital distance (AGD) and nipple retention (Mylchreest et al. 2000; Zhang et al. 2004), are considerably higher than reported exposure levels in the human population (Hauser and Calafat 2005; Koch et al. 2003; Silva et al. 2004). Additionally, most of the end points evaluated in rat studies, such as AGD (Barlow and Foster 2003; Barlow et al. 2004; Caruthers and Foster 2005; Barlow et al. 2004; Caruthers and Foster 2005; Mylchreest et al. 1999, 2000) are difficult to relate directly to TDS in humans.

Therefore, the aim of the present study was to evaluate end points in our in vivo rat model of TDS that can be related more obviously to similar TDS disorders in humans, and to explore the DBP dose sensitivity of these end points. It was also important that all of these end points could be quantifiable to enable objective measurement. The end points that we chose included actual end points of TDS (infertility, cryptorchidism), together with indicators within the testis of dysgenesis (occurrence of dysgenetic areas, MNGs, and abnormal LC aggregation). Other fetal end points, such as testis weight and testicular testosterone levels, were also evaluated. Where relevant, these end points were investigated in...
both fetal and postnatal life, and four different doses of DBP (4, 20, 100, and 500 mg/kg/day) were used. Our results show that it is the fetal end points that are the most sensitive to DBP action.

Materials and Methods

Animals, treatments, sample collection, and processing. Wistar rats were maintained in our own animal facility according to UK Home Office guidelines [Animal (Scientific Procedures) Act 1986], and were fed a soy-free breeding diet (SDS, Dundee, Scotland). Time-mated females [day of vaginal plug = gestation day (GD) 0.5] were treated from GD13.5 to either GD20.5 (fetal samples) or GD13.5 to either GD20.5 (postnatal tissue) with either 0 (control), 4, 20, 100, or 500 mg/kg DBP (Sigma, Dorset, UK) in 1 mL/kg corn oil administered daily by oral gavage. The DBP was 99% pure according to the supplier. Corn oil was obtained from a supermarket and was used as obtained.

Fetal samples. Control and DBP-treated pregnant dams were killed by inhalation of carbon dioxide followed by cervical dislocation on GD21.5. Fetuses were removed, decapitated, and placed in ice-cold phosphate-buffered saline (PBS; Sigma). Testes were removed via microdissection, fixed for 1 hr in Bouins fixative, and then transferred to 70% ethanol. Fixed testes were weighed and then processed into paraffin wax using standard methods. One or more male fetuses from each litter were subsequently used for the quantitative and immunohistochemical studies described below; selection of fetuses for further study was random.

Adult samples. Male rats 90 days of age were killed by inhalation of CO₂ followed by cervical dislocation. Testes were carefully inspected for normality of the epididymis and vas deferens and then removed, weighed, and fixed for 5–6 hr in Bouins fixative, and then transferred into 70% ethanol. Testes were halved after approximately 3 hr fixation to aid penetration of the fixative. Testes were then further cut into four to eight blocks, depending on size, and embedded in paraffin as described above. At necropsy, testicular position was classified as high abdominal (at level of the kidney), mid-abdominal, inguinal, or scrotal, which enabled classification of testes into cryptorchid or scrotal groups. In controls, all testes were scrotal in position. Individual testes from adult animals that exhibited any gross epididymal lesions were excluded from histologic analysis to avoid possible confounding effects of this change on testicular morphology (Barlow and Foster 2003; Mylchreest et al. 1998); this applied to two testes from a total of 13 males. Before dissection, the adult male rats underwent a fertility test. This involved each male rat being housed singly for 1 week with a female rat of proven fertility. Males were classed as fertile if offspring were produced.

For the studies above, animals were treated humanely and with regard for alleviation of suffering. All studies were performed according to the Animal (Scientific Procedures) Act (1986) under Project Licence approval by the UK Home Office.

Testicular testosterone analysis. Testicular testosterone levels were measured by radioimmunoassay, as described previously (Fisher et al. 2003), on GD21.5 in individual testes from males from four to six litters per treatment group. After dissection, testes were snap frozen on dry ice and stored at −70°C before analysis. Testes were defrosted and homogenized individually in 0.5 mL PBS; an aliquot of this solution was then extracted with 2 mL diethyl ether, shaken for 5 min, and then placed in a bath of methanol cooled with dry ice. The nonaqueous portion of the extract was then decanted, dried overnight in a fume hood, and reconstituted in assay buffer. The limit of detection of the assay was 40 pg/testis.

