Developmental Programming: Prenatal and Postnatal Androgen Antagonist and Insulin Sensitizer Interventions Prevent Advancement of Puberty and Improve LH Surge Dynamics in Prenatal Testosterone-Treated Sheep

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Prenatal T excess induces maternal hyperinsulinemia, early puberty, and reproductive/metabolic defects in the female similar to those seen in women with polycystic ovary syndrome. This study addressed the organizational/activational role of androgens and insulin in programming pubertal advancement and periovulatory LH surge defects. Treatment groups included the following: 1) control; 2) prenatal T; 3) prenatal T plus prenatal androgen antagonist, flutamide; 4) prenatal T plus prenatal insulin sensitizer, rosiglitazone; 5) prenatal T and postnatal flutamide; 6) prenatal T and postnatal rosiglitazone; and 7) prenatal T and postnatal metformin. Prenatal treatments spanned 30–90 days of gestation and postnatal treatments began at approximately 8 weeks of age and continued throughout. Blood samples were taken twice weekly, beginning at approximately 12 weeks of age to time puberty. Two-hour samples after the synchronization with prostaglandin F2α were taken for 120 hours to characterize LH surge dynamics at 7 and 19 months of age. Prenatal T females entered puberty earlier than controls, and all interventions prevented this advancement. Prenatal T reduced the percentage of animals having LH surge, and females that presented LH surge exhibited delayed timing and dampened amplitude of the LH surge. Prenatal androgen antagonist, but not other interventions, restored LH surges without normalizing the timing of the surge. Normalization of pubertal timing with prenatal/postnatal androgen antagonist and insulin sensitizer interventions suggests that pubertal advancement is programmed by androgenic actions of T involving insulin as a mediary. Restoration of LH surges by cotreatment with androgen antagonist supports androgenic programming at the organizational level. (Endocrinology 156: 2678–2692, 2015)

Approximately 60 million to 80 million people globally experience difficulty conceiving (1). Among the infertility disorders, polycystic ovary syndrome (PCOS) is one of the most common, affecting approximately 4 million US women and more than 100 million women worldwide (2–5). Women with PCOS are oligo-/anovulatory and functionally hyperandrogenic and have multifollicular ovarian morphology, LH excess, and neuroendocrine deficiencies manifested as reduced sensitivity to steroid feedback (2–6). Women with PCOS are at risk for ovarian hyperstimulation and pregnancies with multiple fetuses (7, 8) and are more likely to develop gestational diabetes (9) and preeclampsia (10). Approximately 70% of these women manifest insulin resistance (4). An increased risk of cardiovascular disease, dyslipidemia, hypertension, diabetes mellitus, and endometrial cancer in PCOS (11) has

Abbreviations: C, control; C*, C and CR groups combined into a single group; C†, control groups into a generation of a combined control group; C+F, control plus flutamide; C+M, control plus metformin; CR, prenatal rosiglitazone treated; C+R, control plus rosiglitazone; CV, coefficient of variation; E2, estradiol; P4, progesterone; PCOS, polycystic ovary syndrome; PGF2α, prostaglandin F2α; QC, quality control; T, prenatal T treated; TF, prenatal T plus prenatal flutamide treated; T+F, T plus flutamide; T+M, T plus metformin; TR, prenatal T plus prenatal rosiglitazone treated; T+R, T plus rosiglitazone.
led to a unique clinical perspective, one that not only recognizes the need for addressing the issue of infertility but also emphasizes the long-term goals of preventing diabetes mellitus, heart disease, cancer, and perhaps most importantly, transgenerational transfer of unwanted traits to offspring. There is a great need for developing approaches to overcome infertility to avoid the negative effects of infertility on the quality of life.

The etiology of PCOS is unknown. Although evidence points to the heritability of PCOS traits (12), increasing evidence suggests many adult defects may originate from abnormal programming of organ differentiation during early development (13). Many believe that androgen excess early in life may provide a hormonal insult, resulting in manifestation of PCOS phenotype in adulthood (14–16). In support of this evidence, polycystic ovarian phenotype is highly associated with conditions in which the fetus has been exposed to higher concentrations of sex steroids before birth. For instance, women with classical 21-hydroxylase deficiency mimic PCOS and exhibit anovulation, ovarian hyperandrogenism, and LH hypersecretion (17). Prenatal T excess in animal models recapitulates the characteristics of women with PCOS (18–20). Gestational T excess advances neuroendocrine puberty and leads to oligoanovulation, functional hyperandrogenism, multifollicular ovarian phenotype, and insulin resistance in precocial models such as sheep and monkeys (14, 18) and altricial rodent models (19). At the maternal level, gestational T treatment, in addition to increasing androgens, perturbs maternal insulin-glucose homeostasis manifested as hyperinsulinemia in sheep (21) and hyperglycemia in monkeys (22). The relative contribution of imposed hyperandrogenic status from administered T and the resulting maternal hyperinsulinemia in programming the development of the PCOS phenotype in prenatal T-treated models is unclear. In addition, the involvement of postnatal functional hyperandrogenism and insulin resistance in maintaining and/or amplifying the severity of the phenotype also remains to be teased apart.

