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The Plasticizer Diethylhexyl Phthalate Induces Malformations by Decreasing Fetal Testosterone Synthesis during Sexual Differentiation in the Male Rat

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Phthalate esters (PE) such as DEHP are high production volume plasticizers used in vinyl floors, food wraps, cosmetics, medical products, and toys. In spite of their widespread and long-term use, most PE have not been adequately tested for transgenerational reproductive toxicity. This is cause for concern, because several recent investigations have shown that DEHP, BBP, DBP, and DINP disrupt reproductive tract development of the male rat in an antiandrogenic manner. The present study explored whether the antiandrogenic action of DEHP occurs by (1) inhibiting testosterone (T) production, or by (2) inhibiting androgen action by binding to the androgen receptor (AR). Maternal DEHP treatment at 750 mg/kg/day from gestational day (GD) 14 to postnatal day (PND) 3 caused a reduction in T production, and reduced testicular and whole-body T levels in fetal and neonatal male rats from GD 17 to PND 2. As a consequence, anogenital distance (AGD) on PND 2 was reduced by 36% in exposed male, but not female, offspring. By GD 20, DEHP treatment also reduced testis weight. Histopathological evaluations revealed that testes in the DEHP treatment group displayed enhanced 3β-HSD staining and increased numbers of multifocal areas of Leydig cell hyperplasia as well as multinucleated gonocytes as compared to controls at GD 20 and PND 3. In contrast to the effects of DEHP on T levels *in vivo*, neither DEHP nor its metabolite MEHP displayed affinity for the human androgen receptor at concentrations up to 10 μM *in vitro*. These data indicate that DEHP disrupts male rat sexual differentiation by reducing T to female levels in the fetal male rat during a critical stage of reproductive tract differentiation.

Key Words: **antiandrogen; phthalate; DEHP; testosterone; testis; sexual differentiation; endocrine disrupters.**

Identifying chemicals that alter endocrine function is a high public and research priority, due to the potential risk such chemicals pose to the reproductive health of humans, fish, and wildlife (Colborn, 1994; Colborn *et al*., 1993; EDSTAC Final Report, 1999; Giesy and Kannan, 1998; Gray *et al*., 1997). Recently, considerable concern has focused on the potential health effects of di(2-ethylhexyl) phthalate (DEHP) exposures to children from toys and other sources, and to pregnant women from dialysis treatment or blood transfusions. In animal studies, DEHP treatment produces developmental and/or reproductive toxicity in a wide range of mammalian species (rats, mice, rabbits, guinea pigs, and ferrets) with a period of susceptibility extending from fetal to pubertal stages of life.

It was initially proposed that some phthalates (i.e., dibutyl phthalate [DBP] and butylbenzyl phthalate [BBP], but not DEHP) were estrogenic (Sharpe *et al*., 1995), based upon *in vitro* data (Harris *et al*., 1997; Jobling *et al*. 1995). However, none of the phthalates that are active *in vitro* display such estrogenic activity *in vivo* (Gray *et al*., 1999; Zacharewski *et al*., 1998). This *in vivo* activity was assessed using assays sensitive to estrogens, including the uterotropic assay, the timing of vaginal opening, induction of female sexual behavior, and vaginal cornification.

This does not mean, however, that the PEs are not endocrine disrupters. Mylchreest *et al*. (1998, 1999) and Gray *et al*. (1999) hypothesized that DBP and DEHP altered fetal development via an antiandrogenic mechanism. This hypothesis is based upon the observation that reproductive tract malformations in androgen-dependent tissues in male rat offspring are similar to effects of antiandrogens such as vinclozolin, procymidone, and flutamide. For example, *in utero* exposure to dibutyl phthalate (DBP) (Gray *et al*., 1999; Mylchreest *et al*., 1999; Wine and Chapin, 1999), DEHP (Arcadi *et al*., 1998; Gray *et al*., 1999), butylbenzyl phthalate (BBP) and diisononyl phthalate (DINP) (Gray *et al*., 2000), have been shown to disrupt differentiation of androgen-dependent tissues in male rat offspring. These alterations include hypospadias and vaginal pouch formation, alterations in androgen-dependent processes (ie., testis descent, retained nipples), and mal-

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formations (i.e., ventral prostate, seminal vesicle, levator ani plus bulbocavernosus muscles, gubernacular cord, and the epididymis). While these observations demonstrate that PEs alter differentiation of androgen-dependent tissues during fetal life, the mechanism by which these effects occur has not been determined.

In contrast to the above effects seen in transgenerational studies, standard developmental toxicity tests of DEHP have not detected any of the aforementioned reproductive malformations (Narotsky *et al*., 1995; Thomas and Thomas, 1984; Tyl *et al*., 1988). In order to evaluate the potential of DEHP to adversely affect sensitive populations such as developing fetuses and children, mechanism of action studies need to be conducted to determine how alterations of the fetal hormonal milieu, as well as other effects, result in reproductive malformations in the offspring.

The purpose of this study was to elucidate the alterations in testicular testosterone production and reproductive development with DEHP exposure during late gestation and neonatal life. The current study used a battery of *in vitro*, *ex vivo*, and *in vivo* assays to examine two hypotheses: (1) DEHP and its major metabolite mono-ethylhexyl phthalate (MEHP) bind to the human androgen receptor (hAR), and (2) testicular testosterone (T) production and levels of T in the fetus are altered by maternal perinatal DEHP exposure. This paper, along with similar studies on DBP (Foster *et al*., 1999) strengthen the characterization of PEs as antiandrogens and contributes to the body of mechanistic information that will enhance our ability to extrapolate the developmental reproductive toxicity and subsequent malformations of PEs in rats to humans and other species.

MATERIALS AND METHODS

In Vitro *Study of Androgen Receptor Binding*

A competitive binding assay was utilized to evaluate the ability of DEHP and its major metabolite, MEHP, to compete with [^{3}H] R1881 (a synthetic androgen ligand, DuPontNEN) for binding to the human androgen receptor (hAR) (MEHP was generously provided by Dr. Paul Foster of CIIT). COS cells (monkey kidney line, ATCC) were transiently transfected with the hAR expression vector pCMVhAR as described by Wong *et al*. (1995). COS cells were plated at 200,000 cells/well in 12-well plates and transfected with 1 mg of pCMVhAR, using diethylaminoethyl dextran (pCMVhAR was generously provided by Dr. Elizabeth Wilson, University of North Carolina). Twenty-four h later, cells were exposed to 5 nM [^{3}H] R1881 in the presence and absence of varying doses of DEHP or MEHP (0.05, 0.2, 0.5, 1.0, 10 μM) for 2 h at 37°C. Nonspecific binding (NSB) was determined by adding 100-fold molar excess of unlabeled R1881 to only NSB assay tubes. Cells were washed in phosphate-buffered saline and lysed in 200 μl ZAP (0.13 M ethylhexadecyldimethylammonium bromide with 3% glacial acetic acid). Radioactivity contained in DEHP- and MEHP-treated cells was determined by scintillation counting with 7 replicate studies performed, and with 4 replicate studies performed for DHT-treated cells.

