Atrazine-induced elevation or attenuation of the LH surge in the ovariectomized, estrogen-primed female rat: role of adrenal progesterone

Jerome M Goldman, Lori K Davis[†], Ashley S Murr and Ralph L Cooper

Endocrine Toxicology Branch, MD72, Toxicity Assessment Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, North Carolina 27711, USA

Correspondence should be addressed to J M Goldman; Email: goldman.jerome@epa.gov

[†]L K Davis is now at Impact Pharmaceutical Services, Research Triangle Park, North Carolina 27709, USA

Abstract

Multiple exposures to the herbicide atrazine (ATRZ) were shown to suppress the LH surge in both cycling female rats and those ovariectomized (OVX) and primed with estradiol (E₂). A single ATRZ administration was found to induce a prompt and marked increase in progesterone (P₄). As exogenous P₄ is known to have a differential effect on the LH surge depending on its temporal relationship with the surge, it was hypothesized that a single treatment in an OVX, E₂-primed rat would augment the surge, whereas several exposures would cause a decrease. Following four daily treatments with 100 mg/kg, LH surge was suppressed. In contrast, a single ATRZ exposure elevated the surge. Two treatments were without effect. The single administration caused a large increase in P₄ at 30 and 60 min that was likely attributable to adrenal secretion. Four exposures also elevated P₄ after the final treatment, although the duration of the increase was shortened. A single treatment with 0, 10, 30, and 100 mg/kg ATRZ showed similar elevations at the highest concentration in P₄, the LH peak, and area under the curve (AUC), whereas four exposures reduced the AUC. An increase at 1 h in the expression of *Kiss1* in the anteroventral periventricular nucleus suggests that this regional kisspeptin neuronal population has a role in the ATRZ augmentation of the surge. These data support the hypothesis that ATRZ-induced changes in adrenal P₄ can either augment or attenuate the surge depending on the temporal proximity of exposure to the rise in LH.

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Introduction

The herbicide atrazine (ATRZ) (chloro-s-triazine), first introduced commercially in 1958, has become one of the most heavily used pesticides in the United States. It is designated as a restricted use pesticide for agriculture and is principally employed for corn, sorghum, and sugarcane to control pre- and post-emergent broadleaf and grassy weeds. In plants, ATRZ's principal mode of action has been determined to be an inhibition of chloroplast photosynthesis (Gysin & Knuesli 1960).

A series of studies in rodents has shown that ATRZ is able to alter reproductive endocrine endpoints in both males and females. A downregulation of Leydig cell steroidogenic gene expression (Pogrmic *et al.* 2009) and reductions in testosterone concentrations have been observed both in serum and in testicular interstitial fluid following 3–4 weeks of exposure (Trentacoste *et al.* 2001, Friedmann 2002), although decreases in body weight with longer treatments were believed to contribute to these endocrine effects. In females, lifetime dietary

exposures to ATRZ were found to cause premature reproductive senescence in female Sprague Dawley rats (Wetzel *et al.* 1994). Exposure by oral gavage for 3 weeks also reported to alter ovarian function in young-adult Sprague Dawley and Long–Evans females (Cooper *et al.* 1996). Subsequent studies in the young-adult females revealed that ATRZ suppresses the amplitude of the surge of LH in the ovariectomized (OVX), estradiol (E₂)-primed female rat (Cooper *et al.* 2000, McMullin *et al.* 2004, Foradori *et al.* 2009) as well the ovulatory surge of LH in the intact proestrus female (Cooper *et al.* 2007). The ovulatory surge of LH represents the hormonal signal that triggers the final stages of ovarian follicular and oocytic maturation, and the ATRZ-induced disruption of gonadotropin secretion contributes to the altered ovarian cycling noted in the earlier studies. This attenuation in the LH surge is apparently due to an impact on the hypothalamic mechanisms underlying the secretion of GnRH and not via a direct effect on pituitary LH secretion (Cooper *et al.* 2000). However, the

entirety of the toxicity pathway involved in this effect remains unknown.

