

Effects of Prenatal Testosterone Propionate on the Sexual Development of Male and Female Rats: A Dose-Response Study

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Testosterone plays a major role in male sexual development. Exposure of females to testosterone *in utero* can induce masculine characteristics such as anovulation, increased anogenital distance (AGD), absence of nipples, retention of male-like tissues, and agenesis of the lower vagina. In addition, high levels of androgens during fetal development can lead to toxic effects such as reduced litter size and viability. The study of the effects of testosterone administration during sexual differentiation provides a foundation for understanding the effects of environmental androgens on fetuses, a sensitive subpopulation. In the current study, we investigated the ability of a range of concentrations of testosterone propionate (TP) administered prenatally to masculinize female and alter male offspring, and measured maternal and fetal T levels. Pregnant Sprague-Dawley rats were dosed by sc injection on gestational day (GD) 14–19 (GD 1 = day of plug) with either corn oil (vehicle; 0.1 ml/rat) or with 0.1 ml of TP solution at 0.1, 0.5, 1, 2, 5, or 10 mg/0.1 ml. Parturition was delayed at 2, 5, and 10 mg TP, litter size was reduced at 5 and 10 mg TP, and pup weight was significantly reduced in both sexes at 0.5 mg TP and higher doses. Viability of offspring was unaffected at any dosage level. Androgenic effects seen at 0.5 mg TP in females included increased AGD at weaning and adulthood, reduced number of areolas and nipples, cleft phallus, small vaginal orifice, and presence of prostate tissue. This dose of TP elevated maternal T levels 10× but had no effect on fetal T levels. At 1 mg TP and above, female AGD on postnatal day (PND) 2 (or postcoital day 24 [gestation length = 22½]) was increased; areolas and nipples were virtually eliminated; levator ani muscle, bulbourethral glands, and seminal vesicles (2 mg TP and above) were present; none of the females developed a vaginal orifice and many females in the 1 and 2 mg TP dose groups developed a greatly distended, fluid-filled uterus after puberty. Maternal T levels at 1 mg TP were elevated 30×, and female fetal T levels showed an 80% increase. Male offspring displayed a reduced AGD and body weight on PND 2 at

0.5 mg TP and higher doses. These effects were not evident by weaning and male offspring displayed no malformations. We conclude that gestational administration of 0.5 and 1 mg TP masculinizes female offspring without greatly affecting pup viability or pregnancy of the dam. This study provides a useful model for *in utero* testing of environmental androgens for their potential to induce developmental abnormalities.

Key Words: testosterone propionate; androgens; masculinization; prenatal exposure; sexual differentiation; anogenital distance; agenesis of the lower vagina; Sprague-Dawley rats.

Sexual differentiation of mammals is dependent on the hormonal status of the fetus during a critical period of gestation. At this stage, the reproductive tract is bipotential and indifferent and development is acutely sensitive to androgens. Testosterone (T) and dihydrotestosterone (DHT) maintain the Wolffian duct system and promote growth and development of male sex accessory glands and external genitalia (Schultz and Wilson, 1974) whereas regression of the female reproductive tract is primarily dependent on Mullerian inhibiting substance (MIS; Josso *et al.*, 1977). Females are also susceptible to the masculinizing effect of androgens since the female reproductive tissues contain functioning androgen receptors (AR; rat, Bentvelsen *et al.*, 1995; Cunha *et al.*, 1991; human, Shapiro *et al.*, 2000). Human females exposed prenatally to higher than normal levels of androgens, as in the case of drugs taken by the mother (Schardein, 1993), develop ambiguous internal and external reproductive organs, or as in the case of congenital adrenal hyperplasia, can develop male-like external genitalia visible at birth, including clitoromegaly and fused labia (Ammi *et al.*, 1992; New and Wilson, 1999), and male play behavior (Berenbaum *et al.*, 2000). Prenatal administration to the rat of exogenous androgens, such as T, methyltestosterone, or testosterone propionate, induces in the female offspring male-like genitalia, increased anogenital distance, (AGD; Greene *et al.*, 1939; Kawashima *et al.*, 1978; McCoy and Shirley, 1992; Rhees *et al.*, 1997; Swanson and van der Werff ten Bosch, 1965), delayed puberty, early constant estrus (Kawashima *et al.*, 1978; McCoy and Shirley, 1992; Slob *et al.*,

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1983), delayed anovulatory syndrome (Slob *et al.*, 1983; Swanson and van der Werf ten Bosch, 1965), and male-like changes in brain nuclei (Ito *et al.*, 1986). In other mammals, prenatal androgens increased AGD (mouse, Gandelman *et al.*, 1979), and induced male-like sexual behavior (guinea pig, Phoenix *et al.*, 1959; rhesus monkey, Goy *et al.*, 1988) in the female.

In addition to their effects on sexual development, prenatal exposure to androgens can result in other adverse effects. High levels of T or other androgens administered to the dam can induce infanticide in rats (Rosenberg and Sherman, 1974) and mice (Mann and Svare, 1983) and can produce negative effects on the offspring such as low body weight (Fritz *et al.*, 1984; Slob *et al.*, 1983) and reduced litter size or low pup viability (Fritz *et al.*, 1984; Popolow and Ward, 1978; Rosenberg and Sherman, 1974), and decreased reproductive capacity of the dam including delayed parturition and a greater number of resorptions (Greene *et al.*, 1939; Swanson and van der Werff ten Bosch, 1965). In addition, women with hyperandrogenism often have low reproductive success (Gustafson *et al.*, 1996; Redmond, 1995).

There is a growing awareness that androgenic chemicals are present in the environment. Female mosquito fish downstream of kraft pulp mill effluent were found with anal fins that had developed into a male-like gonopodium (Davis and Bortone, 1992) and female marine animals and bears have been found masculinized (Vos *et al.*, 2000). More recently, the drug trenbolone acetate, given to beef cattle to improve weight gain and feed, was shown to cause masculinizing effects in female rat offspring including clitoral enlargement and increased AGD (FDA summary, 2000). The above information provides examples of how environmental androgens might alter development.

In light of this information, it is possible that the female reproductive system would be altered by environmental androgen exposure, especially during the prenatal period. The fetus is acutely susceptible to the effects of environmental endocrine disruptors (Gray *et al.*, 1999; Wolf *et al.*, 2000). Therefore, the study of the effects of prenatal testosterone propionate (TP) on male and female offspring will prove a useful model for the study of environmental androgens and allow us to identify sensitive endpoints to monitor in future studies. In the present study, we sought to characterize the effects of prenatal TP in the male and female rat and to define developmental endpoints that are sensitive to disruption of sexual differentiation by TP. In 1 experiment, presented first, we describe *in vivo* effects of various doses of prenatally administered TP in the adult offspring. In a second experiment, we measured the levels of T in the serum of the dam and in the gestational day (GD) 19 male and female whole fetuses after administration of TP to the dam to determine the levels in the fetus responsible for the physical effects of TP observed in the adult rat offspring. This information will be used to determine androgenic, nontoxic doses of TP suitable for coadministration with antiandrogens such as vinclozolin during the gestational period.

MATERIALS AND METHODS

Study 1: Adult Offspring Dose-Response Experiment in Vivo

Twenty-eight timed-pregnant Sprague-Dawley rats (Charles River Labs, Raleigh, NC) were received on GD 3 and housed 1 per cage in polycarbonate cages (20 × 25 × 47 cm) with laboratory-grade pine shavings (heat-treated to remove resins) as bedding. They were acclimated to 68–74°F and 40–50% relative humidity on a reversed light schedule (14 h light:10 h dark; lights off 1100 h EST). They were given Purina LabDiet 5008 (high-energy diet for gestation and lactation) and tap water (Durham, NC municipal water, tested for pesticides and heavy metals) *ad libitum*. On GD 12, dams were weighed, weight ranked, and randomly assigned to dose groups (4 dams per group) that were equilibrated with respect to body weight. On GD 14–19, rats were dosed daily by sc injection with 0, 0.1, 0.5, 1, 2, 5, or 10 mg TP (CAS# 57-85-2; lot# 98H0566) suspended in 0.1 ml corn oil. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. Doses used were on a per rat basis without correction for body weight in order to replicate methods used extensively by other investigators (Greene *et al.*, 1939; Kawashima *et al.*, 1978; Lee and Hutson, 1999; McCoy and Shirley, 1992; Rhees *et al.*, 1997; Swanson and van der Werff ten Bosch, 1965).

Maternal weight was monitored throughout the dosing period. Day of delivery was recorded and GD 23 was designated postnatal day (PND) 1 for all litters, including those that actually delivered on a later day. On PND 2, pups were counted, weighed, sexed if possible (sexing of pups could not be reliably performed in the higher dose groups), and anogenital distance (AGD) was measured. AGD was measured on each pup in a blind fashion using a dissecting microscope fitted with an ocular micrometer reticle. On PND 15, pups were reexamined for sexual phenotype, their sex confirmed or reassigned if necessary, and males and females were checked for areolas in a blind fashion. An areola or a nipple was considered an areola and areola counts were based upon the consensus of 2 technicians. Areolas were described as either faint, smaller than normal, or normal, meaning prominent and easily identified.

On PND 22, pups were weaned, counted, sexed, and measured for AGD in a blind fashion using micron rotary dial calipers (Manostat). Litter mates were assigned an individual identification marking with picric acid stain, distributed 2–3 per cage, and housed under the same conditions as described for dams except that feed provided was switched to Purina LabDiet 5001 (standard rodent diet). Runts (3 animals) were weaned on PND 28, when they were large enough to reach the water dispenser. Dams were sacrificed 1 day after weaning (pup PND 23) by CO₂ asphyxiation followed by decapitation, and uterine implantation sites were counted by visual examination. Female offspring were checked for vaginal opening (VO) from PND 29–38 and male offspring were monitored for preputial separation (PPS) from PND 37–47 as indicators of puberty. From PND 88 to necropsy, females were sacrificed and gross necropsy was performed if they became bloated, weak, and in poor health.