In Vivo *Transgenerational Study in the Rat*

General methods. Pregnant Sprague-Dawley rats (Charles River Breeding Laboratory, Raleigh, NC) of approximately 90 days of age were mated (mating confirmed by sperm presence in vaginal smears) and shipped on the day after mating. Dams were housed individually in clear polycarbonate cages (20 × 25 × 47 cm) with laboratory-grade pine shavings as bedding (Northeastern Products, Warrensburg, NY). The day after mating is designated gestation day (GD) 1, and the day after birth is designated postnatal day (PND) 1. Animals were provided Purina Rat Chow (5008) and filtered (5 microns) water, *ad libitum*, in a room with a 14:10 h (light/dark cycle, lights off at 11:00 A.M.) photoperiod and temperature of 20–22°C, with a relative humidity of 45–55%. Durham, NC municipal water was used for drinking water and was tested monthly for pseudomonas and every 4 months for a suite of chemicals including pesticides and heavy metals.

Treatment administration. Pregnant rats were randomly assigned to treatment groups on GD 14 in a manner that provided similar body weight means and distribution. Dams were dosed daily in the morning by gavage from GD 14 through the morning of necropsy (GD 17,-18,-20, or postnatal day (PND)-2) with vehicle or 750 mg DEHP/kg/d (Aldrich, lot # 48H3537, CAS # 117–81–7) in 2.5 μl of corn oil/g body weight (Sigma- cat # C 8267, lot # 107H1649, CAS # 8001–30–7). In previous studies adult offspring from this treatment regimen had a high incidence of reproductive tract malformations. Thus, this dosing regime was chosen to increase the chances of detecting endocrine alterations during the early stages of late gestation and just after birth.

Experimental design. This study consisted of 2 experimental blocks. Block 1, 11 controls and 11 treated dams: GD 17 (n = 4), GD 18 (n = 4) and PND 2 (n = 3); block 2, 6 controls and 6 treated dams: GD 20 (n = 4) and PND 2 (n = 2). As there were no significant block differences, data for PND 2 are presented as one study. Anogenital distance (AGD) and body weights were measured on all male and female offspring on PND 2, prior to necropsy. AGD was measured in a blinded fashion, using a dissecting scope with an ocular micrometer (magnification ×15).

Dissections. On gestational days 17, 18, and 20, dams were anesthetized using carbon dioxide and euthanized by decapitation. Dams were sacrificed in a random fashion, alternating between control and treated, and dissections for all ages were conducted within a 4-h period in the morning. Fetuses were removed, anesthetized on ice, and necropsied using a dissecting scope. Neonatal pups were euthanized by decapitation. One testis from 2–3 males per litter from GD17 to GD20 was incubated in media to determine T production, and the other testis was frozen whole for determination of T levels (n = 11 to 12 per treatment). After removal of fetal testes, the carcass was frozen and kept at –20°C until extracted for determination of T levels (n = 18 to 20 per treatment). On PND 2, one testis each from 2 to 3 males per litter were incubated in medium to determine T production (n = 11 to 14 per treatment) and the other testis was frozen whole for determination of T levels (n = 12–14 per treatment).

Ex vivo testicular T production. Testes (with torn tunica) were incubated in either 400 μl (GD17, 18, 20) or 1 ml (PND 2) of M-199 medium (Hazleton Biologics, Inc., St. Lenexa, KS) plus 10% steroid-stripped serum (charcoal/dextran-treated fetal bovine serum, Hyclone Laboratories, Logan, UT) for 3 h in a 34°C incubator on a rocker (GD 17, 18, 20) or in a 34°C water bath (PND 2). At the end of the incubation, the medium was removed and placed in a microcentrifuge tube, frozen on dry ice, and stored at –20 °C for T radioimmunoassay (RIA) analysis.

Testicular T extraction. Each testis was placed in a 12 × 75 mm glass test tube with 100 μl of distilled water. One ml (0.5 ml, 2×) of ethyl ether (99% pure) was added and the testis was crushed using a plastic pestle. The homogenized sample was placed in an acetone/dry ice bath until the aqueous portion was frozen. The ethyl ether fraction was poured off into a clean 12 × 75 mm glass test tube and the 2 extractions were pooled and evaporated to dryness in a fume hood overnight. Tubes were parafilm-sealed and kept at room temperature until RIA analysis for T, no longer than 2 weeks.

Whole body T extraction. The fetus was weighed (testes already removed), placed in a 15-ml Falcon tube (Becton Dickinson, Lincoln Park, NJ) to which was added 0.5 ml (on GD 17, 18, 20) or 2 ml (PND 2) of

double-distilled deionized water and homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After homogenization, ethyl ether (2 × 2 ml) was added, samples vortexed for at least 30 s, and centrifuged at 2,200 rpm (1000 × *g*) for 10 min. After centrifugation, the sample was placed in an acetone/dry ice bath until the aqueous portion was frozen. The ethyl ether fraction was poured off into a clean 12 × 75 mm glass test tube and allowed to evaporate in a fume hood overnight. The extraction efficiency of radiolabeled T from whole body homogenate was between 65% and 68%. Tubes were parafilm-sealed and kept at room temperature until RIA analysis for T, no longer than 2 weeks.

RIA analysis for T. Testosterone levels in media and from fetal tissue extracts were measured by RIA using a Coat-a-Count total T kit, according to specifications (Diagnostic Products Corporation, Los Angeles, CA). Tubes containing dried fetal extract were resuspended in 70 μl of zero standard, vortexed for 30 s, and a 50-μl aliquot assayed for T. Testosterone cross-reactivity with 5α-dihydrotestosterone was 7.9%. The interassay coefficient of variation was 7%. Extracted testicular tissues were assayed for T by RIA, as previously described by Cochran *et al*., 1981; Ewing *et al*., 1984; Schanbacher and Ewing, 1975. Briefly, dried testes extracts were resuspended in 300 μl of phosphate-buffered saline with 1% gelatin (PBSG), vortexed for 30 s, and incubated for 10 min in a 45°C water bath. Six hundred μl of PBSG containing T antibody (1:10,000) and tritiated T (10,000 DPM/100 μl, 1 mCi/ml, DuPontNEN) was added to this suspension. Data are presented as litter means for (1) T production as ng T produced per testis per 3 h, (2) testicular T levels as ng T per testis, and (3) for the fetal carcass as both total T (ng T per fetus) and T concentration (ng T/g fetus).

Testis histology. Testes from PND 2 male rats were fixed with 5% glutaraldehyde in 0.05 M collidine containing 0.1 M sucrose (collidine buffer) overnight at 4°C ($n = 4$ control and 6 DEHP males). Tissues were then rinsed ×2 with collidine buffer and post-fixed for 1 to 2 h, depending on the size of the tissue, in 1% aqueous osmium tetroxide in 0.05 M collidine buffer. Dehydration of samples progressed through 15-min incubations in 70, 80, and 95% ethanol, followed by 2 15-min incubations in 100% ethanol, and with a final rinse in propylene oxide at room temperature. Final fixation continued with propylene oxide:EPON incubation for one h, followed by overnight incubation in 100% EPON (poly/Bed 812) at 4°C. Tissues for light microscopy were then embedded in capsules, allowed to harden, sectioned (1 μ), stained with aqueous toluidine blue, and photographed on a Vanox light microscope. Sections were evaluated at the light microscopic level in a treatment-blinded fashion.

