The Effect of Atrazine on Puberty in Male Wistar Rats: An Evaluation in the Protocol for the Assessment of Pubertal Development and Thyroid Function

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Received May 15, 2000; accepted July 24, 2000

Since atrazine (ATR), a chlorotriazine herbicide, has been shown previously to alter the secretion of luteinizing hormone (LH) and prolactin (PRL) through a direct effect on the central nervous system (CNS), we hypothesized that exposure to ATR in the EDSTAC male pubertal protocol (juvenile to peripubertal) would alter the development of the male rat reproductive system. We dosed intact male Wistar rats from postnatal day (PND) 23 to 53 and examined several reproductive endpoints. ATR (0, 12.5, 25, 50, 100, 150, or 200 mg/kg) was administered by gavage and an additional pair-fed group was added to compare the effects of any decreased food consumption in the high dose group. Prepubertal separation (PPS) was significantly delayed in the 12.5, 50, 100, 150, and 200 mg/kg ATR dose groups. PPS was also delayed in the pair-fed group, although significantly less than in the high dose-ATR group. The males were killed on PND 53 or 54, and pituitary, thyroid, testes, epididymides, seminal vesicles, and ventral and lateral prostates were removed. ATR (50 to 200 mg/kg) treatment resulted in a significant reduction in ventral prostate weights, as did the reduced food consumption of the pair-fed group. Testes weights were unaffected by atrazine treatment. Seminal vesicle and epididymal weights were decreased in the high dose-ATR group and the control pair-fed group. However, the difference in epididymal weights was no longer significantly different when body weight was entered as a covariable. Intratesticular testosterone was significantly decreased in the high dose-ATR group on PND 45, but apparent decreases in serum testosterone were not statistically significantly on PND 53. There was a trend for a decrease in luteinizing hormone (LH) as the dose of ATR increased; however, dose group mean LH was not different from controls. Due to the variability of serum prolactin concentrations on PND 53, no significant difference was identified. Although prolactin is involved in the maintenance of LH receptors prior to puberty, we observed no difference in LH receptor number at PND 45 or 53. Serum estrone and estradiol showed dose-related increases that were significant only in the 200 mg/kg-ATR group. No differences were observed in thyroid stimulating hormone (TSH) and thyroxine (T4) between the ATR groups and the control; however triiodothyronine (T3) was elevated in the high dose-ATR group. No differences in hormone levels were observed in the pair-fed animals. These results indicate that ATR delays puberty in the male rat and its mode of action appears to be altering the secretion of steroids and having subsequent effects on the development of the reproductive tract, which appear to be due to ATR’s effects on the CNS. Thus, ATR tested positive in the pubertal male screen that the Endocrine-Disrupter Screening and Testing Advisory Committee (EDSTAC) is considering as an optional screen for endocrine disrupters.

Key Words: atrazine; prepubertal separation; hormones; puberty; aromatase; reproductive tract.

Puberty in mammalian species is a period of rapid interactive endocrine and morphology changes. Whether exposure to environmental compounds can alter pubertal development in animals is a fundamental concern in reproductive toxicology. This concern was recognized by the Endocrine-Disrupter Screening and Testing Advisory Committee (EDSTAC), which acknowledged the need for the development and standardization of a protocol for the assessment of the impact of endocrine-disrupting compounds (EDC) in the pubertal male. The committee recommended inclusion of an assay of this type as an alternative test in the tier-one screening battery (EPA, 1998). The pubertal male protocol, which was designed to detect alterations of pubertal development, thyroid function, and the hypothalamic-pituitary-gonadal (HPG) system’s peripubertal maturation, has been recently reviewed (Stoker et al., 2000). In this modified protocol (see Fig. 1), intact 23-day-old weanling male rats are exposed to the test substance for 30 days, during which pubertal indices are measured. Upon necropsy, reproductive and thyroid tissues are weighed and evaluated histologically, and the serum subsequently is assayed for hormone content.
Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is a chloro-s-triazine herbicide that is employed extensively in agriculture worldwide. It was first introduced in 1958 and provided farmers with an alternative to 2, 4-D for the control of grasses and other weeds (Gianessi, 1998). In the United States, 75 million pounds of atrazine is used each year, more than any other agricultural herbicide. Atrazine is the leading herbicide applied to corn and is used extensively on sorghum and sugar cane (USDA report, 1990-1994). The primary mode of action of atrazine in plants is to inhibit photosynthesis (Gysin and Knuesli, 1998). Atrazine is a chloro-s-triazine herbicide present in the treated female suggests that the primary site of action for the disruption of ovarian function is the CNS. Indeed, we found that atrazine can interfere with hypothalamic catecholaminergic activity in a manner that is consistent with the altered hormone secretion. Following oral atrazine exposure to Long-Evans adult females, hypothalamic norepinephrine concentrations were decreased and dopamine concentrations and turnover were increased (Cooper et al., 1998). Importantly, the changes in hypothalamic catecholaminergic activity and pituitary hormone secretion induced by atrazine are observed both in the presence and absence of endogenous or exogenous estradiol, ruling out any role of the estrogen receptor in this process.

