ABSTRACT

Di(\(n\)-butyl) phthalate (DBP) is commonly used in personal care products and as a plasticizer to soften consumer plastic products. Male rats exposed to DBP in utero have malformations of the male reproductive tract and testicular atrophy characterized by degeneration of seminiferous epithelium and decreased sperm production. In the fetal testis, in utero exposure to DBP reportedly resulted in reduced testosterone levels, Leydig cell aggregates, and multinucleated gonocytes (MNG). We investigated whether exposure in utero to DBP affects rat fetal Sertoli cells and compromises interactions between Sertoli and germ cells in the developing testis. Histological examination showed that MNG occurred at low frequency in the normal fetal rat testis. Exposure in utero at the dose level of DBP above estimated environmental or occupational human exposure levels significantly increased the number of these abnormal germ cells. Postnataally, MNG exhibited aberrant mitoses and were detected at the basal lamina. MNG were not apoptotic in the fetal and postnatal rat testes, as indicated by TUNEL. Sertoli cells in DBP-exposed fetal testis had retracted apical processes, altered organization of the vimentin cytoskeleton, and abnormal cell-cell contacts with gonocytes. The effect of DBP on Sertoli cell morphology at the level of light microscopy was reversed after birth and cessation of exposure. Our data indicate that fetal Sertoli cells are targeted by exposure in utero to DBP and suggest that abnormal interactions between Sertoli and germ cells during fetal life play a role in the development of MNG.

INTRODUCTION

Human male reproductive health problems such as subnormal semen quality, decline in sperm counts, testicular cancers, and congenital malformations of the reproductive tract are reportedly increasing in incidence and are believed to be manifestations of a condition known as testicular dysgenesis syndrome (TDS) [reviewed in Fisher [1]]. TDS disorders have been proposed to result from disrupted development of the male reproductive tract during fetal life [2, 3]. Data in support of this hypothesis came from studies that elucidated the embryonic origin of human germ cell cancer [4–6]. In animal models, fetal disruption of Sertoli and Leydig cell development by genetic manipulation or exposure to toxicants resulted in alterations in the male reproductive tract that resemble human TDS [7–10].

Phthalate esters represent a class of environmental endocrine-active chemicals known to disrupt development of the male reproductive tract in rodents [11–14]. Phthalates are widely used as softeners of consumer plastics and in solvents used in personal care, residential and commercial construction, and automotive products. Detectable levels of at least four phthalate metabolites were found in urine samples collected from the general U.S. population [15, 16]. One of the most significant findings in these studies was that women of reproductive age had higher compared to the general population concentrations of monobutyl phthalate (MBP), a monoester metabolite of di(\(n\)-butyl) phthalate (DBP).

In animal studies, DBP acted as a reproductive toxicant in the males but not females [12, 13]. The tissues, for which development depends on androgens, appeared to be most sensitive to DBP [12, 13]. Male rats exposed to DBP from Gestation Days (gd) 12 to 21 have reduced anogenital distance, retained thoracic nipples, malformed external genitalia, undescended testis, and testicular dysgenesis characterized by degeneration of seminiferous epithelium and decreased sperm production [12–14, 17]. Fetal and neonatal testes in rats exposed to DBP in utero also have multinucleated gonocytes (MNG), but DBP-exposed animals do not develop germ cell cancer [18]. The mechanism of MNG development remains undetermined. The phenotype induced by exposure in utero to DBP and other phthalates in rats resembles human manifestations of TDS and is similar, although not identical, to the phenotype induced by exposure in utero to chemicals that bind to the androgen receptor. DBP and its primary metabolite MBP do not interact with the androgen receptor [19]. Exposure to DBP in utero decreases the level of fetal testicular testosterone and alters expression of genes and proteins regulating steroidogenesis [20–22]. A decrease in fetal testosterone has been generally accepted as a mechanism by which exposure in utero to DBP disrupts development of testosterone- and dihydrotestosterone-dependent male reproductive organs and other tissues. Also, reduction in fetal testosterone indicative of abnormal function of fetal Leydig cells supports a link between altered development of the male reproductive tract during fetal life and the TDS-like phenotype of DBP-exposed adult male rats.