Animal models of PCOS provide a unique resource for dissecting the contribution of androgen and insulin in the programming of PCOS phenotype, developing intervention strategies for preventing the development of the phenotype, and/or treating to ameliorate the severity and progression of the pathological phenotype and hence of high translational relevance. Androgen antagonists and insulin sensitizers have been used as treatment strategies in women with PCOS (23, 24). Our earlier studies examining the impact of prenatal and postnatal intervention with androgen antagonist and insulin sensitizers focused on neuroendocrine anomalies, and found the following: 1) prenatal androgens program sensitivity to the negative feedback actions of estradiol (E2) and the timing of neuroendocrine puberty, evidenced by an increase in LH in the ovariectomized, E2-replaced model (25), and 2) the prevailing functional hyperandrogenism and insulin resistance of ovary-intact females contribute to the dampening of the LH surges seen in response to the E2-positive feedback challenge (26).

Because comparative studies with prenatal T and DHT-treated sheep pointed to the androgenic organization of E2-negative feedback response and estrogenic organization of E2-positive feedback response (27, 28), we tested the following hypotheses in this study: 1) prenatal, but not postnatal intervention with an androgen antagonist or insulin sensitizer, would prevent the pubertal advancement in prenatal T-treated sheep as determined on the basis of the initiation of progestogenic cycles; 2) neither prenatal nor postnatal interventions would restore LH surges in prenatal T-treated sheep; and 3) postnatal intervention with an insulin sensitizer or androgen antagonist would increase the amplitude of LH surges without affecting the timing of the surge.

Materials and Methods

Animals and gestational treatments

The Institutional Animal Care and Use Committee of the University of Michigan approved all procedures used in this study. They are consistent with National Research Council’s Guide for the Care and Use of Laboratory Animals. The study involving multiparous Suffolk breed of sheep was conducted at the University of Michigan Research Facility (Ann Arbor, Michigan; 42°18’N). Details of breeding and feeding regimen have been described earlier (29). Once mated, the females were randomly assigned to treatment groups after blocking for body condition and weight.

Figure 1 summarizes the various treatment groups used and the sequence of the studies performed. For addressing prenatal intervention strategies the following treatment groups were studied: control (C; n = 6); prenatal T treated (T; n = 6); prenatal T plus prenatal flutamide, an androgen antagonist, treated (TF; n = 10); prenatal T plus prenatal rosiglitazone, an insulin sensitizer, treated (TR; n = 7); and prenatal rosiglitazone treated (CR; n = 7). A prenatal flutamide-only group was not included because no differences were found in cycle attributes between control and flutamide-treated groups in previous studies. Postnatal treatment groups included the following: 1) control (same as above); 2) control plus flutamide (C + F; n = 5); 3) control plus rosiglitazone (C + R; n = 7); 4) control plus metformin (C + M; n = 5); 5) T (same as above); 6) T plus flutamide (T + F; n = 8); 6) T plus rosiglitazone (T + R; n = 8); and 7) T plus metformin (T + M; n = 5). Details of prenatal T and flutamide treatments and their impact on neuroendocrine puberty and E2-positive feedback have been previously published (25, 26).

For generating prenatal T-treated females, pregnant sheep were administered 100 mg of T propionate suspended in 2 mL of
corn oil (1.2 mg/kg; Sigma Chemical Co, St Louis, MO) im twice a week from day 30 to day 90 of gestation. This mode of T treatment produces circulating concentrations of T in pregnant sheep and umbilical arterial blood comparable with those seen in intact adult males and 60-day-old male fetuses, respectively (30). The generation of the TF animals involved a cotreatment of T with flutamide (Sigma-Aldrich; 15 mg/kg/d, oral). The generation of the TR animals involved cotreatment of T with rosiglitazone (Avandia; GlaxoSmithKline; 8 mg/kg/d, oral). The generation of the C and T animals involved oral treatment with metformin hydrochloride (TEVA Pharmaceutical Ind Ltd; 7.1 mg/kg, oral), another insulin sensitizer. Only one female offspring was used from each ewe when twins or triplets were born.

