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Atrazine-induced reproductive tract alterations after transplacental and/or lactational exposure in male Long–Evans rats

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Abstract

Studies showed that early postnatal exposure to the herbicide atrazine (ATR) delayed preputial separation (PPS) and increased incidence of prostate inflammation in adult Wistar rats. A cross-fostering paradigm was used in this study to determine if gestational exposure to ATR would also result in altered puberty and reproductive tissue effects in the male rat. Timed-pregnant Long–Evans (LE) rats were dosed by gavage on gestational days (GD) 15–19 with 100 mg ATR/kg body weight (BW) or 1% methylcellulose (controls, C). On postnatal day (PND)1, half litters were cross-fostered, creating 4 treatment groups; C-C, ATR-C, C-ATR, and ATR-ATR (transplacental-milk as source, respectively). On PND4, male offspring in the ATR-ATR group weighed significantly less than the C-C males. ATR-ATR male pups had significantly delayed preputial separation (PPS). BWs at PPS for C-ATR and ATR-ATR males were reduced by 6% and 9%, respectively, from that of C-C. On PND120, lateral prostate weights of males in the ATR-ATR group were significantly increased over C-C. Histological examination of lateral and ventral prostates identified an increased distribution of inflammation in the lateral prostates of C-ATR males. By PND220, lateral prostate weights were significantly increased for ATR-C and ATR-ATR, but there were no significant changes in inflammation in either the lateral or ventral prostate. These results suggest that in LE rats, gestational ATR exposure delays PPS when male offspring suckle an ATR dam, but leads to increased lateral prostate weight via transplacental exposure alone. Inflammation present at PND120 does not increase in severity with time.
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Keywords: Atrazine; Prostate; Lactation; Inflammation; Endocrine disruptor

Introduction

Several herbicides are used to control weed growth on crops grown in the United States. One of the most commonly used herbicides is atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, ATR). Due to its extensive use and detection in surface and ground water (Baker, 1998); ATR has been studied to determine possible adverse effects on endocrine function and reproductive tissues in mammalian models.

Studies of male rats have examined the onset of puberty, hormone levels, and alterations in reproductive organs following exposure to ATR during the early postnatal or peripubertal periods. Stoker and colleagues (1999a) measured the suckling-induced prolactin release at postnatal day (PND)1–4 in Wistar rat dams and found a dose-dependent suppression in the dams' circulating prolactin levels following gavage exposure to 12.5–50 mg/kg ATR/day during that time. These early postnatal exposures to ATR showed lasting effects in the male offspring. Examination of myeloperoxidase activity in lateral prostates of 120-day-old male Wistar offspring exposed during the early postnatal period (25–100 mg/kg ATR/day by gavage to the dam) suggested increased prostate inflammation (Stoker et al., 1999a).

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Because they were able to reverse the effect with ovine prolactin delivery, in conjunction with the ATR exposure to the dam, the authors concluded that the disruption of the offspring's own endocrine system via decreased prolactin in the dam's milk was responsible for altered offspring susceptibility to lateral prostate inflammation. However, prolactin in the dam's milk was not measured.

Several studies have also demonstrated that exposure to ATR postnatally can delay puberty. Stoker and colleagues (2000) determined that puberty, distinguished by preputial separation (PPS), was delayed in Wistar rats treated by gavage from PND 23–53 with 12.5–200 mg/kg ATR. Testicular testosterone was decreased in males treated with high dose (200 mg/kg) ATR when measured at PND45, but not at PND53. They also found that ventral prostate weights were reduced immediately following the dosing period (PND53) in rats given 50–200 mg/kg ATR. Ventral prostate weights were still reduced at PND120 in rats that received 200 mg/kg ATR during the pubertal period. However, those authors also demonstrated a similar decrease in ventral prostate weight in a pair-fed group. Sprague–Dawley (SD) rats treated with 1–200 mg/kg ATR by gavage from PND22–47 had delayed puberty induced by 100–200 mg/kg ATR and reduced serum luteinizing hormone (LH), interstitial fluid testosterone and serum testosterone levels, ventral prostate weights, and seminal vesicle weights when necropsied at PND48. They also found that food restricted control rats displayed reduced hormone concentrations and reproductive organ weights (Trentacoste et al., 2001). In other studies, both chronic (27 days; PND22–48) and acute (3 days; PND46–48) ATR exposures significantly reduced serum and intratesticular testosterone levels in SD rats on PND49 (Friedmann, 2003). In vitro studies with Leydig cells obtained from 49 day old rats showed that ATR reduced testosterone production (Friedmann, 2003).

Previous studies (such as those mentioned earlier) suggest that ATR is an endocrine disruptor capable of reducing LH, prolactin, and circulating or testicular testosterone levels, all potentially by exerting its effects at the hypothalamus level (discussed in Cooper et al., 2000; Stoker et al., 2000; USEPA, 2002). Disruptions of these hormones early in life are thought to affect the growth of reproductive tissues and pubertal progression in male rats exposed postnatally. The effects of gestational only or gestational and lactational ATR exposure have not been studied. The latter part of gestation and early postnatal time periods mark important periods of growth and development of male and female reproductive organs and the brain. Previous studies in our lab have shown this period to be critical for normal development of the mammary gland, another hormone dependent tissue (Fenton et al., 2002; Rayner et al., 2004; Rayner et al., 2005).

The present study examined the effects of short-term prenatal exposure to ATR in Long–Evans rats on pubertal timing, reproductive tissues, and hormone levels in the male offspring. The effects on their female siblings were reported elsewhere (Rayner et al., 2004). Exposure parameters were designed to determine if exposure-induced changes were the result of transplacental ATR exposure or lactationally-driven effects, or both. The lactational effects could be from ATR-induced changes in quality or quantity of delivered milk or residual ATR metabolites in the milk. We report, for the first time, male reproductive tissue effects following gestational exposure. We have also evaluated the effects on the prostate via myeloperoxidase assays and histopathology at PNDs 120 and 220. Our results suggest that late gestational exposure to atrazine and/or the resulting lactational effects of the prenatal exposure induce changes in hormone levels, and weight and inflammation of the prostate, long after the exposure has occurred, presumably due to its effects on developing tissues at the time of the exposure.

Methods

Animals. Long–Evans (LE) rats, shown in previous pilot studies to be sensitive to ATR effects in mammary tissue (S. Fenton, personal communication), were the animals chosen for this study. Timed-pregnant LE rats (sperm positive=Day 0) were purchased from Charles River Breeding Laboratories (Raleigh, NC). Dams were housed one per cage in an AAALAC-International accredited facility and provided food (Purina 5008 Rodent Chow, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. The rats were maintained in a room with a 14:10 h light cycle, 20–24 °C and relative humidity of approximately 50%. Animal protocols were reviewed and approved by the National Health and Environmental Effects Research Laboratory (NHEERL), Institutional Animal Care and Use Committee. Animals tested negative for infectious diseases prior to beginning the study.