In young and adult rodents, exposure to DBP and other phthalates caused pathological and biochemical changes in the testis without affecting the level of testicular testosterone [23]. Although abnormal maturation and death of germ cells are the most common adverse responses in the rat testis exposed to phthalates, the Sertoli cell appears to be
a primary target for a toxic action of these chemicals [23–25]. In young rats, exposure to mono-(2-ethylhexyl) phthalate resulted in collapsed filaments of vimentin cytoskeleton in Sertoli cells and germ cell sloughing [24, 25]. In vitro, phthalates disrupt Sertoli-germ cell contacts in postnatal Sertoli-germ cell cocultures several hours after exposure [26] and alter signaling modulated by follicle-stimulating hormone in cultured Sertoli cells [27, 28].

Abnormal differentiation of Sertoli cells has been proposed to contribute to the development of the TDS-like phenotype in male rats in utero exposed to DBP [1, 7]. In these rats, the adult testes exhibited abnormal spermatogenesis and had Sertoli cells lacking expression of p27kip, a protein marker of mature differentiated Sertoli cells [7]. This finding suggested that DBP targets Sertoli cells in the fetal testis, although morphological or biochemical defects in these cells have not yet been identified. The objective of our study was to determine whether exposure to DBP in utero at the dose levels that reportedly caused gross morphological changes in the adult rat testis also altered morphology of fetal Sertoli cells and their interactions with gonocytes.

METHODS

Animals and Exposure

Timed-mated Sprague-Dawley rats were obtained from Charles River Laboratories, Inc. (Raleigh, NC) on gd 0, defined as the day that sperm were identified in the vagina. The animals were housed and treated according to federal guidelines for the care and use of laboratory animals. The dams were maintained on a standard 12L:12D cycle at 18–25°C and a relative humidity of 30–70%. Water and NIH-07 rodent chow were provided ad libitum. Naive and corn oil-treated dams were used as controls. Naïve and corn oil-treated dams were used as controls. Dams received corn oil or 500 mg kg⁻¹ day⁻¹ DBP (Sigma, http://www.sigma-aldrich.com) in corn oil by oral gavage. Gavage was performed from gd 12 to gd 16 through 20, and dams were killed by CO₂ on gd 17 through 21. Fetuses were removed by Caesarean section. Pups were from dams treated from gd 12 to 21 and were killed by CO₂ on Postnatal Days (pnd) 1, 2, and 5. Males were identified by opening the fetuses’ peritoneal cavities and examining internal sex organs under a dissecting microscope. In gd 17–19 fetuses, both testes were left in situ. In gd 20–21 fetuses and all pups, the left testis with epididymis was removed for Western analysis or fixed for electron microscopy as described here. Fetuses were fixed in modified Davidson fixative, which preserves morphological detail in the testis better than 10% neutral buffered formalin or Bouin fluid [29, 30]. Several gd 21 control and DBP-exposed fetuses were also fixed in 10% neutral buffered formalin, which preserves immunogenicity of vimentin better than modified Davidson fixative [30]. The fetuses were immersed in the fixative for 24 h and transferred to 70% ethanol until dissection. Fixed in situ testes were dissected, processed through graded alcohols, cleared in xylene, and embedded in paraffin. Sections of 5 μm were cut from paraffin-embedded tissues and used for hematoxylin-eosin staining, immunocytochemistry, and terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick labeling (TUNEL). Three corn oil- and three DBP-exposed litters and one testis per fetus or pup were examined at each gestational and postnatal time point. The numbers of fetuses and pups used for evaluations by different methods are shown in Table 1.

Immunodetection of Proteins and 5-Bromo-2-Deoxyuridine (BrdU)

