Male Rats Exposed to Linuron in Utero Exhibit Permanent Changes in Anogenital Distance, Nipple Retention, and Epididymal Malformations That Result in Subsequent Testicular Atrophy

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Prenatal exposure to the herbicide linuron, a weak androgen receptor antagonist, has been shown to perturb androgen-dependent male rat reproductive development as evidenced by slight decreases in anogenital distance (AGD), increased retention of areolae/nipples, and induction of epididymal malformations in combination with testicular atrophy in the adult rat over dose levels ranging from 12.5 to 100 mg/kg/day. Studies were undertaken to determine whether linuron-mediated changes in AGD and nipple retention are permanent, whether linuron is a direct testicular toxicant, and if there was an association between areola/nipple retention and malformations. Pregnant rats were administered corn oil vehicle or linuron by gavage at 0 or 50 mg/kg/day \((n = 8 \text{ controls}, 20 \text{ treated})\) from gestation days 12 to 21. Male offspring were necropsied on postnatal days (PND) 35 and 56. Linuron-exposed male rats exhibited a significant (8%) decrease in AGD on PND 1 and a similar decrease was also observed on PND 56. Linuron-exposed male rats displayed an increase in areola retention on PND 13, as evidenced by 0.6 ± 0.5 and 3.3 ± 0.4 areolae per rat in the control and exposed groups, respectively. Male rats displayed a significant increase in nipple retention on PND 35 and 56 (collectively) of 0 ± 0.5 and 1.7 ± 0.3 nipples per rat in control and exposed groups, respectively. On PND 35, 4/51 rats (3/9 litters) from linuron-treated dams displayed enlarged testes in combination with malformed epididymides. Epididymal malformations were observed in 19/51 rats (6/9 litters) in the linuron-exposed dose group. On PND 56, grossly enlarged and edematous testes were seen in 16/56 linuron-exposed rats (6/9 litters). Epididymal lesions were observed in 23/58 rats (6/9 litters). Microscopically, all linuron-exposed animals that exhibited a testicular lesion on PND 56 also displayed an epididymal lesion. These lesions were not seen in control animals. Approximately 25 and 60% of the male offspring that had malformations of the epididymis and vas deferens did not exhibit either areolae on PND 13 or nipples at necropsy, respectively. These data indicate that in utero linuron exposure to 50 mg/kg/day results in permanent changes in AGD and nipple retention in male rats. Moreover, these findings indicate that linuron-induced testicular atrophy, which is observed in adult rats, is secondary to increased intratubular pressure resulting from obstruction of testicular fluid outflow subsequent to malformation of the epididymides. These data also suggest that although linuron-mediated retention of areolae on PND 13 and nipples at necropsy may be suggestive of altered testosterone-mediated reproductive development seen in adult rats, these endpoints are not predictive.

Key Words: AGD; nipple retention; linuron; male reproductive development; testicular atrophy; in utero exposure; gestational exposure.

In the rat and human, fetal androgen production during gestation is required for normal male sexual differentiation (Schardein, 1993). Exposure to environmental contaminants with endocrine-like activity has been hypothesized to be responsible for the increased prevalence of reproductive and other deficits in humans and wildlife (Gray et al., 1998; Sharpe and Skakkebaek, 1993; Toppari et al., 1996). An emerging class of these endocrine-active chemicals are antiandrogenic, i.e., they block androgen action in vitro and/or alter androgen-dependent processes in vivo (Gray and Kelce, 1996; Gray et al., 1994; Kelce et al., 1998, 1997; LeBlanc et al., 1997; McIntyre et al., 2000; Mylchreest et al., 1998, 2000; You et al., 1998).