For histochemical localization of 3β-HSD activity, testes were removed, placed in OTC compound (Miles, Inc. Elkhart, IN), frozen immediately in liquid nitrogen, and stored at −80°C until sectioned. Testicular samples were taken on GD 20 from surplus males, to evaluate 3β-HSD staining from testes of 4 control males (from 3 litters) and 5 males (3 litters) in the DEHP-treated group. In addition, given the robust differences between the 2 treatment groups in 3β HSD staining from GD 20 fetal testes, testes also were taken from neonatal males on PND 3 from a parallel study treated identically with DEHP (companion paper by Gray *et al*., 2000) for 3β-HSD staining (for control: 1 from 3 litters and for DEHP treated: 1 from 3 litters).

Testes were sectioned and stained as previously described by Payne, 1980. Briefly, cryostat sections (7 μm) of the testis were cut, placed on a glass slide, and stored at –20°C until staining. At the time of 3β-HSD staining, slides were held at room temperature for 10 to 15 min. Stain was applied and slides were incubated for 1 h at 37°C under humid conditions, to decrease evaporation of the staining solution. The solution contained 1 mg/ml etiocholanolone (epiandrosterone-5β-androstan-3β-ol-17-one), 3.3 mg/ml nitroblue tetrazolium salt, 1% triton ×100, and 1.05 mg/ml NAD^+ in Dulbecco's phosphate-buffered saline (D-PBS). Stained sections were washed with distilled water, fixed with 5% formalin containing 5% sucrose, and cover slipped with glycerol/D-PBS (50:50, v/v). Photomicrographs of these testicular cross sections from GD 20 and PND 3 (7 control and 8 treated) were examined by 10 individuals in a treatment-blinded fashion to determine if the intensity of LC staining differed between the control and treated groups.

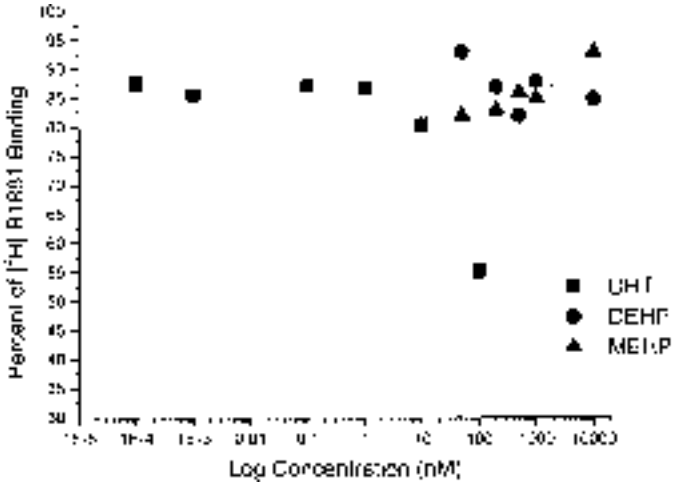

FIG. 1. Binding of DEHP and its major metabolite, MEHP, to the hAR was assessed using the COS-whole cell binding assay, compared to the potent androgen dihydrotestosterone (DHT). Cells were dosed with DEHP and MEHP (0, 0.05, 0.2, 0.5, 1.0, and 10 μM; $n = 7$ replicates) and DHT (0.0001, 0.001, 0.1, 1, and 10 nM; $n = 4$ replicates).

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) using Proc GLM (Statistical Analysis System [SAS version 6.08], SAS Institute, Inc., Cary, NC) using litter means where appropriate. When statistically significant effects ($p < 0.05$, F statistic) were detected in the overall ANOVA model, control means were compared to treated groups using the LSMEANS procedure on SAS (a 2-tailed *t*-test). Endocrine endpoints (testis T, T production, and fetal T) were log-transformed to correct for heterogeneity of variance. AGD data were analyzed for a treatment effect by sex using litter-mean values corrected for body weight by analysis of covariance. Incidences of testicular histological alterations were statistically evaluated by Fisher's exact test, using individual scores.

RESULTS

In Vitro Androgen Receptor Binding Assay

In contrast to the potent androgen dihydrotestosterone (DHT), measured in 4 replicate studies, neither DEHP nor MEHP competed with R1881 (a potent synthetic androgen) for androgen-receptor binding in the COS whole-cell binding assay, at any of the concentrations tested (0.05, 0.2, 0.5, 1.0, 10 μM) in 7 replicate studies (Fig. 1).

Transgenerational Study

Testicular T production. Testicular T production was significantly lower in the DEHP-exposed fetuses compared to control, at all ages measured (Fig. 2). The normal developmental peak in testicular T production, displayed by control male rats, was absent in the DEHP group. Testicular T production in the DEHP-treated group was sustained at a lower level when compared to controls from GD 17 through PND 2 (Fig. 2): litter means of 4 litters per treatment, with 11–12 observations per treatment (2–3 males per litter) at GD 17–20 and 5 litters

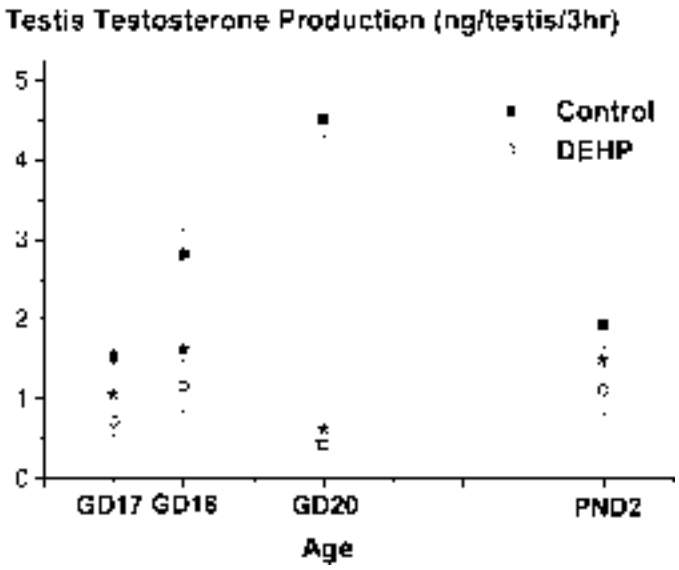

FIG. 2. Basal levels of testosterone production (ng/testis/3 h) during incubation of one testis (GD 17–20) or paired testes (PND 3) for control and DEHP (750 mg/kg/day from GD 14–PND 3) treatment groups from gestational day 17 to postnatal day 2. Graph values represent litter means with standard error bars, and asterisks indicate statistical significance at the $p \leq 0.05$ level, based on litter means analyses ($n = 4$ litters for GDs 17, 18, and 20, and $n = 5$ litters for PND 2).

per treatment, with 12–14 observations per treatment (2–3 males per litter) on PND 2.