Dosing solution. Atrazine (Syngenta Crop Protection, Inc. Greensboro, NC, 97.1% purity) was prepared as a suspension in 1.0% methyl cellulose (Sigma Chemical, St. Louis, MO) in distilled water. Timed-pregnant rats were treated with vehicle or 100 mg ATR/kg BW by oral gavage in 5 mL/kg dosing volume. This selected dose is high given that the maximum contaminant level set by the US Environmental Protection Agency is 3 μg/L, and ATR detection in community water systems from across the United States has ranged from 0.2 μg/L to 4.2 μg/L from 2001–2004 (USEPA, 2006). Because of the potential dilution of health effects stemming from the cross-fostering of pups within the study, this relatively high dose of ATR was chosen based on consistent results on reproductive tissues in previous studies (Laws et al., 2000; Rayner et al., 2004; Stoker et al., 1999a), and so that results could be compared across studies.

Experimental design. The general study design has previously been reported (Rayner et al., 2004). Briefly, 40 pregnant LE dams were treated on gestational days (GD)15–19 (time period during which reproductive tissues are developing) with vehicle (20 dams, C) or 100 mg/kg ATR/BW (20 dams, ATR). On PND1 (day of birth; beginning at lights on—0700 h), litters were weighed, pups counted, and all litters were cross-fostered as described in Fig. 1. Half litters were cross-fostered with half litters from a vehicle or ATR treated dam, creating four exposure parameters with 20 dams in each (Fig 1): birth dam or gestational exposure only (ATR-C), milk source or lactational exposure only (C-ATR), exposure both pre- and postnatal (ATR-ATR), or neither (C-C). Pups remaining with their birth dams were tattooed, and all pups were individually identified and tracked, making sure to record and analyze data from individual pups by their birth dam. Pups were not weighed at birth. Previous studies using this same ATR dosing regimen and dose in this lab have demonstrated no *in utero* effect of ATR on GD20 or 21 weights (Rayner et al., 2005). Pups were individually weighed (N=30 or more pups/group) and litters randomly equalized (without regard to their tattoo) to 10 pups on PND4 (6 female and 4 males when possible).

At weaning, PND22, pups were again individually weighed and two males from each cross-fostered litter in each treatment group were randomly selected for continued evaluation of puberty and necropsy on PND120 and 220, and housed as two males from the same cross per cage (not necessarily siblings). Males were singly housed if aggressive behavior was observed within a cage. All animals were evaluated for pubertal endpoints (N>18 males/group). Half of each group was necropsied on PND120 or 220 (N=9–10/group per day). Extra males were euthanized and discarded after weaning. Pup

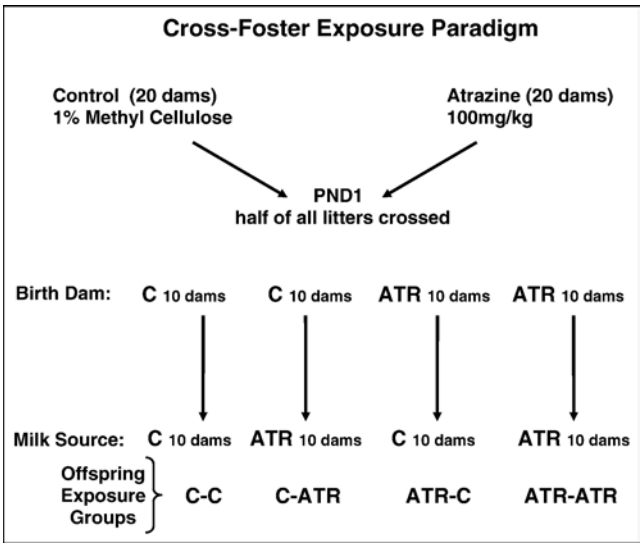

Fig. 1. Half litter cross-foster exposure paradigm. Time pregnant dams were dosed by oral gavage on pregnancy days 15–19 with the described treatments. Within and between each treatment group (Control or ATR), half of each litter was cross with half of another litter creating four exposure parameters: birth dam or gestational exposure only (ATR-C), milk source or lactational exposure only (C-ATR), exposure both pre- and postnatal (ATR-ATR), or neither (C-C). Within each litter, two exposure parameters were represented by the offspring: for example, C-ATR = C-C and C-ATR. Birth dam means were used for data evaluations shown in the text.

mortality between cross-foster and weaning was minimal. It should be noted that by using random culling and selection methods at PND4 and weaning, we significantly decreased our N for each exposure group. Of the 18–22 males remaining per exposure group, there were only 4–5 dams represented of the 10 in the original group.

Preputial separation. Beginning on PND 37, male offspring (N>18 males/group) were evaluated for puberty. The separation of the foreskin of the penis from the glans (preputial separation, PPS) was used to determine if an animal had gone through puberty (Korenbrot et al., 1977). The day of complete PPS and body weight on the day of PPS were recorded.

Necropsy. Necropsies were performed following an overnight and continued stay in a quiet holding area, and by using DecapiCones© (Braintree Scientific, Inc., Braintree, MA) for animal transfer to reduce stress. Males were euthanized on either PND120 (N=10/group) or 220 (N=9–10/group). Following decapitation, trunk blood was collected into serum separator tubes and centrifuged for 30 min at 3000 rpm (4 °C) to obtain serum for hormone assays. The pituitary gland, testes, lateral and ventral prostates, and seminal vesicles of each animal were removed and individually weighed. Gross lesions were recorded. The right lateral and ventral prostates were fixed in 10% neutral buffered formalin for routine histology. Pituitary glands and left lateral prostates were placed on dry ice, frozen, and stored at −80 °C.

Radioimmunoassays. Sera obtained from male offspring on PNDs 120 and 220 were analyzed for prolactin levels by radioimmunoassay using materials supplied by the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases. The prolactin assay was run as previously described in detail by Stoker et al. (2000). Total serum testosterone and androstenedione were measured using Coat-a-Count Radioimmunoassay Kits obtained from Diagnostic Products Corporation (Los Angeles, CA). Serum estrone was measured using the DSL 8700 Estrone Radioimmunoassay kit from Diagnostic Systems Laboratories, Inc. (Webster, TX). All assays were run in duplicate following manufacturer's directions. Inter-assay coefficients of variation for the prolactin, testosterone, adrostenedione, and estrone assays were 5.2%, 4.7%, 4.4%, and 4.9% respectively.