Male rats exposed in utero to antiandrogens often display alterations in androgen-mediated development, as evidenced by decreased anogenital distances (AGD) and retention of areolae and/or nipples, together with clearly adverse responses such as genital malformations and reproductive tract lesions. However, the correlation between end points that signify antiandrogen-mediated perturbations (e.g., decreased AGD and nipple retention) and adverse and irreversible malformations in androgen-dependent development is unclear. Confusion exists about the biological significance of antiandrogen-mediated changes in these end points and how they may be used in risk assessment. Male rats exposed in utero to finasteride, a 5α-reductase inhibitor that blocks the conversion of testosterone (T) to dihydrotestosterone (DHT), display decreased AGD at birth (Clark et al., 1993, 1990). However, these offspring...
displayed catch-up growth and adult animals exposed to low doses of finasteride in utero displayed AGDs similar to control animals at adulthood, suggesting that decreases in AGD seen in early postnatal life are transient. These investigators also indicated that areolae/nipples seen in early postnatal rats were temporary (Clark et al., 1990). In contrast to these previous studies with finasteride, rats exposed to the antiandrogens diethylhexylphthalate, di(n)butyl phthalate, and linuron displayed retained nipples at both PND 13 and at the adult (PND 180–270) necropsy (Gray et al., 1999). Whether antiandrogen-induced changes in these early postnatal end points are predictors and/or indicators of subsequent adverse responses in rat reproductive development is unknown.

Linuron is a pre-and post-emergence herbicide applied to suppress broadleaf and grassy weeds. In a 3-generation reproductive study in rats, animals exposed to 625 ppm (~31.5 mg/kg/day) in the diet exhibited decreased fertility in the F₁ and F₂ generations but not the F₃ generation (U.S. EPA, 1995). In this study, linuron also decreased reproductive performance, as evidenced by decreased pup survival at the 625-ppm dose level and decreased pup weights (male and female) at both the 125-ppm (6.25 mg/kg/day) and 625 ppm dose levels (U.S. EPA, 1995). In a later, 2-generation reproductive toxicity study in rats conforming to relevant guidelines at that time, dietary exposure to 625 ppm (44–54 mg/kg/day) of linuron had no effect on fertility (U.S. EPA, 1995). However, testicular and epididymal pathology (testicular atrophy, intratubular fibrosis, epididymal inflammation, and oligospermia) were observed in the F₁ adults but not in the parental generation, no epididymal malformations were reported (U.S. EPA, 1995). In a recently published, multigenerational study in rats, treatment with 40 mg/kg/day of linuron by gavage delayed the onset of puberty in rats conforming to relevant guidelines at that time, dietary exposure to 625 ppm (44–54 mg/kg/day) of linuron had no effect on fertility (U.S. EPA, 1995). However, testicular and epididymal pathology (testicular atrophy, intratubular fibrosis, epididymal inflammation, and oligospermia) were observed in the F₁ adults but not in the parental generation, no epididymal malformations were reported (U.S. EPA, 1995). In a recently published, multigenerational study in rats, treatment with 40 mg/kg/day of linuron by gavage delayed the onset of puberty and decreased seminal vesicle and cauda epididymal weights in the F₀ generation (Gray et al., 1999). F₁ rats exposed to linuron in utero and via milk during lactation and gavaged after weaning produced fewer pups when mated continuously over 12 breeding cycles (Gray et al., 1999). Male offspring displayed reduced testicular and epididymal weights and decreased spermatozoid numbers. Linuron has been reported to be neither teratogenic nor embryo-toxic when administered by gavage to pregnant rats from GD 6 to 15 at dose levels as high as 100 mg/kg/day (Khera et al., 1978). In a developmental toxicity study in the rat, dams administered linuron in the diet at 625 ppm exhibited decreased body weight and food consumption, increased postimplantation loss, and increased litter and fetal incidences of resorptions (developmental lowest-observable-effect level (LOEL) in rat) (U.S. EPA, 1995). Rabbets exposed in utero to 100 mg/kg/day from GD 7 to 19 by gavage exhibited skeletal variations of the skull (developmental LOEL in rabbit) (U.S. EPA, 1995). Late gestational exposure to linuron during androgen-dependent reproductive development has been shown to cause epididymal malformations, hypospadias, and decreases in AGD, and to induce retention of areolae and nipples in male rats (Gray et al., 1999; McIntyre et al., 2000). Linuron is a weak competitive androgen receptor antagonist in vitro and induces a positive response in the immature and adult Hershberger assay (Cook et al., 1993; Lambright et al., 2000; McIntyre et al., 2000). Although the exact mechanism is unknown, these data suggest that the antiandrogenic effects observed in linuron-exposed rats are the result of altered androgen-receptor-dependent rat reproductive development.