Fetal whole-body T levels and concentrations. Testosterone (T) levels (T/fetus) and concentrations (T/g fetus) in extracts of fetal tissues (whole body, minus testes) were significantly reduced by DEHP treatment at GD 17 and GD 18 as compared to control male tissue extracts (Table 1). Differences in fetal T concentrations were greatest at GD 17, being 71% lower than controls. DEHP reduced T concentrations (ng/g) by 47% at GD 18 and by 32% at GD 20 (Table 1). Fetal T (ng/g) at GD 17 was 0.38 ng T/g in controls versus 0.11 in the DEHP group, while at GD 18 the values were 0.21 ng T/g in controls versus 0.11 in the DEHP group (p value ≤ 0.02). The difference on GD 20 (0.094 versus 0.07 ng/g fetus for control and DEHP, respectively) was not significant. Data are litter means (4 litters per treatment) with 18 to 20 observations per treatment (4 to 5 males per litter) at each gestational period.

Testicular T levels. The level of T in the extract of testicular tissue (expressed either as total T, as in Table 1, or as T/g testis (data not shown) was also reduced by DEHP treatment. A significant reduction in total testicular T levels in the treatment group compared to control occurred at the earliest timepoint measured at GD 17 (Table 1). This level remained significantly reduced throughout the dosing period, PND 2. Levels of testicular T were reduced by approximately 60–85% during fetal and neonatal life (Table 1). Significant differences in testicular T levels between control and treatment groups occurred prior to significant reductions in testicular weight (Fig. 3). These results were assessed using litter means (4 litters per treatment) with 11 to 12 observations per treatment (2 to 3 males per litter) for GD 17–20. Assessment at PND 2

TABLE 1
A Comparison of Body Weight, Testis Weight and Testosterone Levels Between Control and DEHP Exposed Male Rats During Late Gestation and Neonatal Life

Age	Treatment (n of litters)	Litter size	SE	Body weight (mg)	SE	Testis weight (mg)	SE	Testis testosterone (ng/testis)	SE	Testosterone production (ng/testis/3 hr)	SE	Fetal testosterone ng/fetus (ng/g)	SE ng/fetus (ng/g)
GD17	Control (4)	14.8	1.0	498	17	0.58	0.08	0.30	0.06	1.54	0.09	0.18 (0.38)	0.08
				$n = 18$		$n = 12$		$n = 11$		$n = 12$		$n = 18$	(0.17)
	DEHP (4)	13.5	0.3	480	21	0.61	0.01	0.10**	0.01	0.71*	0.18	0.05 (0.11)**	0.01
				$n = 20$		$n = 12$		$n = 11$		$n = 12$		$n = 20$	(0.04)
GD18	Control (4)	13.8	0.7	894	51	0.82	0.04	0.26	0.02	2.82	0.31	0.19 (0.21)	0.01
				$n = 20$		$n = 12$		$n = 11$		$n = 12$		$n = 20$	(0.006)
	DEHP (4)	13.0	0.9	901	33	0.66	0.07	0.10**	0.02	1.16***	0.33	0.19 (0.11)*	0.03
				$n = 18$		$n = 12$		$n = 11$		$n = 12$		$n = 18$	(0.03)
GD20	Control (4)	15.0	1.1	2383	74	1.04	0.05	1.51	0.12	4.51	0.24	0.23 (0.09)	0.03
				$n = 20$		$n = 11$		$n = 12$		$n = 12$		$n = 20$	(0.008)
	DEHP (4)	12.8	0.8	2340	43	0.85*	0.08	0.20[c]	0.06	0.42***	0.05	0.15 (0.07)	0.06
				$n = 20$		$n = 11$		$n = 12$		$n = 12$		$n = 20$	(0.003)
PND2	Control (5)	13.0	1.6	8085	407	7.25	0.37	0.96	0.21	1.92	0.32	NM	–
				$n = 32$		$n = 32$		$n = 12$		$n = 13$			
	DEHP (5)	12.2	0.8	6197	63	3.67***	0.12	0.40**	0.06	1.11*	0.35	NM	–
				$n = 28$		$n = 28$		$n = 14$		$n = 11$			

Note. For endocrine endpoints (Testis T, T production, and fetal T), data were log-transformed to normalize for homogeniety of variance; n = number of individual fetuses or pups per treatment; GD = gestational day, GD1 = first day post conception; PND = postnatal day, PND1 = first day after birth; and NM = not measured.
* p value ≤ 0.05; ** p value ≤ 0.01; *** p value ≤ 0.001. Data were analyzed using litter mean values.

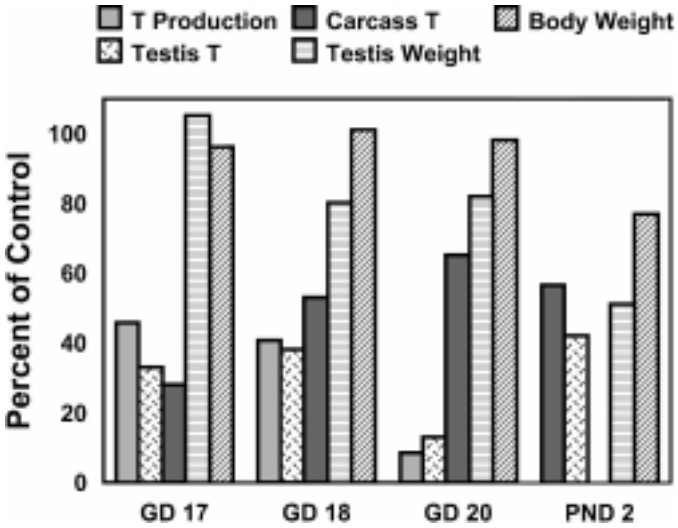

FIG. 3. The endocrine and gravimetric data here demonstrate that dramatic reductions (> 50%) in testosterone (T) synthesis and tissue levels of T in the fetal male rat occur at an age when testis and body weights are unaffected. Testis weights are reduced by about 20% later in life on GDs 18 and 20 and by about 50% at 2 days of age. Body weight is only reduced during neonatal life, an age at which the reproductive tract has nearly completed differentiating from an indifferent state to a male-like condition. Body and testis weights, tissue testosterone levels and testicular testosterone production for control and DEHP (750 mg/kg/day from GD 17 to PND 3) treatment groups from gestational day 17 to postnatal day 2. Body weights were measured in grams with testes already removed. Testes weights were measured in milligrams. Testicular testosterone levels were measured by radioimmunoassay (ng testosterone/testis). Testis testosterone production measured testosterone in incubation media after 3 h (ng testosterone produced per testis in 3 h). Fetal testosterone levels were measure in whole-body extracts without testes (ng testosterone/fetus). Data are presented as percentage of control. Statistical significance, standard errors, untransformed means and sample sizes are presented in Table 1.

used litter means (5 litters per treatment) with 12 to 14 observations per treatment (2 to 3 males per litter).