Based on the effect of atrazine on hypothalamic-pituitary function in the adult female, we hypothesized that this compound would test positive in the male pubertal protocol. Since the compounds tested to date with this protocol have been selected primarily because they are known to affect thyroid hormone synthesis or androgen receptor function, this study would be the first to provide information on a compound with a primarily hypothalamic-pituitary mode of action.

MATERIALS AND METHODS

Animals. Timed-pregnant female Wistar rats were purchased from Charles River, Raleigh, NC, and were shipped to arrive on gestation day 13. Upon arrival, they were housed one per cage in an AAALAC-accredited facility maintained at 22°C and on a 12:12 h light:dark cycle (on 0500 h, off 1700 h). Food (Purina laboratory rat chow 5001) and water were provided ad libitum unless otherwise noted. The day of delivery was designated postnatal day (PND) 0. On PND 3, the pups were culled to 8 to 10 per litter to maximize uniformity in growth rates. On PND 22, all male pups were weaned, weighed to the nearest 0.1 g, and weight-ranked. Pups were then assigned so that treatment groups exhibited similar body weight means and variances (see Table 1). After assignment, similarly treated males were housed 2 per cage and weighed daily throughout treatment. Some males were killed on PND 45 (n = 6 for controls and 200 mg/kg atrazine) to evaluate LH receptor number in the testes and serum testosterone levels. Other males were killed at 120 days-of-age (n = 8 per group) to evaluate recovery of reproductive tract weights and hormone values. All other males were sacrificed on PND 53 or 54 (as justified in the protocol for sacrificing large blocks) for the protocol endpoint measures. For the number of animals in these treatment groups, see Figure 2.

Treatment. All treatments were administered daily by oral gavage from PND 23 to 53 or 54 (for males killed on PND 54) at a volume of 0.5 cc/100
Effect of atrazine on ventral prostate weight at PND 53. Mean ± SEM; a, p < 0.05 when compared to control. The pair-fed mean was not different from the atrazine 200-mg/kg group mean. Numbers in each group are listed below treatment group.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PND 23</td>
<td>56.41 ± 1.46</td>
<td>53.88 ± 3.72</td>
<td>53.18 ± 3.52</td>
<td>56.55 ± 0.81</td>
<td>56.68 ± 1.85</td>
<td>54.27 ± 4.47</td>
<td>56.78 ± 1.28</td>
</tr>
<tr>
<td>PPS</td>
<td>201.32 ± 3.90</td>
<td>196.00 ± 5.76</td>
<td>200.33 ± 8.88</td>
<td>206.60 ± 3.16</td>
<td>198.65 ± 5.04</td>
<td>190.67 ± 7.74</td>
<td>189.32 ± 3.21</td>
</tr>
<tr>
<td>PND 43</td>
<td>207.63 ± 5.18</td>
<td>190.00 ± 7.89</td>
<td>195.5 ± 8.99</td>
<td>202.50 ± 4.98</td>
<td>187.88 ± 8.98</td>
<td>189.50 ± 10.38</td>
<td>177.70 ± 8.02</td>
</tr>
<tr>
<td>PND 53</td>
<td>280.75 ± 4.30</td>
<td>266.3 ± 10.3</td>
<td>265.20 ± 12.03</td>
<td>275.00 ± 6.80</td>
<td>262.00 ± 8.18</td>
<td>251.00 ± 6.38</td>
<td>232.80 ± 7.48</td>
</tr>
</tbody>
</table>

Note. Mean body weight in grams ± SEM. Numerical doses of atrazine in mg/kg. PF, pair-fed; PPS, preputial separation day; PND, postnatal day; a, p < 0.05 as compared to control. None of the PF group means were different when compared with the 200 mg/kg-atrazine group.

g body weight between 0800 and 0900 h. Atrazine (Novartis, Greensboro, NC; 97.1% purity) was administered as a suspension in a 1% solution of methyl cellulose (Sigma Chemical, St. Louis, MO). Groups of rats were treated with atrazine (12.5, 25, 50, 100, 150, or 200 mg/kg body weight/day) in a suspension of methyl cellulose. Control males received the 1% solution of methyl cellulose only. A group of 10 pair-fed males was also evaluated to determine the extent to which a reduction of food intake might contribute to alterations in the parameters included in this protocol. Beginning on the first day of dosing (PND 23), the daily food consumption of 10 of the 200-mg/kg atrazine-dosed males was monitored. Then on the following day, each pair-fed male was fed the same amount of food as the corresponding dosed animal. This feeding regimen was continued until the males were killed on PND 53.

Preputial separation. The separation of the foreskin of the penis from the glans, preputial separation (PPS), is an early reliable marker of the progression of puberty that normally occurs between 40 and 50 days-of-age, with an average of 43 days, depending on the rat strain (Korenbrot et al., 1977). In the present study, PPS was monitored beginning on PND 33, until all males showed separation. All males were monitored daily at approximately the same time each day. A partial separation with a thread of cartilage remaining was recorded as “partial”, but only the day of complete separation was used in the data analyses. Partial separation was not a factor in this study.