A number of additional chlorotriazine studies have shown that ATRZ or its metabolites will stimulate the secretion of both E_2 and progesterone (P_4), in addition to inducing an increase in adrenal corticosterone. The increase in E_2 was primarily reported from *in vitro* studies (Sanderson *et al.* 2000, Pogrmic-Majkic *et al.* 2010, Tinfo *et al.* 2011), although a few publications with rodents have reported either marginal effects on aromatase activity or small statistically significant elevations in circulating E_2 (Rivest *et al.* 2010, Victor-Costa *et al.* 2010). In contrast, P_4 and corticosterone have been found to rapidly increase in response to single or multiple oral exposures of ATRZ or its metabolites (Fraites *et al.* 2009, Taketa *et al.* 2011). In female rats, there is a marked elevation in both steroids within 15 min following a single exposure (Fraites *et al.* 2009, Laws *et al.* 2009). Such changes in circulating E_2 , P_4 , and corticosterone are known to feed back to the hypothalamus and pituitary and have an impact on the LH surge. In this regard, E_2 and P_4 serve complementary roles in initiating the surge, with each having a different temporal relationship with the rise in LH (e.g. Mahesh & Muldoon 1987). During the normal rat estrus cycle, circulating concentrations of E_2 rise during diestrus II and peak around mid-day on proestrus. This leads to an upregulation of the participant mechanisms of GnRH secretion, a contribution that is essential to the initiation of an LH surge. Around the time of the surge, P_4 provides a synergistic complement to E_2 , augmenting LH concentrations many-fold above levels induced by E_2 alone. However, if P_4 is elevated over a period of days preceding the surge, even for brief periods as in the case of chlorotriazine exposure, it will have a negative influence on the central mechanisms that control LH and impair the surge (e.g. Caligaris *et al.* 1971, DePaolo & Barraclough 1979).

P_4 is a secretory product of both the ovaries and the adrenals, and this study was designed to examine the temporal relationship between an ATRZ-induced increase in adrenal P_4 and the generation of the surge, using an OVX, E_2 -primed rat model. In contrast to the many studies demonstrating that 3 days of ATRZ exposure is able to impair the estrogen-induced release of LH (Cooper *et al.* 2000), it is hypothesized that a single oral exposure to ATRZ given to such females on the day of the anticipated estrogen-induced surge will promptly increase circulating P_4 and enhance the amplitude of the LH surge. In contrast, daily ATRZ treatments administered over multiple days will cause a more extended exposure to P_4 and attenuate the surge, as previously observed.

One family of neuropeptides that has emerged as a major upstream regulatory factor for GnRH secretion is the kisspeptins. Kisspeptin (Kiss1) neurons in the hypothalamic anteroventral periventricular nucleus

(AVPV) send fibers to GnRH neurons, and it has become apparent that this input serves as a powerful impetus for elicitation of the LH surge (e.g. Gottsch *et al.* 2004, Navarro *et al.* 2005). This study examined the relationship between the expression of *Kiss1* mRNA in the AVPV and the surge following 1 and 4 days of ATRZ exposure.

Materials and methods

Animals

Long-Evans hooded female rats (~60 days old) were purchased from Charles River Laboratories (Raleigh, NC, USA). Each rat was housed individually in a clear polycarbonate cage (20×25×47 cm) with heat-treated pine shavings and provided PMI LabDiet Formulab 5008 and filtered tap water *ad libitum*. The housing rooms were maintained at 20–22 °C and 45–55% relative humidity under a 14 h light:10 h darkness photoperiod (lights on 0500 h).

All animal care, handling, and treatment procedures conformed to NIH standards for laboratory animal research and were approved by the Institutional Animal Care and Use Committee at the National Health and Environmental Effects Research Laboratory.

Chemicals

ATRZ (97.1% purity) was generously provided by Syngenta Crop Protection, Inc. (Greensboro, NC, USA). Dosing solutions were prepared as suspensions in 1% methylcellulose (Sigma Chemical Co.) in reverse-osmosis purified water. The treatment regimens are described below.