Testis and body weights. While paired-testis weight during late gestation was not significantly reduced in DEHP males on GD 17 and GD 18, the testes of treated males were significantly smaller (by 18%) than control testes on GD 20 (Fig. 3). On PND 2, testes of DEHP-treated males were 49% smaller than control-male testes (Table 1). However, DEHP did not affect body weight until PND 2, at which time DEHP male pups were approximately 23% smaller than control pups (Table 1, Fig. 3). These results were assessed using litter means from 4 litters for GD 17-20 per treatment, with 11 to 12 observations for testis weight (2 to 3 males per litter) and 18–20 observations for body weight (4 to 5 males per litter) at GD 17–20. Assessment at PND 2 used litter means (5 litters per treatment) with 28 to 32 observations for both body weight and testis weight (2 to 10 males per litter). Testis weights, adjusted for body weight by analysis of covariance, were also significantly reduced in the DEHP-treated ($n = 9$ litters), 3-day-old pups used for 3β HSD staining (data from 1 male per litter) when compared to controls ($n = 10$ litters and pups): 7.90 ± 0.21 mg in controls to 6.03 ± 0.36 mg ($p < 0.01$) in the DEHP group. Body weight in male pups at PND 3 was reduced from 8.99 ± 0.23 g in the control group to 8.34 ± 0.34 g in the DEHP group.

Anogenital distance (AGD). AGD at 2 days of age, adjusted for body weight by analysis of covariance, was significantly reduced in males from the DEHP group, measuring 3.35 ± 0.12 mm (mean ± SE) in the control versus 2.14 ± 0.10 mm in treated males. AGD was not reduced in treated females as compared to females from the control group (Fig. 4). These results were assessed using litter means from 5 litters per treatment with 28 to 32 observations for males (2–10 pups per litter) and 23 to 33 observations for females (3 to 8 pups per litter).

Testicular histopathology. Histological alterations were seen at PMD 2 in both the interstitium and the seminiferous cords of all 4 animals examined from the DEHP but not in any of the 6 control animals ($p < 0.01$, by Fisher's exact test). Leydig cells (LC) were identified in sections as cells with centrally located, round nuclei and abundant amounts of cytoplasm containing lipid droplets (Figs. 5A–5D). In controls, LCs were often found individually and in small clusters (5 to 6 cells) scattered throughout the interstitium (Figs. 5A and 5B). In the DEHP group, LC hyperplasia was randomly scattered throughout the interstitium of all testes examined (Fig. 5C). The hyperplastic LCs formed dense aggregates, frequently containing over 20–30 Leydig cells, and were often localized around blood vessels (Fig. 5D). The altered LCs were angular and small with less cytoplasm, though they still contained characteristic lipid droplets. Confirmation of the presence of LC aggregates in DEHP-exposed testes was conducted using the well characterized 3β-HSD staining technique, which is restricted to this cell type in the testis. On GD 20 and PND 3, LC staining for 3β HSD was uniformly dispersed in control testes

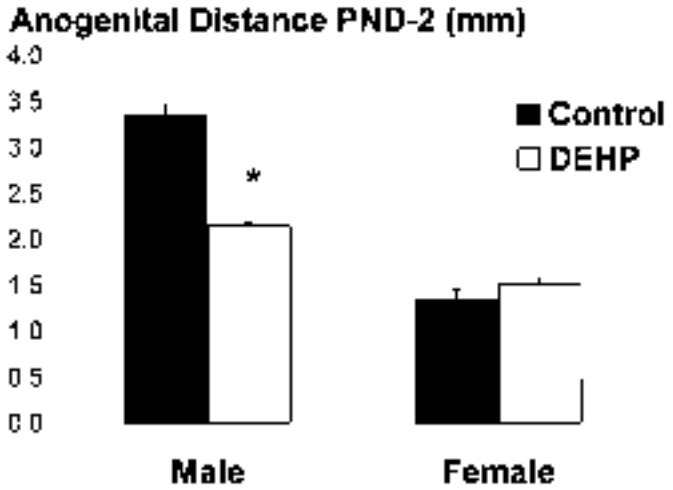

FIG. 4. Anogenital distance of postnatal day 2 male and female rats for control and DEHP treatment (750 mg/kg/day from GD 14 to PND 2). Asterisks indicates statistical significance at the $p \leq 0.05$ level, based on litter means analyses adjusted for body weight by analysis of covariance ($n = 5$ litters).