Myeloperoxidase assay. The left lateral prostates collected on PNDs120 and 220 were analyzed for myeloperoxidase (MPO) activity. Tissues were homogenized and prepared as described in detail by Stoker et al. (1999a) for myeloperoxidase assays. The supernatant (250 μL) of the tissue homogenate was assayed using the APLCO Myleperoxidase ELISA kit (American Laboratory Products Company, Windham, NH) following the manufacturer's directions. Duplicate samples were loaded onto a 96-well plate coated with anti-MPO antibody. A peroxidase-conjugated polyclonal anti-MPO antibody was added followed by tetramethylbenzidine and a stop solution. The plate was read on a Spectra Max Plus Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA) at an excitation wavelength of 450 nm. The standard curve used in the assays had correlation coefficients of R = 0.99 (PND120) and R = 1.0 (PND220). The data were used to calculate the MPO concentration in each sample according to the manufacturer's directions.

Histology and pathology. The right lateral and ventral prostates which were fixed in 10% neutral buffered formalin, and were processed by routine methods to paraffin block. Five slides were prepared from each gland; 5 μm sections were taken following step-sectioning through the gland (skipping 20 μm segments). They were stained with hemotoxylin and eosin (H and E) for microscopic examination. All 5 slides from a single prostatic lobe were examined without knowledge of treatment and graded based on the distribution (% of gland affected) and severity (magnitude of cellular infiltrate) of the inflammation. Distribution scores were as follows: 0–none, 1–10% or less of gland affected, 2–approximately 25%, 3–approximately 50%, 4–75% or greater of the gland affected. For severity of inflammation in organ: 0–no infiltration of inflammatory cells, 1–minimal interstitial infiltration of inflammatory cells, 2–moderate interstitial inflammation with or without focally marked accumulations of perivascular collections of inflammatory cells, 3–early micro-abscess formation, mild numbers of inflammatory cells in acinus with some degradation of colloid, 4–micro-abscess, 5–micro-abscess with moderate to marked interstitial inflammation. If two or more slides per gland were found to have inflammation a score was given and averaged according to the most severe lesion per slide. No score was given if a lesion was found on only one slide. In addition, the cellular component of the inflammatory process was identified. Lymphocytes were most commonly present. If neutrophils made up more than 10% of the inflammatory cell population then both the diagnosis of lymphocytes and neutrophils were made. If neutrophils were more than 90% of the cell population then only the diagnosis of neutrophils was made. The prostates were also examined for the presence of edema which was characterized by expansion of the interstitial spaces between the glandular acini with eosinophilic proteinaceous material present.

Statistical analysis. Birth dam means (vs. litter means, because half litters were crossed) were calculated for PPS, as well as male offspring body and tissue weights. Data were evaluated for age and treatment effects by analysis of variance (ANOVA) using least-squares regression model (LSM) (Statistical Analysis System (SAS), SAS Institute, Inc. Cary, NC). Means were evaluated and treatment groups compared to each other. Means and adjusted means relative to body weight were calculated for organ weights. These means were compared with control means and among treatment groups. Body weights, serum hormone concentrations, and MPO concentrations were analyzed by one-way ANOVA. Visual evidence of prostate abnormalities was analyzed in a contingency table using Fisher's exact test in Graphpad Instat (Graphpad Software, San Diego, CA). Prostate pathology distribution and severity scores were analyzed for treatment and age effect by t-test and Dunnett's multiple comparison in JMP (SAS). Significant treatment effects were indicated when $p<0.05$.

Results

Dam weight gain

Dam weight was recorded throughout the dosing period (GD15–19) and weight gain compared among the groups. Mean body weight of the ATR dams on GD19 (346.7 ± 7 g) was reduced 13% when compared to control dams (396.7 ± 7 g).

Control dams gained an average of 53.3±1.6 g during the 5-day dosing period and ATR-treated dams gained only 19.3±3.6 g during the same time period. This reduced weight gain was found to be statistically significant ($p<0.0001$), and is comparable to decreased dam weight gain observed in a previous ATR study (Rayner et al., 2005).

Growth and puberty

The male offspring in the ATR-ATR group were 6.5% smaller ($p<0.001$) when compared to offspring in the C-C and C-ATR groups on PND4 (8.6±0.1 g ATR-ATR vs. 9.2±0.1 g C-C, 9.0±0.2 g ATR-C, and 9.2±0.1 g C-ATR). This small decrement in weight was also reported in ATR-ATR exposed female siblings (Rayner et al., 2004), but is not due to lack of weight gain *in utero* (Rayner et al., 2005). It is suspected that ATR or its metabolites may have affected dam milk quantity or quality (nutritional impact), or residual ATR metabolites in milk may have been present and having effect on the pups at that stage. At weaning, PND22, the treatment groups were all similar in body weight (56.9±1.0 g C-C, 54.6±1.1 g ATR-C, 54.6±1.0 g C-ATR, and 56.0±0.9 g ATR-ATR). Body weights in the C-ATR males were significantly reduced, but other males were not different at PND120 (Table 1). Body weights of males in the ATR-C and ATR-ATR groups were also reduced at this time point, but were not considered statistically different. Animals from the ATR-C group were significantly smaller ($p<0.05$) than animals in the control and C-ATR groups at PND220 (Table 1). However, these inconsistent changes in body weight at later life time points were not considered to be due strictly to ATR exposure (due to lack of clear trend), but possibly due to random offspring selection at the various necropsy dates.

Male offspring were evaluated for preputial separation (PPS) as a physical indication of puberty. Control males displayed PPS on PND 41.3±0.3 (Fig. 2A). A significant 2 1/2 day delay was seen only in males in the ATR-ATR group (43.7±0.5, $p<0.01$). This delay was also significant when compared to males in the remaining groups. Body weights recorded at time of PPS were decreased in the ATR-ATR animals ($p<0.01$ vs. C-C, Fig. 2B). While a significant reduction in body weight was not seen in the remaining ATR groups, there was a trend for body weight reduction (3–7%) at time of puberty, in addition to a 1 1/2 day ($p=0.07$) delay in mean day of PPS in the C-ATR group.

Reproductive tissue weights

Reproductive tissues and pituitaries were removed from male rats on PND 120 or 220. There were no significant weight differences among the groups for pituitary glands in rats euthanized at PND120 (Table 1). The pituitary glands from the prenatally ATR exposed males weighed significantly more than those taken from controls at PND220. However, when controlling for body weight of the animals in the distinct treatment groups, pituitary gland weights of animals in the ATR-C and ATR-ATR treatment groups were significantly increased compared to control animals ($p<0.03$).