Immunohistochemistry. Specific proteins were detected by immunohistochemistry using methods that have been described previously (Fisher et al. 2003). Sections (5 μm) were mounted onto coated slides (BDH Chemicals, Poole, UK), dewaxed, and rehydrated. Slides were incubated in 3% (vol/vol) hydrogen peroxide in methanol to block endogenous peroxidase activity, then washed in Tris-buffered saline (TBS: 0.05 M Tris, 0.85% NaCl, pH 7.4 at room temperature). Non-specific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (Sigma). Sections were incubated with the primary antibodies 3β-hydroxysteroid dehydrogenase (3β-HSD; 1:4000; gift from I. Mason, Edinburgh, UK) or smooth muscle actin (SMA, 1:2000; Sigma) overnight at 4°C. Specific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% bovine serum albumin (Sigma). Sections were then incubated for 1 hr in Tris-buffered saline (0.05 M Tris, 0.85% NaCl, pH 7.4 at room temperature). Sections were then incubated with the appropriate secondary antibody conjugated to biotin, at a dilution of 1:500 (rabbit anti-mouse or swine anti-rabbit; DAKO, Cambridgeshire, UK). The biotinylated antibody was linked to horseradish peroxidase (HRP) by 30-min incubation with avidin-biotin–HRP complex (ABC-HRP; DAKO). Antibody localization was detected by application of diaminobenzidine (liquid DAB; DAKO) until staining in control sections was optimal; the reaction was stopped by immersing slides in distilled water. Slides were counterstained with hematoxylin, dehydrated, and mounted using Pertex mounting media (Cell Path; Hemel Hempstead, UK).

Analysis of MNGs. We identified MNGs using routine blue staining of GD21.5 testis sections from one or more testes from animals selected at random from five to nine litters per treatment group. Slides were dewaxed and rehydrated as for immunohistochemistry. The toluidine blue stain (BDH Chemicals) was filtered and applied to slides at a 50% dilution with distilled water. Once staining was optimal the slides were immersed in distilled water and then dehydrated and mounted. To analyze the occurrence of MNGs, we used an Olympus BH-2 microscope (Olympus Optical, London, UK) fitted with a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). One complete testis cross-section from each animal was analyzed, and the percentage of seminiferous cord cross-sections that contained one or more MNGs was recorded. We did not record the number of MNGs per cord or the number of nuclei per MNG. The analyzer was blinded with respect to the treatment status of animals.

LC Cluster analysis. LC aggregation was objectively quantified using previously described methods (Mahood et al. 2005). Testes from one or more fetuses per litter at GD21.5, from five to six litters per treatment group, were randomly selected and serially sectioned. DBP treatment results in unequal distribution of LC throughout the testis due to their abnormal migration/aggregation into more central regions (Mahood et al. 2005). To avoid sampling errors because of this unequal distribution, we selected three sections per testis that corresponded to uniform intervals through the serially sectioned testis (25, 50, and 75% intervals, which were thus 20–30 sections apart from each other); these sections were immunostained for 3β-HSD as described above.

Quantification of LC clustering in these sections was undertaken using Image-Pro Plus 4.5.1 software and equipment (Media Cybernetics UK, Wokingham, Berkshire, UK). Specimens immunostained for 3β-HSD were of sufficient homogeneity, high contrast, and low background to allow computer-assisted thresholding and subsequent computer-assisted counting of LC (3β-HSD–immunopositive) clusters and determination of LC cluster area. Digital images of complete testis sections were captured at ×40 magnification. The software was used to trace around each section, creating an area of interest; the area of each section could then be calculated. Computer-assisted thresholding was then used to identify and analyze clusters of 3β-HSD–immunopositive cells, generating data on cluster number and the proportion of each section occupied by LC clusters, as described in detail elsewhere (Mahood et al. 2005). To take account of the decrease in LC size in DBP-exposed males (Mahood et al. 2005), each LC cluster was expressed as a percentage of the total LC cluster area in each animal. Clusters were then arbitrarily assigned to one of three groups: small clusters, accounting for ≤5% of the total
LC cluster area per testis; medium clusters, accounting for 5.1–14.9%; and large clusters, which individually accounted for a 15% of the total LC cluster area per testis.