On PND 112–158, females (3 per litter where possible) were euthanized by CO₂ asphyxiation followed by decapitation, shaved on the ventral and lateral surfaces of the trunk for viewing nipples, and necropsied in blocked fashion by treatment group. Blood was collected in sterile 13 ml vacutainer serum separation tubes (Becton Dickinson, Lincoln Park, NJ), centrifuged at 1000 × g at 8°C for 15 min and serum collected and stored at –70°C for subsequent measurement of estradiol (E₂) levels. Most of the endpoints measured that showed statistical significance at necropsy are summarized in Table 1. Phallus width and length, not included in table, were significant in some dose groups (measured on 3 per litter). Other endpoints, all of which were not significant, include weights of liver, right and left kidney, and paired adrenals (measured on 2 per litter), pituitary weight, and weight of filled or drained uterus (continued on 3 per litter). Uteri were classified as normal or having hydrometrocolpos, a condition marked by severe distention and fluid retention of both the uterus and upper vagina. Ovaries were observed fresh for the presence of corpora lutea (CL). Observation of bulbourethral glands (BUG) was included in the necropsy when this structure was noticed, after 1 female from each of the 0, 0.1, 0.5, and 1 mg TP dose groups had been necropsied. Considering the

TABLE 1
Endpoints Monitored at Necropsy in Female Offspring Exposed to Testosterone Propionate (TP)
on GD 14–19, in Order of Sensitivity

LOAEL	Endpoint	Description	n
0.5	Number of nipples	Number of nipples visible on rat after shaving	3 per Litter
0.5	Cleft phallus	Cleft or split through the dermis on ventral side of phallus	All on study
0.5	Vaginal thread	Thread of tissue transecting the vaginal orifice	All on study
0.5	Vaginal-genital distance (VGD)	AGD-AVD difference	3 per Litter
0.5	Prostate	Presence of tissue similar in appearance and location to that in a male	All on study
1	Anogenital distance (AGD)	Distance (mm) from cranial edge of anus to base of phallus	3 per Litter
0.5	Anovaginal distance (AVD)	Distance (mm) from anterior edge of anus to posterior edge of vaginal orifice	3 per Litter
1	Vaginal agenesis	Absence of lower vagina, or vaginal orifice (Vorfice)	All on study
1	Levator ani (LA)	Presence of tissue similar in appearance and location to that in a male	All on study
2	Bulbourethral glands (BUG)	Presence of tissue similar in appearance and location to that in a male	All on study
2	Seminal vesicle (SV)	Presence of tissue similar in appearance and location to that in a male	All on study
5	Kidney to ovary distance (Kd-Ov)	Shortest distance (mm) from edge of kidney to edge of ovary when pulled taut, side and downward movement is restricted (usually at an angle; see Fig. 6)	3 per Litter
5	Vertical kidney to ovary distance (vtKd-Ov)	Vertical distance (mm) from caudal tip of kidney to ovarian pelvis when ovary is pulled taut (will be a straight vertical line; see Fig. 6)	2 per Litter (begun after 1 per litter)
10	Paired ovary weight	—	2 per Litter

Note. LOAEL, lowest observable adverse effects level for each endpoint, in the current study, in mg/rat.

nonexistent incidence of BUG in the low dose groups, probably no BUG were overlooked.

A test was performed on 3 intact reproductive tracts *in situ* in the 2 and 5 mg TP dose groups. Tracts were selected from middle and high dose groups to represent severely affected urogenital tracts (2 and 5 mg TP group) and a tract with suspected hydrometrocolpos (2 mg TP dose group) for the determination of uterine fluid flow, as follows. Saline (~2 cc) was injected at a rate of approximately 0.5 cc/s into the uterus using a 3 cc syringe fitted with a 22 gauge needle, and the needle kept in place in the uterine wall during observation. The entire reproductive tract including the phallus was viewed and any distention or leakage of fluid was noted and recorded.

Reproductive tissues (uterus, ovary, phallus, prostate, levator ani [LA], seminal vesicle [SV], BUG, any male structures, vaginal threads, pituitary) were stored in Bouin's fixative for 24 h and rinsed and stored in 70% ethanol for histological assessment (see histopathology section).

Males (2 per litter) were sacrificed by decapitation and necropsied on PND 161–172. Endpoints measured were weights of glans penis, pituitary, liver, right and left kidneys, paired adrenals, right and left testes, left epididymis, right caput + corpus epididymis, right cauda epididymis, ventral prostate (VP), SV, and LA + bulbocavernosus (LA/BC). The remaining males were necropsied on PND 177 and 178 for 1 endpoint that showed a statistically significant difference, the glans penis weight, and these data represented all males, or 3–6 males per litter per dose group.

Radioimmunoassay. Adult female offspring serum from the necropsy was assayed for E₂ using the E₂ RIA kit #TKE21 (Diagnostic Products Company, Los Angeles, CA) with the supplied protocol.

Histopathology of female tissues. Reproductive tracts were dissected from the carcass intact with associated accessory organs (prostate, SV, phallus, and LA) attached, fixed in Bouin's solution for 24 h, rinsed with water, and stored in 75% ethanol. Accessory organs were observed *in situ* and trimmed by the pathologist. All tissues (distended uteri [n = 5], malformed uteri [n = 3], BUG [n = 8] and ovaries, prostates, LAs, SVs, [1 per litter each]) were paraffin embedded with VIP tissue processor, sectioned 3 microns thick to produce 1 section, stained with Harris hematoxylin and eosin Y (Anatich, Battle Creek, MI), and evaluated for pathologies and the identity of male

organs by the pathologist. CLs were identified in ovarian sections (1 section per ovary, 1 ovary per female, 1 female per litter) as large round masses of unorganized, luteal granulosa cells. Ovarian sections were scored for CL abundance and for antral follicle abundance in a blind fashion. Scoring system was designed as follows: no CLs (or antral follicles), score = 0; if 1 to 2, score = 1; if 3 to 6, score = 2; if 7 to 10, score = 3; if ≥ 11, score = 4.

Study 2: Maternal/Fetal Testosterone Level Experiment in Vivo

Eight timed-pregnant Sprague-Dawley rats (Charles River Labs, Raleigh, NC) were received on GD 4 (GD 1 = day of sperm positive smear) and housed 1 per cage. Conditions were the same as described for the above experiment during the gestational period. On GD 13, dams were weighed, weight ranked, and randomly assigned to treatment groups that were equilibrated with respect to treatment group. Dams were dosed on GD 14–19 by sc injection in the nape of the neck with 0.1 ml of 0 (n = 3), 0.5 (n = 2), or 1 (n = 2) mg TP/0.1 ml corn oil dose solution. These doses were selected based on their masculinizing effects in the female offspring without any toxicity in the dam. On GD 19, 1 h after dosing, dams were euthanized in blocked fashion by CO₂ asphyxiation followed by decapitation. Blood and fetuses were collected not sooner than 1 h after dosing to allow TP to undergo distribution and metabolism and reach probable peak T levels in both dam and fetus, as TP has a longer half-life than that of T (Rhees *et al.*, 1997; Sommerville and Tarttelin, 1983). Each dam was euthanized and its fetuses collected before the next dam was euthanized. Fetuses were removed from the uterus, held on ice in a small plastic petri dish, sexed by opening of their abdominal wall under a dissecting microscope and viewing of internal reproductive organs, and saved in 15 ml plastic round-bottomed Falcon tubes (Becton-Dickinson, Lincoln Park, NJ). Fetuses were stored at –20°C for ~1 week until extracted and assayed for T levels. To avoid interference of the fetal carcass collection protocol, maternal blood was collected on a separate set of dams (n = 6; 2 per dose group) at a later date. To avoid contamination of blood samples with the TP dose solution from the neck, blood was collected by cardiac puncture. Dams were maintained under the same housing conditions described above. Dams were selected for blood collection in blocked fashion by treatment group (1 representative of each dose group constitutes a block). Blood was collected by heart puncture while dam

TABLE 2
Effects of Increasing Doses of Testosterone Propionate (TP) Administered
on GD 14–19 on Dam Fertility and Pup Weight and Viability

Endpoint	0	0.1	0.5	1	2	5	10
Maternal wt. gain (g)	50.78 ± 3.14	55.26 ± 4.67	35.45 ± 4.45	36.45 ± 4.63	31.0 ± 2.25*	33.68 ± 5.44*	26.03 ± 4.17***
No. late/No. normal delivery (%)	0/4 (0)	0/3 (0)	0/4 (0)	1/4 (25)	2/4 (50)	3/4 (75)*	3/4 (75)*
Mean no. days late	0	0	0	1	0.75	1	1.33
No. whole litter loss by PND 5	0	0	0	1	0	0	1
Live litter size (PND2)	14.0 ± 0.58	12.67 ± 0.67	12.0 ± 0.91	8.5 ± 2.90	10.75 ± 2.46	7.5 ± 1.89*	6.25 ± 2.14**
No. uterine implants	15.25 ± 0.43	14.0 ± 0.82	15.5 ± 1.12	15.5 ± 1.80	15.5 ± 1.12	15.0 ± 2.23	13.75 ± 2.04
Pup wt (g) PND 2	7.43 ± 0.32	7.89 ± 0.26	4.76 ± 0.22‡	5.23 ± 0.43‡	4.83 ± 0.21‡	4.97 ± 0.37‡	5.26 ± 0.42**
Pup wt at weaning (g) (male)	50.07 ± 1.41	57.53 ± 2.40	44.35 ± 3.74	47.42 ± 2.94	44.92 ± 4.04	47.37 ± 6.02	42.65 ± 5.44
Pup wt at weaning (g) (female)	49.85 ± 1.93	54.5 ± 1.49	42.63 ± 2.11	44.52 ± 4.01	38.46 ± 6.74	42.80 ± 4.93	40.02 ± 2.47
Litter size at weaning	13.5 ± 0.50	11 ± 1.0	10.75 ± 1.65	8.00 ± 2.97	8.25 ± 2.46	7.25 ± 2.14*	6.0 ± 2.08*
% Viability to weaning	96.7	86.5	88.2	73.2	73.3	87.5	74.1

Note. Values are litter means ± SE unless otherwise described. PND, postnatal day; wt, weight.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; ‡ $p < 0.0001$; all compared to control values.

was under halothane anesthesia and dams died by exsanguination. Maternal blood was centrifuged and serum was stored at -70°C for 1 week until assayed for T levels. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted.