There was a significant difference in lateral prostate weights at both time points. At PND120, the lateral prostates of the ATR-ATR offspring group weighed significantly more ($p<0.01$) than

Table 1
Effect of 100 mg/kg ATR* on reproductive organ weights at PND120 and PND220

	C-C[1]	ATR-C	C-ATR	ATR-ATR
PND120				
Body wt (g)′	661.6±17.3	623.4±18.5	605.7±13.9[a]	616.0±18.3
Pituitary (mg)	10.08±0.66	9.65±0.71	9.64±0.49	10.34±0.34
Left testis (g)	1.99±0.04	1.98±0.03	1.87±0.08	1.88±0.07
Right testis (g)	1.92±0.04	1.92±0.03	1.87±0.04	1.85±0.07
Seminal vesicle wet (g)	1.64±0.25	1.45±0.10	1.43±0.08	1.58±0.07
Seminal vesicle dry (g)	0.728±0.04	0.698±0.04	0.700±0.02	0.774±0.03
Ventral prostate (mg)	524.0±42.4	469.9±33.6	451.1±28.0	532.3±32.9
PND220				
Body wt (g)	777.3±26.6	703.5±23.5[a,b]	804.1±31.5	739.3±10.8
Pituitary (mg)	8.41±0.98	10.38±1.05[c]	10.52±0.81	12.9±0.72[d,e]
Left testis (g)	1.97±0.06	1.89±0.07	2.01±0.06	2.10±0.07
Right testis (g)	1.92±0.06	1.85±0.07	1.99±0.05	2.03±0.07
Seminal vesicle wet (g)	1.67±0.12	1.74±0.09	1.92±0.15	1.86±0.10
Seminal vesicle dry (g)	0.775±0.04	0.851±0.03	0.944±0.05	0.819±0.05
Ventral prostate (mg)	545.5±26.9	576.5±23.8	579.6±44.1	525.3±56.3

*ATR=atrazine, PND=post natal day. Data presented as Dam mean±S.E. [1]Dam-milk source.
′Body wt of animals euthanized at PND120 or PND220 only.
PND120- Dam $N=5–6$, with $n=10$ offspring per exposure group.
PND220- Dam $N=4–5$, with $n=9–10$ offspring per exposure group.
[a] Significant treatment effect by ANOVA; $p<0.05$ vs. C-C.
[b] Significant treatment effect by ANOVA; $p<0.01$ vs. C-ATR.
Analysis indicated is shown for BW as covariate.
[c] Significant treatment effect by ANOVA; $p<0.03$ vs. C-C.
[d] Significant treatment effect by ANOVA; $p<0.01$ vs. C-C.
[e] Significant treatment effect by ANOVA; $p<0.03$ vs. C-ATR.

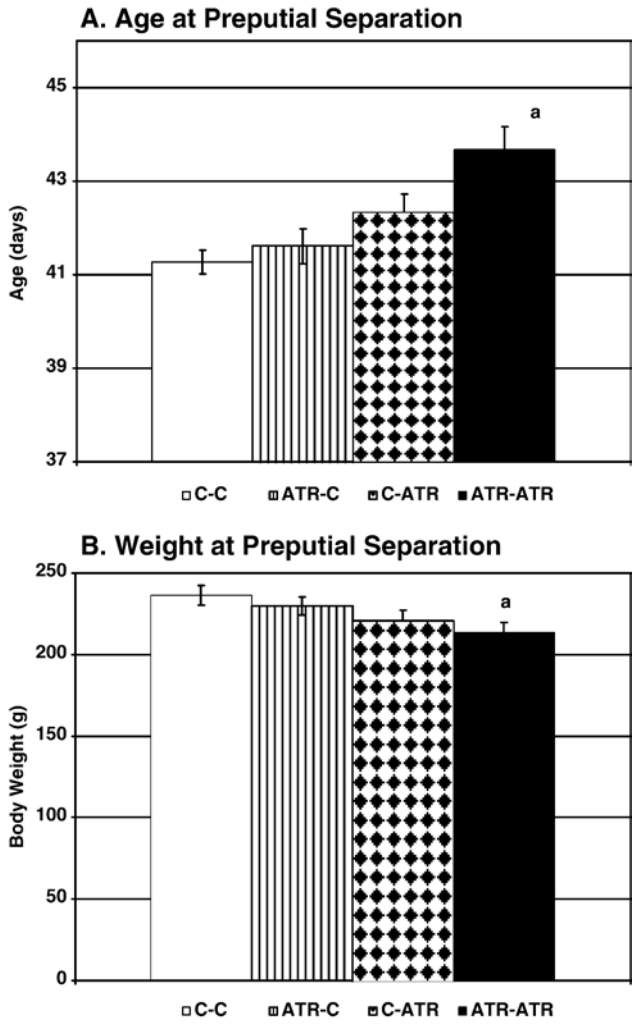

Fig. 2. Effect of gestational exposure to 100 mg/kg BW/day on age and weight at time of preputial separation (PPS). (A) Age (days) at the time of PPS. Data presented as dam mean ± S.E. Groups listed as dam-milk source. Significant treatment effect by ANOVA (LSM). [a]Different from C-C, $p<0.0001$. (B) Dam mean ± S.E. of body weight (g) at the time of PPS. [a]Different from C-C, $p<0.01$.

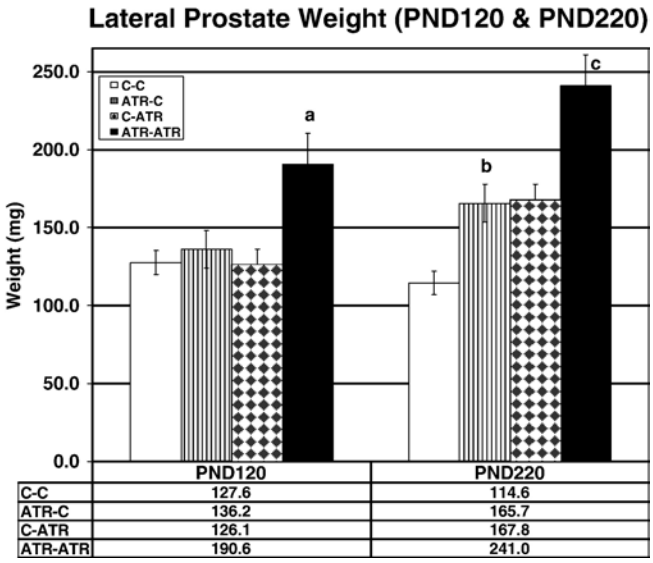

Fig. 3. Total lateral prostate weight at PNDs 120 and 220. Data presented as dam mean ± S.E (mg). Groups listed as dam-milk source. Significant treatment effect by ANOVA (LSM). [a]Different from C-C, ATR-C, and C-ATR, $p<0.001$. [b]Different from C-C $p<0.03$. [c]Different from C-C, ATR-C, and C-ATR, $p<0.03$.

those in any other group (Fig. 3). When lateral prostate weights were compared using body weight as a covariate, they were still found to be significantly different ($p<0.01$) from those of the other groups. The average ATR-ATR lateral prostate weight was 49% higher than control on PND120. At PND220, lateral prostate weights were significantly increased in animals within all ATR exposure groups compared to control animals, 45–110%. When adjusted for BW (statistical differences shown in Fig. 3), animals in ATR-C and ATR-ATR groups had significantly increased lateral prostate weights ($p<0.01$). While lateral prostate weights from the ATR-ATR males were significantly different from all other groups ($p<0.03$), weights from males in the ATR-C group were considered different from C-C and ATR-ATR ($p<0.03$). The greater average body weight in the PND220 C-ATR group reduced the apparent significance of the lateral prostate weight effect. Significant changes in organ weight were not present for other male reproductive tissues from the various ATR exposures (Table 1, whether or not body weight was a covariate).