Incidence of dysgenesis. We assessed the incidence of dysgenesis in each of the treatment groups in adult male rats by visual analysis of SMA immunostained sections from four to eight blocks per testis for each animal. Sections were checked for the occurrence of Sertoli cell–only (SCO) tubules and focal dysgenic areas in each testis (cryptorchid and scrotal). Testes were obtained from one or more animals from at least five separate litters from each treatment group.

Image capture. Images were examined and photographed using a Provis microscope (Olympus Optical, London, UK) fitted with a Kodak DCS330 digital camera (Eastman Kodak, Rochester, NY, USA). Images were compiled using Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA, USA).

Statistical analysis. We used data for each of the fetal end points and for adult testis weight to derive a mean value for each litter; litter means ± SE were then computed and used for statistical analyses. Data were analyzed by one-way analysis of variance followed by the Bonferroni post-test. The incidence of infertility, cryptorchidism, SCO tubules, and focal dysgenic areas in adult animals was analyzed using Fisher’s exact test. For the latter datasets, we analyzed both data from individual animals and litter means, in view of the wide variation (0–100%) in incidence between different litters for some of the measured end points. Data for LC cluster number per testis were log transformed before statistical analysis to normalize variances. All analyses were performed using GraphPad Prism (version 4; GraphPad Software Inc., San Diego, CA, USA).

Results

Infertility, cryptorchidism, testis weight, and testicular testosterone. In adult animals prenatally exposed to 500 mg/kg DBP, we found a 75% infertility rate, dropping to 33% in the 100-mg/kg group and to 14% in the 20-mg/kg group. However, only the 500 mg/kg DBP treatment group had significantly reduced fertility compared with the controls when data were analyzed for individual animals (Figure 1A) or when analyzed using incidence of infertility per litter (p = 0.03). The 500-mg/kg DBP treatment group was also the only treatment group to have a significantly elevated incidence of cryptorchidism compared with the control group, with 90% of animals exhibiting either unilateral or bilateral cryptorchidism compared with no animals in the control group (Figure 1B); this difference was equally evident when data were analyzed as incidence of cryptorchidism per litter (p = 0.005). In all other treatment groups, we found only one case of cryptorchidism (unilateral), and this was in the group exposed to 100 mg/kg DBP. Exposure to 500 mg/kg DBP during gestation resulted in a significant decrease in testis weight compared with control animals both at GD21.5 and in adulthood (Figure 2A,B), although the reduction in adulthood was attributable solely to the high incidence of cryptorchid testes (Figure 2C). Animals exposed to 4, 20, or 100 mg/kg DBP did not show any significant change in testis weight at either GD21.5 (Figure 2A) or in adulthood (Figure 2B). Testicular testosterone levels in GD21.5 animals were significantly decreased in both the 500- and 100-mg/kg DBP treatment groups compared with control values (Figure 3). We found no significant effect of DBP treatment on fetal body weight or on litter size (data not shown).

Occurrence of MNGs at GD21.5. All treatment groups, including the control group, had MNGs within the seminiferous cords at GD21.5 (Figure 4A,B). Prenatal exposure to either 500 or 100 mg/kg DBP resulted in a significant increase in the occurrence of MNGs compared with controls; also,
we found an increase in the occurrence of MNGs in animals exposed to 20 mg/kg DBP (Figure 4C), although it was not statistically significant.

**LC clustering/aggregation at GD21.5.** Changes in LC distribution were obvious in both the 100- and 500-mg/kg DBP treatment groups (Figure 5D,E) compared with controls (Figure 5A); these changes were most pronounced in the highest dose group, with large LC clusters being evident in the center of the testes (Figure 5E). LC distribution in testis sections from the 4- and 20-mg/kg treatment groups were not obviously different from controls (Figures 5B,C). Objective analysis of LC aggregation revealed a significant decrease in total LC cluster number per testis section in animals exposed to either 100 or 500 mg/kg DBP (Figure 6A). This pattern was similarly reflected in the data for the percentage of total cluster area accounted for by small clusters (Figure 6B). LC clusters of medium size were evident in all treatment groups but were only significantly increased above control values in the 100-mg/kg DBP group (Figure 6C). The occurrence of large LC clusters was evident only in groups of males exposed to 20, 100, or 500 mg/kg DBP (Figure 6D). However, compared with control animals, the number of large LC clusters was significantly increased only in the 500-mg/kg group, in which approximately 40% of the total LC cluster area was accounted for by clusters of this size (Figure 6D).