Postnatal treatments were initiated at 8 weeks of age (puberty in sheep occurs at ~28 wk of age). Postnatal androgen antagonist treatment involved daily oral administration of flutamide as packed capsules (15 mg/kg/d) with the dose adjusted on the basis of weekly body weight measures. Postnatal insulin sensitizer treatments involved an oral daily administration of rosiglitazone (8 mg, ~0.11 mg/kg) or metformin (500 mg, 7.1 mg/kg). The insulin sensitizer doses used are comparable with that given to women with PCOS (31, 32). In earlier studies, rosiglitazone treatment beginning in postpubertal life prevented the deterioration of reproductive cycles in prenatal T-treated females (33). Similar studies have not been undertaken with metformin.

At the time of delivery, birth weight and anogenital distance of offspring were recorded. Twice-weekly blood samples were taken beginning approximately 12 weeks of age from all females to monitor changes in progesterone (P4) and time puberty. For studying the impact of intervention on generation of periovulatory LH surges, all animals were synchronized by administering two injections of prostaglandin F2\alpha (PGF2\alpha; 5 mg/mL; Lutalyse; Pfizer Animal Health) 11 days apart. Animals were studied once during the first and once during the second breeding season (7 and 19 mo of age; Figure 1) to assess whether the interventions prevented progressive deterioration. Blood samples were procured beginning 2 hours before the second PGF2\alpha injection and

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**Figure 1.** Schematic showing the treatment groups used in the study (A) and the sequence of studies undertaken (B).
Plasma P4 concentrations were measured in twice-weekly sam-
plings. QC pools averaged 14.7%, 5.7%, 5.2%, and 4.2%, respectively. The interassay CV for the same
products was 6.1%, and 5.3%, respectively. Plasma concentrations were measured in twice-weekly sam-
plings using a solid-phase RIA kit (Coat-A-Count P; Diagnostic Products Corp) as described earlier (35). All samples were as-
sayed in duplicate 100-μL aliquots. The assay sensitivity was
0.007 ± 0.001 ng/mL (n = 45 assays; mean ± SEM) and
traassay CVs, based on the two QC pools measuring 1.6 ± 0.03,
13.4 ± 0.2, were 6.6% and 6.7%, respectively, and the interas-
say CVs were 10.5% and 9.8%, respectively.

Statistical analysis

Birth weight and anogenital distance

For determining differences in birth weight and anogenital
distance, the C and CR groups were combined into a single group
(C^a, n = 30) due to the lack of differences between them. Dif-
fences in birth weight and anogenital distance among the pre-
natal groups (C^a, T, TF, and TR) were assessed using a one-way
ANOVA followed by a Dunnett’s post hoc test.

Puberty and body weight at puberty

Puberty was defined as the first day of twice-weekly samples
that reached a P4 value greater than 0.5 ng/mL. The lack of
differences among the control groups (C, CR, C+F, C+R, and
C+M) in the time of puberty and body weight at puberty allowed
the generation of a combined control group (C; n = 31). The differences in the time of puberty and body weight at puberty
among the prenatal groups (C, T, TF, and TR) and the postnatal
groups (C, T+F, T+R, and T+M) were tested using an ANOVA
followed by a Dunnett’s post hoc test.

LH surge characteristics

The onset of the LH surge was defined as the time when LH
concentration increased 2 times the assay sensitivity above the
baseline and remained elevated for a minimum of 8 hours. The
end of the surge was defined as the first time point below the
onset threshold or the first point before an increase was seen after
the LH concentration had already reached below 2 times the
baseline. In addition, the LH concentrations had to remain above
the threshold for at least 8 hours to be defined as a surge. A
Pearson’s χ^2 test was used to determine the differences in the
percentages of female lambs exhibiting LH surges. The time of
onset of surge, surge duration, surge amplitude, timing of the
surge peak, and total LH concentration were analyzed using a
one-way ANOVA followed by a Dunnett’s post hoc test. For the
surge amplitude, a power analysis was also carried out using the
effect size test (36, 37), which allows comparison of the means
with respect to the magnitude of difference when the sample size
is small. Statistical results are reported as a Cohen's d value,
and 0.2, 0.5, and 0.8 are considered as small, medium, and large
effect sizes, respectively.

All analyses were carried out after appropriate transformations
to account for heterogeneity of variances and corrections for mul-
tiple comparisons. Significance was defined as P < .05. All results
are presented as mean ± SEM or as box plots (minimum, first
quartile, median, third quartile, and maximum). All analyses were
carried out using IBM SPSS for Windows version 20.0.