For immunocytochemistry, sections were deparaffinized in three changes of xylene and rehydrated in a series of graded alcohol. Antigen retrieval was performed using DAKO Target Antigen Retrieval buffer (DAKO, http://dakouusa.com) in a decoocking chamber. Nonspecific antigens were blocked with serum from appropriate species from Vectastain Elite ABC systems (Vector Laboratories Inc., http://www.vectorlabs.com). Primary antibody (ab) against E-, P-, and N-cadherin and NCAM from Santa Cruz (Santa Cruz Biotechnology, http://www.scbt.com), and anti-vimentin ab from DAKO were used at 1:200 dilution for 1 h at room temperature. Slides were washed three times in PBS, and biotinylated secondary ab from appropriate species provided in Vectastain Elite ABC systems were applied for 1 h. After three washes in PBS, slides were incubated with streptavidin-conjugated Alexa Fluor fluorophores (Molecular Probes, http://www.probes.com) for 40 min at room temperature. Excessive fluorophores were removed by washing in PBS and slides coverslipped using Vectashield mounting media (Vector) with a counterstain (DAPI or propidium iodide). Controls to confirm ab specificity included preimmune sera and ab incubated with a corresponding immunizing peptide (4-fold excess) for 1 h at room temperature. Immunostaining for BrdU was conducted as previously described [30]. For PCNA detection, a PCNA staining kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s protocol. Staining was analyzed and recorded on a BX61 Olympus or confocal Zeiss LSM 510 META epifluorescence microscope. Western analysis was performed as described in Klymenova et al. [31] using anti-vimentin ab from Santa Cruz at 1:100 dilution.

TUNEL

TUNEL was performed using a ApopTag Red apoptosis detection kit (Chemicon International, http://www.chemicon.com) according to the manufacturer’s protocol for indirect detection using a fluorescent- or DAB-labeled antibody. Hematoxylin was used as counterstain for DAB-labeled slides. Fluorescence-labeled slides were coverslipped using “Hard Set” Vectashield mounting media (Vector) with DAPI as a counterstain.

Transmitting Electron Microscopy (TEM)

Dissected gd 21 testes were nicked and placed for 2 h in 0.15 M sodium phosphate, pH 7.4, containing 2% paraformaldehyde and 2.5% glutaraldehyde. After initial fixation, testes were cut into approximately 1 mm³ pieces and stored in fixative at 4°C until processing. The samples were postfixed in 0.15 M sodium phosphate buffer containing 1% osmium tetroxide and 1.25% potassium ferrocyanide, dehydrated, and embedded in Polybed 812 epoxy resin (Polysciences, http://www.polysciences.com). Representative areas were selected on toluidine blue-stained sections, and 70-nm sections were cut from these areas and collected on 200-mesh cop-
FIG. 1. Morphology of Sertoli and germ cells in the rat testis exposed to di(n-butyl) phthalate (DBP) in utero. Hematoxylin-eosin-stained sections of corn oil-exposed (A and C) and DBP-exposed (B and D) testes on Gestation Day (gd) 21 (A and B) and Postnatal Day (pnd) 1 (C and D). Thin arrows point to multinucleated gonocytes (MNG) present in both control and DBP-exposed fetal testes, the thick arrow indicates an interstitial cell cluster found only in DBP-exposed testes, and asterisks indicate seminiferous tubules with Sertoli cells exhibiting cytoplasmic changes, which were visible only in exposed testes. PCNA and 5-bromo-2-deoxyuridine (BrdU)-stained sections of DBP-exposed testes on gd 20 (E and F, respectively). TUNEL of pnd 1 and 2 testes exposed in utero to DBP (G and H, respectively). Solid arrows point on nonapoptotic MNG, and dashed arrows indicate TUNEL-positive morphologically normal gonocytes. Original magnification A and B, ×400; C and D, ×440; E, ×520; F–H, ×500.

FIG. 2. Dynamics of the number of tubular cross sections per testicular section in the normal and di(n-butyl) phthalate (DBP)-exposed fetal rat testes. Asterisks indicate statistical significance between control and treated groups. On each gestation and postnatal day, each group had three dams.

RESULTS

Exposure to DBP In Utero Affected Development of Fetal Rat Sertoli and Germ Cells

Histological analysis of gd 17–21 and pnd 1, 2, and 5 testes was performed on matching control and DBP-exposed hematoxylin-eosin-stained cross sections. No morphological changes were found in gd 17 DBP-exposed testes. Histopathologically, DBP-exposed testes were very similar from gd 18 to 21 and therefore are described together. In these testes, DBP-induced abnormalities consisted of cytoplasmic changes in Sertoli cells, clustering of gonocytes in the middle of the tubules, altered morphology of seminiferous tubules, and clusters of interstitial cells (Fig. 1, A and B). Cytoplasmic changes in Sertoli cells were first noted on gd 18, persisted through gd 20, and were most extensive on gd 21, as illustrated in Figure 1B. All DBP-exposed fetuses were affected. The number of tubular cross sections per testicular section was significantly lower in DBP-exposed fetal testes on gd 18–21 (Fig. 2). Aggregates of interstitial cells and MNG previously described in DBP-exposed fetal rat testes [17, 32] were also observed in gd 18–21 DBP-exposed testes (Fig. 1B).