Necropsy. Males were killed on either PND 45, 53 (or 54), or 120 days-of-age. On the day before necropsy (PND 53 or 54), males were placed in a holding room adjacent to the room used for necropsy. This room was maintained under the same lighting conditions as the animal room, but the location allowed for each animal to be decapitated immediately (typically less than 15 s after removal from their home cage) to minimize stress-induced changes in hormones. Following decapitation, blood was collected and the pituitary, testes, ventral and lateral prostates, epididymides, and seminal vesicles with coagulating gland (with fluid) were removed and weighed. The clotted blood was centrifuged at 1260 × g for 30 min, and the serum harvested and stored frozen at –80°C for subsequent hormone assays. The anterior pituitary was removed, frozen on dry ice, and stored at –80°C for subsequent hormonal analyses. The epididymides, left testis, and thyroid gland (kept intact with the bracketing trachea) were removed and fixed in 10% neutral buffered formalin for 24 h, before transferring to 70% ethanol until later processing for histology could be performed (H&E stain). The right testis was decapsulated, frozen on dry ice, and stored at –80°C for subsequent LH receptor assays. Levator ani and bulbocavernous muscle weights were not evaluated in this study, although this is a required endpoint in the male pubertal protocol.

Additional males (6 control and 6 200-mg/kg atrazine) were killed on PND 45 in the same manner as described above, specifically for determination of LH receptors and intratesticular and serum testosterone content.

Radioimmunoassays. Serum and anterior pituitary hormones were analyzed for luteinizing hormone (LH), prolactin (PRL), and thyroid-stimulating hormone (TSH) by radioimmunoassay. The assays were performed using the following materials supplied by the National Hormone and Pituitary Agency for LH, PRL, and TSH, respectively: iodination preparation I-9, I-6, I-9; reference preparation RP-3, RP-3, RP-3; and antisera S-11, S-9, S-6. Labeled hormone were then added to each tube, and the tube was vortexed and incubated at 5°C for 24 h. One hundred μl with coagulating gland (with fluid) were pipetted with appropriate dilutions to a final assay volume of 500 μl with 100 mM phosphate buffer containing 1% bovine serum albumin (BSA). Standard reference preparations were serially diluted for the standard curves. 200 μl of primary antiserum in 100 mM potassium phosphate, 76.8 mM EDTA, 1% BSA and 3% normal rabbit serum were pipetted into each assay tube, vortexed, and incubated at 5°C for 24 h. One hundred μl of the iodinated hormone were then added to each tube, and the tube was vortexed and incubated for 24 h. Second antibody (goat anti-rabbit gamma globulin, Calbiochem, at a dilution of 1:100/μl μl was then added, vortexed, and incubated for 24 h. The samples were centrifuged at 1260 × g for 30 min, the supernatant was aspirated, and the sample tube, with pellet, was counted on a gamma counter. Historical intra-assay coefficients of variation for the assays were 2.4, 2.7, and 3.1%, and inter-assay coefficients of variation were 9.1, 7.2, and 8.5%, for LH, PRL, and TSH, respectively.

Serum testosterone, estradiol, total T3, and total T4 were measured using Coat-a-Count radioimmunoassay kits obtained from Diagnostic Products Corporation (Los Angeles, CA). The serum estrone was measured using a 3rd Generation Estradiol [125I] Double antibody kit from Diagnostic Systems Laboratories, Inc. (Webster, Texas).
RESULTS

Body Weight

The effect of atrazine on body weight on PND 53 is shown in Table 1. The high dose of atrazine (200 mg/kg) caused a significant decrease in body weight (17%). We also compared the body-weight means of the treatment groups on the day of preputial separation. None of the atrazine treatment-group means were different from controls; however, there was a significant decrease in the pair-fed group, which was significantly different from the control group (Table 1). Table 1 also displays the body-weight means on what was determined to be the average day of preputial separation between the controls and treated males (PND 43-Table 1) to allow a comparison at a single time point. At this time the body weight of the 200 mg/kg-atrazine group was also significantly lower than control (14%). Finally, as anticipated, the body weights of the pair-fed animals were significantly below the control and comparable to that observed in the 200 mg/kg group on both PND 43 (20%) and 53 (14%). Thus, the pair-fed animals provide a basis for discerning the effect attributable to a reduction in body weight per se vs. the effect of treatment with atrazine.

By PND 120, the body weights in all of the atrazine-treated groups had returned to control levels (data not shown).

Reproductive Tract and Organ Weights

Lateral prostate and testicular weights were not different from control in any of the atrazine or pair-fed rats killed on PND 53 (data not shown). However, ventral prostate weight was significantly decreased in a dose-dependent fashion following exposure to atrazine at 50 mg/kg or greater (Fig. 2). Ventral prostate weight was also decreased in the pair-fed group. When the ventral prostate weights of the atrazine and pair-fed groups were analyzed as a covariable with body weight on PND 53, these decreases were still significant. Seminal vesicle and epididymal weights on PND 53 were also decreased in the 200 mg/kg-atrazine and pair-fed groups. When these measures in the atrazine and pair-fed groups were analyzed as covariables with body weight, the decrease in seminal vesicle weight (Fig. 3), but not the epididymal weight (data not shown), was still significant. Also, there were no differences observed between the pair-fed and 200-mg/kg atrazine group in the weights of these 2 tissues.