Table 1. Birth Weight, Weight at Puberty, and Anogenital Distance of the Different Treatment Groups

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Birth Weight, n</th>
<th>Weight at Puberty, n</th>
<th>Anogenital Distance, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.9 ± 0.2 (29)</td>
<td>50.0 ± 1.0 (29)</td>
<td>1.1 ± 0.2 (30)</td>
</tr>
<tr>
<td>T</td>
<td>4.7 ± 0.5 (6)</td>
<td>46.3 ± 4.8 (5)</td>
<td>15.5 ± 1.3 (6)^a</td>
</tr>
<tr>
<td>TF</td>
<td>5.4 ± 0.4 (12)</td>
<td>55.9 ± 2.7 (11)</td>
<td>1.0 ± 0.1 (12)</td>
</tr>
<tr>
<td>TR</td>
<td>4.2 ± 0.7 (10)</td>
<td>46.3 ± 3.1 (10)</td>
<td>15.1 ± 0.7 (10)^a</td>
</tr>
<tr>
<td>T+F</td>
<td>4.4 ± 0.4 (8)</td>
<td>52.4 ± 4.6 (8)</td>
<td>15.9 ± 0.6 (8)^a</td>
</tr>
<tr>
<td>T+R</td>
<td>4.7 ± 0.6 (8)</td>
<td>50.7 ± 3.2 (8)</td>
<td>16.1 ± 0.9 (8)^a</td>
</tr>
<tr>
<td>T+M</td>
<td>4.7 ± 0.6 (5)</td>
<td>45.6 ± 4.9 (5)</td>
<td>16.2 ± 1.0 (5)^a</td>
</tr>
</tbody>
</table>

^a Significant difference from controls.

Results

The birth weight of offspring did not differ between the C
and T groups (C: 4.9 ± 0.2 vs T: 4.7 ± 0.5 kg) or among
intervention groups (Table 1). The body weight of animals
at puberty also did not differ across treatment groups (Ta-
ble 1). As expected, the anogenital distance in the prenatal
T-treated female offspring was greater than in the controls
(C: 1.1 ± 0.2 vs T: 1.5 ± 1.3 cm) (Table 1). Cotreatment
with the androgen antagonist, but not the insulin sensi-
tizer, prevented this masculinization. As expected, there
was no effect of the postnatal androgen antagonist or the
insulin sensitizer treatment on the anogenital distance.

Effects of interventions on timing of puberty

Representative biweekly profiles of progestogenic cy-
cles marking the timing of puberty from the prenatal
(C, CR, T, TF, and TR) and postnatal groups (C+F, C+R,
C+M, T+F, T+R, and T+M) are shown in Figure 2 and the
mean (±SEM) timing of puberty in Figure 3. Puberty in

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Control lambs occurred at 27.5 ± 0.3 weeks of age (calendar month October 16) (Figure 3A). Prenatal insulin sensitizer treatment or postnatal androgen antagonist, rosiglitazone, or metformin treatment had no effect on pubertal timing (Figure 3A). Prenatal T treatment significantly advanced puberty (22.5 ± 2.5 wk, average in calendar month September 14) (Figure 3B). Either prenatal interventions, androgen antagonist or insulin sensitizer treatment, prevented this advancement (weeks of age: TR, 26.5 ± 1.0; TF, 25.4 ± 0.7; calendar months: TR, October 10; TF, October 5) (Figure 3B). Similarly, postnatal androgen antagonist or insulin sensitizer treatments also prevented pubertal advancement (weeks of age: T+R, 27.7 ± 0.7; T+F, 28.8 ± 0.9; T+M, 28.4 ± 1.1; calendar months: T+F, October 23; T+R, October 17; T+M: October 22) (Figure 3C). Rosiglitazone and metformin, the two insulin-sensitizing interventions used as treatment interventions, were equally efficacious in preventing the pubertal advancement. Overall, the prenatal T females entered puberty earlier than controls with all interventions, preventing this advancement.
Effects of interventions on periovulatory LH surges