By pnd 1, morphological appearance of Sertoli cells in the exposed and control testes was indistinguishable (Fig. 1, C and D). On pnd 2 and 5, testes exposed in utero to DBP also had histologically normal Sertoli cells (data not shown). However, the number of tubular cross sections per testicular sections in pnd 1, 2, and 5 testes exposed in utero to DBP remained significantly lower compared to age-matched control testes (Fig. 2). Aggregates of interstitial cells were visible in pnd 1, 2, and 5 testes exposed to DBP in utero (data not shown), consistent with previously reported observations [17, 32].

MNG were visible on hematoxylin-eosin-stained sections on gd 19–21 in both corn oil control and DBP-treated testes (Fig. 1, A and B). However, the DBP treatment significantly increased the number of MNG per testicular section and the number of nuclei per MNG (Table 2). Subjectively, in the DBP-exposed testes, tubules with morphologically abnormal Sertoli cells appeared to have more MNG. Although the number of MNG per testicular section and the number of nuclei per multinucleated cell significantly increased from gd 19 to 21, these multinucleated cells were

Statistical Analysis

Control and DBP-treated groups had three randomly (by body weight) assigned dams per gestational or postnatal day. The number of tubular cross sections per section and MNG were counted on hematoxylin-eosin-stained slides. The mean number of tubular cross sections was counted on three consecutive sections of the right testis and adjusted for size of the section, which was determined using ImagePro software (Media Cybernetics, http://www.mediacy.com). Normality of data distribution was assessed using JMP software (SAS, http://www.sas.com). Significant differences between treatment groups and between gestation days were established by two-way ANOVA using JMP software and litter as the experimental unit.
TABLE 2. Occurrence of MNG in the fetal rat testis following exposure to DBP in utero.

<table>
<thead>
<tr>
<th>No. of fetuses, MNG, and nuclei</th>
<th>Day of gestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Corn oil</td>
<td></td>
</tr>
<tr>
<td>Total number of fetuses analysed</td>
<td>14</td>
</tr>
<tr>
<td>Number of fetuses with MNG per % of litters affected</td>
<td>0</td>
</tr>
<tr>
<td>Mean number of MNG per section in an affected fetus</td>
<td>0</td>
</tr>
<tr>
<td>Mean number of nuclei per MNG</td>
<td>0</td>
</tr>
<tr>
<td>DBF</td>
<td></td>
</tr>
<tr>
<td>Total number of fetuses analyzed</td>
<td>14</td>
</tr>
<tr>
<td>Number of fetuses with MNG per % of litters affected</td>
<td>0</td>
</tr>
<tr>
<td>Mean number of MNG per section in an affected fetus</td>
<td>0</td>
</tr>
<tr>
<td>Mean number of nuclei per MNG</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.05) between corn oil- and DBP-exposed fetuses.

b Significant difference (P < 0.05) between gd 19 and 21.

negative for PCNA (Fig. 1E) as well as for BrdU (Fig. 1F). On hematoxylin-eosin-stained sections, mitotic figures were visible in both control (as expected) and DBP-exposed gonocytes on gd 17 (data not shown) but not on gd 18–21 as illustrated in Figure 1, A and B.

As indicated by TUNEL assay, MNG were not apoptotic at any gestational or postnatal time point examined, as illustrated in Figure 1, G and H. Apoptosis in other cell populations in the fetal and postnatal rat testes including morphologically normal gonocytes was also not affected by exposure in utero to DBP (Fig. 1, G and H).

After birth, morphologically normal gonocytes were observed at the basal lamina in both control and DBP-exposed testes (Fig. 3, A and B). In DBP-exposed testes, some MNG were found at the basal lamina (Fig. 3, B and E), but no MNG in the control testes were observed in the middle of seminiferous tubules or basally. As expected for pnd 5 [33–35], some basal and centrally located gonocytes in the normal rat testis were mitotic (Fig. 3C). In DBP-exposed testes, mitoses occurred in single- and multinucleated germ cells (Fig. 3D). Mitotic figures in MNG located in the middle of tubules or near the basal lamina appeared disorganized and contained an increased number of chromosomes (Fig. 3, D and E). On pnd 5, MNG exhibiting morphologically abnormal mitosis were not apoptotic (Fig. 3F).