At necropsy, seven of 10 offspring in the ATR-ATR group had macroscopic abnormalities characterized by nodular pale white foci present on the outer surface of the lateral prostates at PND120 (unilateral; three on right lateral, four on left lateral prostate, Table 2) which was statistically different from C-C and ATR-C group as both lacked macroscopic alterations. The C-ATR group had four of 10 males with macroscopic prostate alterations. All were found on the right lateral prostate. At PND220, visible abnormalities were present in all ATR exposed groups. The majority were found on the right lateral prostate. As shown, ATR-C males had 22% and C-ATR had 40% displaying macroscopic nodules. ATR-ATR had 56% with alterations which was significantly different from C-C group which had no visible foci.

Histopathology

At PND120, the distribution of inflammation in the lateral prostates of males from the C-ATR group was significantly

Table 2
Visual evidence of prostate abnormalities at necropsy

Dam-milk source	PND 120	PND220
C-C	0/10	0/8
ATR-C	0/10	2/9
C-ATR	4/10	4/10
ATR-ATR	7/10[a]	5/9[b]

Data presented as # of animals exhibiting prostate abnormalities/total # of animals per group.
PND120- Dam $N=5–6$, with $n=10$ offspring per exposure group.
PND220- Dam $N=4–5$, with $n=9–10$ offspring per exposure group.
Analysis of abnormalities—2 sided p-values.
[a] $p=0.003$ vs. C-C and ATR-C.
[b] $p=0.03$ vs. C-C.

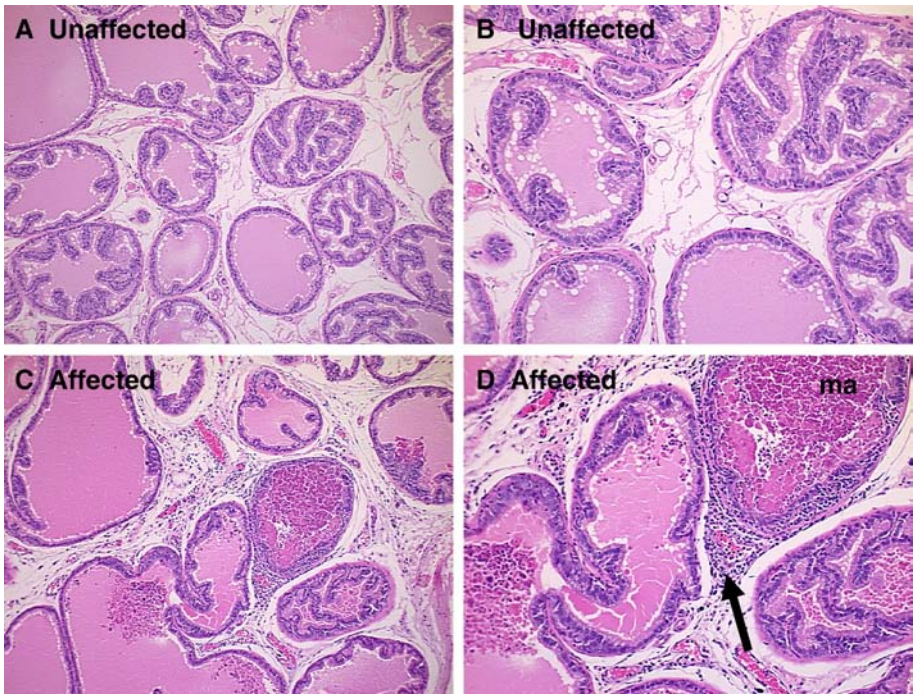

Fig. 4. Histological sections of lateral prostates at PND120. (A, B) Unaffected offspring (C-C) displaying few inflammatory cells. (C, D) Affected offspring (C-ATR) found to have statistically significant distribution of inflammation. Note the infiltration (arrow) of inflammatory cells and micro-abscess (ma) formation. A, C at 100×; B, D at 200× magnification.

increased compared to control males ($p<0.05$, Fig. 4, Table 3). The inflammation was localized to focal areas of the tissue. Affected glands had multiple acini affected with inflammation present in the interstitial space and acinus, and micro-abscesses (Fig. 4). The C-ATR and ATR-ATR glands had neutrophils as the predominant inflammatory cells. The background incidence of inflammation in the ventral prostate was fairly high in that eight of 10 control animals presented evidence of inflammation at PND120. Neutrophils were more prominent in the ventral prostate (Fig. 5) in all dose groups, and were associated with the higher overall incidence of inflammation in the ventral prostate (Table 3). The incidence of inflammation in the ventral prostate was not treatment related and presented a wider tissue distribution than the lateral prostates. The severity of inflammation in the ventral prostate was also not affected by ATR exposure.

At PND220, no significant increases in prostate inflammation were detected relative to controls (Table 4), however, the incidence, distribution, and severity of inflammation in the ventral prostate were decreased compared to PND120. The lateral prostates were not different from control. There was no increase in incidence or severity of prostate inflammation over time following ATR exposure.

Myeloperoxidase assay (MPO)

The MPO was assayed separately for PND120 and 220, and the values of the internal standards varied by less than 1%

Table 3
Prostate pathology on PND120

Group*	Incidence	Distribution (total)	Distribution (affected)	Severity (total)	Severity (affected)	Neutrophils	Total incidence inflammation
Lateral prostate inflammation							
C-C	0/10	0	0	0	0	0	8/10
ATR-C	0/10	0	0	0	0	0	8/10
C-ATR	3/10	0.5±1.0[a]	1.7±1.2	1.1±2.1	3.7±2.3	2/3	7/10
ATR-ATR	1/10	0.1±0.3	1.0	0.5±1.5	5.0	1/1	6/10
Ventral prostate inflammation							
C-C	8/10	1.1±0.07	1.4±0.5	1.2±0.9	1.5±0.7	3/8	
ATR-C	8/10	1.7±1.3	2.1±1.0	1.1±1.1	2.0±0.8	4/8	
C-ATR	7/10	1.4±1.2	2.0±0.8	1.3±0.9	1.9±0.4	2/7	
ATR-ATR	6/10	1.1±1.1	1.8±0.8	1.0±1.1	1.7±0.8	1/6	

* Group=Dam-milk source data presented as dam mean±S.E. PND120- Dam $N=5–6$, with $n=10$ offspring per exposure group.
All significant effects vs. Control. Total incidence=lateral and ventral lobes.
[a] Significant treatment effect by *t*-test; $p<0.05$.