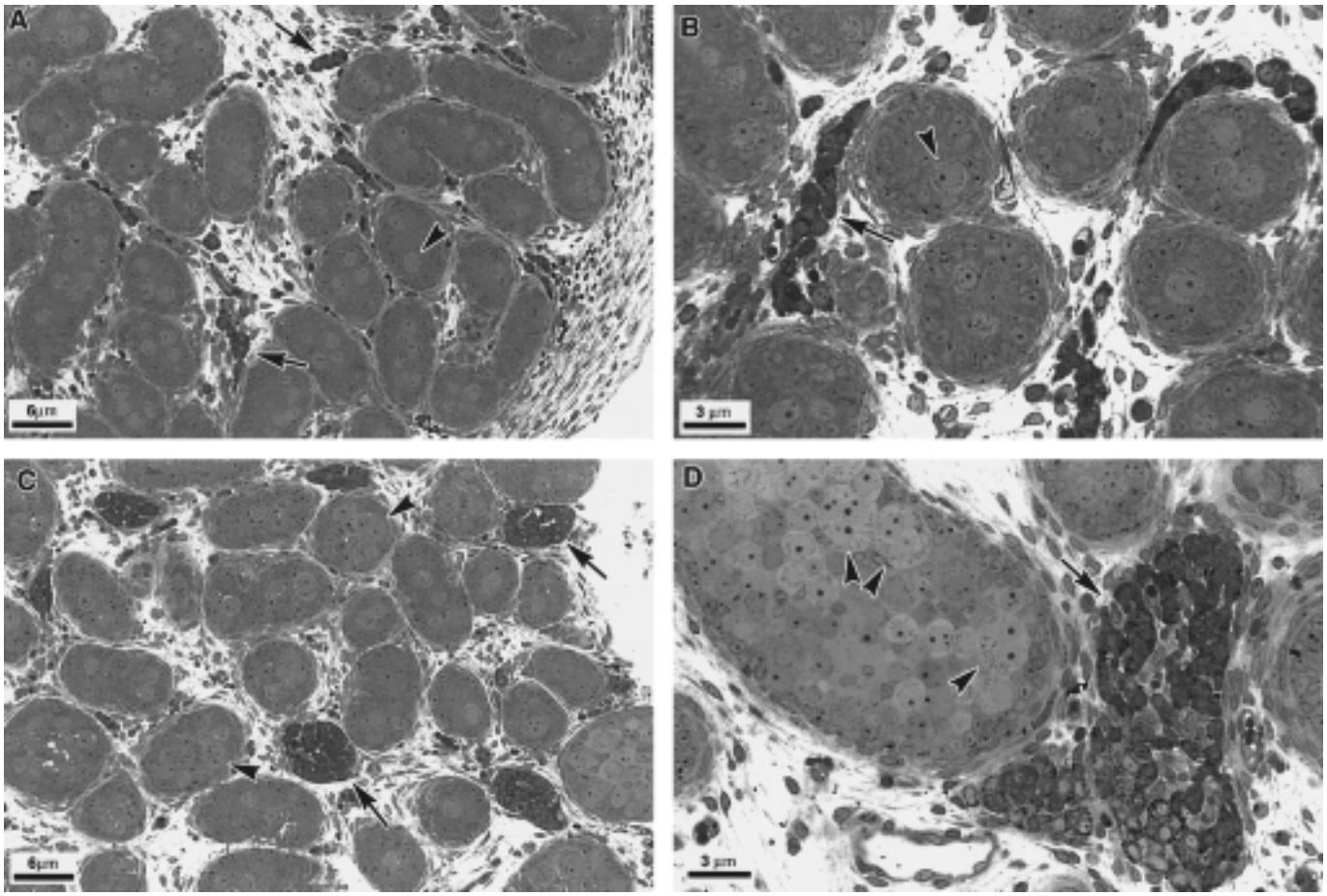

FIG. 5. (A) Photomicrograph of the histology of a semi-thin EPON embedded, toluidine-blue stained, 1-μ cross section of a testis from a control male. (B) Higher magnification of a control male testis cross-section. The Leydig cells, containing lipid droplets, are scattered individually throughout the interstitium and form multiple small aggregates (arrow). Seminiferous cords generally contain several gonocytes of similar size per cross-section (arrowhead). (C) Testis from a treated male. Multiple areas of Leydig cell hyperplasia are scattered throughout the interstitium (arrows). The areas of hyperplasia contain many more cells then the small aggregates seen in control testes. Cross sections of seminiferous cords contain numerous gonocytes, some of which are enlarged and contain multiple nuclei (arrowheads). (D) Higher magnification of testis from DEHP-treatment group. The photomicrograph contains a large area of Leydig-cell hyperplasia (arrow) surrounding a blood vessel and a seminiferous cord with numerous gonocytes. Several of the gonocytes are enlarged and multinucleated (arrowheads). Panels (A) and (C), scale bars represent 6 μm; panels (B) and (D), scale bars represent 3 μm.

($n = 7$) in contrast to the DEHP ($n = 8$) testes ($p < 0.01$, by Fishers exact test). Large clusters of LCs were present in the testes of all treated males, while highly dispersed, smaller clusters were seen in testes from control males (Figs. 6A and 6B). When the LC clusters/staining intensity was used to classify the testes in control or treated groups, 7 of 10 observers correctly classified all 15 tissues, while the other 3 successfully classified 14 of 15 tissues.

Additionally, seminiferous cords from controls on PND 2 contained an average of 3–5 gonocytes that were consistent in size (Figs. 5A and 5B) while, the seminiferous cords of treated males frequently contained more numerous gonocytes (Figs. 5C and 5D), sometimes resulting in an apparent increase in testis cord diameters. While the gonocytes were usually of consistent size, similar to controls, there were modest numbers of cells scattered through the population that were markedly increased in size and contained multiple nuclei (Figs. 5C and 5D). Most often, they contained 2–3 nuclei, but several numbers contained 4, and occasional enlarged germ cells contained 5–6 nuclei. Enlarged gonocytes with multiple nuclei were also frequently seen in tubules of normal diameter.

Maternal toxicity. During 11 days of dosing, from GD 14 to PND 2 (24 days post conception), maternal weight of treated dams was significantly altered compared to control dams on gestational days 16, 17, and 18 (Fig. 7A). Maternal weight gain of treated dams compared to controls was significantly reduced on gestational days 17, 18, and 20 as well as on PND 2 (Fig. 7B). As dams were sacrificed on different days, subsequent results were assessed using a subset of the original dams: each group used 17, 13, 9, and 5 dams for GDs 17, 18, 19 and 20, and GD 21 to PND 2, respectively. The number of live pups was not reduced by maternal DEHP treatment (Table 1) when examined by gestational day or overall (14.0 pups versus 12.8, NS, pooled across gestational days).

DISCUSSION

In the current study, we found that DEHP and a major metabolite, MEHP, did not bind hAR *in vitro* at concentrations of up to 10 μM (Fig. 1). However, when DEHP was administered at 750 mg/kg/day from GD 14 on, testicular T production, testicular T, and fetal T levels were reduced to female levels during sexual differentiation. Hence, in contrast to the well-characterized environmental antiandrogen vinclozolin, neither the parent compound DEHP nor a major metabolite MEHP appear to be AR antagonists.