**Contacts Between Sertoli Cells and Gonocytes**

**Impaired in Fetal Rat Testes Exposed to DBP**

Histologically, the lesion observed in fetal Sertoli cells in DBP-exposed testes was reminiscent of morphological changes observed in Sertoli cells from postnatal rats treated with phthalates [23]. We investigated whether abnormal morphology of fetal Sertoli cells in the DBP-exposed testis was associated with altered cytoskeleton and impaired contacts between these cells and gonocytes.

Previous studies demonstrated the presence of cadherins (the plasma membrane glycoproteins mediating calcium-dependent cell adhesion) at the sites of Sertoli and germ cell contacts in the fetal testis [36]. We performed immunostaining with anti-E-, P- or N-cadherin ab on sections from gd 17–21 and pnd 1, 2, and 5 naive, corn oil-, and DBP-exposed rat testes. E-cadherin staining within seminiferous cords was weak, although it was readily detectable in the rat small intestine used as positive control tissue (data not shown). In naive testes, patterns of P- and N-cadherin staining were the same on each fetal or postnatal time points examined, and there was no difference in staining for these cadherins between naive and corn oil-treated testes. The staining was localized at the lateral and apical aspects of the plasma membrane of Sertoli cells, as illustrated in Figure 4, A and B. As indicated by cadherins staining, the lateral and apical processes of fetal Sertoli cells in naive
and corn oil-exposed gd 17–21 testes extended around every gonocyte and separated them (Fig. 4, A and B). In contrast to the controls, Sertoli cells showed retracted lateral and apical cytoplasmic processes and adjacent gonocytes in direct contact with each other in DBP-exposed fetal testes (Fig. 4C). There were no cadherin-marked Sertoli cell apical processes around gonocytes in gd 17–21 DBP-exposed testes. By confocal microscopy, we detected redundant multilayered in z-direction (depth of the section) cadherin staining at the apical portion of the plasma membrane. Staining at the apical portion of Sertoli cells was defined on each z-plane, as illustrated in Figure 4C. As we proceeded in the z-direction, on each z-plane, cadherin-marked contours of retracted lateral and apical processes of Sertoli cells changed slightly in the x- and y-directions. Gonocytes in the DBP-exposed testes had only limited contact with Sertoli cells at the apical aspect in the area where the Sertoli cell plasma membrane was convoluted. Although staining pattern for cadherins was dramatically changed in DBP-exposed fetal rat testes, there were no changes detectable by epifluorescent microscopy in the intensity of cadherin staining between control and DBP-exposed fetal testes on any gd examined.

On pnd 1, 2, or 5, P- and N-cadherin staining was indistinguishable between naive, corn oil-, and DBP-exposed testes. As illustrated in Figure 4D, on pnd 2 DBP-exposed testes had cadherin-marked apical processes of Sertoli cells in the middle of the tubules and around gonocytes.

Lack of contact with apical and lateral processes of Sertoli cells did not have a dramatic effect on the integrity of the plasma membrane of the gonocytes in DBP-exposed fetal testes. NCAM was expressed circumferentially on the germ cell plasma membrane in both control and exposed testes on gd 17–21 (Fig. 4, E and F). Intensity of staining was preserved in control and DBP-exposed testes on all gd examined, as well as pnd 1, 2, and 5. MNG in DBP-exposed testes had circumferential NCAM expression at the plasma membrane similar to normal gonocytes (Fig. 4E). Confocal microscopy of NCAM-stained sections was used to obtain a three-dimensional visualization of a multinucleated gonocyte. This visualization demonstrated that multinucleated gonocytes were single cells of spherical form containing several nuclei (data not shown).