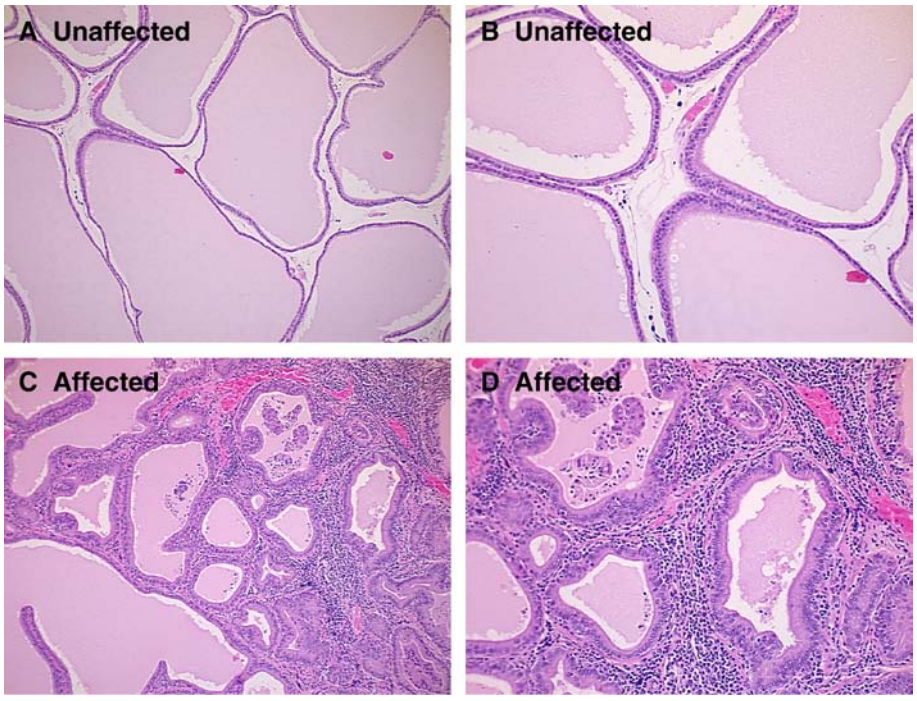

Fig. 5. Histological sections of ventral prostates at PND120. (A, B) Unaffected offspring. (C-C). (C, D) Affected offspring (ATR-C). This affected gland received a severity of inflammation grade of 3-mild numbers of inflammatory cells in acinus with some degradation of colloid. A, C at 100×; B, D at 200× magnification.

between runs. The positive and negative controls used in the assay resulted in the expected values. While total lateral prostate weights were increased at both PND120 and 220, MPO assay data showed that the inflammation in the left lateral prostates of offspring of treated dams was not different from controls at PND120 (Table 5). Even though, ATR-ATR males had 14% more MPO than control males, the difference lacked statistical significance. This small increase was consistent with the observed increase in macroscopic alterations in that group of animals. A general increase in MPO concentration was noted in the lateral prostates within a treatment group between PND120 and 220. A significant decrease was seen between MPO concentrations in the ATR-ATR group and C-C group ($p<0.002$) at PND220. ATR-C animals had MPO concentrations similar to C-C while those in C-ATR groups were lower. This is inconsistent with the observations of visible abnormalities, but could possibly be explained by the use of the left lateral prostate for MPO and not the right, in which the majority of visible abnormalities were observed at necropsy, or because neutrophils were not always the predominant inflammatory cell present. There was a correlation in very low MPO values in individual animals whose tissues had visible abnormalities, further supporting this conclusion.

Serum hormone measurements

Serum hormone concentrations of total testosterone, androstenedione, estrone, and prolactin by RIA are presented in Table 6. There were no statistically significant differences in serum hormone concentration among the groups at PND120 for

Table 4
Prostate pathology on PND220

Group*	Incidence	Distribution (total)	Distribution (affected)	Severity (total)	Severity (affected)	Neutrophils	Total incidence inflammation
Lateral prostate inflammation							
C-C	1/8	0.1±0.4	1.0	0.5±1.4	4.0	1/1	2/8
ATR-C	2/9	0.3±0.7	1.5±0.7	0.9±1.8	4.0±1.4	2/2	4/9
C-ATR	3/10	0.2±0.6	2.0	0.5±1.6	5.0	1/3	3/10
ATR-ATR	2/9	0.2±0.4	1.0	0.8±1.8	4.0	2/2	4/9
Ventral prostate inflammation							
C-C	1/8	0.1±0.4	1.0	0.1±0.4	1.0	1/1	
ATR-C	2/9	0.2±0.4	1.0	0.4±1.0	2.0±1.4	0/2	
C-ATR	1/10	0.1±0.3	1.0	0.3±0.9	3.0	1/1	
ATR-ATR	3/9	0.4±0.7	1.3±0.6	0.9±1.4	2.6±0.6	1/3	

* Group=Dam-milk source data presented as dam mean±S.E. PND220- Dam $N=4–5$, with $n=9–10$ offspring per exposure group. Total incidence=lateral and ventral lobes.

Table 5
Concentration (ng/mL) of myeloperoxidase in left lateral prostate at PNDs 120 and 220

	C-C[1]	ATR-C	C-ATR	ATR-ATR
PND120	44.5±2.75	41.3±1.83	45.3±2.50	51.9±3.61
PND220	224.1±29.1	228.7±35.6	155.3±22.8	84.7±16.2[a]

Data presented as dam mean±S.E. [1]Dam-milk source. PND120- Dam $N=5–6$, with $n=10$ offspring per exposure group. PND220- Dam $N=4–5$, with $n=9–10$ offspring per exposure group.
[a] Significant treatment effect by ANOVA; $p<0.01$ vs. C-C.

testosterone, androstenedione, estrone, and prolactin. There was a 35–40% increase in mean testosterone levels in C-ATR and ATR-ATR males when compared to control males, but these levels were within what is regarded to be a physiological range. The same males from these two groups also had slightly increased (7–10%) androstenedione levels. These increases were not statistically different. All three ATR exposed groups had non-significant increases in estrone (10–20%) and prolactin (14–31%) levels.