By PND 120, all reproductive tract organ weights had returned to control levels in all treatment groups, except for the ventral prostate, which was still significantly different from the controls in the 200 mg/kg-atrazine group (784.80 ± 46.1 in the controls vs. 638.13 ± 25.3 in the 200-mg/kg group).

Preputial Separation

Preputial separation was significantly delayed in the 12.5, 50, 100, 150, and 200 mg/kg-atrazine groups by 2.3, 1.8, 1.7, 1.6, and 3 days as compared to controls (Fig. 4). The 25-mg/kg

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**LH-receptor measurements.** The LH-receptor assay employed was modified from the method published by Hauger et al., 1977. The decapsulated testes were thawed and homogenized in 0.25 M sucrose/0.01 M HEPES (pH 7.4) using a teflon-glass homogenizer (1 testis/4.5 ml). The homogenate was centrifuged at 20,000 × g for 30 min (4°C) using a Beckman JA 20.1 rotor (12,000 rpm). A sample of the supernatant was frozen for subsequent testosterone measurement. The pellet was resuspended in 2 ml ice-cold assay buffer (Dulbecco’s phosphate-buffered saline, GIBCO) using a glass dounce homogenizer (Dulbecco’s PBS, pH 7.4). Aliquots of this homogenate were removed and frozen for subsequent protein measurement. A 100 µl aliquot (with a range of 100–200 µg of protein) of this suspension was used to determine hCG-binding capacity. The LH-binding assay used polycarbonate tubes (12 × 75 mm) that were presoaked in 1 ml of 5% BSA overnight to prevent nonspecific binding. A saturating concentration of [125I]hCG was determined and used to measure receptor number in the presence and absence of excess unlabeled hCG. The binding was expressed as fmol/µg protein, based on the specific activity of the [125I]hCG.

**Histology.** To initially screen the tissue samples, only tissues from the high-dose atrazine (200 mg/kg) and control groups were submitted to Experimental Pathology Laboratory, Inc. for processing and histopathological evaluation. Following paraffin embedding, each 4–6 µmr section was stained with hematoxylin and eosin for examination. For the thyroid tissues, each slide to be evaluated contained a transverse section of the thyroid gland (bracketing the trachea).

**Statistical analyses.** Data were analyzed for age and treatment effects by ANOVA using the General Linear Model (GLM) procedure (Statistical Analysis System (SAS), SAS Institute, Inc., Cary, NC), and for homogeneity of variance using Bartlett’s test (GraphPad InStat, GraphPad Software, San Diego, CA). Since no significant age effect was observed for necropsy data obtained on PND 53 and 54, these data were pooled for further analyses. When significant treatment effects (p < 0.05) were indicated by GLM, the Dunnett’s t-test was used to compare each treatment group with the control. The Tukey multiple-comparisons test was used to compare the pair-fed and atrazine 200-mg/kg groups. Necropsy organ weights were analyzed by ANCOVA and least squares mean comparison using the body weight at PND 53 as the covariable. For quantification of linear association between atrazine treatments and LH concentrations, a Pearson correlation test was used (GraphPad, InStat).
group mean was not statistically different from the control mean; however, it was nearly significant \((p = 0.07)\). PPS was also delayed by 2 days in the pair-fed animals as compared to controls. The pair-fed group was significantly different from the 200 mg/kg-atrazine group, with a shorter delay in PPS. Thus, there were changes in PPS in atrazine treatments, which did not affect body weight, and a more severe delay in the highest-dose group which did decrease body weight.

**Hormone Analysis**

**Steroids.** Although there appeared to be a decrease in the serum testosterone in the 25 to 200 mg/kg-atrazine group on PND 53 and in the 200-mg/kg group on PND 45, the values were not statistically different from controls. This may be due to the increased variability of testosterone at these ages (Table 2). However, intratesticular testosterone was significantly decreased on PND 45 in the 200-mg/kg group. Intratesticular testosterone on PND 53 in the 200-mg/kg group was not significantly different from the control. Figure 5 depicts serum estradiol and estrone concentrations on PND 53 in the males examined in this study. There was an increase in the concentration of both hormones as the dose of atrazine increased; however, this increase was statistically significant only at the 200-mg/kg level. The serum concentration of these 2 hormones in the pair-fed animals was not different from the control group (Fig. 5).