As indicated by histological examination and cadherin immunocytochemistry, Sertoli cells in DBP-exposed fetal testes were most affected on gd 21. To better characterize this DBP-induced lesion in fetal Sertoli cells, we stained gd 21 testes with anti-vimentin antibodies and conducted TEM. Anti-vimentin immunostaining followed by confocal microscopy revealed abnormal vimentin cytoskeleton features in fetal Sertoli cells after developmental exposure to DBP (Fig. 4, G and H). Control testes had vimentin microfibrils in Sertoli cell processes extended toward the apical aspect of the cell (Fig. 4G). In DBP-exposed testes, vimentin was visible only at the basal aspect of Sertoli cells (Fig. 4H). Western analysis detected no differences in expression of vimentin between corn oil- and DBP-exposed gd 21 testes (data not shown). Examination of gd 21 testes by TEM confirmed histological and immunocytochemical observations of abnormal morphology of Sertoli cells in DBP-exposed testes. In control testes, the cytoplasmic processes of Sertoli cells were visible between gonocytes; these processes separated and surrounded individual gonocytes (Fig. 5A). The Sertoli cells processes were retracted in DBP-exposed testes, and gonocytes were assembled together in the middle of the seminiferous cord (Fig. 5B). The plasma membrane of DBP-exposed gonocytes appeared normal (Fig. 5B, insert), which was consistent with the results of NCAM staining. The convolution of the apical Sertoli cell plasma membrane seen in DBP-exposed testes by confocal microscopy was also apparent by TEM. Ultrastructurally, Sertoli cells with retracted cytoplasmic projections exhibited overlapping layers of thinned cytoplasm adjacent to gonocytes (Fig. 5, C and D).

Our observations of features seen in fetal and neonatal rat testes exposed in utero to 500 mg kg⁻¹ day⁻¹ DBP are summarized in Table 3.

**DISCUSSION**

Previous studies demonstrated that in utero exposure to DBP decreased levels of fetal testosterone that caused ab-

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**FIG. 4.** Localization of cadherins, NCAM, and vimentin in the rat fetal testis following exposure to di(n-butyl) phthalate (DBP) in utero. P-cadherin in naive (A) and N-cadherin in corn oil-exposed (F) Gestation Day (gd) 18 rat testis. P-cadherin in DBP-exposed gd 18 and Postnatal Day (pnd) 2 (C and D, respectively) testes. Arrows point to Sertoli cell plasma membrane-localized cadherin. NCAM in corn oil- and DBP-exposed (E and F, respectively) gd 21 testes. Arrows indicate plasma membrane NCAM, localization of which in single nuclei gonocyte (in both control and DBP-exposed testes) and multinucleated gonocyte (MNG) (in DBP-exposed testes) was similar. Vimentin in corn oil- and DBP-exposed (G and H, respectively) gd 21 testes. Arrows point to long vimentin filaments in normal Sertoli cells and condensed vimentin in DBP-exposed testes. Original magnification ×520.
normal development of testosterone-dependent tissues in the rat [12, 14, 17, 37–39]. In this species, gestational exposure to DBP also disrupted development of fetal male germ cells [17, 40]. Now we have shown that exposure in utero to 500 mg kg$^{-1}$ day$^{-1}$ DBP altered the morphology of fetal rat Sertoli cells. This altered morphology manifested as retracted cytoplasmic processes, convoluted apical plasma membrane, and abnormal vimentin cytoskeleton features. Also, contacts between fetal Sertoli and germ cells were disrupted in DBP-exposed testes. The impaired contact between Sertoli and germ cells was not associated with germ cell apoptosis in the fetal rat testis. This contrasts with the response in the adult rat testis, in which the collapse of vimentin filaments and lack of normal contact between Sertoli and differentiating germ cells following exposure to heat or testicular toxicants were associated with death of spermatocytes [23].

The morphological changes seen in fetal Sertoli and germ cells exposed to DBP (retraction of Sertoli cell processes, convoluted apical plasma membrane, adjacent gonocytes in direct contact) were very similar to those observed in mature rat Sertoli cells exposed to di(n-pentyl) phthalate [41]. Histopathological changes in DBP-exposed fetal Sertoli cells first detected on hematoxylin-eosin-stained sections were confirmed by immunocytochemistry and TEM, which rules out the possibility that these changes were a fixation artifact. Similar to the regression of cytoplasmic rarefaction and re-formation ectoplasmic specialization in mature Sertoli cells 48 h after cessation of exposure to di(n-pentyl) phthalate [41], morphology of Sertoli cells was restored (at the level of light microscopy) in the developing testis after cessation of gestational exposure to DBP. The postnatal reversibility of the effect of DBP exposure in utero has also been reported for testicular testosterone production [7, 39] and expression of smooth muscle actin in peritubular myoid cells [7]. However, the number of tubular cross sections per section remained lower in DBP-exposed postnatal testes even 3 days after cessation of exposure. Previously, histological examination of adult testes in rats exposed to DBP in utero revealed convoluted