Male rats exposed to linuron in utero exhibit dose-dependent epididymal abnormalities (epididymal hypoplasia and agenesis). These alterations are associated with ipsilateral testicular atrophy in the adult rat (Gray et al., 1999; Lambright et al., 2000; McIntyre et al., 2000).

Whether the observed linuron-mediated testicular lesions in adult rats are a direct effect of linuron on the testes or are secondary to malformations of the epididymides is unclear. Therefore, the objectives of this study were to determine whether (1) linuron-induced changes in AGD and areola/nipple retention in rats are permanent; (2) linuron-induced epididymal malformations are associated with increased testicular size (the result of restricted or obstructed testicular fluid outflow from the testis, causing subsequent seminiferous epithelial degeneration and testicular atrophy); and (3) there is an association between rats that display reproductive tract lesions and either areolae on PND 13 or permanent nipple retention. To test these hypotheses, pregnant rat dams were treated with either corn oil (vehicle control) or 50 mg/kg/day of linuron from GD 12 to 21. This dose level was selected from previous dose-response studies (McIntyre et al., 2000) to maximize effects on the fetus while minimizing any toxicity to the dam. Male offspring were uniquely identified at birth. Male rats were euthanized on PND 35 (low testicular fluid outflow) and 56 (high fluid outflow) (Setchell et al., 1994), and male offspring were inspected for permanent decreases in AGD and retention of areolae on PND 13 and nipples at necropsy.

MATERIALS AND METHODS

Animals. This study was conducted in accordance with Federal guidelines for the care and use of laboratory animals (National Research Council, 1996) and was approved by the Institutional Animal Care and Use Committee at the CIT Centers for Health Research (CIT). Animals were housed in the CIT animal-care unit, a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Animals were kept in a HEPA-filtered, mass air-displacement room with a 12-h light-dark cycle at 18–26°C and relative humidity of 30–70%. Animals had access ad libitum to deionized water and rodent chow (NIH-07, Zeigler Brothers, Gardner, PA). Time-mated, 8- to 10-week-old, nulliparous CRL:CD (SD)Br rats were obtained from Charles River Laboratories, Inc. (Raleigh, NC) on GD 0 (defined as the day that sperm was found in the vagina of the mated female). Animal allocation to treatment groups was done by body weight randomization to ensure unbiased weight distribution among groups. Individual dams and offspring were housed in polycarbonate cages on ALPHA-dri bedding (Shepherd Specialty Papers, Kalamazoo, MI) until weaning (PND 21), at which time animals were group-housed, up to 4 per cage, by sex and treatment. Male offspring were euthanized by CO₂ asphyxiation on PND 21.

Treatment. Sperm-positive animals, 8 control dams and 20 dams receiving 50 mg linuron/kg/day, were gavaged daily (0800–1030) from GD 12 to 21.
with either 2 ml/kg/day corn oil (Sigma) or 2 ml/kg/day linuron (50 mg/kg/day). This dose level was chosen based on a previous study in which pregnant rat dams receiving 50 mg/kg/day of linuron from GD 12 to 21 displayed limited maternal toxicity (decreased weight gain during treatment). However, adult male offspring exhibited approximately a 12% incidence in epididymal abnormalities and testicular atrophy (McIntyre et al., 2000). Dams were examined daily for clinical signs of toxicity.