**Incidence of focal dysgenesis in adulthood.** Visual analysis of SMA-immunostained testis sections from each treatment group revealed that all cryptorchid testes examined from the 500-and 100-mg/kg DBP groups had SCO tubules present (Table 1). Of the 11 cryptorchid testes examined in the 500-mg/kg DBP group, 7 had one or more focal dysgenetic areas. In testes from control animals, we found neither SCO tubules nor areas of focal dysgenesis (Figure 7A). SCO tubules were found in all DBP-exposed groups except the 4 mg/kg treatment group (Table 1). However, not all testes examined in each group had SCO tubules present. Interestingly, we found that a similar number of testes in the two highest dose groups (500 and 100 mg/kg) had SCO tubules (~66% of testes examined); this incidence was statistically significant when data were analyzed for individual animals (Table 1) or based on the incidence per litter (i.e., any animal per litter exhibiting SCO tubules; \( p = 0.04 \)). Focal dysgenetic areas were detected only in the 500- and 100-mg/kg DBP animals (Figure 7B,C); 55% of testes examined in the 500-mg/kg group and approximately 33% in the 100-mg/kg dose group had areas of dysgenesis present. However, only the incidence of dysgenetic areas in the 500-mg/kg DBP group achieved statistical significance when analyzed for individual animals (Table 1); this was of borderline significance when evaluated per litter (\( p = 0.05 \)).

**Discussion**

We and others have shown that fetal exposure of male rats to DBP, or certain other phthalates, results in a high incidence of disorders such as cryptorchidism, hypospadias, and infertility (Barlow and Foster 2003; Mylchreest et al. 1998, 2000; Fisher et al. 2003; Gray et al. 1999; Mylchreest et al. 1998, 1999, 2000). These disorders are collectively similar to those reported in human TDS patients, and as such, the focus of the present study was to investigate DBP-induced changes in fetal life and in adulthood that were considered relevant to TDS. To date, studies investigating the effects of DBP on male reproductive development have used end points that may be relevant to human TDS (occurrence of cryptorchidism, decreased fertility), or that are not relevant (nipple retention in male rats), or that are presently of uncertain relevance (decreased AGD) (Barlow and Foster 2003; Barlow et al. 2004; Carruthers and Foster 2005; Mylchreest et al. 1998, 2000; Mylchreest et al. 1998, 1999, 2000; Zhang et al. 2004). Similarly, changes in gene and protein expression after DBP exposure, which have been found to be more sensitive to the effects of DBP (Lehmann et al. 2004), cannot be related directly to TDS other than when the changes relate to expression of genes involved in LC hormone production. These include reduction in the expression of genes and proteins involved in cholesterol transport and steroidogenesis leading to a concomitant...
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The percentage occurrence of small (B), medium (C), and large (D) LC clusters are shown for each treatment. Figure 7. Photomicrographs of scrotal testes from adult rats exposed in utero to (A) corn oil (control), or to DBP doses of (B) 500 or (C) 100 mg/kg/day DBP. Although the majority of each cross-section of scrotal testes in DBP-exposed animals had normal seminiferous tubules, some animals exhibited one or more focal dysgenetic areas comprising malformed seminiferous tubules as visualized by immunostaining for SMA to identify peritubular myoid cells. Note the occurrence of unusually large numbers of LCs surrounding the malformed tubules (B,C). Bar = 100 µm.

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SCO tubules formed in the testes of DBP-exposed rats (Mahood et al. 2005, 2006). This model hypothesizes that DBP-induced abnormal migration and aggregation of LCs within the fetal testis “traps” isolated SC, gonocytes, and presumably peritubular myoid cells within them (Fisher et al. 2003). Postnatally, after cessation of DBP treatment, these non-segregated clusters of cells then try to form seminiferous cords; we propose that this process results in the formation of dysgenetic areas focally within the testes, surrounded by otherwise normal tissue with complete spermatogenesis (in scrotal testes). The results from the present dose–response study support our proposed model because dysgenetic areas were seen in adulthood following fetal exposure to either 100 or 500 mg/kg/day DBP, and it is at these doses that abnormal LC aggregation was induced in fetal life. The dose-dependent nature of fetal LC aggregation was grossly evident in the testicular images and it is at these doses that abnormal LC cessation of DBP treatment, these non-MNGs occur throughout the fetal testis and they (Fisher et al. 2003). Postnatally, after exposure to di-n-butyl phthalate during late gestation in rats. Toxicol Lett 98:87–92; doi:10.1016/S0378-4274(99)00107-8.