Experiment 1: single ATRZ concentration

This experiment was designed to evaluate the effect of a single dosage of ATRZ administered on the day of the expected LH surge in the OVX, E_2 -primed female rat compared with multiple exposures to the herbicide. Previous experiments had demonstrated that ATRZ at 100 mg/kg was able to suppress the LH surge following three daily treatments (Cooper *et al.* 2000). Consequently, this dose was initially employed to evaluate the temporal influence of the herbicide on the LH surge in OVX, E_2 -primed female rats. This animal model, using OVX rats implanted with E_2 -containing capsules, has been shown, under controlled E_2 concentrations, to display daily LH surges over the course of 6 or 7 days (Legan & Karsch 1975, Legan *et al.* 1975).

After a period of acclimation, vaginal lavages from each female were taken daily for at least 2 weeks to assess cycling status, as described elsewhere in detail (Goldman *et al.* 2007). Only those animals having at least two consecutive 4- or 5-day estrus cycles were used in experiments. These animals were deeply anesthetized under ketamine:xylazine anesthesia (70:10 mg/kg), bilaterally OVX, and implanted subcutaneously in the right flank with a 6 mm Silastic tubing capsule (1.57 mm ID×3.18 mm OD) containing E_2 benzoate (Sigma; 4 mg/ml in sesame oil), as described previously (Goldman *et al.* 2008).

Figure 1 Study design for Experiment 1. Animals are OVX and implanted with an E₂ capsule (hatched arrow) on day 0 (d0), 3 days prior to sampling for blood by tail nick. Dosing is indicated for one, two, or four daily exposures at 1300 h by a shaded arrow, and blood samplings (1400, 1600, 1800, and 2000 h) are shown by the small black triangles. The light and dark portions of the 14 h light:10 h darkness photoperiod over the 4 days are represented by respective white and shaded blocks.

Capsules were soaked in saline prior to implantation to preclude an initial uneven rise in circulating E₂ concentrations before values fell to a relatively constant level.

One, two, or four daily treatments of 100 mg/kg ATRZ and the vehicle controls were administered at 1300 h by oral gavage (5 ml/kg body weight) prior to blood samples being taken. For animals receiving four exposures, the first day of ATRZ treatment preceded OVX by no more than 1½ h prior to surgery. This dosing time is within the critical window for the generation of the neural signals triggering the LH surge (Everett & Sawyer 1950, Greig & Weisz 1973). Three days after surgery, serial blood samples were taken for all animals at 1400, 1600, 1800, and 2000 h in order to encompass the peak in the LH surge, which typically occurs between 1600 and 1800 h. For the first three samples, ~250 µl aliquots of blood were gently expressed from a nick in a lateral tail vein into small serum separation tubes. The animals were then killed by decapitation at 2000 h and trunk blood collected. This experimental paradigm is shown in Fig. 1. After each collection, blood was centrifuged at 1160 g (4 °C) for 30 min and serum collected and frozen (–60 °C) in siliconized centrifuge tubes for later analysis of serum LH.

In order to assess ATRZ-related elevations in P₄ that are present prior to the 1400 h sampling, a number of additional females were killed 30 and 60 min after a single or the last of four daily treatments. Trunk blood was collected and serum separated and frozen for P₄ immunoassay.

Experiment 2: dose–response assessments

In order to determine whether the effects of ATRZ on the LH surge are dose responsive in nature, OVX, E₂-primed animals were gavaged daily with 0, 10, 30, or 100 mg/kg ATRZ for 1 or 4 days. The surgical procedure, dosing regimen, and serial blood sampling for LH were as described for Experiment 1. Additional females from each 1- and 4-day group were killed by decapitation at 1 h after dosing for determinations of serum P₄.

Experiment 3: Kiss1 genetic expression

For the genetic expression of the kisspeptin (*Kiss1*) gene, additional OVX, E₂-primed Long–Evans hooded females were gavaged daily (1300 h) as before for 1 or 4 days with 0 or 100 mg/kg ATRZ and killed by decapitation at 1400 h. Brains were promptly removed, frozen, and sectioned (800 µm). AVPV punches were taken with a previously chilled 18-gauge stainless steel tube (1 mm diameter), expelled, and immediately snap frozen using dry ice. Brain punches from Long–Evans adult females (pooled female AVPVs) and males (pooled male cerebellar tissue) were used as positive and negative controls respectively.