**Pituitary hormones.** Pituitary weights were decreased in the atrazine 200-mg/kg group at PND 53 as compared to controls (data not shown), but were not different by PND 120. On PND 53, serum LH showed a significant trend for a dose-dependent linear decrease from controls to 200 mg/kg of atrazine \((r = -0.93, p < 0.0024)\). However, none of the mean serum LH values were significantly different from the control mean (see Table 3). The variability of serum LH in animals at this age made comparisons difficult. No difference was observed between the pair-fed group and the control group, 0.318 ± 0.04 vs. 0.339 ± 0.10, respectively. There were no differences in mean pituitary LH concentrations between any of the treatment groups and controls (Table 3).

Although there appeared to be a decrease in serum prolactin at the 2 highest atrazine doses, there was no statistically significant difference between the mean serum prolactin in any of the treatment groups and the control (Table 3). Again, no differences were observed between the pair-fed group and the control group, 2.371 ± 0.70 vs. 1.937 ± 0.44, respectively. There were no differences in mean pituitary PRL concentrations between any of the treatment groups and controls (Table 3).

**Thyroid hormones.** Serum TSH or T4 concentrations were not different among any of the atrazine-treated groups and the controls (Table 3). However, there was a dose-related increase in serum-T3 concentration, but this difference was significant only in the 200 mg/kg-atrazine group. Again, there was no difference between the pair-fed and control animals for any of the thyroid hormone measures.
LH Receptors

Although LH receptor analysis is not part of the male pubertal protocol, we included this analysis to determine whether or not possible decreases in prolactin may have resulted in a decrease in the normal upregulation of receptors during puberty. No effect was observed between the number of LH receptors in the control and the 200 mg/kg atrazine-dose groups on PND 45 or 53 (Fig. 6).

Histology

High dose atrazine and control thyroids, epididymides, and testes were examined histologically. Approximately 33% of the 200 mg/kg-dose group of atrazine showed minimal hypospermia (defined as rats in which the overall density of spermatazoa within the epididymal ducts appeared to be decreased compared to control samples). This minimal hypospermia rated a 1 on a scale of 1 to 5, with 5 being severe. This observation is probably a result of the length of the delay in puberty in the high-dose group of atrazine and the day of sacrifice. No significant differences between control and 200-mg/kg atrazine-treated males were observed in the thyroid gland sections.

DISCUSSION

The results of the present study demonstrate that atrazine causes a delay in pubertal progression and reproductive-tract development in the male rat when administered during the juvenile and peripubertal period, according to the recommended Tier 1 male pubertal protocol. This protocol was designed to identify compounds that alter puberty by affecting estrogen and androgen, and thyroid and hypothalamic-pituitary-axix (HPA) functions. In this study, the LOAEL for atrazine’s effect on preputial separation was 12.5 mg/kg. This dose is lower than that found to inhibit suckling-induced prolactin secretion in lactating females and altered reproductive function in the male offspring (12.5 mg/kg twice a day) following 3 days of treatment (Stoker et al., 1999), and lower than the dose of 50 mg/kg/day for three days that suppressed the estrogen-induced LH and prolactin surges in ovariectomized adult female rats (Cooper et al., 2000). In this regard, the delay in preputial separation observed in the present study occurs at doses lower than the doses reported in other studies examining effects in male rats. Additionally, the LOAEL in this study was approximately one-half the daily exposure required to induce premature reproductive senescence and mammary gland tumor development in females maintained on an atrazine diet of 400 ppm (approximately 22.5 mg/kg/day) (Eldridge et al., 1999). Interestingly, with the range of doses tested in this study, we did not determine a NOEL.

Although the identification of the mode of action of a test chemical is not a stated purpose of the male pubertal protocol, we can evaluate the alterations observed on pubertal development in the present study based on previous reports. Atrazine has been reported to affect several endocrine endpoints, which must be taken into consideration when evaluating the effects in this male assay. The evidence available suggests that atrazine

| Table 2: Effect of Atrazine on Serum and Intratesticular Testosterone on PND 45 and 53 |
|---------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                                | CON    | 12.5   | 25     | 50     | 100    | 150    | 200    | PF     |
| **PND 45**                     |        |        |        |        |        |        |        |        |
| Serum                          | 0.957 ± 0.20 | –     | –     | –     | –     | –     | 0.676 ± 0.38 | –     |
| Test                           | 19.020 ± 1.0 | –     | –     | –     | –     | –     | 12.470 ± 1.8* | –     |
| **PND 53**                     |        |        |        |        |        |        |        |        |
| Serum                          | 2.22 ± 0.43 | 2.22 ± 1.14 | 1.13 ± 0.37 | 1.59 ± 0.26 | 1.87 ± 0.38 | 1.25 ± 0.32 | 1.55 ± 0.21 | 2.43 ± 0.77 |
| Test                           | 17.75 ± 3.3 | –     | –     | –     | –     | –     | 12.40 ± 2.0 | 15.35 ± 1.6 |

*Note. Mean ± SEM; Test., intratesticular; –, measures not taken.
*p < 0.05 as compared to control mean.