Fisher JS, MacPherson S, Marchetti N, Sharpe RM. 2003. Human testicular dysgenesis is an indirect measure of such effects. Similarly, the occurrence of abnormal LC aggregation in the fetal testis is potentially of direct evidence to adult testis function, as our evidence MNGs was also observed at GD21.5 in the 100 mg/kg and the 500 mg/kg DBP dose groups (Figure 4), although it is still unknown if these abnormal germ cells contribute to the dysgenetic phenotype of the adult DBP-exposed animals and what their precise relevance, if any, is to human TDS (Ferrara et al. 2006). The cause of MNGs is unknown, but it is not due to abnormal/incomplete cell division because it occurs at ages when the germ cells are quiescent and not dividing (GD19.5–20.5; Ferrara et al. 2006); we have confirmed this using a number of different proliferation markers (Ferrara et al. 2006). It seems likely that the mechanisms underlying the induction of MNGs are separate from those involved in the formation of focal dysgenetic areas, because MNGs occur throughout the fetal testis and are equally evident in normally formed seminiferous cords as in areas destined to form dysgenetic areas (Fisher et al. 2003).

A dose–response study investigating changes in gene and protein expression in the fetal testis established a dose of DBP of 50 mg/kg/day as the lowest observed adverse effect level (LOAEL), and 10 mg/kg/day as the no observed adverse effect level (NOAEL) (Lehmann et al. 2004). In the present study, we did not use a dose of 50 mg/kg/day, but we have established a LOAEL of 100 mg/kg/day for fetal end points and a NOAEL of 20 mg/kg/day, although there does appear to be a clear trend towards effects on the occurrence of MNGs at a dose of 20 mg/kg/day DBP; this may have reached statistical significance if more litters had been studied. Taken together, the available data suggests that doses of 20–50 mg/kg/day DBP may cause changes in fetal rat testis development that are of likely relevance to the postulated origins of human TDS disorders (i.e., disruption of fetal testis organization/function). These levels of exposure are still much higher than the suggested environmental exposure levels of the human population (Hauser and Calafat 2005; Koch et al. 2003; Silva et al. 2004). Although this contrast suggests, at face value, that the human male fetus is at low risk from exposure to DBP alone, it is important to consider the combined risk of exposure to DBP and other phthalates that can exert similar effects (e.g., diethylhexyl phthalate), or indeed other endocrine-disrupting chemicals. Recent studies have reported that pairs of phthalates (Borch et al. 2004) or combination of a phthalate with another anti-androgen with a different mode of action (Hochkiss et al. 2004) produce dose-additive effects on male reproductive malformations in rats. Therefore, studies investigating the dose–response relationships and dose-additive effects of phthalates and other endocrine-disrupting chemicals on male reproductive development need to be a focus of future research in order to fully evaluate the risk of human exposure.

We hope that the new fetal end points described in the present study (occurrence of MNGs and abnormal LC aggregation), which can be objectively quantified, will prove a useful resource in future studies. It can be argued that the fetal occurrence of MNGs induced by DBP is irreproducible because it has to overcome direct consequences for the fetus (which does not reproduce) or the adult. However, because MNGs may reflect altered SC function in fetal life (Ferrara et al. 2006), and it is the number (and function) of the SC that predetermines sperm count and fertility in adulthood, MNGs may provide a sensitive (indirect) measure of such effects. Similarly, the occurrence of abnormal LC aggregation in the fetal testis is potentially of direct evidence to adult testis function, as our evidence suggests that it is an important cause of SCO tubules and focal dysgenetic areas in the testis in adulthood (Mahood et al. 2006). As the latter features are clearly relevant to human TDS (Hoei-Hansen et al. 2003; Sharpe 2006), it would be sensible to concentrate on such end points or to identify other fetal end points that have proven consequences that are detectable in adulthood.

References


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