RNA was extracted in TriReagent (Molecular Research Ctr, Cincinnati, OH, USA) following the manufacturer's instructions. RNA (25 ng/µl) was reverse transcribed using a qScript cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA). In addition, a water sample was taken through the cDNA synthesis reaction to test for non-specific amplification and

Figure 2 The effect of 0 or 100 mg/kg ATRZ on serum concentrations of P₄ (ng/ml ± S.E.M. at 30 min and 1 h after a single or fourth daily treatment). Numbers listed above each column indicate group sizes. ***P* < 0.01 for comparisons to 0 mg/kg groups.

reagent contamination. *Kiss1* primer (accession no. Rn00710914_m1) along with a β -actin housekeeping primer (Rn00667869_m1) for normalization were obtained from Applied Biosystems. Quantitation by RT-PCR was performed using TaqMan Gene Expression Assays from Applied Biosystems. 20 $\times$ primer/probe mixture and 2 $\times$ Taqman Mastermix were combined with molecular grade water and the cDNA. Each reaction was 10 μ l in a 384-well PCR plate (Applied Biosystems). In addition to cDNA samples, water was added as 'no template controls' to duplicate wells for each gene. Each plate was run on the Applied Biosystems 7900HT real-time PCR machine in the sequence 95 $^{\circ}$ C for 10 min followed by 40 cycles at 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min.

Hormone assays

In both experiments, sera from all time points were analyzed for LH to determine dose-related effects on the surge peak as well as the area under the curve (AUC). Serum concentrations of E_2 , P_4 , and LH were determined by RIA. E_2 and P_4 Coat-a-Count RIA kits were purchased from Siemens Healthcare Diagnostics (Deerfield, IL, USA) and run according to the manufacturer's protocols. Analytical sensitivities were 8 pg/ml and 0.02 ng/ml respectively for the E_2 and P_4 assays. Steroid

intra- and inter-assay coefficients of variation (CV) were <10%. The LH RIA was performed using materials provided by Dr A Parlow from the National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Torrance, CA, USA). Individual tracers were radiolabeled with 125 I (PerkinElmer NEN Radiochemicals, Waltham, MA, USA) using chloramine-T and labeled hormone was separated from free iodide by gel filtration chromatography. Iodination parameters, reference, and antibody dilutions were carried out according to recommendations accompanying each kit, and all RIAs used goat anti-rabbit gamma-globulin (Calbiochem/EMD Chemicals, Gibbstown, NJ, USA) as secondary antibody. A 24-h co-incubation (4 $^{\circ}$ C) of sample and primary antibody prior to the addition of labeled hormone for an additional 24 h increased the assay sensitivity to 0.11 ng/ml. All samples were run in duplicate, and the intra- and inter-assay CV were <12%.

Statistical analysis

Serial LH samples for both Experiments 1 and 2 were analyzed by a repeated measures ANOVA (SAS 9.1, Cary, NC, USA). Because the time of appearance of a surge peak can vary among animals (typically, as previously indicated, at the 1600

Figure 3 Effects of oral administration of 0 or 100 mg/kg ATRZ on the LH surge (ng/ml $\pm$ S.E.M.) following 1 (A), 2 (B), or 4 (C) daily exposures in OVX, E_2 -primed females. The left panels show plots of samples taken at 1400, 1600, 1800, and 2000 h. Values for individual animals within a dose group were shifted along the horizontal axis for a correspondence to the presence of the LH peak. The panels at right represent ATRZ effects on areas under the curve (AUC) for the one, two, and four daily treatments. The percentages of females in each group exhibiting a surge were 0 mg/kg – 100%, 100 mg/kg – 91% (one exposure); 0 mg/kg – 100%, 100 mg/kg – 88% (two exposures); and 0 mg/kg – 100%, 100 mg/kg – 80% (four exposures). For each of these days, the difference in percentage from controls was not statistically significant. Group sizes for those females with a surge are shown in parentheses. * P < 0.05 for comparisons to 0 mg/kg controls at that sampling time.