Androgen-dependent reproductive end points. On the day of delivery, which was considered to be postnatal day (PND) 1, pups were counted and examined for signs of clinical toxicity. Pups were uniquely identified by foot tattoo, and the AGD was measured using a dissecting microscope with an eyepiece reticle (accuracy of 0.05 mm). An individual investigator who was unaware of animal exposure measured the AGD for all pups. Pup litter weights (by sex and litter) and individual pup body weights after weaning were collected weekly. On PND 13, a single investigator, who was unaware of animal exposure, inspected male pups for the presence and number of either areolae or nipples. No distinction was made between the retention of an areola or nipple on PND 13.

Necropsy of F1 animals. Pups were weaned on PND 21, and dams were euthanized by CO2 asphyxiation. The male offspring from one-half of the control and linuron-treated dams were necropsied on PND 35 and the other half on PND 56. Rats were euthanized by decapitation, and trunk blood was collected. Following blood collection, the ventral surface of the animal was shaved for counting the number of nipples, and the AGD was measured with a dial caliper (on PND 56 only). The external genitalia, scrotum, prepuce, and penis were visually inspected. Gross internal examination of the reproductive tract included inspection of the testes, epididymides, vasa deferentia, prostate, seminal vesicles, and coagulating glands. Additionally, the liver, kidneys, and adrenal glands were grossly examined. Body weight and weight of the epididymides and testes were collected. Tissues (right testis and epididymis) were fixed in 10% neutral buffered formalin (for ancillary immunohistochemical studies), processed, paraffin-embedded, sectioned (5 μm), and stained with hematoxylin and eosin. The left testes and epididymides were retained for ancillary studies.

Statistical analysis. The litter was the experimental unit, unless otherwise noted. Statistical analyses were conducted using JMP (version 4.0.0, SAS Institute, Cary, NC). Retained nipples on PND 35 and 56 were combined and compared to control. Normality (Shapiro-Wilk) and homogeneity (Bartlett) assumptions were tested prior to analysis. Pups were nested by dam to yield litter means. Either ANOVA or ANCOVA was used to test for significance of treatment effects, and the covariates are defined in the figure legends. If the p value for treatment effects was less than 0.05, post hoc comparisons of either Dunnett’s (for ANOVA) or contrasts of least-square means were used to assess the significance of treatment differences. The Bonferroni correction was applied for post hoc ANCOVA analyses, as appropriate.

RESULTS

Effects of Linuron on the Dam

Dam body-weight gain over the 10-day dosing period was significantly decreased in the linuron-dosed group by approximately 30% when compared to vehicle-treated control animals. Dam body weight of linuron-exposed animals on GD 21 was significantly decreased by 5% as compared to control animals (data not shown). All dams were pregnant and littered normally. Neither litter size nor pup weight was affected by linuron exposure (data not shown). Two dams in the 50-mg/kg/day-dose group cannibalized their offspring within 24 h.

Linuron-Induced Perturbations in AGD, and Retention of Areolae/Nipples

On PND 1, male offspring from linuron-treated dams displayed a significant decrease in the nested litter mean AGD of male offspring by approximately 8% when compared to control animals (Fig. 1). On PND 56, the litter-mean AGD of linuron-exposed animals displayed a statistically significant decrease of 5% relative to control animals (Fig. 1). On PND 13, the nested litter mean for the number of retained areolae per pup from vehicle-treated control dams had a value of less than one. Male offspring exposed prenatally to linuron exhibited a litter mean of 3.3 areolae per pup (Fig. 2). At necropsy, male offspring exposed to the vehicle control displayed a low incidence of retained nipples (one control male rat displayed 2 nipples at necropsy with 1 areola present on PND 13), and the nested litter mean was 0.04 nipples per rat. Linuron-exposed animals displayed a nested litter mean of 1.7 nipples per rat, and this increase was significant as compared to vehicle control-exposed animals (Fig. 2).

On PND 13, the urethral opening of one linuron-exposed animal was observed at the base of the sex papilla (Fig. 3). At necropsy on PND 56, this animal displayed a hypospadias with a cleft in the ventral surface of the penis and exposure of the os penis (Fig. 3). Two linuron-exposed animals also exhibited cryptorchid testes on PND 56.