Figure 4 summarizes the percentage of females that showed estrous synchronization after the PGF$_{2\alpha}$ administration and the percentage of animals showing preovulatory LH surges during the first and second year of reproductive life. During the first year, only 66.7% of prenatal T animals showed estrous synchronization as opposed to 100% of controls (C vs T; $P < .05$). All TF animals showed estrous synchronization, whereas only 57.1% of the animals in the TR group responded with estrous synchronization (C vs TR; $P < .05$). When all animals were considered, 50% of prenatal T and 42.9% of TR animals had LH surges compared with 100% control and TF animals having LH surges. Among those that showed estrous synchronization, 75% of prenatal T- and TR-treated females had LH surges. There were no differences in the percentage of animals that showed estrous synchronization in the postnatal intervention groups compared with controls. In the second year, 83% of the prenatal T females that synchronized after a PGF$_{2\alpha}$ administration had LH surges (C vs T; $P < .05$). No significant differences were found in the percentage of synchronization among the prenatal or postnatal groups with the exception of T+F (60% synchronization). However, the overall percentage of T females that had an LH surge was lower compared with controls (C: 100% vs T: 50%; $P < .05$). The percentage of females with LH surges was also lower in the postnatal groups, T+F (60%) and T+R (63%), compared with the controls. Among those that showed estrous synchronization, only those females in the T group had a lower percentage of females with LH surges compared with the C group (C: 100% vs T: 60%; $P < .05$). Overall, only prenatal androgen antagonist intervention fully restored synchronization and LH surge potential in prenatal T-treated females to the level of the control group.

Representative patterns of periovulatory changes in LH from the prenatal and postnatal intervention groups during the first and second year of reproductive life are shown in Figures 5 and 6, respectively. As expected, prenatal T-treated animals had no surges or showed progressive deterioration (animal number 063 in Figure 5, animal number 251 in Figure 6). Although none of the pre- or postnatal treatments affected LH surges in control animals, all TF animals had LH surges.

The mean time of onset of LH surge and LH surge amplitude, based on those that had an LH surge, are summarized in Figure 7. Because the number of animals having LH surge was small in the prenatal T-treated group, the composite data from the control and prenatal T-treated animals from several breeding cohorts including animals used in this study (n = 3 cohorts), are also presented (gray bars). Although composite data showed a significant delay in the timing of the LH surge in the prenatal T-treated females relative to controls in year 1, this variable did not achieve statistical significance in the current study due to the small sample size in either year 1 or 2. Due to the high variability in the timing of the LH surge in the prenatal T group,
the effect size analyses did not discern any differences between the control and prenatal T-treated groups. However, the effect size analyses found robust differences between the control and all intervention groups (C vs TF: 1.32; C vs TR: 1.18; C vs T+F: 1.47; C vs T+R: 1.03; C vs T+M: 3.18), which is supportive of the surge delay, and it was more pronounced in the postnatal metformin intervention group. Similar direction of changes was also evident in year 2.

Similarly, whereas the composite data in year 1 showed a significant reduction in the LH surge amplitude, the small number of prenatal T-treated animals that had a LH surge in this study precluded the achievement of significance in the ANOVA test. However, the effect size analysis found robust effect relative to the LH surge amplitude between the control and prenatal T-treated offspring (C vs T: $d$ = 2.2; Cohen’s $d$ values of 0.2, 0.5, and 0.8 reflect small, medium, and large effect sizes, respectively). Minus the postnatal metformin-treated group in which a small effect was evident (effect size: $d$ = 0.26), all other prenatal or postnatal interventions had no impact in preventing/ameliorating the reduction in the LH surge amplitude evident in the prenatal T-treated females ($d$ = T vs TF: 0.004; T vs TR: 0.04; T vs T+F: 0.02; T vs T+R: 0.05). This is further corroborated by the fact that a robust effect size was evident when all intervention groups were compared with the controls (C vs TF: 1.9; C vs TR: 1.6; C vs T+F: 1.4; C vs T+R: 1.5; C vs T+M: 1.3). In year 2, the LH surge magnitude was reduced in all postnatal intervention groups relative to controls, with the greatest suppression evident in the prenatal T-treated group. Overall, in considering the impact of interventions in overcoming LH surge disruptions induced by prenatal T excess, although all interventions failed to normalize the time delay in LH surge onset, cotreatment with androgen antagonist was the only intervention that helped prevent the loss of LH surges in prenatal T-treated females.

**Discussion**

Findings from this study provide evidence that prenatal or postnatal intervention with either androgen antagonist or insulin sensitizer prevents pubertal advancement induced...
by prenatal T excess. Similar outcomes were achieved with rosiglitazone and metformin, an antidiabetic drug that decreases hyperglycemia by suppressing glucose production by the liver (38). In contrast, only the prenatal androgen antagonist, not the insulin sensitizer intervention, restored preovulatory LH surges. Conversely, both interventions failed to prevent the time delay in LH surges or normalize magnitude of LH surges. The implications of these find-
ings in terms of interventions and potential relevance to reproductive dysfunctions seen in women with PCOS are addressed below.