or 1800 h sampling times), the four LH values for each animal were shifted as a group along the horizontal axis so that analyses could be performed on the peaks (or highest LH concentrations), along with times designated as -6, -4, -2, +2, +4, and +6 h from the peak. Common baseline concentrations were determined for each treatment group, and animals that failed to show at least a twofold peak elevation above baseline were eliminated from analyses of the surge. E_2 and P_4 concentrations in Experiment 1 were analyzed by Student's *t*-test, whereas those in Experiment 2 were assessed by the Kruskal–Wallis procedure followed by Dunn's multiple comparison test. The AUCs for the sampling times were determined using MedCalc Software (Mariakerke, Belgium) and then analyzed by *t*-test (Experiment 1) or ANOVA with Dunnett's test (Experiment 2). For the 4-day treatments in Experiment 2, differences among dose groups in the distribution of AUCs along the range of obtained values were evaluated by χ^2 analysis. Statistical analyses of PCR data used ΔCq values, and graphed results indicate fold changes ($2^{-\Delta Cq}$) from controls (Livak & Schmittgen 2001). Statistical significance for all analyses was set at $P \leq 0.05$.

Results

Experiment 1

Figure 2 shows the effect of 100 mg/kg ATRZ on serum P_4 concentrations at 30 min and 1 h following one and four daily exposures. For the one-time exposure, there was a prompt eightfold elevation over controls that persisted over the course of 1 h. At 4 days, the concentrations, while markedly elevated at 30 min, decreased to near control levels by 1 h. No differences between one and four daily treatments with ATRZ were present in circulating E_2 concentrations from E_2 implants (1 day – 0 mg/kg

61.5 ± 3.8 pg/ml; 1 day – 100 mg/kg 64.9 ± 7.8 ; 4 days – 0 mg/kg 61.2 ± 5.6 ; 4 days – 100 mg/kg 72.7 ± 5.3).

In response to a single daily ATRZ treatment, there were significant elevations in LH present at the surge peak (Fig. 3A). When calculated as AUC, the effect on LH was statistically significant ($P \leq 0.05$) at 181% of controls. Following 2 days of dosing, a statistical difference at the peak was no longer present (Fig. 3B), while the AUC, although not statistically significant, had fallen to 62% of controls, a marked drop from a single day of treatment. Serial samples taken after 4 days of treatment showed a significant decline in LH at the peak ($P \leq 0.05$) compared with the vehicle controls (Fig. 3C). The decrease in AUC was statistically significant ($P \leq 0.05$) at 54% of controls.

Experiment 2

Using the same paradigm employed in Experiment 1, dose–response assessments of P_4 and E_2 were conducted at 1 h following one and four daily exposures of ATRZ (Fig. 4). Following a single daily exposure, there was a dose-related increase in P_4 , with concentrations in the 30 and 100 mg/kg group statistically significant at $P < 0.01$. No differences in circulating E_2 were present across the dose range. By the fourth day of dosing, no differences in serum P_4 were present, with the lowered concentrations in the 100 mg/kg group consistent with effects seen in Experiment 1 (Fig. 2). Levels of E_2 were again comparable across dose groups.

For the single exposure to 100 mg/kg, there was a significant increase at the LH peak along with post-peak elevations at 2 and 4 h (Fig. 5A). As in Experiment 1, the AUC was also increased at this dose (Fig. 5B).

Figure 4 Serum concentrations ($\pm$ S.E.M.) at 1 h of P_4 (A and C) and E_2 (B and D) after 1 day or the last day of four daily treatments (1300 h) with 0, 10, 30, or 100 mg/kg ATRZ. Group sizes are shown at the base of the P_4 columns. ** $P < 0.01$ for comparison to 0 mg/kg controls.

Figure 5 LH surge (ng/ml $\pm$ S.E.M.) following a single oral ATRZ dose of 0, 10, 30, or 100 mg/kg in OVX, E_2 -primed females (A). Values for individual animals within a treatment group were shifted along the horizontal axis according to the presence of the LH peak. The percentages of females in each group exhibiting a surge were 0 mg/kg – 93%, 10 mg/kg – 87%, 30 mg/kg – 100%, and 100 mg/kg – 100%. The lower panel (B) indicates the AUC for these dose groups, with group sizes located at the base of each column. * $P < 0.05$; ** $P < 0.01$ for comparisons to 0 mg/kg controls at the corresponding time relative to the LH peak or to the control AUC.