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**TABLE 3. Dynamics of cellular changes induced in the developing rat testis by exposure in utero to DBP.**

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Day of gestation</th>
<th>Postnatal day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Sertoli cell apical vacuolation (H&amp;E)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Retracted Sertoli cells processes and convoluted</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sertoli cell plasma membrane (cadherin staining)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collapsed vimentin cytoskeleton</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lower number of tubular cross-sections</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Occurrence of MNG</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Aberrant mitosis in MNG</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Abnormal apoptosis</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*–, no effect; +, minor; ++, moderate; ++++, substantial; ND, not determined.

* Convoluted Sertoli cell plasma membrane was also detected by TEM.
tubules with either small or mildly dilated lumina [14, 17]. In both studies investigators noted that testicular cross sections of DBP-exposed testes had elongated, misshaped tubular cross sections that were not observed on matching sections from control animals [14, 17]. Collectively, these and our data suggest that the effect of gestational exposure to DBP on formation of seminiferous tubules is permanent and is likely to contribute to the testicular dysgenesis that develops in the adult testis of rats developmentally exposed to this phthalate. Whether the lower number of tubular cross sections per section indicates shorter or less coiled tubules or fewer tubules in DBP-exposed fetal testes is unclear. We are currently conducting a three-dimensional reconstruction of seminiferous tubules in the fetal rat testis to assess coiling and the number of tubules in the control and DBP-exposed fetuses.

Impaired contact with Sertoli cells did not significantly alter integrity of the germ cell plasma membrane in either morphologically normal gonocytes or MNG in DBP-exposed fetal and postnatal rat testes, as assessed by TEM and immunostaining against NCAM. Plasma membrane-localized NCAM is thought to regulate postnatal migration of germ cells to the basal lamina [42]. Lack of apparent alterations in NCAM expression on the plasma membrane of DBP-exposed gonocytes was consistent with ability of some of these cells to migrate to the basal lamina. Barlow and Foster [17] suggested that decreased spermatogenesis in adult rats in utero exposed to DBP may have resulted from failure of gonocytes to migrate to the basal membrane postnatally. We found that exposure to DBP in utero did not prevent migration of some gonocytes including MNG to the basal membrane on pnd 5. Quantification of basal gonocytes in exposed testes will clarify whether exposure to DBP interferes with postnatal gonocyte migration.

Our studies demonstrated that MNG occurred at low frequency during normal development of the rat testis. However, exposure in utero to DBP resulted in a significant increase in the number of these abnormal germ cells. In contrast to Mylchreest et al. [38], our study revealed lack of PCNA staining in DBP-exposed testes on gd 19–21. Different fixation and immunostaining procedures may account for this discrepancy. We observed intense nonspecific PCNA staining in gonocytes that could not be blocked by the immunizing peptide in both control and DBP-exposed gd 21 testes fixed in 10% neutral buffered formalin. However, no BrdU staining was visible in gonocytes in these testes, while Sertoli and interstitial cells were BrdU positive. In fetal testes fixed in modified Davidson fixative, the staining patterns for PCNA and BrdU were identical. Therefore, we concluded that gonocytes (including MNG) in the DBP-exposed fetal rat testis did not proliferate on gd 19–21. The lack of mitotic figures on gd 18–21 in gonocytes in DBP-exposed testes supports this conclusion. Our data indicate that abnormal division between gd 19 and 21 is not the cause of an increase in the number of MNG and number of nuclei per MNG in DBP-exposed fetal testes. We propose that loss of normal contact and physiological support from fetal Sertoli cells injured by exposure to DBP plays a role in the degeneration of gonocytes and formation of MNG.

Whether collapse of vimentin cytoskeleton in DBP-exposed fetal testes caused or resulted from aberrant contact between Sertoli and germ cells remains to be determined. In the adult rat testis, the vimentin cytoskeleton in Sertoli cells can be altered by an experimentally induced decrease in intratesticular testosterone [43] or exposure to testicular toxicants [24, 25]. These treatments also lead to apoptotic death of germ cells, and Show et al. [43] hypothesized that changes in biochemical properties of vimentin may reflect a general mechanism regulating loss of physiological support of differentiating germ cells by Sertoli cells. However, changes in cell-cell contacts between Sertoli and germ cells and collapse of the vimentin cytoskeleton in fetal and adult Sertoli cells following toxic insult may be secondary and reflect abnormal function of these cells.