Linuron-Induced Effects Observed on PND 35

At necropsy on PND 35, linuron-exposed offspring exhibited grossly abnormal testes and agenesis of the epididymides (partial to complete) in approximately 8% (3/9 litters) and 40% (6/9 litters) of male offspring, respectively (Table 1). Unilateral and bilateral agenesis (partial to complete) of the vasa defer-
entia was observed in 22% (6/9 litters) of the linuron-exposed animals (Table 1). Rats that exhibited malformed vasa deferentia also had epididymal malformations. These lesions were not observed in control animals. The weight of grossly normal testes and epididymides was similar to that of tissues from vehicle control-exposed rats (Table 1). Microscopic assessment of the right testes and epididymides showed a strong association with the gross postmortem examination. Affected testes (7/51 rats, 5/9 litters) from linuron-exposed animals exhibited mild to moderate dilation of the seminiferous tubular lumina and concomitant dilation of the seminiferous tubules (Table 2). Seminiferous epithelium appeared thinner with abnormal spermatogenesis. In all but 2 incidences (one each on PND 35 and 56), each lesioned testis was associated with a histologically abnormal epididymis. In both of these incidences, the testicular lesion was mild, and the testis weight was unaffected. With the exceptions noted above, the ipsilateral testes of animals that exhibited malformed epididymides on PND 35 were microscopically indistinguishable from that of a control testis (Fig. 4). Histologically, affected epididymides on

FIG. 2. Male rats exposed to 50 mg of linuron/kg/day in utero from gestation days 12 to 21 displayed retention of areolae on postnatal day (PND) 13 and permanent post-weaning nipples (PND 35 and 56). Results are presented as nested least-square litter means ± SE. *Significantly different from control; p < 0.05.

FIG. 3. Male rats exposed to 50 mg of linuron/kg/day in utero from gestation days 12 to 21 exhibit malformations of the external genitalia. Photographs of the sex papilla on PND 13 (A, B) and penis on PND 56 (C, D), control animals (A and C) and a linuron-exposed animal (B and D). Note the urethral opening at the base of the sex papilla on postnatal day (PND) 13 in the linuron-exposed animal (arrow). On PND 56, the penis of a linuron-exposed animal had a hypospadias, a cleft in the ventral surface of the penis with exposure of the os penis.
PND 35 exhibited partial to complete agenesis and a decreased number of ductules that were often dilated and surrounded by an inflammatory infiltrate.

**Linuron-Induced Effects Observed on PND 56**

At necropsy on PND 56, linuron-exposed offspring exhibited grossly abnormal testes and agenesis of the epididymides (partial to complete) in approximately 36% (6/9 litters) and 40% (6/9 litters) of the individuals, respectively (Table 1). Partial to complete agenesis of the vasa deferentia was observed in 26% (6/9 litters) of the linuron-exposed rats (Table 1). Approximately 29% (6/9 litters) of the linuron-exposed rats displayed unilateral or bilateral enlargement of the testes (Table 1, Fig. 5). These testes weighed, on average, 70% more than those from control animals (Table 1). Small testes were also observed in 7% of the linuron-exposed animals (3/9 litters). These testes weighed on average 39% less than the vehicle controls (Table 1). Grossly normal testes from linuron-exposed rats appeared to weigh less than testes from control animals (Table 1). However, this did not reach statistical significance ($p = 0.06$). Microscopic examination of the right testes and epididymides correlated with the gross examination. Affected testes (22/58 rats, 7/9 litters) from linuron-exposed animals were characterized by mild to moderate dilation of the lumina with concomitant dilation of the seminiferous tubules TABLE 2