However, even though P_4 was found to be elevated at 30 mg/kg, this dose did not have a corresponding effect on the surge. Following the four daily exposures, the apparent decline in the LH surge (Fig. 6A) and AUC (Fig. 6B) from control levels did not reach statistical significance.

In response to four daily ATRZ treatments, the lack of a significant reduction on the LH AUC in the 100 mg/kg group was investigated further by grouping the distribution of the AUC concentrations into segments along the range of obtained values (Fig. 7). It is evident that a few animals in this dose group did exhibit a sizable effect on LH even though 62.5% of females had AUCs below 10 ng/ml, masking an overall decrease. In fact, five of the ten animals in this segment had values of 5 ng/ml or less. This is in contrast to control animals that showed 61.5% of AUCs falling between 10 and 20 ng/ml. A χ^2 analysis of the groups resulted in a probability of 0.057, which

although marginally beyond the 0.05 cutoff, is strongly suggestive of an effect and would be in agreement with the suppression reported in Experiment 1.

Experiment 3

A single exposure to 100 mg/kg ATRZ caused a 2.25-fold increase ($P < 0.05$) at 1 h in the expression of *Kiss1* (Fig. 8), whereas there was no difference from controls 1 h after four daily treatments at this dose. Consistent with data shown in Figs 1 and 3, 100 mg/kg caused a prompt and marked elevation in circulating P_4 that fell to near control levels on the fourth day of treatment. Serum concentrations of E_2 from implanted capsules were also comparable across day and dose and were consistent with levels presented for Experiments 1 and 2.

Discussion

A number of previous studies (Cooper *et al.* 2000, McMullin *et al.* 2004, Foradori *et al.* 2009) demonstrated that daily ATRZ treatments administered over 3–5 consecutive days in steroid-primed, OVX rats caused a significant attenuation in the LH surge. However, this is the first report to show that a single dose of ATRZ will increase the amount of LH released if a female is exposed in the hours immediately preceding the expected rise in LH. This augmentation is in agreement with the synergistic relationship between E_2 and P_4 at this time and demonstrates that the increase in circulating P_4 in response to an ATRZ-induced activation of the adrenal axis has a clear impact on the central regulation of LH. As E_2 concentrations are comparable across the dose groups, there does not appear to be an effect of treatment on E_2 clearance or metabolism.

In the cycling rat, there are multiple sites of P_4 production independent of the ovaries. Adrenal secretion occurs in concentrations that are typically comparable to ovarian release (Fajer *et al.* 1971, Shaikh & Shaikh 1975). There is also evidence that local P_4 synthesis is present in hypothalamic astrocytes and has been shown to occur in response to estrogen feedback (Micevych *et al.* 2003), linking this effect to generation of the LH surge. However, under the present paradigm, the large and prompt ATRZ-induced elevation in circulating P_4 implicates adrenal P_4 as a principal driving factor in the LH results observed. It then becomes clear that in a cycling female, the herbicide is able to affect P_4 secretion from both ovaries and adrenals. By themselves, the ovaries will secrete significant amounts of P_4 in response to ATRZ. It had previously been shown that cultures of granulosa cells treated with ATRZ *in vitro* responded with an increase in P_4 (Tinfo *et al.* 2011). In addition, data from our laboratory (J M Goldman, L K Davis and A S Murr, unpublished observations) have demonstrated that 4 days of ATRZ exposure to immature female rats treated with pregnant mare serum gonadotropin caused

Figure 6 LH surge (ng/ml $\pm$ S.E.M.) following four daily oral treatments with 0, 10, 30, or 100 mg/kg in OVX, E₂-primed females (A). Values for individual animals within a treatment group were shifted along the horizontal axis according to the presence of the LH peak. The percentages of females in each group exhibiting a surge were 0 mg/kg – 88%, 10 mg/kg – 100%, 30 mg/kg – 100%, and 100 mg/kg – 93%. The lower panel (B) indicates the AUC for these dose groups, with group sizes located at the base of each column.

marked dose-related elevations in P₄ from excised pre-ovulatory follicles maintained in culture.

