Disorders of male reproductive health are common and perhaps increasing in the Western world (Sharpe and Skakkebaek 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 Skakkebaek 2003; Skakkebaek et al. 2001). Based on this evidence, these disorders have been hypothesized to constitute a testicular dysgenesis syndrome (TDS), including infertility, cryptorchidism, focal “dysgenetic areas,” and Sertoli cell–only tubules in the adult testis. Humans are widely exposed to DBP, but at much lower levels than those causing adverse effects in rats.

**Objectives:** The objective of this study was to evaluate end points affected by DBP action in rats in fetal and adult life that are relevant to human TDS, and to compare their dose sensitivity.

**Methods:** Pregnant rats were gavaged daily with corn oil (control) or with 4, 20, 100, or 500 mg/kg DBP. We examined adult end points of TDS (infertility, cryptorchidism) and indicators within the testis of dysgenesis (abnormal Leydig cell (LC) aggregation, multinucleated gonocytes (MNGs)), as well as conditions that may result from these indicators in adulthood (occurrence of focal dysgenetic areas). Fetal testis weight and testicular testosterone levels were also evaluated.

**Results:** The fetal end points analyzed (testicular testosterone levels, abnormal LC aggregation, occurrence of MNGs) were most sensitive to disruption by DBP, as all were significantly affected at a dose of 100 mg/kg/day DBP, with a trend toward effects occurring at 20 mg/kg/day DBP; adult end points were affected consistently only by 500 mg/kg/day DBP.

**Conclusions:** The fetal end points we evaluated can be objectively quantified and may prove helpful in evaluating the health risk of exposure to DBP and other phthalates, as well as identifying DBP-sensitive fetal events that have adult consequences/end points that are identifiable in human TDS.

**Keywords:** cryptorchidism, di(n-butyl) phthalate, dose response, dose sensitivity, dysgenetic areas, infertility, Leydig cell aggregation, male reproductive development, multinucleated gonocytes, testicular dysgenesis syndrome. *Environ Health Perspect* 115(suppl 1):55–61 (2007). doi:10.1289/ehp.9366 available via [http://dx.doi.org/](http://dx.doi.org/) (Online 8 June 2007)

This article is part of the monograph “Endocrine Disruptors—Exposure Assessment, Novel End Points, and Low-Dose and Mixture Effects.”

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We thank M. Fiskin for expert animal husbandry. This work was supported in part by grants QLK4-199-01422 and QLK4-CT-200-00603 from the European Union.

The authors declare they have no competing financial interests.

Received 22 May 2006; accepted 8 February 2007.
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 GD21.5 (postnatal tissue) with either 0 (controls), 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 terted daily by oral gavage. The DBP was 99% Dorset, UK) in 1 mL/kg corn oil adminis-

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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 dysgenetic 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 dysgenetic 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 6D). 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; Emaj 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; Emaj 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...
decrease in fetal testicular testosterone levels, as seen in the present study at doses ≥ 100 mg/kg DBP, and as has also been reported to occur at doses as low as 50 mg/kg/day DBP (Barlow et al. 2003; Lehmann et al. 2004; Shultz et al. 2001). The reduction in AGD and the retention of nipples in male rats exposed during gestation to DBP are presumed to be effects of DBP that are secondary to decreased testicular testosterone production (Fisher et al. 2003; Mahood et al. 2005; Mylchreest et al. 2002; Parks et al. 2000; Shultz et al. 2001). For this reason, and because dose–response studies using these end points are already well reported (Mylchreest et al. 1998, 1999, 2000; Zhang et al. 2004), we did not measure AGD or nipple retention in the present study. Instead, our aim was to evaluate objectively quantifiable end points that we considered of direct relevance to either the manifestation (adult end points) or origins (fetal end points) of TDS.

Our results indicate that it is the fetal end points analyzed (testicular testosterone levels, abnormal LC aggregation, occurrence of MNGs) that appear to be most sensitive to disruption by DBP. All three of these end points were affected significantly by a DBP dose of 100 mg/kg/day; it also appears that a dose of 20 mg/kg/day probably also has an effect on some of these parameters, although this did not achieve statistical significance. Only exposure to 500 mg/kg/day DBP resulted in a significant decrease in testis weight in fetal and adult life, but even in the latter, the observed decrease was secondary to the occurrence of cryptorchidism. Thus, segregation of adult testis weights into scrotal and cryptorchid testes in the 500-mg/kg dose group showed that the decrease in mean testis weights seen at this dose was due completely to the small size of the cryptorchid testes in these animals. In this regard, it is also notable that in the 500-mg/kg/day DBP treatment group, adult scrotal testes, which were normal in weight and largely normal for gross morphology and completeness of spermatogenesis, clearly exhibited focal abnormalities (focal dysgenesis, SCO tubules). The other postnatal end points investigated in this study (incidence of infertility and cryptorchidism) were significantly increased above control levels only in the 500 mg/kg/day group, indicating that these are insensitive end points to use when investigating the effects of lower dose DBP exposure in fetal life.

We have recently proposed a model to explain the origin of dysgenetic areas and.
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 (Figure 4), although this latter feature is clearly relevant to human male reproduction to di(n-butyl) phthalate (Fisher et al. 2006). In the present study, 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 2005). Abnormal Leydig cell aggregation in the fetal testis of male rats exposed to di(n-butyl) phthalate: an antiandrogenic effect (Ferrara et al. 2003). Pathogenesis of male reproductive malformations in the male rat. Toxicol Pathol 32:79–90; doi:10.1080/01926230390265894.

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