**Incidence of Microscopic Testicular and Epididymal Lesions on Postnatal Days (PND) 35 and 56**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>0 mg/kg/day Linuron</th>
<th>50 mg/kg/day Linuron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lesion incidencea</td>
<td>Weightb</td>
</tr>
<tr>
<td>PND 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>—</td>
<td>132.4 ± 5.8</td>
</tr>
<tr>
<td>Testis Normal</td>
<td>—</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>Testis Smallc</td>
<td>0/23 (0/4)</td>
<td>NA</td>
</tr>
<tr>
<td>Testis Enlargedc</td>
<td>0/23 (0/4)</td>
<td>NA</td>
</tr>
<tr>
<td>Epididymis Normal</td>
<td>—</td>
<td>0.056 ± 0.002</td>
</tr>
<tr>
<td>Epididymis Malformedc</td>
<td>0/23 (0/4)</td>
<td>NA</td>
</tr>
<tr>
<td>Vasa deferentia Normal</td>
<td>—</td>
<td>0.223 ± 0.004</td>
</tr>
<tr>
<td>Epididymis Malformedc</td>
<td>0/23 (0/4)</td>
<td>NA</td>
</tr>
<tr>
<td>Vasa deferentia Malformedc</td>
<td>0/23 (0/4)</td>
<td>NA</td>
</tr>
</tbody>
</table>

aIndividual incidence; litter incidence is in parentheses.
bNested litter means. For tissues, the sum of left and right tissue divided by 2 with body weight as a covariate. NA, not available.
cUnilateral or bilateral.
dMean weight of individual affected testes.
eAgenesis of the epididymal body, head, or tail or partial to complete agenesis of the vas deferens.
fTwo animals from one litter displayed unilateral cryptorchid testes. These small testes were omitted from analysis.
gSignificantly different from control, $p < 0.05$; **$p = 0.06$.  

PND 35 exhibited partial to complete agenesis and a decreased number of ductules that were often dilated and surrounded by an inflammatory infiltrate.

**Note.** Individual incidence; litter incidence is in parentheses.

*Right side only.*

Testicular lesions were characterized by mild to moderate dilation of the lumina and concomitant dilation of the seminiferous tubules. These tubules exhibited abnormal spermatogenesis with small numbers of multinucleated spermatids on PND 56.

One animal each on PND 35 and 56 exhibited mild (<5% of the tubules affected) seminiferous tubule degeneration in the absence of an apparent epididymal lesion.

The spectrum of lesions included partial to complete agenesis, decreased number of ductules, dilation of ductules, and a decreased number or absence of spermatids on PND 56.

**TABLE 1**

**Incidence of Gross Observations and Tissue Weights on Postnatal Days (PND) 35 and 56**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>0 mg/kg/day Linuron</th>
<th>50 mg/kg/day Linuron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lesion incidencea</td>
<td>Weightb</td>
</tr>
<tr>
<td>PND 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>—</td>
<td>132.4 ± 5.8</td>
</tr>
<tr>
<td>Testis</td>
<td>Normal</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>Testis</td>
<td>Smallc</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>Testis</td>
<td>Enlargedc</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>Epididymis</td>
<td>Normal</td>
<td>—</td>
</tr>
<tr>
<td>Epididymis</td>
<td>Malformedc</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>Vasa</td>
<td>Normal</td>
<td>—</td>
</tr>
<tr>
<td>deferentia</td>
<td>Malformedc</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>PND 56</td>
<td>—</td>
<td>335.0 ± 7.5</td>
</tr>
<tr>
<td>Testis</td>
<td>Normal</td>
<td>1.37 ± 0.03</td>
</tr>
<tr>
<td>Testis</td>
<td>Smallc</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>Testis</td>
<td>Enlargedc</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>Testis</td>
<td>Cryptorchid</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>Epididymis</td>
<td>Normal</td>
<td>—</td>
</tr>
<tr>
<td>Epididymis</td>
<td>Malformedc</td>
<td>0/23 (0/4)</td>
</tr>
<tr>
<td>Vasa</td>
<td>Malformedc</td>
<td>0/23 (0/4)</td>
</tr>
</tbody>
</table>

aIndividual incidence; litter incidence is in parentheses.

bNested litter means. For tissues, the sum of left and right tissue divided by 2 with body weight as a covariate. NA, not available.
cUnilateral or bilateral.
dMean weight of individual affected testes.
eAgenesis of the epididymal body, head, or tail or partial to complete agenesis of the vas deferens.
fTwo animals from one litter displayed unilateral cryptorchid testes. These small testes were omitted from analysis.
gSignificantly different from control, $p < 0.05$; **$p = 0.06$. 