**FIG. 6.** Effect of atrazine on fmol of testicular LH receptors bound per μg protein on PND 45 and 53. Mean ± SEM; n = 6 per group.
is not directly estrogenic (Connor et al., 1996; Eldridge et al., 1994; Tennant et al., 1994), but may or may not possess some anti-estrogenic properties (Graumann et al., 1999; Tennant et al., 1994b; Tran et al., 1996). However, neither of these observations is consistent with the delay in male pubertal development seen in this study, because natural and environmental estrogens typically delay preputial separation if administered prior to puberty (Anderson et al., 1995; Chapin et al., 1997). There is evidence that atrazine may enhance estrogenic activity by stimulating the activity of aromatase, the enzyme that converts androgens to estrogens. In vivo, atrazine stimulates aromatase activity in human adrenocortical carcinoma cells (Sanderson et al., 2000). In vivo, this herbicide induced gonadal-adrenal mesonephros aromatase activity in male hatching alligators (Crain et al., 1997). The dose-related increase in serum estradiol and estrone and decrease in testicular testosterone observed in the present study are consistent with a stimulatory effect on aromatase, although this enzyme was not assayed directly. It is not clear whether this apparent atrazine-stimulated increase in serum estrogens contributed to the pubertal delay observed in this study. Saksena and Lau (1979) showed that, prior to PND 22, serum estradiol and estrone concentrations were high in the male rat (i.e., >200 pg or 200 ng for estradiol and estrone, respectively). However, there is a dramatic decrease in the serum levels of these two hormones between PND 22 and PND 32. Furthermore, Lephart and Ojeda (1990) reported that hypothalamic aromatase activity decreased in the peripubertal animal and that this decrease was associated with the decline in sensitivity to testosterone feedback that occurs as puberty progresses. Thus, one might speculate that a persistent elevation in serum estrogens contributed to the atrazine-induced pubertal delay. However, the increase in serum estrogens was only significant at the highest dose of atrazine, which was far greater than those required to alter other endpoints such as preputial separation. At the same time, the possibility that localized tissue differences in aromatase activity (i.e., within the CNS) contributed to the delay in puberty remains to be determined. Thus, further mechanistic and time-point evaluations would be required to determine the exact contribution of this alteration to the delay in puberty that we observed in this study.

In addition to atrazine’s effects on estrogenic activity, atrazine has also been reported to inhibit androgen receptor function in vitro (Danzo, 1997). A delay in preputial separation and reproductive tract development is a well-known effect of environmental anti-androgenic compounds when administered in the male pubertal assay (e.g., Monosson et al., 1999). We are unaware of any in vivo studies that have implicated a direct anti-androgenic effect of this herbicide, although these results would be consistent with the pubertal delay observed in the present study.

In addition to steroidal effects of atrazine, there have been several studies implicating the CNS or hypothalamus as the primary target site of atrazine’s effect on reproductive function. The alteration of neurotransmitters by atrazine has been observed previously in both female and male rats, with a resultant decrease in norepinephrine (NE) and an increase in dopamine (DA) within three hours following treatment by gavage (Cooper et al., 1998). This alteration in neurotransmitter concentrations appears to underlie the suppression of LH and prolactin that has been reported in previous studies using the ovariectomized, estradiol-primed female rat (Cooper et al., 2000; Simpkins et al., 1998) and in the lactating dam (Stoker et al., 1999). Such changes in pituitary hormone secretion, as seen with the dose-related decrease in luteinizing hormone in this study, may explain the delay in puberty observed in the present study. In addition, it has been shown that an increased turnover rate in hypothalamic GnRH, NE and DA precedes the dramatic increase in testosterone (Matsumoto et al. 1986) prior to the onset of puberty.

LH stimulates testosterone secretion by the Leydig cells. At the same time, LH secretion varies only slightly as puberty approaches. However, there is an increased sensitivity of the testes to LH prior to puberty, due to other hormonal influences, such as increased prolactin secretion, that facilitate an upregulation of LH receptors (Kamberi et al., 1980; Odell et al., 1973; Vihko et al., 1991). Thus, a combined suppression of LH release and possibly LH receptor function would result in