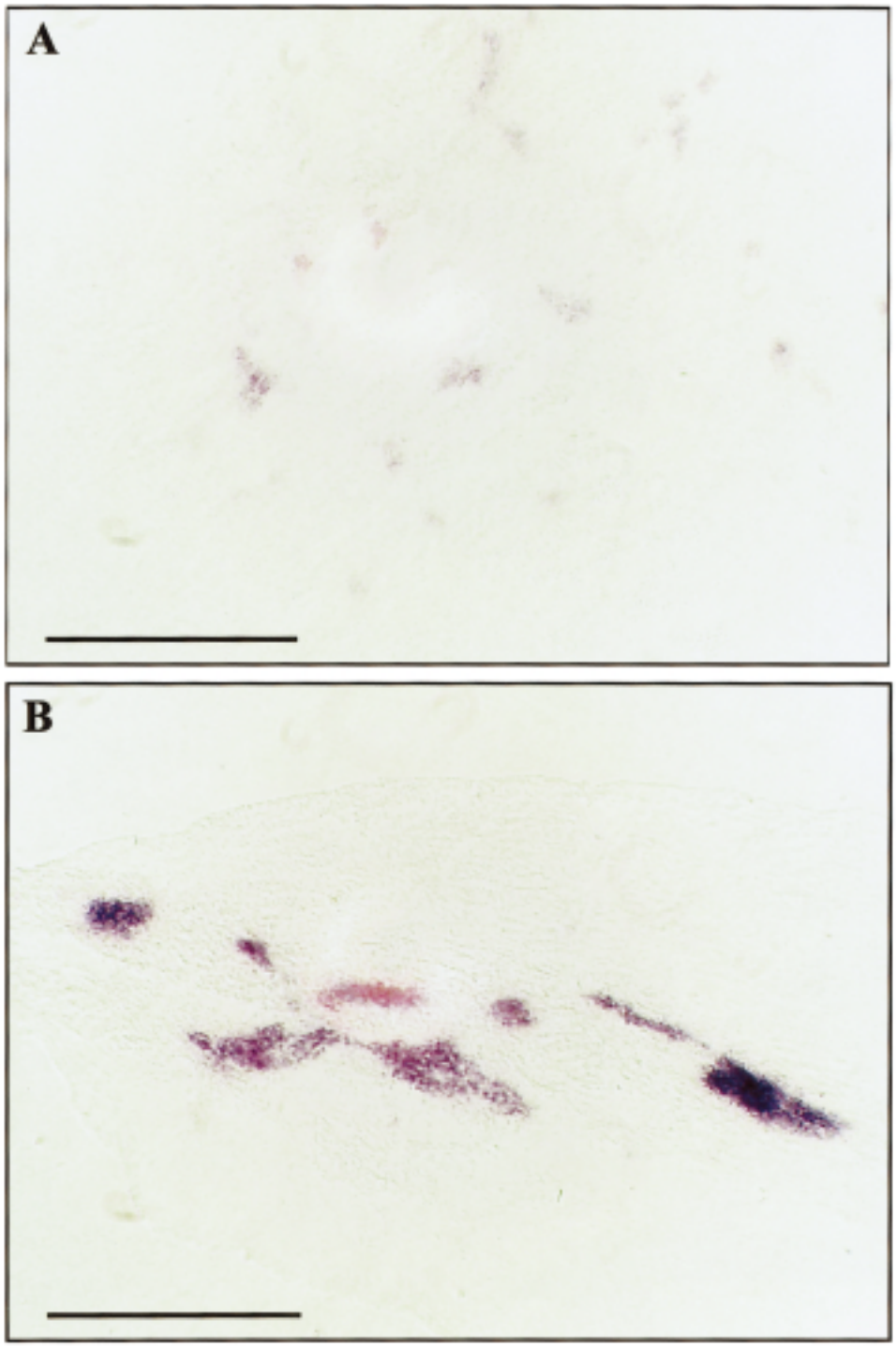

FIG. 6. Immunohistochemical staining of 3β HSD enzyme activity, which is highly specific for Leydig cells in the testis, from 7-μm frozen cross-section of testis of control and DEHP-treated male rat offspring. This revealed that the Leydig cells in 3-day-old treated males on gestational day 20 appeared to be hyperplastic and arranged in relatively large clusters, as compared to the Leydig cells of control male rat testes at the same ages. (A) displays a cross-section of one control male testis at 3 days of age, compared to the testis (B) of a male rat from a litter in which the dam was dosed with 750 mg DEHP/kg/d from day 14 of gestation to day 4 of lactation (as per Gray *et al.*, 2000). Scale bars represent 300 μm.

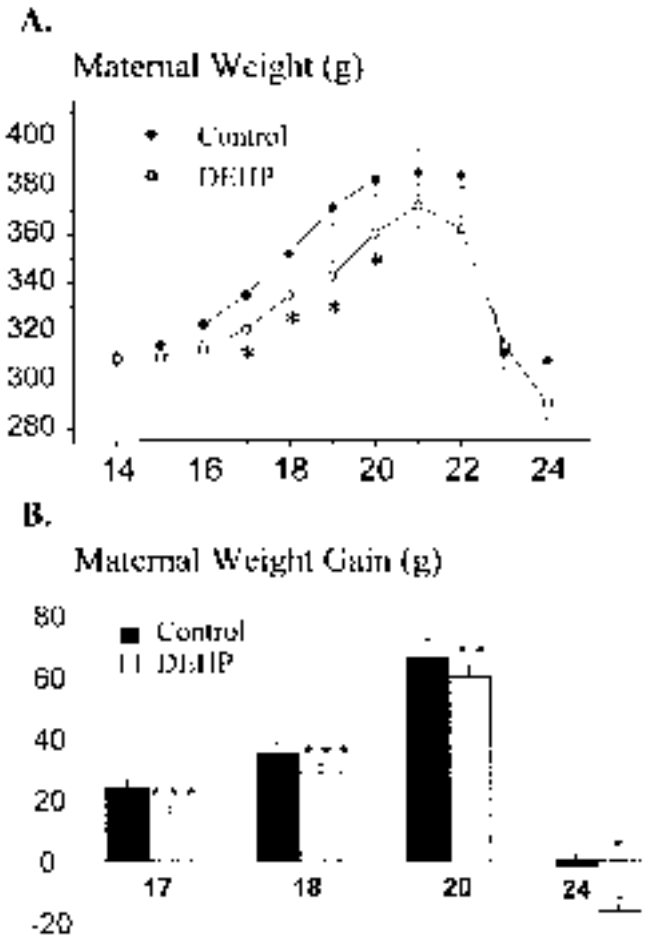

FIG. 7. Maternal weight and maternal-weight gain over the dosing period starting on GD 14 and ending on PND 2. (A) Maternal weight (g) over the dosing period (GD14–PND2) for control and DEHP (750 mg/kg day)-treated dams. (B) Maternal weight gain (g) over the dosing period (GD 14–PND 2) for control and DEHP (750 mg/kg/day)-treated dams. Data are presented as gram weight; *indicates statistical significance, $p \leq 0.05$; **indicates statistical significance, $p \leq 0.01$; and ***indicates statistical significance, $p \leq 0.001$. These results were assessed using 17 dams from control and 17 dams for treated from GD 14 to GD 17. As dams were sacrificed on different days, subsequent results were assessed using a subset of the original dams: control used 13, 9, and 5 dams and DEHP used 13, 9, and 5 for GDs 18, 19, 20, and 21 to PND 2, respectively.

Testicular T and T production peaked in our control male rats on GD 20, similar to previous study results assessing whole fetal T levels (El-Gehani *et al*., 1998a,b; Weisz and Ward, 1980). However, DEHP-treated male fetuses displayed only a marginal increase in testicular T and T production from GD 17 to GD 20 (Fig. 2), with levels that were near female tissue levels (Parks, unpublished). Adult male offspring of dams, dosed identically to doses in this study, possessed malformations of androgen-dependent tissues including severe hypospadias, vaginal pouch formation, cryptorchidism, and nipple retention (Gray *et al*., 1999, 2000). To our knowledge, results from this study are the first to indicate that the antiandrogenic effects *in utero* of DEHP correlate with reductions in tissue T levels and T production during a critical period of sexual differentiation in the male rat fetus.

Along with the reductions in tissue T levels in male rats, the AGD of our DEHP-exposed males was significantly (after adjustment for body weight by analysis of covariance) reduced at 2 days of age (Fig. 4). While body weight was reduced in both male and female neonates, AGD was reduced only in males (down 36%). AGD is a sexual dimorphism that results from the sex difference in fetal androgen (DHT) levels (Rhees *et al*., 1997). This result indicates that perinatal DEHP treatment inhibited the effects of dihydrotestosterone on the differentiation of this sexually dimorphic endpoint.

It is important to note that dramatic alterations in T synthesis and tissue T levels were initially seen at GD 17, whereas testis and body weights were only markedly affected in neonatal but not fetal life (Fig. 3). Furthermore, while the effects of low testosterone levels on the reproductive tract are permanent, body weight was only transiently affected, such that differences in body weights between control and DEHP-treated male rat offspring were absent later in life (Gray *et al*., 1999). Thus, it is clear that the effects of DEHP on T-production and sexual differentiation do not result from systemic or general fetotoxicity, as none is evident at GDs 17, 18, or 20 (Fig. 3).

During fetal and neonatal life, the "fetal" Leydig cells (LC) produce T. At approximately two weeks after birth, the fetal LCs regress and are thought to be replaced by progenitors to the "adult" LC (Hardy *et al*., 1990). The effect of DEHP on Leydig cell T production appears to be transient and may be unique to the fetal LC, because T levels are not consistently reduced when these offspring reach maturity (Gray *et al*., 2000). The cellular and molecular differences between these LC lineages may account for the enhanced sensitivity of the fetal LC, compared to the adult LC, to DEHP-induced reductions in T production. On the other hand, altered fetal LC function could result from altered Sertoli-cell paracrine factors that regulate LC differentiation and T production. While one might expect that a reduction in LC numbers would be concurrent with reduced T levels, this does not appear to be the case. LC numbers appeared to be increased, rather than decreased, in DEHP-treated testes as compared to controls, and DEHP-treatment altered the pattern of fetal LC organization (Figs. 5 and 6). In exposed males, on PND 2 there were areas of LC hyperplasia in the testicular interstitium, whereas the LCs of control animals were arranged in smaller aggregates that were more uniformly distributed through the interstitial region of the testis. Even though T production of the testes was reduced, 3β HSD activity in the LCs of DEHP-treated males was increased, rather than decreased. On GD 20 and PND 3, LC staining for 3β HSD was uniformly dispersed in small clusters in control testes, in contrast to the DEHP-exposed testes in which there was an apparent increase in areas of the interstitium of the testis staining for 3β HSD, indicating an

apparent increase in the numbers of LCs as compared to controls.