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The seminiferous epithelium appeared thinner, with inappropriate, abnormal, or no spermatogenesis occurring (Fig. 6). The rete testis was often dilated. This was similar to but more severe than the testicular lesions observed on PND 35. Similar to what was observed on PND 35, microscopic examination of the epididymides revealed partial to complete epididymal agenesis and a decreased number of ductules that were dilated and surrounded by an inflammatory infiltrate. Animals exposed in utero to linuron displayed a slight but significant decrease in the weight of grossly normal epididymides (Table 1).

At necropsy, 25% of the male rats that displayed agenesis of epididymides or vasa deferentia did not exhibit retained areolae when examined on PND 13. The relationship between retained areolae and malformations was similar for both epididymides and vasa deferentia (Fig. 7A). Increased areolae retention was not associated with a concomitant increase in percentage of animals that exhibited malformations (Fig. 7A). Male rats that did not display malformations of the reproductive tract exhibited an areolae response similar to that of affected animals (Fig. 7B).
Similarly, approximately 60% of the male offspring that had malformed epididymides and vasa differentia did not exhibit nipples at necropsy, whereas the remaining 40% of male offspring exhibited 1–6 permanent nipples (Fig. 7B). Increased permanent nipple retention was not associated with an increase in the percentage of animals exhibiting malformations (Fig. 7B). Male rats that did not display reproductive tract malformations exhibited a nipple response similar to that of affected animals (Fig. 7B).

**DISCUSSION**

In this study, male offspring of pregnant rats exposed to 50 mg of linuron/kg/day from male GD 12 to 21, the critical window for male reproductive development, exhibited permanent decreases in AGD and in retention of nipples. On PND 35, when fluid outflow from the testis is low (Setchell et al., 1994), male offspring predominantly exhibited malformations of the epididymis and vas deferens. In contrast, on PND 56, when fluid outflow from the testis is high (Setchell et al., 1994), rats that displayed malformations of the epididymis and vas deferens also exhibited testicular lesions. In the linuron-exposed group, one animal exhibited hypospadias, and 2 other animals had cryptorchid testes, both of which are known androgen-dependent malformations. On an individual basis, neither the retention of areolae on PND 13 nor the presence of permanent nipples at necropsy was significantly associated with malformations of the epididymis or vas deferens.

In our current study, male rats exposed in utero to linuron exhibited a significant decrease in AGD on PND 1 and PND 56. It has been previously demonstrated that rats exposed prenatally to linuron exhibit a decrease in AGD measured shortly after birth, and this magnitude of decrease on PND 1 (8%) is similar to what we previously observed under the same dosing regimen (McIntyre et al., 2000). These data demonstrate that small decreases in AGD as a result of antiandrogen exposure are reproducible from experiment to experiment within a given laboratory. Studies in our laboratory as well as other laboratories have demonstrated that prenatal exposure to vinclozolin and flutamide can also induce permanent decreases in AGD (Gray et al., 1994, McIntyre et al., 2001). In contrast,
previous investigators utilizing finasteride have suggested that these decreases are, in part, transient (Clark et al., 1990). In the current study, rats exposed to linuron displayed an 8% decrease (relative to control) in AGD on PND 1 and 5% on PND 56. In addition, we have previously shown that rats exposed to 6.25 mg/kg/day of flutamide exhibit a 43% decrease in AGD on PND 1 but only 29% at adult necropsy (McIntyre et al., 2001). The discrepancy in magnitude of response of these findings at different ages probably reflects postnatal growth of the rodent perineum and genital area. Exposure to linuron during gestation resulted in an increased number of areolae on PND 13, and this increase correlated with an increased number of nipples at necropsy. This finding confirms and extends the finding of previous investigators that demonstrated in utero exposure to linuron (in addition to other antiandrogens such as dibutyl phthalate, diethylhexyl phthalate, and flutamide), results in permanent retention of nipples (Gray et al., 1999; McIntyre et al., 2001). Since the definition of a malformation is traditionally considered to be a permanent structural change that is either rare or life threatening, it could be argued that the presence of nipples and decreased size of the perineum in male rats constitute true malformations and are therefore adverse effects that could be used in risk assessment.