Fetal testosterone extraction. T was extracted from fetal tissue as described previously (Parks *et al.*, 2000). Fetuses were thawed and homogenized individually in 500 μl distilled deionized water with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After homogenization, 2 ml ethyl ether (Fisher Scientific, Pittsburgh, PA) were added to each tube, tubes were vortexed for 30 s, and centrifuged at 2000 rpm ($1000 \times g$) at 8°C for 10 min. Following centrifugation, each tube was held 1 at a time in an acetone/dry ice bath until the bottom aqueous layer froze, and the supernatant (ether layer) was then transferred to a 12 \times 75 mm glass tube. The ether extraction was performed twice. Glass tubes of ether extract were dried in a fume hood overnight. Tubes were stored for up to 2 weeks until analyzed by radioimmunoassay.

Radioimmunoassay (RIA) of dams and fetuses. Each tube of dried fetal extract was resuspended by vortexing for 30 s in 70 μl of 0 standard buffer provided in the Coat-A-Count Total Testosterone RIA kit #TKTT5 (Diagnostic Products Company, Los Angeles, CA). Fifty μl of the 70 μl fetal resuspension were transferred to the antibody-coated tubes in the RIA kit and T levels were determined according to the manufacturer's protocol. Tubes were read for 1 min each in a gamma counter (CliniGamma 1272, LBK-Wallac, Finland). Counts are programmed to report a ng/ml value. This value was adjusted for volume of fetal extract by multiplying by 0.07 (70 $\mu\text{l}/1$ ml). Maternal serum was vortexed and 50 μl of straight and 50 μl of 5 \times diluted serum was assayed in duplicate by RIA for T following the DPC Total Testosterone RIA kit protocol.

Statistics. Data were analyzed by ANOVA on a litter means basis using the PROC GLM (general liner models) function on SAS (for Windows 95, version 3.0.554, Cary, NC). Weights and distance measurements (kidney to ovary distance, Kd-Ov; AGD, ano-vaginal distance, AVD; phallus length) were analyzed with and without body weight as a covariate. Percentage data (% late delivery, % with nipples) were performed with arcsine transformation of the individual (dam) means or litter (pup) means. When significant differences were found for a main effect, a 2-tailed *t*-test was used to test differences

between treatment groups using least square means. Maternal endocrine data were log transformed to reduce heterogeneity of the variance. Uteri were classified as normal or having hydrometrocolpos both subjectively based on size and quantitatively based on weight. Counts and categorical data on malformations (cleft phallus, vaginal thread, absence of vaginal orifice, vaginal orifice-phallic cleft not separate, presence of prostate, SV, LA, BUG, hydrometrocolpos) and number of females dead before necropsy were analyzed on an individual basis using Fisher's exact test or chi square as appropriate.

RESULTS

Study 1: Dose Response in Vivo

Prewaning maternal and pup data. Maternal weight gain during the dosing period decreased with increasing dose of TP and was significant in the 2, 5, and 10 mg dose groups (Table 2). One dam (in the 0.1 dose group) was not pregnant and was not included in the weight gain assessment. Late delivery, in which dams delivered all their pups by any day later than the expected GD 23, occurred at 1 mg TP and higher doses and was significant in the 5 and 10 dose groups (Table 2). Two dams who delivered late lost their entire litters within 2 days (2 days after late delivery = PND 5; Table 2). Two other treated litters consisted of only a few runts; 2 pups in a 2 mg group litter and 1 pup in a 5 mg group litter. Live litter size on PND 2 was reduced in the 5 and 10 mg TP dose groups, and remnants of pups were found in nearly all treated group cages. Pup weight on PND 2 was significantly reduced at 0.5 mg and higher doses of TP, although this reduction was a transient effect. By weaning, PND 22, pup weight was not different in TP-exposed pups (Table 2). Although litter size was reduced at birth and weaning in the high dose groups (5 and 10 mg TP),

viability to weaning was unaffected. Pups too small to reach water supply on PND 22 (runts) were weaned on PND 28.

AGD. On PND 2, sex was determined and AGD recorded for males and for females of each litter in the 0, 0.1, and 0.5 mg TP dose groups (Fig. 1h). Sexing of the pups by AGD became more difficult and unreliable above the 0.5 mg dose. This dilemma is reflected in the AGD data shown in Figure 1. AGD measurements (AGDs) were obtained for each pup and all AGDs were grouped into ranges of AGDs. For each dose, AGD ranges were plotted along the X-axis and number of pups in each range was plotted along the Y-axis. The resulting line graphs then illustrate the frequency distribution of AGD measurements. In the control group (Fig. 1a), 2 populations of pups are displayed as 2 distinct peaks in the graph. One peak to the left represents a population of pups with smaller AGDs, identified as females, and the other peak represents a population of pups with larger AGDs, identified as males. With increasing dose of TP (Fig. 1b–1g), both peaks move closer to the middle ranges and identification of the pups as male or female based on their AGD becomes more difficult and eventually impossible. At 0.5 mg TP, the mean AGD on PND 2 for males is significantly reduced ($p < 0.05$) while the female AGD is unaffected ($F[2,8] = 0.88, p = 0.4496$; Fig. 1h; Table 3). At 1 mg and higher doses, it is clear that both male and female AGDs were affected on PND 2, as the mean female AGD increased and the mean male AGD decreased.

At PND 22, AGD in males was not affected, while AGD in females was significantly increased at 1, 5, and 10 mg doses of TP (Table 3). With body weight covariance analysis, AGD in females was significantly increased at 0.5 mg TP ($p < 0.05$) and in all higher doses ($p \leq 0.0001$; Table 3). This effect persisted to necropsy (PND 112–158; Table 4) and is therefore permanent under the conditions of this study.

Areolas. Areolas were described in 2 degrees of severity: faint and normal. Normal was limited to only those areolas that were prominent. One hundred percent of females in the 0 and 0.1 mg dose groups displayed the full compliment of normal areolas (12). In the 0.5 mg dose group, although 100% of the females displayed areolas and most females displayed the full number of areolas (mean = 11.9 areolas), many of these areolas were faint and the average number of normal areolas was significantly reduced (mean = 8.6; Table 3). In the 1 mg TP and higher dose groups, virtually all females lacked normal areolas (Table 3), and displayed only a few (mean < 2) faint areolas if any. Areolas were not detected in any male offspring.

Weaning, puberty, and viability. Viability from birth to weaning (PND 22) and body weight at weaning were unaffected in either sex by any dose of TP (Table 2).

The ages at PPS in all males and VO in females in the 0.1 and 0.5 mg dose groups were unaffected by TP treatment ($F[6,15] = 0.80$; $F[3,7] = 2.03$, respectively; Table 3). However, inspection of females for VO revealed an absence of the vaginal orifice in 100% of females in the 1 through 10 mg TP

dose groups (Fig. 2; Table 5), absence of the vaginal orifice in 1 female in the 0.5 mg dose group, and a vaginal orifice so small in 1 female in the 0.5 mg TP dose group VO could not be determined. Shortly after puberty, female offspring from the middle range of TP dose groups (in this case the 1, 2, and 5 mg TP groups) appeared to have distended abdomens and began dying (11 in the 1 mg group, 10 in the 2 mg dose group, and 1 in the 5 mg TP dose group died before scheduled necropsy). Thereafter, females with distended abdomens that appeared lethargic were sacrificed for gross necropsy (3 females in the 1 mg dose group, 1 female in the 2 mg dose group; Fig. 3a). Necropsy of each female revealed an extremely large, distended, fluid-filled uterus and upper vagina (hydrometrocolpos), some uteri weighing as much as 94 grams, with no other gross abnormalities aside from the absence of a vaginal orifice (Fig. 2). This condition was suspected of being the cause of death of the females that had died.

Female necropsy. At necropsy of female offspring (PND 112–158), uterine weights varied greatly in the middle dose groups (1, 2, and 5 mg TP) due to the degree of uterine enlargement and fluid accumulation in some individuals (Fig. 3b; Table 4). Of the females sacrificed both before and during scheduled necropsy, 5 out of 9 females in the 1 mg group, 2 out of 10 females in the 2 mg group, and 1 out of 20 females in the 5 mg group displayed an abnormally distended, fluid-filled uterus, or hydrometrocolpos. No female from any other dose group, including the 10 mg dose group, displayed such a condition. Females that had died before the necropsy period without being inspected are suspected of having had hydrometrocolpos.