### TABLE 3
Effect of Atrazine on Thyroid Hormones, Prolactin (PRL), and Luteinizing Hormone (LH)

<table>
<thead>
<tr>
<th></th>
<th>sTSH (ng/ml)</th>
<th>T4 (ng/ml)</th>
<th>T3 (ng/ml)</th>
<th>sLH (ng/ml)</th>
<th>pLH (ug/mg)</th>
<th>sPRL (ng/ml)</th>
<th>pPRL (ug/mg)</th>
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<tr>
<td>Control</td>
<td>1.35 ± 0.24</td>
<td>60.17 ± 7.59</td>
<td>104.38 ± 124.7</td>
<td>0.339 ± 0.10</td>
<td>0.987 ± 0.08</td>
<td>1.937 ± 0.44</td>
<td>0.808 ± 0.10</td>
</tr>
<tr>
<td>ATR 12.5</td>
<td>1.34 ± 0.17</td>
<td>64.88 ± 4.50</td>
<td>110.38 ± 113.5</td>
<td>0.305 ± 0.06</td>
<td>1.16 ± 0.07</td>
<td>1.791 ± 0.76</td>
<td>0.817 ± 0.10</td>
</tr>
<tr>
<td>ATR 25</td>
<td>1.59 ± 0.17</td>
<td>62.3 ± 7.21</td>
<td>1245.8 ± 150.6</td>
<td>0.271 ± 0.09</td>
<td>0.982 ± 0.07</td>
<td>1.597 ± 0.36</td>
<td>0.986 ± 0.23</td>
</tr>
<tr>
<td>ATR 50</td>
<td>1.44 ± 0.21</td>
<td>76.9 ± 6.86</td>
<td>1416.4 ± 160.1</td>
<td>0.241 ± 0.03</td>
<td>0.792 ± 0.08</td>
<td>1.964 ± 0.40</td>
<td>0.844 ± 0.06</td>
</tr>
<tr>
<td>ATR 100</td>
<td>1.59 ± 0.33</td>
<td>61.23 ± 10.47</td>
<td>1277.8 ± 154.5</td>
<td>0.246 ± 0.05</td>
<td>0.978 ± 0.12</td>
<td>2.760 ± 0.79</td>
<td>0.627 ± 0.07</td>
</tr>
<tr>
<td>ATR 150</td>
<td>1.103 ± 0.21</td>
<td>66.63 ± 8.16</td>
<td>1440.8 ± 162.7</td>
<td>0.192 ± 0.05</td>
<td>1.397 ± 0.17</td>
<td>1.011 ± 0.11</td>
<td>0.977 ± 0.26</td>
</tr>
<tr>
<td>ATR 200</td>
<td>1.083 ± 0.13</td>
<td>75.31 ± 4.97</td>
<td>1709.3 ± 161.5</td>
<td>0.179 ± 0.03</td>
<td>1.00 ± 0.17</td>
<td>1.179 ± 0.23</td>
<td>0.915 ± 0.17</td>
</tr>
<tr>
<td>Pair–fed</td>
<td>1.12 ± 0.12</td>
<td>70.13 ± 3.89</td>
<td>1091.9 ± 157.9</td>
<td>0.318 ± 0.04</td>
<td>1.31 ± 0.21</td>
<td>2.371 ± 0.70</td>
<td>0.858 ± 0.06</td>
</tr>
</tbody>
</table>

*Note. Mean concentrations ± SEM.
*p < 0.05 as compared to control mean.
decreased testosterone synthesis by the Leydig cell. This decrease in testosterone secretion would delay androgen-dependent tissue development and preputial separation. In support of this potential atrazine-induced effect on the regulation of steroidogenesis, we did observe a dose-dependent trend for decreased serum LH concentrations on PND 53, but serum prolactin concentrations were not different at the time the males were terminated.

As mentioned, atrazine can suppress prolactin secretion by the pituitary (Cooper et al., 2000; Stoker et al., 1999). Prolactin has been shown to play various roles during puberty. It is involved in the upregulation of LH receptors on the Leydig cells of the testes (Haifiez et al., 1971; Johnson and Brown, 1974; Kamberi et al., 1980; Kolena and Sebokova, 1983; McNeilly et al., 1979; Purvis et al., 1979; Zipf et al., 1978). Normally, prolactin levels are low until PND 20 and then rise significantly between 25 and 40 days-of-age in the rat, with maximum prolactin levels coinciding with the start of accelerated growth in the prostate and seminal vesicle (Negro-Vilar et al., 1973). For example, treatment with a dopaminergic agonist such as bromocriptine, which inhibits prolactin secretion, will decrease by one half the number of LH receptors per one million Leydig cells and reduce the in vitro steroid response of these cells to hCG, when administered after PND 30 (Aragona et al., 1977; Purvis et al., 1979). Thus, prolactin plays an important role in maintaining the functional integrity of rat Leydig cells.

When we examined the LH receptors present on the Leydig cells in this study from PND 45 and PND 53 males, there were no significant differences between control, pair-fed, and high-dose atrazine males. LH receptors appear on the fetal testes around GD 15, and their concentration rises significantly between PND 15 and 38 (Ketelslegers et al., 1978). We might have observed an alteration in LH receptor numbers if we had done a more detailed time-point analysis of the receptor levels (i.e., every 5 days, once dosing began on PND 23). This would provide a better comparison, since the normal rise in LH receptors observed in untreated males occurs between PNDs 15 to 38. One explanation for seeing no difference in the atrazine-treated males’ receptor number at PND 53 may have been due to regulation of LH receptor number by decreases in testosterone or LH, with a corresponding upregulation of LH receptors on the Leydig cells by the day of sampling.