Rats exposed to linuron exhibited abnormal T-mediated development of the epididymis and vas deferens, and the spectrum of target tissues was similar to that reported previously (Gray et al., 1999; Lambright et al., 2000; McIntyre et al., 2000). In contrast, male offspring from linuron-treated dams displayed minimal alterations in some DHT-mediated end points such as testicular descent (2 animals from 1 litter) and development of the external genitalia (hypospadias). Gestational exposure to 100 mg/kg/day of linuron to pregnant rats has been shown to induce a low incidence of epispidias, a less severe form of hypospadias, in the male offspring (Lambright et al., 2000). In our study, prenatal linuron exposure resulted in a 40 and 20% incidence of malformations of the epididymis and vas deferens, respectively. This incidence is higher than that previously reported and likely reflects the steepness of the dose-response curve for linuron-induced teratogenesis (McIntyre et al., 2000). Nevertheless, this finding confirms our previous report that male rats exposed to linuron in utero display malformations (McIntyre et al., 2000). This dose level is similar to the developmental LOEL in the rat of 625 ppm, a dose level that increased postimplantation loss and increased fetal incidences of resorptions (U.S. EPA, 1995).

The relative absence of concomitant testicular and epididymal lesions in linuron-exposed rats on PND 35 correlates with low testicular fluid outflow (Setchell et al., 1994). In contrast, on PND 56 the appearance of both testicular and epididymal lesions is associated with high fluid outflow from the rat testis (Setchell et al., 1994). These data indicate that linuron-induced testicular atrophy observed previously on PND 100, and in 4 animals in the current study at PND 56, is the progressive outcome of increased intratubular pressure resulting from obstruction of testicular fluid outflow and secondary to malformed epididymides (Lambright et al., 2000; McIntyre et al., 2000). Although slight decreases in grossly normal testicular and epididymal weight on PND 56 were observed, suggesting that in utero linuron exposure may affect spermatogenesis directly, these data demonstrate that T-dependent development of the epididymis and vas deferens is the primary target of in utero linuron exposure. Studies are currently under way in our laboratory to determine whether in utero linuron exposure decreases subsequent sperm levels in the adult rat.

A prognostic association between linuron-induced malformations and retained areolae on PND 13 or retained nipples at necropsy was not evident. This finding suggests that linuron-mediated blockade of DHT-dependent nipple regression has a
different threshold from that of linuron-mediated alterations in T-mediated reproductive development. This observation brings into question the predictive usefulness and validity of an end point of androgen perturbation in which 20 and 40% of the animals that exhibited malformations did not display either retained areolae or nipples, respectively. Moreover, nipple retention has been shown to be an insensitive indicator of altered T-mediated development, as evidenced by a study examining the effects of gestational exposure to flutamide and subsequent androgen-dependent reproductive development in the male offspring. This study demonstrated that a female-like nipple response would be required before epididymal malformations would be observed in male offspring (McIntyre et al., 2001).

In summary, the current study demonstrates that changes in AGD and nipple retention resulting from prenatal linuron exposure to 50 mg/kg/day are permanent. Testicular atrophy that has been previously reported in linuron-exposed adult animals exhibiting ipsilateral epididymal agenesis is the result of restricted fluid outflow from the testis. Late gestational exposure, coupled with retaining the full male litter complement, may be more sensitive than traditional protocols in detecting teratogenic responses of environmental antiandrogens. Areola retention on PND 13 and nipple retention at necropsy are not predictive of linuron-induced malformations in T-dependent tissues.

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