Females that had no vaginal orifice did have a cervix and upper vagina. In the middle dose groups (1, 2, and 5 mg TP), the upper vagina ended blindly alongside the dorsal aspect of the urethra. In the higher dose groups (mostly 5 and 10 mg TP), the formation of the end of the upper vagina was difficult to determine visually, but the vagina was apparently continuous with and opened into the urethra, as explained subsequently. A test was performed on rats in 2 middle dose groups (2 and 5 mg TP) to determine whether the fluid in the uterus could escape. Saline injected into the uterus of 1 female in the 2 mg TP dose group did not escape but further distended the uterus (uterus initially appeared to be only slightly distended, not having hydrometrocolpos). Saline injected into the uterus of other females without hydrometrocolpos in the 2 mg (1 female) and 5 mg TP (1 female) dose groups passed into the bladder and exited via the urethra to the tip of the phallus, indicating that fluid in the uterus could escape in some females in the high dose groups with no vaginal orifice by exiting through the urethra. In addition to the hydrometrocolpos observed in the middle dose groups, 3 females in the high dose groups (1 in the 5 mg and 2 in the 10 mg TP dose group) had malformed uteri such that the ends of the horns were hard and curled or

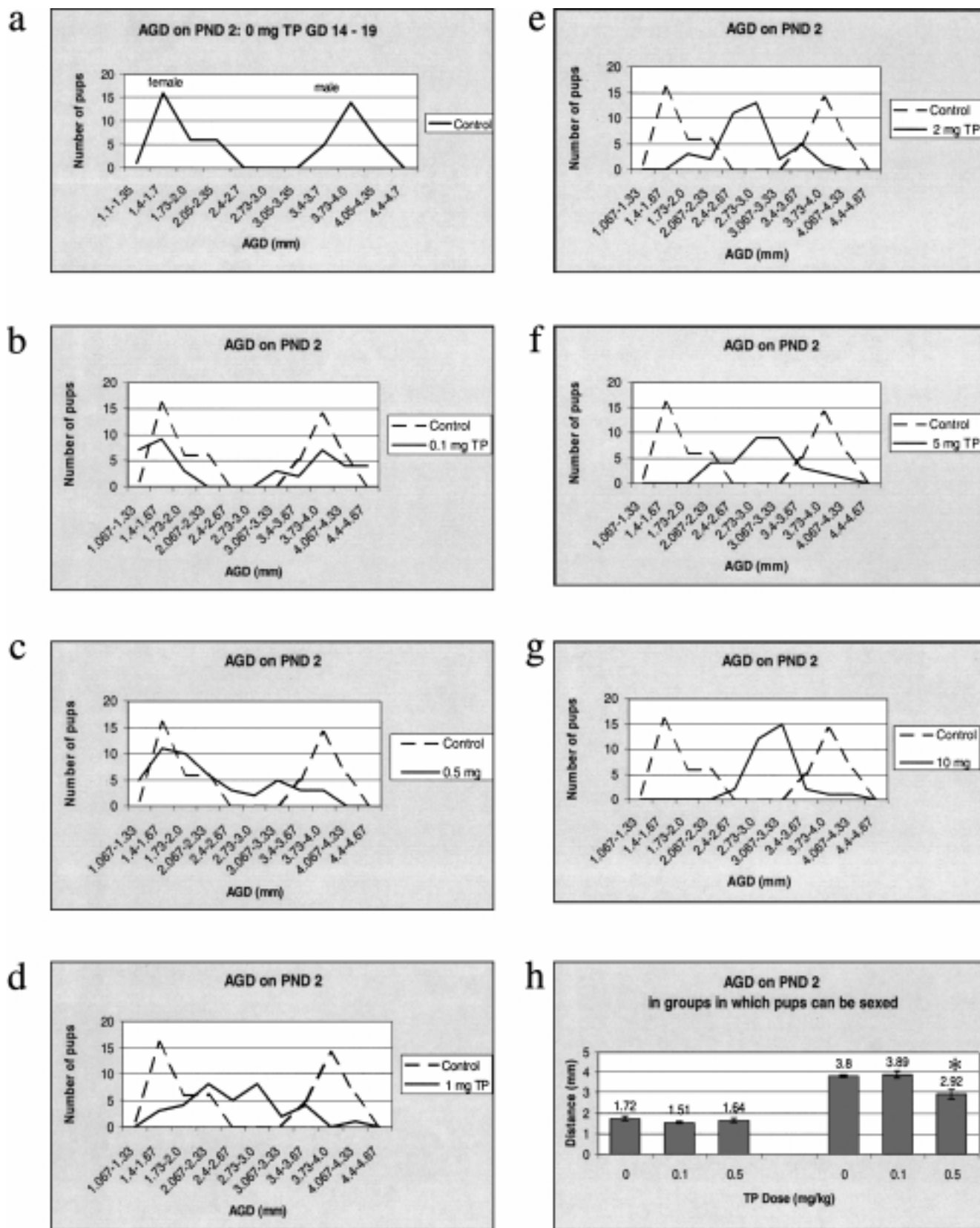


FIG. 1. Effect of testosterone propionate administered prenatally (gestational days 14 through 19) on anogenital distance (AGD) in 2 day old pups; dose groups: (a) control, (b) 0.1 mg TP, (c) 0.5 mg TP, (d) 1 mg TP, (e) 2 mg TP, (f) 5 mg TP, (g) 10 mg TP, (h). (a–g) Frequency distribution histograms of AGD in pups. Solid line in each graph represents the distribution of measurements for the dose group represented; dashed line represents control group distribution for comparison. (h) Mean AGD \pm SE for the lower dose groups, in which sexing of pups was possible. AGD was significantly reduced in male pups in the 0.5 mg TP dose group. * $p < 0.05$.

TABLE 3
Effects of Increasing Doses of Testosterone Propionate (TP) Administered on GD 14–19
on Androgenic Endpoints during Prepubertal Development

Endpoint	0	0.1	0.5	1	2	5	10
AGD on PND 2 (male)	3.80 ± 0.06	3.89 ± 0.21	2.92 ± 0.26*	ND	ND	ND	ND
AGD on PND 2 (female)	1.72 ± 0.099	1.51 ± 0.11	1.64 ± 0.107	ND	ND	ND	ND
AGD on PND 22 (male)	16.88 ± 0.38	17.34 ± 0.73	15.12 ± 1.14	16.25 ± 0.46	15.60 ± 0.92	16.28 ± 0.68	15.77 ± 0.58
AGD on PND 22 (female)	10.30 ± 0.28	10.44 ± 0.59	10.70 ± 0.33[*]	12.32 ± 0.61*[*‡]	11.85 ± 0.99[*‡]	13.81 ± 0.27***[*‡]	13.81 ± 0.41***[*‡]
% Areolas (female)	100	100	100	50.0 ± 0.19*	25.0 ± 0.19***	26.2 ± 0.14***	30.0 ± 0.15***
No. areolas/pup (all)	12.03 ± 0.03 ^a	12.0 ± 0	11.92 ± 0.08	1.25 ± 0.66‡	0.65 ± 0.58‡	0.52 ± 0.29‡	0.53 ± 0.29‡
No. normal areolas/pup	12.03 ± 0.03 ^a	12.0 ± 0	8.61 ± 1.87**	0‡	0‡	0.095 ± 0.095‡	0‡
Age at VO	34.74 ± 0.48	34.56 ± 0.78	34.05 ± 0.34	NV	NV	NV	NV
Age at PPS	43.62 ± 0.43	42.64 ± 1.83	45.11 ± 0.40	44.62 ± 1.37	45.39 ± 0.31	44.36 ± 1.52	44.28 ± 0.53

Note. Values are litter means ± SE. AGD, anogenital distance; PND, postnatal day; ND, no data—sexes could not be distinguished; VO, vaginal opening; PPS, preputial separation; NV, no vaginal orifice. Symbols in brackets represent significance when analyzed by covariate analysis with body weight.

^a One rat in the control group had 13 nipples.

p* < 0.05; *p* < 0.01; ****p* < 0.001; ‡*p* ≤ 0.0001; all compared to control values.

crumpled, or in 1, the portion of the uterus in which the 2 horns meet had become hardened and enlarged.

Every female in the low dose groups (0, 0.1, and 0.5 mg TP) had nipples and had nearly all 12 nipples. However, at 0.5 mg TP the number of nipples was significantly reduced to 11.25. At 1 mg TP and higher doses the percentage of females having nipples and the mean number of nipples per rat was drastically reduced to near 0 (Table 4).

AGD at necropsy increased in a dose-dependent fashion and was significant (by covariate analysis with body weight) at 1 mg TP and higher doses (Table 4). Although AGD was not increased in the 0.5 dose group, many females displayed an array of genital malformations such as cleft phallus, vaginal thread, and a joined vaginal orifice-cleft phallus (Table 5). In the latter case, the perimeter of the vaginal orifice ran contin-

uous with the cleft of the phallus so that the 2 were nearly indistinguishable. In those females that did have a complete vaginal orifice, the orifice appeared closer to the phallus than in control females, less well defined, and smaller in diameter (not quantitated). The measurement of AVD (Table 1) in the 0.5 mg dose group reflected this trend (*p* = 0.0532), and the difference between the AGD and the AVD, or the vaginal-genital distance (VGD), indicating the distance from the vaginal orifice to the phallus, was significantly smaller at 0.5 mg TP (*p* < 0.01; Tables 1 and 4). These malformations were unique to the 0.5 mg dose group, with the exception of a partially cleft phallus that was observed in 1 animal in the 1 mg TP dose group (Table 5).