**Puberty**

Sheep are seasonal breeders, with annual cycles controlled primarily by the annual photoperiodic cycle (39). Puberty, defined as the awakening of the hypothalamic-pituitary gonadal axis, occurs after an escape from enhanced sensitivity to the negative feedback effects of E2 (40) in October in Ann Arbor, Michigan, in the Suffolk sheep used in this study. Findings from this study using ovary-intact animals indicate that puberty, defined as onset of first progestogenic cycle, occurred earlier in prenatal T-treated sheep despite the body weight of animals at puberty being similar. Similar advancement was not seen in our earlier studies (41), possibly due to differences in sample size and body condition of animals. Pubertal advancement seen in this study is consistent with extensive investigations carried out with the neuroendocrine model (ovariectomized plus E2 replaced), which showed that an increase in LH pulse frequency, indicative of neuroendocrine puberty, occurred earlier in prenatal T-treated females (27, 42).

Although the precise mechanisms by which puberty is controlled are not fully elucidated, environmental and metabolic cues, such as nutrition and body weight, clearly

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**Figure 6.** Representative patterns of LH in control (C), prenatal T (T), and postnatal intervention groups (T+F, T+R, T+M) and corresponding controls (C+F, C+R, C+M) after administration of two injections of PGF2α given 11 days apart during year 1 (top panel) and year 2 (bottom panel) of their reproductive life. Time zero represents timing of PGF2α injection.
to prevent defeminization of the timing of puberty by T. This was indeed the case, with TF animals achieving puberty at the same time as controls. A similar outcome was also seen with neuroendocrine puberty in the ovariectomized plus E$_2$ replaced model after prenatal androgen antagonist cotreatment (2.5). The fact that prenatal insulin sensitizer cotreatment also prevents pubertal advancement indicates that metabolic and steroidal pathways synergize in programming pubertal timing.

Central to the neuroendocrine mechanisms associated with puberty in the sheep is the reduction in sensitivity to E$_2$-negative feedback inhibition, allowing gonadotropin release at levels needed to stimulate ovaries to function like an adult (46). In the female sheep, this occurs at approximately 25–30 weeks of age, much later than in males (8–10 wk of age) (39). Although early increases in circulating LH mark neuroendocrine puberty in the neuroendocrine model (42) and is predictive of precocious initiation of ovulatory cycles in prenatal T-treated females, the timing of early initiation of progestogenic cycles in this study in prenatal T-treated females did not coincide with the time of the expected neuroendocrine puberty (~10 wk) as reported earlier (42). Therefore, whereas the prenatal T treatment appears to have advanced the neuroendocrine mechanisms controlling negative feedback inhibition of E$_2$, it may not have had a similar impact on establishing the timing of E$_2$-positive feedback mechanism, or alternatively, the ovary is defective and does not produce the preovulatory estradiol rise necessary for inducing positive feedback response.

**Preovulatory LH surge defects**

Feedback actions of ovarian steroids control reproductive neuroendocrine function with positive feedback action of E$_2$ providing the trigger for initiation of the preovulatory LH surge (46), which can be blocked by P$_4$ (47). Prenatal exposure to T reduces sensitivity to these feedbacks, thereby contributing to the absence of an LH surge in the neuroendocrine model or absence/attenuation of LH surges in the ovary-intact prenatal T-treated female sheep (42). Our findings of a lack of LH surges in a subset of T females and compromised surges in the rest are consistent with our previous reports (27) and indicative of a partial disruption of the surge mechanism by prenatal T excess in ovary-intact females. Altered programming at the hypothalamic level reducing responsiveness to E$_2$ and changes in the pituitary sensitivity to GnRH combined with an inability of the ovary to generate proper preovulatory E$_2$ signal may have all contributed to this defect. Our earlier studies have indeed found prenatal T disrupts E$_2$-positive feedback (14, 27, 28, 42), increases pituitary responsiveness to GnRH (48), and produces a highly ele-
vated ovarian E2 release (49) that is conceivably stemming from the multifollicular phenotype (50).