The apparent increase in LC number may be a compensatory response to the decreased levels of testosterone production or altered paracrine Sertoli cell secretions. In the pubertal and neonatal rat, DEHP exposure directly affects Sertoli cell function (Dostal *et al*., 1988; Li *et al*., 1998). The Sertoli cell secretes paracrine factors that are necessary for the stimulation and maintenance of testosterone synthesis and normal fetal LC function. Hence, altered Sertoli cell function could direct LCs to proliferate instead of to differentiate and produce testosterone (Sharpe, 1993). On the other hand, LC clustering could prevent the cells from receiving the appropriate paracrine factors from the Sertoli cells. However, as none of the exposed males displayed retained Mullerian-duct derivatives, the secretion of at least one Sertoli cell hormone, Mullerian inhibiting substance (MIS), was not significantly altered by DEHP treatment. It is likely that the effect of DEHP on T production reflects the direct action of MEHP on the testis, as opposed to an alteration of hypothalamic-pituitary function, because initiation of testosterone synthesis by the fetal rat testis does not appear to be regulated by pituitary luteinizing hormone (LH) secretion (El-Gehani *et al*., 1998b). Histological examination of the testes also revealed alterations in the seminiferous cords within which the Sertoli cells reside. Gonocytes within the seminiferous cords were more numerous in the treated animals and some of these cells contained multiple nuclei. Although the changes in testicular histology are dramatic and rather novel because they have only been described in GD 20 and PND 2 males, it is uncertain if these alterations provide clues about the direct mode of action of DEHP in the fetal male or if they are merely one of the many indirect effects of DEHP treatment. Examination of testicular histology earlier in fetal life, especially at the onset rather than at the end of sexual differentiation, will reveal the true significance of these alterations.

Based upon results of the current study, we hypothesize that DEHP, or a metabolite, reduces T production either by directly acting on the LCs to reduce T synthesis, or by interfering with Sertoli-cell paracrine factors that regulate LC differentiation and function. Regardless of the mechanism, if the LCs in exposed males continued to divide rather than differentiate for only a brief period of sexual differentiation, this could delay the onset of LC T production and lead to malformations of the reproductive tract, external genitalia, and other androgen-dependent tissues (nipples and levator ani muscle).

The fact that the phthalates are not AR antagonists but act as antiandrogens during fetal life by reducing T levels, probably explains why the profiles of effects induced by DEHP (Gray *et al*., 1999) or DBP (Mylchreest *et al*., 1998, 1999) differ from those seen after *in utero* treatment with vinclozolin (Gray *et al*., 1999) and procymidone (Ostby *et al*., 1999) which act as AR antagonists. DEHP and DBP induce more severe effects on the T-dependent tissues like the epididymis than do the AR antagonists (Gray *et al*., 1999, 2000; Mylchreest *et al*., 1998, 1999).

Determining the mechanism of testicular effects of perinatal DEHP exposure in the rat, and the dosage levels that produce such effects, are essential steps in extrapolating these effects to humans. While some of the effects of DEHP, acting via peroxisome proliferating mechanisms, have been dismissed as irrelevant to human health (Koop and Juberg, 1999), peroxisome proliferator-activated receptor alpha (PPARα) is not required for PE-induced testis and kidney lesions, as indicated by the observation that DEHP treatment induces pathology in these organs in PPARα-knockout mice (Ward *et al*., 1998).

It is clear that the testicular effects of PEs are not limited to rodent species. PEs have been reported to cause reproductive abnormalities following *in utero* exposure in rabbits (Higuchi *et al*., 1999), guinea pigs (Gray *et al*., 1982), ferrets (Lake *et al*., 1976), and frogs (Higuchi *et al*., 1999). In addition, the lack of sensitivity of the hamster testis to PEs is often overstated (Koop and Juberg, 1999). Testicular lesions are displayed in the testes of hamsters treated with DEHP, with even more severe testicular lesions with MEHP treatment of male hamsters (Gray *et al*., 1982).

Not only are these effects seen in several different species, but they are seen at relatively low dosage levels. Arcadi *et al*. (1998) reported that *in utero* DEHP exposure at a concentration as low as 3 mg/kg/day induced testicular alterations in the rat offspring such as decreased testes weight and delayed appearance or absence of elongated spermatids. Poon *et al*. (1997) observed that administration of DEHP at about 37 mg/kg/day caused effects on the Sertoli cells of the testis, which they described as adverse (selected as the NOAEL by the European Commission [EC], 1998). Without including exposures from other routes or sources, the EC estimated a margin of exposure (MOE) for DEHP of 19 from infant mouthing of toys alone, which is about 5-fold lower than a more acceptable MOE of 100 (Faustman and Omenn, 1996). In this regard, the EC has proposed banning the use of several phthalates, including DEHP, in mouthing toys (ENS, 1999). While there are many sources of DEHP, maternal and neonatal dialysis treatments and blood transfusions during development can result in high serum MEHP and DEHP levels. A comprehensive assessment of the risk of PEs will need to include the most sensitive life stages, along with an assessment of aggregate and combined phthalate exposure levels.

It is noteworthy that the developmental effects of the PEs in rodents include testicular cancer, hypospadias, undescended testes, and permanently reduced sperm production, a list that resembles the reported adverse trends in human male reproductive health that is frequently associated with exposure to environmental contaminants (Toppari, 1998). Since human serum contains MEHP after DEHP exposure, we should be concerned about the ability of this metabolite to alter human reproductive function by affecting testicular function during development. While only a few studies have evaluated PE exposure in nonhuman primates (Rhodes *et al*., 1986; Kurata *et al*., 1998), none of these reported testicular effects from DEHP

treatment. However, these studies were designed to investigate the effects of PE exposures during adult life and did not include exposure during the most sensitive stages of life, which appears to be from reproductive-tract differentiation *in utero* until puberty.

The results of this study contribute to understanding of the mechanism of action of PEs by identifying alterations in testicular steroid production with *in utero* DEHP exposure. These data also emphasize the need for additional mechanistic information to decrease uncertainty in extrapolation of the effects of PEs from rats to humans. The more we know about the cellular and molecular mechanism of action of DEHP in the fetal rodent testis, the greater certainty we will have concerning the likelihood that the effects are relevant to human development. Since both the process of steroidogenesis and the role of androgens in sexual differentiation are highly conserved among all mammalian species, the effects seen here are not likely to be unique to rodents. In summary, our results demonstrate that (1) inhibition of testicular testosterone production to female levels in the male during sexual differentiation is likely the direct cause of the malformations in the androgen-dependent tissues of male rat offspring, and (2) these malformations appear to result from an AR-independent mechanism of antiandrogenicity.

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