Phallus length was significantly increased in the middle dose groups (2 and 5 mg TP; Table 4). Phallus width was increased

TABLE 4
Effects Found at Necropsy (PND 112–158) in Female Offspring Exposed to Testosterone Propionate on GD 14–19

Endpoint	0	0.1	0.5	1	2	5	10
% Nipples	100	100	100	0‡	8.33 ± 0.08‡	5.00 ± 0.08‡	6.67 ± 0.07‡
No. nipples/adult female	12.0 ± 0	11.92 ± 0.08	11.25 ± 0.48*	0‡	0.17 ± 0.17‡	0.08 ± 0.08‡	0.27 ± 0.27‡
Phallus length ^a	3.53 ± 0.26	3.05 ± 0.14	3.04 ± 0.07	3.61 ± 0.31	4.69 ± 0.21***	4.10 ± 0.19*	3.48 ± 0.25
Phallus width ^a	4.49 ± 0.08	4.02 ± 0.12	4.90 ± 0.13	5.17 ± 0.13*	4.96 ± 0.17	4.73 ± 0.29	5.28 ± 0.12**
AGD ^a	24.12 ± 0.38	23.71 ± 0.46	23.41 ± 0.63	25.94 ± 0.74*	26.69 ± 0.98**	29.52 ± 1.43‡	31.38 ± 2.12‡
AGD-AVD difference	7.47 ± 0.61	7.66 ± 0.70	4.43 ± 0.61**	—	—	—	—
Uterine wt (filled)	646.92 ± 45.27	594.03 ± 89.76	455.24 ± 96.07	28,633.6 ^b	8624.90 ± 7927.7	10,632.5 ± 10,209.2 ^c	361.13 ± 96.05
Uterine wt (drained)	535.23 ± 30.46	518.87 ± 49.01	355.40 ± 34.00	1347.46 ± 553.36	1469.44 ± 939.5	1234.27 ± 1029.7	423.13 ± 87.13
Ovary wt (paired) ^a	102.97 ± 6.00	102.92 ± 7.06	83.01 ± 7.32	70.72 ± 2.22	81.57 ± 6.61	84.72 ± 13.71	82.93 ± 23.65*
Body wt	332.09 ± 17.88	342.44 ± 19.15	293.69 ± 11.14	290.43 ± 36.13	320.67 ± 20.89	319.69 ± 33.08	350.83 ± 25.38

Note. Values are litter means ± SE, 2–3 individuals per litter. AGD, anogenital distance; AVD, anovaginal distance (see text for description); wt, weight. Organ weights given in mg, body weight in g.

^a Analyzed by covariate analysis with body weight.

^b Value represents mean of 4 individuals from one litter, therefore one litter mean and no SE.

^c One individual had a filled uterus wt of 92,046.0 mg. The mean filled uterus weight of the other individuals on a litter means basis was 466.5 mg.

p* < 0.05; *p* < 0.01; ****p* < 0.0005; ‡*p* < 0.0001; all compared to controls.

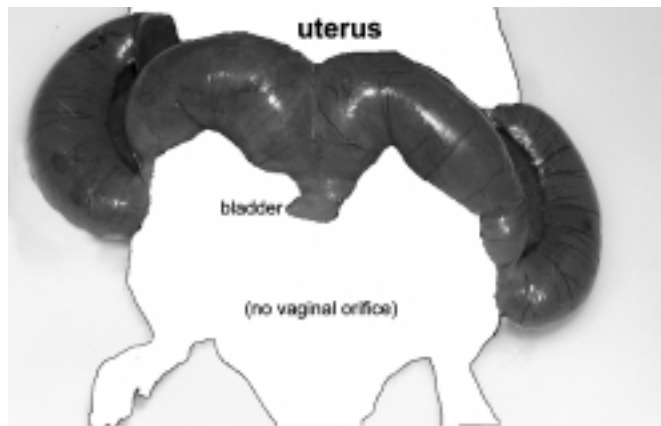


FIG. 2. Severe distention and fluid retention of the uterus (hydrometrocolpos) with vaginal atresia in postpubertal females exposed prenatally to middle range doses of testosterone propionate (TP; gestational days 14–19). Female was exposed prenatally to 1 mg testosterone propionate and sacrificed at postnatal day 74 after presenting a distended abdomen. The uterus was filled with brown, viscous, odorous fluid.

in the 1 and the 10 mg dose groups only, with or without body weight as a covariate—these results did not reveal a pattern and were variable, possibly due to body fat, and thus were considered less indicative of a response (Table 4). The internal shaft of the phallus appeared thicker and more developed, more masculine, with increasing dose of TP and included penile bulbs at their base to which LA were usually attached.

Male structures such as prostate, SV, LA, and BUG or cowper's glands, were present in females in the higher dose groups to a significant degree (Table 6, Fig. 4). Prostatic tissue appeared on the ventral side of the urethra close to the base of the bladder, a location in which it is found in the male, and its

TABLE 5
Malformations in Female Offspring Exposed to 0.5 mg TP/dam on GD 14–19

Endpoints	0	0.5	1
No. cleft phallus/no. necropsied (%)	0/28	15/26 (57.7)**	1/6 (16.7)
No. VOr in cleft/no. necropsied (%)	0/28	7/26 (26.9)** ^a	0/6
No. vaginal thread/no. necropsied (%)	0/28	7/26 (26.9)*	0/6
No. absent VOr/no. weaned females (%) ^b	0/28	1/26 (3.85)	19/19 (100)**

Note. Percentages are on an individual basis. VOr, vaginal orifice.

^a The 7 with VOr in cleft were not necessarily the same 7 that had vaginal thread. Animals displaying 1 particular malformation often, but not always, displayed another malformation.

^b Data collected on PND 29–38 while checking for VOp.

* $p = 0.004$; ** $p < 0.0001$; all compared to control values. Analyzed by Fisher's Exact test.

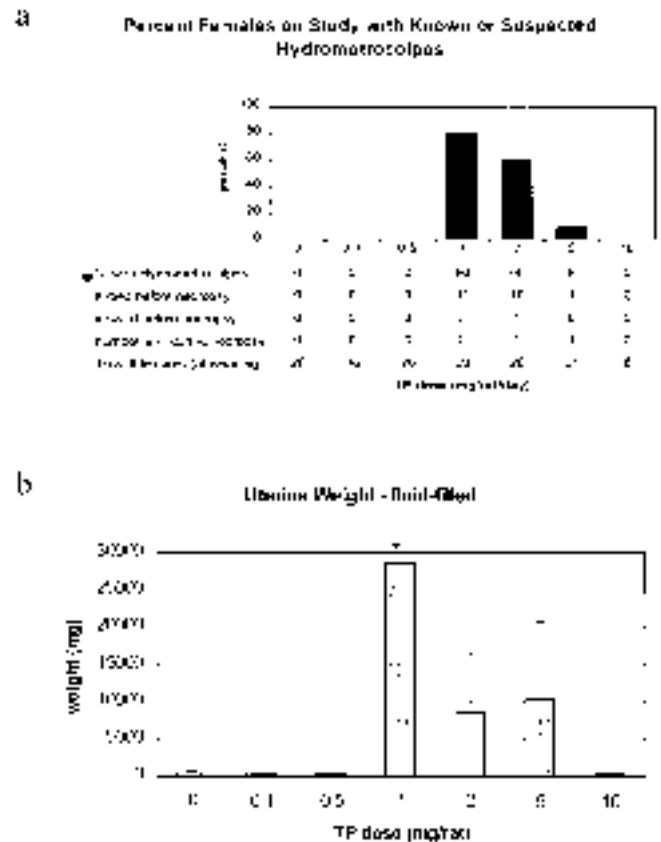


FIG. 3. Effects of prenatal testosterone propionate (TP; gestational days 14–19) on (a) hydrometrocolpos and mortality and (b) uterine weight at necropsy (PND 112–158). H, hydrometrocolpos. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, compared to controls; analyzed using Fisher's exact test.

incidence was significant at doses as low as 0.5 mg TP. The prostate appeared to increase in size with increasing dose (not quantitated) and to acquire dorsolateral lobes. The SV appeared to be attached to either side of the cervical area of the uterus, and the BUG resided in a pocket within the perineal muscles at location corresponding to the location of the BUG in the male. The identity of these structures was confirmed histologically (1 per litter; Figs. 4c–4f). Some females in the 1 mg TP and higher dose groups also had the appearance of a gubernacular cord upon dissection, or at least the presence of a stream of fat and other connective tissue issuing out from an invagination in the muscle wall of the perineal region on either side of the upper vagina, an area corresponding to the scrotum.

The right and left Kd-Ov distances and right and left vertical Kd-Ov (vtKd-Ov) distances were significantly increased in the 2 highest dose groups (5 and 10 mg; Fig. 5), indicating elongation of the ovarian ligament(s). In some cases, it appeared the ligament connecting the ovary to the dorsal body wall behind the kidney, or cranial suspensory ligament, was not just elongated but absent, as no tension could be produced upon

TABLE 6
Presence of Male Reproductive Organs and CL and Antral Follicles in Females Exposed to Testosterone Propionate (TP) on GD 14–19

Endpoint	0	0.1	0.5	1	2	5	10
No. females necropsied	28	16	26	6	9	20	8
No. with prostate (%) ^a	0	0	14 (53.8)**	5 (83.3)**	6 (66.7)**	20 (100)**	8 (100)**
No. with SV (%) ^a	0	0	0	0	1 (11.1)	19 (95.0)**	8 (100)**
No. with LA (%) ^a	0	0	0	1 (16.7)	3 (33.3)*	20 (100)**	7 (87.5)**
No. with BUG (%) ^b	0	0	0	0	3 (33.3)*	2 (10.0)	2 (25.0)†
CL score ^c	3.5 ± 0.58	3.67 ± 0.58	3.5 ± 0.58	3.5 ± 0.71	3.5 ± 1.00	3.25 ± 0.96	2.667 ± 1.53
Antral score ^c	2.75 ± 0.96	3.67 ± 0.58	3.75 ± 0.50	3.5 ± 0.71	2.75 ± 1.26	3.5 ± 0.58	3.67 ± 0.58
Estradiol levels at necropsy (ng/ml) ^d	67.97 ± 8.0	33.61 ± 3.47	51.91 ± 9.79	26.29 ± 9.35	35.58 ± 9.52	66.22 ± 6.18	41.19 ± 16.15

Note. Percentages are on an individual basis, not a litter means basis. SV, seminal vesicle; LA, levator ani; BUG, bulbourethral gland.

^a Identity of organs confirmed by pathology at 1/litter.

^b Identity of every BUG confirmed.

^c Scores based on CL or antral follicle abundance in ovarian sections (see text for details). Values are means ± SD.

^d Overall F value not significantly different. Data analyzed by Fisher’s exact test unless otherwise noted.

p* < 0.05; *p* < 0.0001; compared to control values. †*p* = 0.0103 by Pearson’s χ^2 test, not different by Fisher’s Exact.

pulling the ovary without tearing the fat lying between the ovary and kidney.