The finding that flutamide treatment restored LH surges in all TF animals supports the involvement of an androgenic contribution to this trait. These findings fail to support the premise that LH surge defects in prenatal T-treated sheep are programmed only by estrogenic actions stemming from aromatization of T to estrogen. This predication arose from the presence of normal E2-positive feedback response (14, 27, 28, 42) and preovulatory LH surges (51) in prenatal DHT-treated sheep. The findings from this study raise the possibility that the effects of DHT may not be mediated via androgenic actions but are likely due to DHT getting converted to 3β-androstenediol and acting through estrogen-β receptors (52, 53). A more likely possibility is that both androgens and estrogens synergize in organizing the surge system. This is supported by the finding that despite all TF females having LH surges, the LH surges were not of comparable magnitude with the controls. Similar conclusions were also drawn on the basis of LH surges generated in response to an E2-positive feedback challenge (26).

The doses of androgen antagonist and insulin sensitizers used appear to have been sufficient to achieve the desired blockade. In terms of androgen antagonist, the chosen dose was effective in blocking the action of both exogenous and endogenous androgens on phenotypic virilization in both males and prenatal T-treated female sheep, confirming earlier findings (45), whereas the external genitalia of females treated with prenatal T and TR were masculinized with a well-developed penis and scrotum; the external genitalia of all females and males treated prenatally with T plus flutamide were phenotypically female (data not shown). This is exemplified by the fact that the anogenital distance of TF females, which is used as the index for the degree of masculinization (54), is similar to that of controls. The 8-mg dose (0.114 mg/kg body weight) of rosiglitazone is within the range used effectively in women with PCOS (0.107–0.133 mg/kg in women of a 60–75 kg weight range) (31, 32). Biochemical and enzyme analyses carried out earlier found this treatment dose did not affect hepatic function and/or the overall health of sheep (33). The rosiglitazone treatment used has been shown to normalize the insulin to glucose ratio during pregnancy (21) and improve reproductive function in prenatal T-treated females (33). This is the first use of metformin in this model, and the dose used is within the range used in clinical PCOS (31).

In direct opposition to the postnatal insulin sensitizer treatment increasing LH surge amplitude during E2-positive feedback challenge in prenatal T-treated animals (26), the endogenously triggered LH surge in this study failed to show such amplification. These differences may relate to differences in the developmental stage of the animals being studied: prepubertal (16 wk) in the positive feedback challenge study (26) and postpubertal (7 mo) or adult (19 mo) in the present endogenously triggered LH surge study with pubertal hormones potentially contributing to the dissociation. In sheep, inhibin is a facilitator of LH release (55). It is conceivable that changes in inhibin dynamics in prenatal T-treated females may have compensated for the neuroendocrine deficit, culminating in similar amplitude of the LH surge. In previous studies we found a positive correlation existed between E2 and inhibin A in control but not in cycling prenatal T-treated females (49).

In addition to neuroendocrine programming, the findings from this study indicate that part of the defect stems from failure of T females to luteinize in response to the PGF2α injections and to initiate the cascade of preovulatory hormonal changes responsible for triggering preovulatory LH changes. Considering that PGF2α is very effective in synchronizing estrus in sheep (56, 57), the failure of some of the prenatal T-treated females to respond to PGF2α suggests a continued presence of luteinized follicles or corpora lutea that are compromised. Follicular persistence (41, 58, 59) coupled with LH surge delay (49) may have contributed to the compromised follicular environment and to the development of a defective corpus luteum. Extensive investigations carried out at the ovarian level have found steroid receptor balance, AMH, adiponectin, and pro-/antiapoptotic signals are disrupted in prenatal T-treated sheep and supportive of a compromised follicular environment (60–63).

**Efficacy of prenatal and postnatal interventions in overcoming surge disruptions**

A higher percentage of prenatal T-treated animals having an LH surge after a PGF2α synchronization compared with the overall percentage of animals with LH surge (synchronized plus unsynchronized) is likely related to the presence of defective corpus luteum or luteinized follicles that continue to produce P4 or the oligoanovulatory nature of these animals (58, 59). In sheep, as in humans, P4 blocks the generation of LH surge (47). The significant decline after PGF2α synchronization in the percentage of prenatal T-treated animals having LH surges in year 2 compared with year 1 is consistent with the previous finding of progressive deterioration of the reproductive system (58, 64). The finding that cotreatment with flutamide prevented the decline in the percentage of animals having an LH surge during both years, whereas rosiglitazone treatment showed improvement only in year 2, may be a function of differences in the prevailing endocrine milieu. Al-
ternatively, puberty-related changes in hormonal milieu might have unmasked and activated some programmed events in the TR animals.