The results reported by Fraites *et al.* (2009) showed that just 15 min after a single oral 75 mg/kg exposure in intact rats, ATRZ was able to induce a rapid spike in P₄ to $\sim$ 19 ng/ml, a concentration that was greater than fourfold over controls. At 15 min after the last of four daily ATRZ doses, Fraites *et al.* found that the mean P₄ concentration was 7 ng/ml, markedly lower than the 1-day value, suggesting a decline in the P₄ response with multiple exposures. Although the present data did not show a significant decline in the P₄ response to 100 mg/kg at 30 min between the one and four daily exposures, there was an evident attenuation between them at 1 h, with values for the 4-day group at this dose approximating control levels in all experiments.

The dramatic decline in P₄ between 30 min and 1 h for the 4-day treatment may be attributable to a combined effect of the decrease with multiple ATRZ exposures in the duration of adrenal P₄ secretion coupled with a rapid

clearance, as the mean half-life for P₄ in OVX rats has been reported to be a bit <3 min (Pepe & Rothchild 1973). Even though this treatment-induced elevation in P₄ is present over an abbreviated interval of time, P₄ administration under E₂ priming has been shown to cause a large elevation at 30 min in hypothalamic protein kinase C (PKC) activity (Balasubramanian *et al.* 2008). This effect is initiated at the plasma membrane and is non-genomic. Such an increase in PKC, moreover, was able to induce a rapid rise in secretion from immortalized GnRH neurons in culture that was followed at 16 h by a decline in GnRH mRNA (Wetsel *et al.* 1993).

The biphasic influence of P₄ requires the participation of E₂ at both the hypothalamic and pituitary levels. Rising E₂ concentrations during the cycle, or in response to an E₂ prime in OVX females, induce an increase in the number of P₄ receptors at both sites (Roselli & Snipes 1983, Camp & Barraclough 1985, Turgeon *et al.* 1999), augmenting the influence of elevated P₄ just prior to the surge. In the days subsequent to a short-term P₄ treatment, or in response to continued exposure, P₄ will then downregulate its own receptors, leading to an attenuation of the LH surge (Barraclough *et al.* 1986, Turgeon & Waring 2000). In fact, in cycling animals, it is this post-ovulatory rise in luteal P₄ (present for a short time during the typical rodent cycle or for a more extended period during pregnancy) that prevents the

Figure 7 Areas under the curve for the four daily treatments separated into the number of animals within each treatment group falling within segments along the range of obtained AUC values. A χ^2 analysis indicated a probability for a significant shift along that range of $P=0.057$.

Figure 8 The expression of AVPV *Kiss1* ($\pm$ S.E.M.) 1 h after a single-day ATRZ exposure (100 mg/kg) or the last day of four daily treatments at this dose. Results are normalized relative to controls as described in Livak & Schmittgen (2001). Serum concentrations of P₄ (ng/ml) and E₂ (pg/ml) are located above each column, and group sizes are indicated at the column base. * $P < 0.05$ compared to controls for that day.

prompt re-appearance of the LH surge (Banks & Freeman 1978), something that is seen daily in OVX rats exposed to E₂ only. We are not aware of any study evaluating the effect of ATRZ on hypothalamic or pituitary P₄ receptors. However, it is of interest that Tennant *et al.* (1994) reported that 2-day treatments with ATRZ, simazine, or the common metabolite diaminochlorotriazine significantly diminished P₄ receptor binding capacity in cytosol fractions prepared from uteri of OVX rats pretreated subcutaneously with 1 μ g E₂.

Using OVX rats, there are dose-related differences among previously published studies and the present one in the effectiveness of multiple ATRZ exposures on the LH surge. However, the significant LH suppression by lower dosages found by McMullin *et al.* (2004) and Foradori *et al.* (2009) may well be attributable to the fact that these studies employed priming with both E₂ and P₄. This injection of P₄ would then add to the succession of ATRZ-induced P₄ elevations, resulting in a lowering of the smallest effective dosage.