Paired ovary weight was slightly but significantly reduced at 10 mg TP when normalized to body weight (*p* < 0.05), but otherwise not affected (Table 4). Upon gross visual inspection at necropsy, ovaries from every female of each dose group appeared to have CLs. CL score and antral follicle score in histological sections of ovaries (1 per litter) was not different between dose groups (Table 6). Liver, right and left kidney, paired adrenal gland, and pituitary weights were not affected by TP as compared to controls (by covariate analysis with body weight; *F*[7,16] = 7.15, 3.68, 3.20, 1.14, and 1.78, respectively; data not shown).

E₂ levels from female offspring at necropsy were unaffected (*F*[6,16] = 2.63; Table 6). E₂ levels and visual inspection of uteri and ovaries suggest females from every dose group had estrous cycles.

Male necropsy. Male necropsy revealed only a reduction in glans penis weight, highly significant in the middle dose groups (in this case 0.5, 1, and 2 mg TP) and slightly significant at 10 mg TP (Table 7), graphically approximating a U-shaped dose-response curve. No other effect was found in the male offspring at necropsy (Table 7).

Study 2: Maternal and Fetal T Levels

Maternal serum T levels (in ng/ml) increased in a dose dependent fashion and were significantly elevated at 0.5 and 1 mg TP/dam (other doses were not analyzed). In contrast, fetal T levels (in ng/fetus) rose significantly only in the female at 1 mg TP and not at 0.5 mg TP, despite the increased T level in the dam at this dose (other doses not analyzed). Male fetal T levels were not affected. The elevation in female fetal T levels

at 1 mg TP was increased to near normal male fetal T levels (Fig. 6).

DISCUSSION

Presented herein is a comprehensive dose-response study of the reproductive and developmental effects of prenatal TP exposure that provides a model on which to base testing studies for environmental androgens. We have also determined doses of TP that would effectively masculinize the female rat without inducing overt maternal or fetal toxicity.

A major outcome of this study is the identification of endpoints in the female sensitive to TP that can be used to detect *in utero* exposure to androgenic chemicals (Table 1). Endpoints in the female that were most sensitive to maternal sc TP administration, found at the 0.5 mg TP dose, include malformations of the external genitalia, inhibition of areolar and nipple development, and prostate development. Less sensitive endpoints, found in the middle dose ranges, include AGD, complete absence of nipples, complete absence of a vaginal orifice, precocious death and hydrometrocolpos, and LA development. Least sensitive endpoints, induced only at the 2, 5, and 10 mg TP dose levels, include SV and BUG development, and elongation of the ovarian ligament. In addition, our data revealed a remarkable inverted U-shaped dose-response curve for uterine condition that resulted in a similar level of mortality at the corresponding dose levels. A similar cascade of effects was induced by various doses of TP in the early study by Greene *et al.* (1939). In addition to those endpoints, we included AGD, areola and nipple count and incidence, and hormone levels, and we used a larger number of offspring for the study.

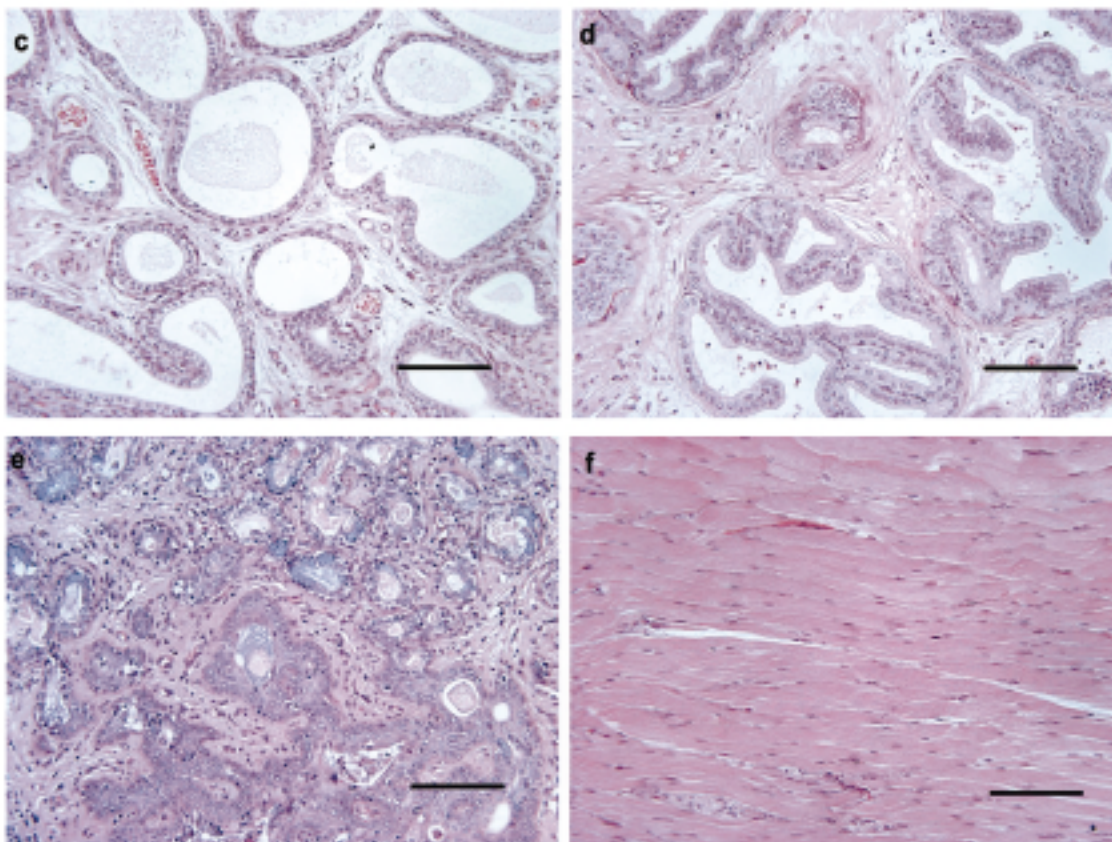
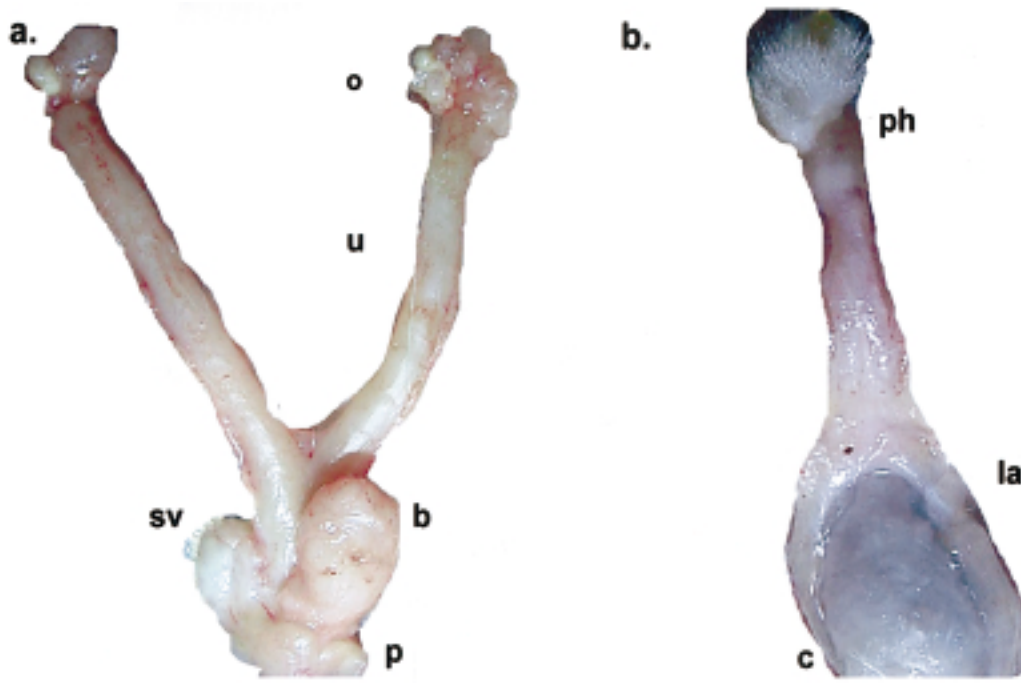
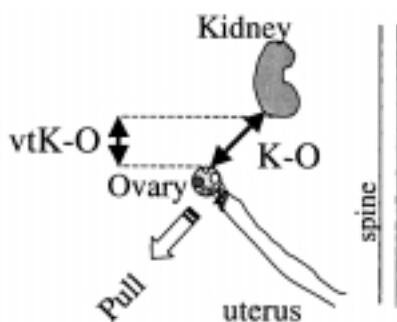


FIG. 4. Development of male organs in females prenatally exposed to 10 mg TP (gestational days 14–19). o, ovaries; u, uterus; b, bladder; p, prostate; sv, seminal vesicles; ph, phallus; la, levator ani; c, colon. (a) Note normal appearance of uterus and follicles in ovaries indicating cyclicity, with male reproductive organs attached to tract. Seminal vesicles were found attached to sides of uterus at cervical area; prostate tissue formed on ventral and sometimes lateral sides of urethra at base of bladder. Such affected animals also had no vaginal orifice and commonly developed the levator ani muscle (b) attached to the penile bulbs of the inner shaft of the penis. (c–f) Photomicrographs of sections of male reproductive organs found in testosterone propionate-exposed (gestational days 14–19) females. (c) Seminal vesicle from a female in the 10 mg TP dose group, (d) prostate from a female in the 0.5 mg TP group, (e) bulbourethral gland from a female in the 2 mg TP dose group, (f) levator ani muscle from a female in the 2 mg TP dose group. Bar in each photo represents 100 μm .