The delay in preputial separation that we observed in the 12.5 to 200 mg/kg-atazine groups did not appear to be dose-dependent. The 12.5 mg/kg-dose group delayed PPS by 2.3 days. The 25 mg/kg-dose group was not statistically different from the control mean; however, it was nearly significant (p = 0.07). Fifty to 150 mg/kg treatment delayed preputial separation approximately 1.5 days and 200 mg/kg delayed PPS by approximately 3 days. The longer delay in the 12.5-mg/kg group may have been less accurate due to a lower n of 6 in the 12.5, 25, and 150 mg/kg groups, as compared to more than 20 in the other groups. Since the development of the size of the penis, cornification of the epithelium of the prepuce, and preputial separation in immature rats are regulated by androgens (Marshall, 1966), a decrease in testosterone during the juvenile period can delay preputial separation (Lyons et al., 1942) and reduce the size of androgen-dependent tissues such as the ventral prostate and seminal vesicles. Although serum testosterone levels in the atrazine-treated males appeared to be lower than controls, there was no statistical difference due to the variability in all groups. Variability in this measure may be due to changes in secretion of testosterone at this age. Normally, testosterone levels rise gradually from PND 20 to 40, and abruptly double by PND 50 (Matsumoto et al., 1986; Monson et al., 1999). For this reason, a more complete evaluation would include a time-point analysis, with testosterone measurements at 5-day intervals following the first doses of atrazine (i.e., PND 28, 33, 38, 43, 48).

Because a dose of 200 mg/kg of atrazine had been shown to result in a decrease in food consumption and body weight (6.5 to 8.5%) in a 6-week gavage study in Sprague-Dawley females (Eldridge et al., 1999), we included a food-deprived or “pair-fed” group, which consumed the same amount of food as the high-dose-atazine group. This allowed us to discern the effects of decreased food consumption from the effects of the endocrine alterations seen with delaying pubertal development. The pair-fed group exhibited delayed preputial separation, but to a significantly lesser extent than the 200 mg/kg atrazine-dose group. The ventral prostate and seminal vesicle weights were also decreased in the pair-fed group, with the effect on epidiymal weight disappearing when analyzed by ANCOVA using body weight at necropsy as the covariate. Nutritional status and body weight are known to have effects on reproduction and puberty, which are suggested to reflect the metabolic signals in the brain that serve as indices of the metabolic state. It has also long been suggested that a low body weight may contribute to a delay in puberty (Kennedy and Mitra, 1963), with food restriction resulting in a delay in pubertal onset and refeeding reversing the delay (Kennedy and Mitra, 1963). The idea that metabolic alterations associated with weight loss or decrease in growth rate are inhibitory to the reproductive system may be related to substances in the body that can alter the release of GnRH such as insulin, amino acids necessary for precursors of neurotransmitter synthesis, and essential fatty acids (Ojeda and Urbanski, 1994). However, in this study we observed effects of atrazine on preputial separation and reproductive tract development at doses that did not affect body weight. Interestingly, the delay observed in the pair-fed animals was not as severe as that observed in the atrazine 200-mg/kg group (which was significantly longer than that in the pair-fed rats). Thus, the effects noted in all dose groups would indicate that body weight per se does not appear to be the only factor involved in the delay in preputial separation observed. At the highest dose level, the effect of atrazine and decreased body weight appeared additive. Also, although we observed no effect of pair feeding on any hormonal endpoint examined, there have been...
several studies indicating that food restriction in the adult male can result in decreased LH and testosterone (Leonhardt et al., 1999). Interestingly, although we observed no change in estradiol or estrone levels at PND 53 in the pair-fed rats, one study reported that aromatase activity was reduced in the medial basal hypothalamus of underfed male rats (Seidl and Pirke, 1987), indicating that hypothalamic metabolism of testosterone is decreased during periods of severe food restriction. However, this alteration was not apparent in this study.

In addition to its effects on reproductive tissue, estrogen also has a direct effect on the CNS regions that regulate appetite by suppressing food intake (Reynolds and Bryson, 1974). Thus, the significant increase in serum estrogen concentrations in the 200 mg/kg-atazine group observed in the present study may have been a factor in the decreased food consumption and body weight in this group. This body weight issue should be taken into consideration in evaluating the effects of compounds screened in this protocol. The inclusion of a pair-fed group was helpful in discerning the effects of such chemicals in this male pubertal screen. It is difficult to differentiate the toxic effects of an increase in estrogen or an estrogenic compound from the endocrine effects on pubertal progression.

In summary, atrazine at doses of 12.5 to 200 mg/kg tested positive in the male pubertal assay causing a delay in preputial separation and reproductive tract development. The measure of testosterone, luteinizing hormone, and prolactin, which are optional endpoints in this male assay, proved to be quite variable on PND 53, and do not appear to be robust endpoints for detecting endocrine-disrupting chemicals. Importantly, this assay was able to detect alterations in pubertal development with a compound that appears to work primarily through a disruption of the CNS control of pituitary function.

Note added in proof: In a recently dosed group of male rats, a dose of 6.25 mg/kg of atrazine was found to have no effect on the day of PPS as compared to the control when exposed in the same male pubertal protocol (data not shown).

ACKNOWLEDGMENTS

The authors express their gratitude to the National Hormone and Pituitary Agency for the gift of the radioimmunoassay materials. We would also like to thank Keith McElroy for his technical contributions.

REFERENCES

Danzo, B. J. (1997). Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environ. Health Perspect. 105, 294–301.
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