In cycling females, an ATRZ-induced elevation in P₄ would likely have an impact on the cycle, at least in the short term. Although a single high dose of ATRZ given on the day of proestrus did not attenuate the LH surge and affect ovulation, multiple exposures beginning on diestrus day 1 were able to block the appearance of vaginal proestrus and suppress ovulation (Cooper *et al.* 2000). Moreover, in rats treated daily for 4 weeks with 40 mg/kg and above (Eldridge *et al.* 1999), there was a statistically significant increase in the number of days spent in diestrus, an effect consistent with the response to a previous elevation in circulating levels of P₄.

In women, the contraceptive effects of exogenous sex steroids have been known for years, and a number of newer progestin-only oral contraceptives have been described to impact both the hypothalamus and pituitary (e.g. Faundes *et al.* 1991, Couzinnet *et al.* 1999).

However, it is still an open question whether ATRZ, via an effect on P₄, is able to adversely affect a woman's cycle. More recently, a preliminary epidemiological report indicated menstrual irregularities in women within a geographic region of extensive ATRZ usage compared with an area where the usage is much more limited (Cragin *et al.* 2011). The follicular phase was found to be lengthened, although there did not appear to be an association during this phase with urinary levels of the P₄ metabolite pregnanediol 3-glucuronide (Pd3G). In contrast, luteal phase Pd3G was reduced, suggesting that long-term ATRZ exposures resulted in insufficient P₄ secretion during this portion of the cycle. Such a decrease, at least outwardly, seems consistent with the previously indicated attenuation in P₄ following multiple days of ATRZ treatment reported by Fraites *et al.* (2009) and with the current differences in P₄ at 1 h (in all of the present Experiments) between single and four daily exposures in response to the 100 mg/kg treatment.

The elevation in expression of the *Kiss1* gene suggests that the increase in the surge at 1 day in response to ATRZ involves an impact of kisspeptin on GnRH secretion. Kisspeptin neurons are known to possess both E₂ and P₄ receptors (Clarkson *et al.* 2008, Clarke 2011), and AVPV membrane P₄ signaling molecules are believed to mediate rapid non-genomic responses to E₂ + P₄ (Intlekofer & Petersen 2011). The lack of a change in expression following four daily ATRZ exposures also suggests a possible receptor downregulation, along with an involvement of other neuroendocrine factors influencing the LH AUC at this time.

In response to ATRZ, there is an increase in circulating levels of ACTH, which consequently triggers a marked secretion of corticosterone from the adrenals (Laws *et al.* 2009). Previous studies have indicated that elevated corticosterone concentrations could suppress the surge under a variety of conditions (Kam *et al.* 2000,

Fraites *et al.* 2009, Laws *et al.* 2009). However, this does not appear likely for a single ATRZ exposure. Employing the same OVX, E₂-primed model in a study of the pesticide chlordimeform, our laboratory demonstrated that the administration of a single dose of corticosterone in the hours prior to the surge did not alter LH secretion (Goldman *et al.* 1991). The resulting serum concentrations of corticosterone in that study approximated those reported by Fraites *et al.* (2009) following a single 75 mg/kg ATRZ treatment. These findings would argue against the possibility that a single morning ATRZ-stimulated rise in corticosterone would modify the afternoon surge in this model. However, if the same dose of corticosterone was given on the day prior to the expected LH surge, there was a significant suppression in the mean peak concentration (Goldman *et al.* 1991). Thus, combined with the present P₄ data, these observations indicate that the effect of repeated ATRZ treatments may well reflect a collective action of both these adrenocortical steroids.

In summary, the results of this study demonstrate for the first time that there is a bidirectional shift in the effect of ATRZ on the LH surge, which is consistent with the temporal influence of a treatment-related elevation in P₄. In helping to define a mode of action, these observations also suggest that the activation of the hypothalamic–pituitary–adrenal axis plays a significant role in the toxicity pathway associated with the ATRZ-induced disruption in LH regulation. The extent to which the observations in this acute study are pertinent to the interference of pituitary–ovarian function following a chronic exposure, however, remains to be determined.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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