Endpoint	0	0.1	0.5	1	2	5	10
Rt. Kd-Ov distance	23.78 ± 0.84	24.82 ± 1.88	22.32 ± 0.68	19.51 ± 5.51	25.66 ± 1.80	34.61 ± 2.59 ***	40.33 ± 1.41 ‡
Lt. Kd-Ov distance	27.11 ± 0.60	29.01 ± 3.14	21.43 ± 1.45	20.11 ± 4.11	27.39 ± 1.18	37.27 ‡ ± 3.37	36.12 ** ± 1.27
Rt. vtKd-Ov distance	13.51 ± 1.05	11.16 ± 3.34	13.98 ± 2.42	18.6 ^a	19.65 ± 4.21	33.06 ^a ± 3.41	39.76 ^a
Lt. vtKd-Ov distance	10.76 ± 1.70	13.53 ± 4.73	8.97 ± 0.69	14.8 ^a	14.22 ± 3.17	36.03 ^a ± 3.76	34.70 ^b

FIG. 5. Kidney to ovary distance, or ovarian ligament length, in females exposed prenatally to testosterone propionate (TP) on gestational days 14–19. Increased ovarian ligament length is an indicator of androgenic activity. Diagram illustrates the distances measured between the kidneys and ovaries. Data are displayed below diagram. Values are litter means ± SE. ^an = 1 female; ^bn = 4 females from 1 litter. Kd-Ov, kidney to ovary distance; vtKd-Ov, vertical kidney to ovary distance (see Materials and Methods section for detailed description of measuring technique). *p < 0.05, **p < 0.01, ***p < 0.0005, ‡p ≤ 0.0001, all as compared to controls.

Sensitive Endpoints in Female Offspring

Prostate. Prostatic tissue was displayed in roughly half the female offspring at 0.5 mg TP, while 0.1 mg TP was without effect. It is possible that some effects, such as presence of prostate, could have been seen at 0.1 mg TP if a greater number of litters was used, however, determining a no observed adverse effect level (NOAEL) was not the primary interest in this

study. Earlier studies involving prenatal TP exposure have also reported presence of prostate tissue at doses lower than those capable of inducing development of other male sex accessory glands (Greene *et al.*, 1939; Hamilton and Gardner, 1937). Development of the prostate is DHT-dependent (Imperato-McGinley *et al.*, 1992; Schultz and Wilson, 1974) and the high induction of prostate tissue compared to seminal vesicle or other T-dependent Wolffian derived tissues may result from the greater potency of DHT as compared to T.

Nipple development. Formation of the external portion of the mammary anlagen (i.e., the areola and nipple) during sexual differentiation in the rat is prevented by the presence of DHT (Goldman *et al.*, 1976; Imagawa *et al.*, 1994; Imperato-McGinley *et al.*, 1992; Topper and Freeman, 1980). The drastic reduction in areola or nipple number observed at 1 mg TP coincided with elevated T levels observed in the female fetus and thus T or its metabolites may have been responsible for the suppression of areola and nipple formation. The appearance and reduced number of areolas and nipples proved to be a sensitive endpoint for androgenicity in the female as evidenced by its significance at 0.5 mg TP and the drastic reduction at 1 mg TP. The presence of areolas in the preweaning male rat is a sensitive indicator of antiandrogenicity as well (Clark *et al.*, 1990; Gray *et al.*, 1999; Ostby *et al.*, 1999; Wolf *et al.*, 2000).

External genitalia. Another set of effects seen at the 0.5 mg TP dose is altered morphology of the genitalia. These effects include decreased size of the vaginal orifice, reduced distance from the phallus, absence of the vaginal orifice in 1 female in this dose group, and persistent cleaving of the phallus. These malformations have been reported previously in the rat prenatally exposed to TP (Greene *et al.*, 1939; Swanson and van der Werff ten Bosch, 1965). These anatomical alterations are similar to the underdeveloped state of the reproductive tracts and urogenital sinus early in development. The vagina and urethra are nearer each other and open into the invaginated urogenital sinus, and the phallus is still cleft, or hypospadiac. We also observed the presence of vaginal thread, or persistent isthmus of tissue across the diameter of the vaginal orifice. The

TABLE 7
Minimal Effect of Exposure to TP on GD 14–19 on Reproductive Organs of Male Offspring at Necropsy (PND 161–178)

Endpoint	0	0.1	0.5	1	2	5	10
Body weight (g)	613.26 ± 15.70	656.37 ± 25.23	571.87 ± 12.10	566.06 ± 26.77	552.66 ± 23.08	578.11 ± 68.85	571.00 ± 14.87
Right testis (g)	1.802 ± 0.058	1.847 ± 0.043	1.671 ± 0.029	1.688 ± 0.063	1.665 ± 0.090	1.712 ± 0.153	1.673 ± 0.109
Right caput epi. (g)	0.329 ± 0.019	0.349 ± 0.013	0.299 ± 0.004	0.322 ± 0.008	0.307 ± 0.017	0.299 ± 0.008	0.305 ± 0.014
Right cauda epi. (g)	0.329 ± 0.020	0.338 ± 0.005	0.307 ± 0.016	0.287 ± 0.013	0.320 ± 0.007	0.344 ± 0.018	0.301 ± 0.007
VP (g)	0.579 ± 0.045	0.685 ± 0.040	0.603 ± 0.032	0.715 ± 0.021	0.649 ± 0.076	0.640 ± 0.044	0.697 ± 0.071
SV (g)	1.900 ± 0.155	2.026 ± 0.199	1.698 ± 0.119	1.626 ± 0.088	1.705 ± 0.077	1.811 ± 0.096	1.714 ± 0.025
LA/BC (g)	1.344 ± 0.069	1.393 ± 0.078	1.214 ± 0.041	1.172 ± 0.034	1.205 ± 0.042	1.338 ± 0.078	1.262 ± 0.042
Glans penis (mg)	125.1 ± 1.48	118.9 ± 1.90	111.32 ± 1.45**	111.4 ± 1.69**	110.99 ± 1.73**	122.08 ± 1.67	117.69 ± 1.68*

Note. Values represent litter means ± SE. epi., epididymis; VP, ventral prostate; SV, seminal vesicle; LA/BC, levator ani/bulbocavernosus muscle group. *p < 0.05; **p < 0.0001; compared to control values.

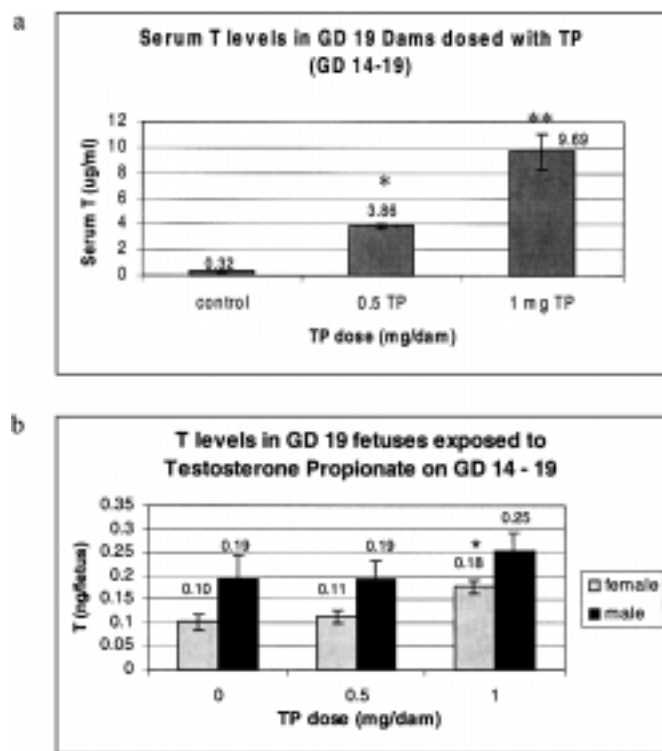


FIG. 6. T levels in dams and fetuses at gestational day (GD) 19, after sc administration of 0.5 or 1 mg TP/dam (GD 14–19). (a) T levels in dam serum are increased in a dose-dependent fashion at 0.5 mg ($p < 0.05$) and 1 mg ($p < 0.01$) TP/rat; $n = 2$ dams per treatment group; values represent litter means \pm SE. Analyzed using log transformed data due to variability among litters. (b) T levels in fetal homogenates increased only at 1 mg TP/dam in female offspring ($p < 0.05$); $n = 2$ litters in control group, 3 litters each in 0.5 mg TP and 1 mg TP groups; mean litter size = 13 fetuses. Values represent litter means \pm SE. * $p < 0.05$, ** $p < 0.01$.

female cleft phallus and the vaginal thread have been associated with prenatal estrogen exposure (Henry *et al.*, 1984; Vannier and Raynaud, 1980) and with TCDD or PCB exposure (Flaws *et al.*, 1997; Gray *et al.*, 1997; Wolf *et al.*, 1999) as well. Cleft phallus is also an effect of antiandrogens in the male (Gray *et al.*, 1994; Imperato-McGinley *et al.*, 1992; Ostby *et al.*, 1999; Wolf *et al.*, 2000). Therefore, the presentation of cleft phallus and vaginal thread alone is not indicative of androgen action, but of endocrine disruption, and must be considered with other endpoints in order to identify the mode of action of the disrupter.

Anogenital distance. The effect of TP treatment on female AGD was permanent, being increased throughout life. The significant increase at weaning in the female at 1 mg TP, or at 0.5 mg TP when adjusted for body weight, attest to the sensitivity of this effect (Table 3).