All postnatal interventions appeared to have some beneficial effect in terms of increasing the percentage of animals responding to PGF<sub>2α</sub>, barring the T+F group in year 2. Elimination of luteinized follicles, which blocks proper synchronization response, might have accounted for these beneficial effects. However, this improvement in synchronization was not reflected in the total percentage of animals showing LH surges. The beneficial effect of postnatal insulin sensitizer treatment is consistent with our earlier findings (33) in which postpubertal treatment with rosiglitazone helped ameliorate the progressive deterioration.

Although both prenatal and postnatal interventions helped increase the percentage of animals producing LH surges, none of the interventions normalized the surge characteristics. The surge delay seen in prenatal T-treated animals persisted across all prenatal and postnatal intervention groups. Similarly, although a nonsignificant increase in LH surge amplitude was evident with postnatal treatments, they were not of normal amplitude. Surge delays have been shown to be detrimental to oocyte competence and successful pregnancies (65–67). Whether PCOS women manifest delayed and dampened surges and, hence, a compromised ovulatory signal, as is the case in prenatal T-treated sheep, remains to be determined. A recent assessment of efficacy of insulin sensitizers in PCOS women found no improvement in live birth rates after metformin treatment, despite improvement in clinical pregnancy rates (68). These findings from this prenatal and postnatal intervention study indicate that none of the interventions tried are optimal for restoring normal periovulatory dynamics. Whether an intervention combining both an androgen antagonist and an insulin sensitizer would provide better normalization remains to be determined. Combination of androgen receptor blockade and insulin sensitization were reported to provide significant endocrine-metabolic benefits in both nonobese (69, 70) and obese women with PCOS (71).

**Efficacy of rosiglitazone vs metformin**

The findings that rosiglitazone and metformin were similarly efficacious in preventing advancement in puberty and improving surge attributes suggest the effects are mediated via improved insulin sensitivity, although these two drugs have different modes of action. Rosiglitazone, which belongs to the thiazolidinedione class of drugs, improves glycemic control by selectively binding to the peroxisomal proliferator-activated receptor-γ receptors, thereby making the cells more responsive to insulin (72, 73). Metformin, a biguanide, suppresses hepatic glucose production, increases insulin sensitivity, and enhances peripheral glucose uptake (38). Both drugs have been used as treatment strategies in PCOS women (24, 68, 74–76), whose characteristics prenatal T-treated sheep recapitulate (14, 20, 28).

**Impact of birth weight**

The lack of difference in birth weight between control and prenatal T-treated females in this study indicates that birth weight is not a contributing factor in mediating the programming effects of prenatal T excess on pubertal advancement and LH surge defects. Failure of gestational T excess in reducing birth weight in this study as opposed to our earlier studies (29, 30) may relate to differences in sample size, inadequate statistical power for comparing the large number of treatment groups in this study, differences in the number of animals with multiple fetuses, and the ratio of male to female fetuses between cohorts, and more likely, differences in the body condition of the mother at the time of breeding.

**Translational relevance**

Because infertility can have profound negative effects on women’s quality of life and psychosocial well-being (77–80), there is a great need to overcome it. Considering that the reproductive and metabolic characteristics of prenatal T-treated sheep, the model used in these studies, bear striking resemblance to PCOS (14, 20, 28), beneficial effects of treatment and prevention strategies undertaken in this model are likely to be of translational relevance. Evidence points to androgen excess early in life providing a hormonal insult that results in the manifestation of PCOS in adulthood (17, 81, 82). A cordocentesis study in humans found that fetal serum T levels around midgestation are elevated to levels in the normal male fetal range in approximately 40% of female fetuses (83). The prenatal T sheep that manifest the PCOS phenotype are also exposed to T at levels found in the male fetuses (30). Other studies point to a pubertal onset of PCOS (84).

In recent years, the emphasis on PCOS research is shifting more and more to hyperandrogenic adolescents and offspring of women with PCOS, who appear to be at risk for developing PCOS (85–87). As such, initiation of treatment during prepubertal years in individuals manifesting hyperandrogenism or insulin defects would be optimal in preventing progressive deterioration and preservation of fertility potential. Because studies involving prenatal intervention help address the contribution of androgen and insulin in programming the pathology, appropriate strategies can be targeted toward prevention. Because early puberty has been found to be associated with behavioral disorders, adult metabolic diseases, metabolic syndrome,
and even breast cancer (88), the finding that pubertal advancement can be prevented by prenatal or postnatal interventions in this precocial model is likely to be of clinical relevance to human PCOS and beyond. Although the pharmacological interventions tried in this study may not be appropriate for human use during pregnancy and in prepubertal children, these findings identify the involvement of androgenic and insulin pathways in the timing of puberty and LH surge generation and as potential targets for interventions via lifestyle modifications.

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