Retraction of Sertoli cell processes, collapse of the vimentin cytoskeleton, and altered contacts between Sertoli cells and gonocytes in DBP-exposed fetal testes were consistent with altered expression of genes encoding for proteins regulating the cytoskeleton and cell-cell contacts. Changes in various isoforms of troponin, tropomyosin, and myosin heavy chain polypeptide 6 were detected by RNA microarrays in gd 19 rat testis exposed to DBP from gd 12 to 18 [44]. These investigators also found decreased expression of connexin 43 and increased expression of testin in DBP-exposed fetal testes. In the adult testis, connexin 43 is involved in gap junctions between Sertoli and differentiating germ cells [45]. Decreased production of connexin 43 in DBP-exposed fetal testis may be related to the observed disruption of membrane contacts between fetal Sertoli cells and gonocytes. Increased expression of testin has been associated with both physiological and chemically induced disruption of Sertoli-germ cell junctions [46, 47], although the role of this glycoprotein in Sertoli-germ cell interactions is unclear.

MNG remained in the postnatal rat testes exposed to DBP in utero even after restoration of Sertoli cell morphology. We did not observe basal MNG postnatally in the control testes, probably because of low occurrence of MNG in unexposed rats. About pnd 5, MNG in exposed testes entered mitosis, which appeared disorganized because of an increased number of chromosomes and possibly failure to form the mitotic spindle. Such abnormal mitosis had not resulted in apoptosis of MNG by pnd 5. In postnatal rat testes exposed to DBP in utero, Barlow and Foster [17] observed MNG in the middle of seminiferous cords on pnd 7 but not 16, which suggests that death of these cells occurred during this time interval. Studies are being conducted to determine the fate of MNG between pnd 7 and 16. Loss of MNG that did not migrate to the basal lamina probably will have no effect on testicular differentiation in DBP-exposed rats since postnatal death of gonocytes that have not migrated to the basal lamina occurs in the normal rat testis. Death of basal MNG may result in depletion of spermatogonia and subsequent lack of spermatogenesis, giving rise to the Sertoli-cell-only tubules observed in adult male rats exposed in utero to DBP [7, 14].

One of the features of human TDS is testis germ cell cancer. This type of cancer is the most prevalent cancer in young men, and its incidence continues to increase [48, 49]. Testicular cancer is generally believed to arise from congenital carcinoma in situ that is characteristically aneuploid [50, 51]. Similar morphology and common biochemical features of carcinoma in situ and gonocytes [52] as well as the correlation between presence of MNG in cryptorchid testes in boys and development of testicular cancer later in life [53] led to the hypothesis that aneuploid embryonic germ cells are the precursors of germ cell tumorigenesis [5, 52]. In DBP-exposed rats, polyplloid MNG persisted 5 days after birth and migrated to the basal lamina, where they may become progenitors of genetically altered spermatogonia. Our data demonstrated that occurrence of MNG sig-
ificantly increased following exposure in utero to the high dose of DBP. However, testicular germ cell cancer has not been reported in rats exposed in utero to various dose levels of DBP, including those used in our study. This suggests that such cancer is a rare event or that rats have an efficient mechanism of elimination of genetically abnormal gonocytes. Thus, rats exposed in utero to DBP can serve as a useful model to study mechanisms underlying development of TDS as well as mechanisms of eliminating polyploid embryonic germ cells. In the present study, we examined cellular responses in the fetal rat testis exposed in utero to a dose level of DBP that reportedly caused significant pathological changes in the rat male reproductive tract [1, 7, 12–14, 17, 32]. In ongoing studies at CIIT, we are extending our observations to include dose levels that closely mimic estimated occupational and environmental human exposures.

ACKNOWLEDGMENTS

The authors thank Drs. David Dorman and Kamin Johnson for a thorough scientific review and Dr. Barbara Kuyper for an editorial review of this manuscript. Assistance of CIIT’s animal care and necropsy personnel is greatly appreciated. The authors thank Otis Lyght for help with BrdU staining, Victoria Madden for preparation of grids, and Earl Tewsbury for help with TEM.

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