Internal Reproductive Effects

Female urogenital tract. A threshold for several developmental processes was apparent at 1 mg TP. The most unusual

effect was hydrometrocolpos, or fluid retention and gross distention of the uterus and upper vagina, an effect associated with vaginal atresia (Fig. 2). This condition has been reported in humans at birth and was associated with vaginal atresia (Janus and Godine, 1986; Nguyen *et al.*, 1984). Moderately masculinized female rats in early studies (Greene *et al.*, 1939; Hamilton and Gardner, 1937) were found to have a greatly distended, fluid filled uterus. With moderate masculinization, development of the external portion of the vagina does not occur while the upper vagina ends blindly. With further masculinization, development of the urethra and associated genital ducts is directed by androgens in what can be considered a male-like fashion, joining with the urethra. Greene *et al.* (1939) showed with histology that the upper vagina of affected females was connected to the urethra via a fistula through which the contents of the uterus and vagina could flow freely. A similar fistula, between the vagina and the bladder, has also been reported in a human infant presenting absent vaginal orifice and mild hydrometrocolpos (Takeda *et al.*, 1997). In the case of Greene *et al.* the uterus and upper vagina were not distended as the fluid can escape through this fistula and out the urethra, to the tip of the masculinized phallus. Indeed, in our study, saline injected into the uterus in some females with vaginal atresia but no hydrometrocolpos exited through the urethra.

It appears that the females with hydrometrocolpos displayed estrous cyclicity despite the masculinization of their external genitalia. Estrous cyclicity is evidenced by the apparent uterine activity and the presence of CLs observed in fresh and histological sections of ovaries. Doses of prenatally administered TP that induce a similar degree of masculinization of the external genitalia in the rat do not alter the pattern of gonadotropin release (Rhees *et al.*, 1997; Swanson and van der Werff ten Bosch, 1965). It also appears that females without hydrometrocolpos, specifically those in the higher dose groups, also displayed estrous cyclicity. Evidence of estrous cyclicity in these females is provided by the presence of CLs in fresh ovaries and the unaffected CL and antral follicle score from ovarian histological sections. In addition, serum E_2 levels and ovarian weights in adult female offspring were not significantly different among dose groups. The sensitive developmental period for masculinization of gonadotropin release is not the prenatal period but the early postnatal period (Diaz *et al.*, 1995; Rhees *et al.*, 1997). Prenatal androgen treatment is less effective than neonatal androgen treatment in inducing early androgen syndrome, an anovulatory syndrome (Huffman and Hendricks, 1981; Slob *et al.*, 1983; Swanson and van der Werff ten Bosch, 1965). Neonatal androgen treatment can cause delayed anovulatory syndrome (DAS; Hendricks *et al.*, 1977) characterized by lack of estrous cyclicity and reduced numbers of ovulatory follicles associated with reduced ovarian weight due to lack of CL in the adult female offspring, effects not observed in this study.

The number of females in this study who died before necropsy by dose group is reflected by the number of females with

hydrometrocolpos by dose group. Both plots depict an inverted U-shaped dose response curve. We hypothesize that the uteri of females in the middle dose groups were responding normally to circulating estrogens by cycling and producing fluid but that the fluid had no outlet for drainage and accumulated, leading to death. The inverted U-shape of this graph thus can be explained by a continuous increase in masculinization—the partial masculinization of the genitalia at middle dose groups, characterized by agenesis of a vaginal orifice with no outlet for drainage of uterine fluid, and a more complete masculinization of the genitalia at higher doses, characterized by a severely reorganized urogenital tract permitting drainage of uterine fluid and alleviation of the hydrometrocolpos. U-shaped dose-response curves generated from *in vivo* experiments are rare, but have been reported from studies on gestationally administered estrogens, DES and E₂, in mice (vom Saal *et al.*, 1997). The issue of *in vivo* U-shaped dose response curves is currently under discussion (NTP, 2000).

Elongation of ovarian ligament, as measured by kidney-to-ovary distance, occurred only in the high dose groups. This measurement was increased in a previous study in which females were exposed *in utero* to androgens (Lee and Hutson, 1999) and indicates masculinization, although only with high levels of androgen.

Male reproductive tissues in female offspring. The pattern or degree of induction of male reproductive tissues in females by prenatal androgen exposure in this study closely follows that observed in past studies (Greene *et al.*, 1939; Hamilton and Gardner, 1937). However, the induction of the LA and histology of these male tissues was not studied before. The LA is present in the male rat and is located in the perineal region attached to the penile shaft, but is not present in the normal female rat. LA development is T dependent (Tobin and Joubert, 1991). The LA has been induced in the female by neonatal T treatment in a previous study (Tobin and Joubert, 1991). In our study, the LA developed only in those females whose phallus had been sufficiently masculinized to form a penile shaft and penile bulbs from which the LA muscle is attached. The bulbocavernosus muscle (BC), another T dependent muscle of the male rat anatomically associated with the LA, did not appear in treated females at any dose. Thus, our study shows organization and development of the LA and BC muscles appear to be differentially regulated with the LA more sensitive to T than is the BC. This was shown elsewhere by differences in AR levels in these 2 muscles altered by castration (Antonio *et al.*, 1999).

The SV appeared rudimentary in all but 1 observed case. In previous studies, prenatal androgens induced well developed SV, but only at doses capable of inducing the vas deferens and epididymides as well (Wistar rat; Greene *et al.*, 1939). In the current study, vas deferens and epididymis were not observed in any female at any dose. Bulbourethral glands have been

reported at doses that induce SV (Greene *et al.*, 1939), as they were in the current study.

Masculine development of the phallus and internal penile shaft in the female offspring occurred in association with vaginal atresia. The increase in phallus length itself at 2 and 5 mg TP, although not a robust effect, was indicative of masculinization. Phallus length was not increased at 1 or 10 mg TP, although this may be due to a lower number of individuals in these 2 dose groups.

Male Offspring

The decrease in male AGD on PND 2 at 0.5 mg TP was not only a transient effect, but it was not significantly reduced at this dose when analyzed by covariance with body weight. The body weight of all pups at 0.5 mg and higher doses of TP was reduced. However, the body weight of male pups on PND 2 did not further decrease in higher TP dose groups from that in the 0.5 mg TP dose group, while AGD did further decrease at 1 mg TP and again at 2 mg TP, indicating AGD is independent of body weight and the reduction in AGD is most likely a true effect. AGD could not be determined by sex at higher doses of TP and thus male AGD could not be analyzed by covariate analysis with body weight at these doses. Interestingly, decreased AGD can also be produced in the male offspring of antiandrogen-treated dams (Gray *et al.*, 1994; Ostby *et al.*, 1999; Wolf *et al.*, 2000). However, these males also displayed nipples and other malformations not observed in our TP exposed males.

The only permanent effect seen in the male was a reduction in glans penis weight at 0.5, 1, 2, and 10 mg TP, but not at 5 mg TP. The graphical representation of these data approximates a U-shaped dose-response curve. Reduction in glans penis weight or phallus size is often associated with antiandrogenicity (Clark *et al.*, 1990; Ostby *et al.*, 1999; Prahallada *et al.*, 1997), although it was reported in male offspring of dams fed androgenic Trenbolone acetate prior to pregnancy (FDA, 2000). However, this effect could not be replicated in more recent studies by the authors of this manuscript (unpublished data). As this effect was the only permanent response seen in any of the androgen-dependent tissues in the male offspring, this response needs to be replicated before one can conclude that it is not spurious statistical vagary.

Maternal Toxicity

Adverse effects on reproduction were observed in the dam and neonates at high dose levels of TP. At the 2, 5, and 10 mg TP dose levels, significant adverse effects on maternal reproductive capacity were evident in the decreased weight gain through the dosing period, the delay in parturition, and the decreased litter size. These are well known effects of pre- or perinatal androgen treatment (Fritz *et al.*, 1984; Greene *et al.*, 1939; Huffman and Hendricks, 1981; McCoy and Shirley, 1992; Poplow and Ward, 1978; Rhee *et al.*, 1997; Rosenberg

and Sherman, 1974; Swanson and van der Werff ten Bosch, 1965). The decrease in live litter size is due not to reduced number of implantation sites, but to late fetal resorption (Greene *et al.*, 1939; our unpublished observations) and pup death at birth as evidenced by the pup carcass remnants on PND 1.

T Levels

Fetal body T concentration was significantly increased at 1 mg TP in the GD 19 female fetus, the dose associated with the profound alterations in sexual development. In a similar study, Hotchkiss (2001) found increased fetal T levels in GD 18 females exposed to 1.5 and to 2.5 mg/kg TP, doses similar to our 0.5 and 1 mg doses. Fetal T level was not elevated in the GD 19 female fetus in our study at 0.5 mg TP, a dose of TP that elicited androgenic responses in the female. However, maternal serum T concentrations rose to 10 times normal levels in this dose group. The difference in T level elevation between dams and fetuses may be explained by the finding that T administered to the rat dam is not delivered to the fetus at equivalent levels, but is metabolized or blocked at the placenta (Vreeburg *et al.*, 1981). In addition, TP given to the rat dam is metabolized by the dam and placenta to other androgens, of which androsterone is the most abundant in the fetus, followed by 3 α -androstenediol and epiandrosterone (Slob *et al.*, 1983). Greene *et al.* (1939) showed that sc injection of androsterone to the dam can masculinize female rat offspring, thus androsterone may directly or indirectly be a hormone responsible for the malformations of the female genitalia, undetected by the T radioimmunoassay. However, androsterone has low affinity for the androgen receptor and may be converted in the fetus to a more potent androgen. T may also be metabolized to DHT, an androgen known to mediate the tissues and processes affected in the female offspring in this study. DHT levels in the fetus were not analyzed in this study, but will be addressed in future studies.

We have concluded that 0.5 mg and 1 mg TP given on GD 14–19 are effectively androgenic while not severely compromising to litter size, viability, or pregnancy and parturition, and illustrate sensitive endpoints to monitor for identification of androgenic chemicals.

It is noteworthy that few of the above endpoints presented herein are included in the standard multigenerational protocols. The standard multigenerational protocols do not include evaluation for the presence of nipples in male or female offspring or examination of female offspring for ventral prostate, levator ani, or other male tissues. In addition, inclusion of AGD is restricted to the F2 generation only after an alteration of puberty is found in the F1 generation. We suggest consideration be given to including some of the more sensitive endpoints in the USEPA Tier 2 testing phase for chemicals that display androgenic activity in Tier 1 screening.

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