By PND220 (Table 6), mean testosterone and estrone levels for all animals had decreased from PND120 levels, but ATR exposed animals' hormone levels still remained higher than controls. Testosterone levels in all ATR exposed groups were 20–60% higher than control levels. ATR-C and C-ATR males had slightly increased (26–29%) androstenedione levels vs. controls. However, these effects are not thought to be ATR driven due to lack of consistent effects in ATR-ATR group. Steroid hormone ratios, particularly those controlled by aromatase and 17-β hydroxysteroid dehydrogenase, were evaluated but no statistical differences were found at either time point. Prolactin levels in all ATR-exposed animals were significantly lower than those in the C-C group (33–55%). Prolactin levels in the ATR-ATR group were less than half of that measured in controls. Although the prolactin levels were significantly different, the values reported are within normal physiological range for animals of that age, with the ATR-ATR at the low end and the C-C males at the high end of the spectrum.

Discussion

These studies demonstrated that a 5-day exposure to 100 mg ATR/kg BW/day during late gestation resulted in effects present at 7 months after the exposure period had ended in Long–Evans rat offspring. This was specifically the case for those male offspring that nursed from a dam exposed to ATR during that period of gestation. The developing lateral prostate and anterior pituitary gland appeared to be sensitive to the altered nutritional or exposure effects derived transplacentally or via nursing an ATR exposed dam. Nursing from an ATR-treated dam appeared to work in combination with *in utero* exposure to enhance delays in puberty, serum prolactin, pituitary weight, and lateral prostate weight compared to gestational exposure alone. Similar to what was observed for mammary gland (Rayner et al., 2004), continual exposure of the developing offspring to ATR is not required for altered gland development (prostate enlargement) or altered hormone status in later life. The males exposed to ATR prenatally (ATR-C group) had enlarged prostates and anterior pituitary glands at 7 months of age and lower serum prolactin levels than controls. Nursing from an ATR-exposed dam (ATR-ATR group) appeared to enhance the effects of gestational exposure resulting in prostate enlargement 3 months earlier. Nursing from an ATR-exposed dam (C-ATR group) significantly increased distribution of inflammation in the lateral prostate. The inflammation caused by ATR does not progress or become more severe over time. Many findings presented in the present study cannot be compared to other publications, as most reports of reproductive gland weights or effects in adult male LE rats ended at or before PND 120 or in late life. Therefore, it is not possible to predict from these data the full effects that perinatal ATR exposure may manifest in the adult, aged rat prostate.

Only some data from the males nursing from ATR-exposed dams in our study were consistent with the previous reports of lactational or peripubertal ATR exposures. Studies have shown that extended peripubertal ATR exposures delayed puberty (Stoker et al., 2000, Trentacoste et al., 2001) and lactational ATR exposures increased the incidence and severity of lateral prostate inflammation at PND120 (Stoker et al., 1999a). The present study also demonstrated a delay in puberty in males with combined gestational and nursing-derived exposure. The female siblings of these males exposed prenatally and lactationally and those exposed only through nursing also displayed delayed puberty. However, the females had increased weights at time of puberty (Rayner et al., 2004). It is possible that the lower body weight of the males contributed to the delayed puberty observed as suggested by Kennedy and Mitra (1963). It is also possible that early hormonal changes or early postnatal brain development (specifically in the median eminence), critical to regulating the timing of PPS, were altered by ATR exposure and led to delays. However, we did not measure hormone concentrations

Table 6
Serum hormone concentration at PND120 and PND220

	C-C[1]	ATR-C	C-ATR	ATR-ATR
PND120				
Testosterone (ng/mL)	1.00±0.17	1.04±0.24	1.41±0.29	1.35±0.31
Androstenedione (ng/mL)	0.360±0.04	0.353±0.04	0.395±0.05	0.386±0.04
Estrone (pg/mL)	43.5±3.01	52.1±4.82	50.4±4.30	47.9±3.80
Prolactin (ng/mL)	2.97±0.39	4.10±0.71	3.31±0.34	3.38±0.39
PND220				
Testosterone (ng/mL)	0.613±0.18	0.982±0.22	0.737±0.14	0.819±0.15
Androstenedione (ng/mL)	0.318±0.05	0.410±0.03	0.400±0.04	0.315±0.05
Estrone (pg/mL)	16.9±2.21	20.7±2.79	21.4±2.96	25.1±2.91
Prolactin (ng/mL)	5.72±0.86	3.81±0.38[a]	3.86±0.55[a]	2.55±0.19[b]

Data presented as dam mean±S.E. [1]Dam-milk source.
PND120- Dam $N=5–6$, with $n=10$ offspring per exposure group.
PND220- Dam $N=4–5$, with $n=9–10$ offspring per exposure group.
[a] Significant treatment effect by ANOVA; $p<0.02$ vs. C-C.
[b] Significant treatment effect by ANOVA; $p<0.01$ vs. C-C.

of the dam or offspring prior to PND120 in these animals and the brain control center(s) for PPS or vaginal opening (visible signs of puberty) are just beginning to be understood. Atrazine may affect the enzymes or local growth factors in the median eminence that regulate LHRH release (Ojeda et al., 2000), the levels of ghrelin (a negative regulator of LH secretion and a marker of energy insufficiency; Fernandez-Fernandez et al., 2005), or possibly the circulating levels of leptin in the offspring were altered due to decreased appetite and weight gain in the dams during the perinatal period. Plasma leptin levels correlate with pubertal timing in rats (Leonhardt et al., 2003). Altered maternal prolactin following ATR-exposure (Cooper et al., 2000) may influence these or other critical elements of pubertal timing in the offspring. Although we have no data to support any of these potential mechanisms of action for perinatal ATR exposure on pubertal delays, they are all consistent with known ATR modes of action (CNS and appetite-related effects).

Our data also agrees with previous reports (Laws et al., 2000; Stoker et al., 2000) that a 100 mg/kg/day dose of atrazine induces pubertal delay. These data suggest that either low levels of ATR or its metabolites may still be present in the milk, or that the milk supply or quality (nutritional impact) is compromised resulting in growth and puberty delays. Atrazine is reported to be rapidly eliminated from the body of adult non-lactating rats with 100% being recovered within 3 days (USEPA, 2002). In fact, Timchalk and colleagues (1990), report a half-life of radiolabeled ATR in the male Fischer rat to be 11 h. Current studies in our laboratory, using similar exposure conditions, have detected ATR and ATR metabolites in body fluids of dams as late as PND4, suggesting that some effects may be contributed by residual ATR metabolites (J. Rayner, personal communication). This is not to say that the nutritional impact of the milk was not altered, also. The mode and mechanism of action of atrazine on pubertal timing is unknown and deserves further investigation, focusing on exposure during the early postnatal period.

Lateral prostate inflammation was present in 120 day old control pups following suckling from dams with prenatal ATR exposure. This effect was insignificant at PND220 suggesting the inflammation may be a transient effect, or that much longer exposures to increased circulating prolactin (and potentially steroid hormones) are needed to increase the incidence and severity of the effects on the prostate. Evidence of inflammation in the lateral prostate after nursing from an ATR-exposed dam was consistent with findings by Stoker et al. (1999a). However, none of the ATR exposure paradigms caused an increased inflammation in the ventral prostate in our study (difficult to assess at PND120 due to high background inflammation) and the combined exposure did not lead to increased inflammation of either prostatic lobe evaluated. The visual lateral prostate abnormalities noted at necropsy were increased by prenatal and nursing-derived ATR exposure (ATR-ATR group; Table 2), but were located on the surface of the gland and may not have penetrated deep within the gland, as relatively small focal areas seem to be affected in the lateral prostate. The five slides per animal for pathological examination (step-sectioned through gland) covered a small portion of the entire gland and it is possible that we may have missed inflamed sites located deeper or more superficial in the gland. We also used a conservative approach in assessing "inflammation" as 2 of the 5 slides had to reveal evidence of inflammation before we scored it as a positive. With that said, many reported prostate inflammation studies score a single slide for pathology. It is possible that not having both lateral prostate glands (one used for MPO and one for pathology) available for evaluation could have increased variability resulting in discrepant MPO concentrations and pathology. Judging by the visible lesions seen in our study, the lateral lobes of the prostate were not affected equally. Furthermore, the inconsistencies of a predominance of neutrophils in the affected prostate glands (at either timepoint) suggests that the inflammation may be chronic in some animals, and not an acute response as would be suggested by polymorphonuclear cells (neutrophils) at the inflammation site. This, too, would potentially result in a discrepancy between the pathology results and MPO outcomes. Future studies should section and evaluate the entire prostate gland and not rely strictly on MPO if the response is not known to have an acute neutrophilic response.

Hormone changes are known to be influential on early prostate development. vom Saal and colleagues (1997) observed enlarged prostate glands in adult male mice exposed to estrogenic compounds (DES and bisphenol A) via late gestational exposure. They suggested that low doses of estrogenic compounds during fetal growth could permanently alter prostate development and lead to enlarged prostates later in life. The prostate was found to be sensitive to the intrauterine position of the male pups. When located between two females during gestation, the prostate size was increased from enhanced development of prostatic buds in the ventral region, presumably due to elevated serum estradiol levels (vom Saal et al., 1997). A similar phenomenon was reported in male rat fetuses that had a greater number of prostatic androgen receptors when they were located between two males in the uterus (Timms et al., 1997). In addition, the dorsolateral region of the prostate had enhanced development compared to the ventral prostate (also observed in the present study). These data suggest that estradiol increased prostatic androgen receptors during fetal development increasing the organ's sensitivity to androgen due to a permanent imprinting effect (Timms et al., 1997). Perinatal xenoestrogen exposures are thought to act through both prolactin-dependent and -independent mechanisms to alter prostate growth and chronic inflammation of the adult male rat (Gilleran et al., 2003).

The present study on early life ATR exposure evaluated circulating hormone concentrations at about 4 and 7 months of life. We detected hormone levels that were both consistent with and that differed from previous work. Stoker and colleagues (1999c) found no statistical differences in steroid hormones at PND120 when they examined male Wistar rats treated PND22–32 with pimozide, bisphenol A, or estradiol. They did find that prolactin levels in their treated animals were higher at PND29. By PND120, serum prolactin levels were not different between the groups. Males exposed to pimozide and bisphenol A also had increased lateral prostate weights at PND120. Perinatal

methoxychlor exposure was shown to increase anterior pituitary prolactin levels (but not serum prolactin levels) and lateral prostate weights at PND90 (Stoker et al., 1999b). In males exposed to ATR via lactation, Stoker and colleagues (1999a) reported significantly lower concentrations of serum prolactin in PND120 males from dams exposed to 50 mg/kg ATR co-treated with ovine prolactin, but there were no lateral prostate differences among the treatment groups, suggesting that the addition of exogenous prolactin alleviated the adverse effects of ATR on pup prostate development. Stoker and colleagues (2000) found no differences in steroid or pituitary hormones at PND120 and no differences in lateral prostate weights at PND120 in male Wistar rats exposed to varying amounts of ATR during PND23–53. Our hormonal observations began at PND120, and no statistical differences were noted at that time, although trends for increase in testosterone and prolactin were noted. However by PND220, there was a decrease in serum prolactin and coincidently, there was an increase in anterior pituitary gland weight in males born to ATR treated dams. While ATR has not been classified as an estrogen mimic, it is considered to be an endocrine disruptor and has been shown to suppress prolactin and luteinizing hormone (Cooper et al., 2000). ATR may disrupt placental and early postnatal hormones leading to permanent changes in early prostate development resulting in growth with age, as observed by the lateral prostate weight increases in the present study.

The results of the present study suggest that the developing lateral prostate gland in the Long–Evans rat is sensitive to ATR-induced changes that are transplacental or via nursing. Our data also suggest that lactational exposure to these ATR-induced changes increases lateral prostate inflammation in not only a sensitive rat strain (Wistar), but Long–Evans, also. A combination of pre- and early postnatal ATR exposures delayed puberty, enhanced lateral prostate gland weight, and decreased serum prolactin. The significance of this decrease in prolactin is not known, nor do we have information on serum prolactin patterns in aged male LE rats. However, all prolactin levels at PND220 are thought to be within normal biological range, and effects are thought to be treatment-related. The exposure parameters responsible for the pubertal outcomes are consistent with what we previously reported for the female siblings (Rayner et al., 2004). However, the males may have been more sensitive to ATR as they also exhibited slight decrements in body weight at the time of puberty that were not observed in the female pups.

In these studies, we used the rat dam as the experimental unit of analysis because the dam was given the treatment dose. At this time, we are determining how much of the actual dose and what form (parent compound or metabolites) reaches the offspring either transplacentally or via the milk. The exposure period ended 2 days before the dams gave birth and any exposure to ATR or its metabolites occurring during lactation should have been far less than the dose given to the dam as the compound is being rapidly metabolized and excreted. We hypothesize that a sufficient dose of ATR can occur via the milk following a late prenatal exposure to cause the effects seen in the male offspring in this study. The alternative hypothesis, that ATR exposure to the dams inhibited milk production or quality resulting in decreased nutritive value of the milk to the pup is also being pursued. Studies to determine if ATR changes the fetal growth and development of the prostate gland in males would be helpful in shedding light on the mechanism behind increased prostate weights in adulthood.

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