

# Response to the gonadotropin releasing hormone agonist leuprolide in immature female sheep androgenized *in utero*

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## ABSTRACT

Similar to women with Polycystic Ovary Syndrome (PCOS), female sheep treated prenatally with testosterone (T-females) are hypergonadotropic, exhibit neuroendocrine defects, multifollicular ovarian morphology, hyperinsulinemia and cycle defects. Hypergonadotropism and multifollicular morphology may in part be due to developmentally regulated increase in pituitary responsiveness to GnRH and may culminate in increased ovarian estradiol production. In this study, we utilized a GnRH agonist, leuprolide, to determine the developmental impact of prenatal testosterone exposure on pituitary-gonadal function and to establish if prenatal exposure produces changes in the reproductive axis similar to those described for women with PCOS. Eight control and eight T-females were injected intravenously with 0.1 µg of leuprolide acetate per kilogram of body weight at 5, 10 and 20 weeks of age. Blood samples were collected by means of an indwelling jugular vein catheter at 0, 3, 6, 9, 12, 18, 24, 30, 36, 42 and 48 hours after leuprolide. Area under the curve (AUC) of LH response to leuprolide increased progressively between the three ages studied ( $P < 0.05$ ). AUC of LH in T-females was higher than in control females of the same age at 5 and 10 weeks of age ( $P < 0.05$ ), but similar at 20 weeks of age. AUC of estradiol response was lower at 10 but higher at 20 weeks of age in T-females compared to controls of the same age ( $P < 0.05$ ). Our findings suggest that prenatal T treatment alters the pituitary and ovarian responsiveness in a manner comparable to that observed in women with PCOS.

**Key terms:** androgenized females, fetal programming, PCOS, leuprolide, female sheep

## INTRODUCTION

Experimental exposure to testosterone during fetal life in mammals, such as sheep and monkeys, or pathological exposure to high levels of androgens in humans produces a series of changes in the reproductive axis that become evident in their post-natal life (Abbot et al., 1998; Birch et al., 2003; West et al., 2001; New, 2003). Recently, it has been established that the prenatal androgen exposure (PAE) in females born to testosterone (T)-treated sheep or monkey mothers is associated with growth retardation, infertility, obesity and

insulin resistance during adulthood, although some of these characteristics could be expressed early in the extra-uterine life (Eisner et al., 2000; Recabarren et al., 2003; 2005; Birch et al., 2003). Ovarian acyclicity, ovarian follicular disruption in prenatally T-treated sheep manifested as a reduction in number of 2-mm follicles, an increase in the size of large follicles and prolonged persistence, associated with hypergonadotropism comparable to those seen in PCOS, have been observed as well, all of which may be initiated early during post-natal life or during fetal development (Sharma et al., 2002; Birch et al., 2003;

West et al., 2001; Padmanabhan et al., 1998).

The GnRH agonist test has been used in human clinical studies to define hypogonadotropic hypogonadism of the constitutional delay of puberty (Street et al., 2002; Lanes et al., 1997) and in humans and animals to evaluate the gonadal axis prior to puberty (Elsholz et al., 2004; Aravindakshan et al., 2000) and after puberty (Ghizzoni et al., 1996). The GnRH agonist produces a transitory hyperstimulation of the gonadotrope, which simultaneously allows the stimulation of the gonad by the endogenous gonadotropins (Bo-Abbas et al., 2001; Rosenfield et al., 1996.) The objective of the present study was to evaluate and compare the pituitary and ovary response to the GnRH agonist leuprolide in immature female sheep, comparing control sheep with prenatally testosterone-treated female sheep of 5, 10 and 20 weeks of age, thus including the late postnatal and early prepubertal period in order to determine the developmental impact of prenatal testosterone exposure on pituitary-gonadal function and to establish if prenatal androgen exposure produces changes in the reproductive axis similar to those described for the Polycystic Ovary Syndrome (PCOS) in humans.

## MATERIALS AND METHODS

### *General management of mothers and lambs*

Forty adult female Suffolk sheep were mated after a synchronized estrus following treatment with intravaginal progestagen pessaries (Eazy Breed) and prostaglandin (Genestren, Drug Pharma, Chile), in early March, during the natural breeding season. Females were randomly assigned to one of the two treatments. One group of 20 pregnant sheep received biweekly intramuscular injections of 60 mg of testosterone propionate (Sigma, USA) dissolved in cottonseed oil (Kosut et al., 1997), beginning at 30 days of pregnancy until day 90. The remaining 20 pregnant sheep received the vehicle. Pregnant sheep were maintained under regular husbandry

protocols supervised by the veterinary staff at the sheep facility of the Faculty of Veterinary Medicine, University of Concepción, Chillán, Chile. At birth, lambs were left undisturbed with their mothers for 4 hours to permit mother and newborn bonding, then the umbilicus was cleaned and disinfected, and the newborn lambs were weighed. The characteristics of the external genitalia were observed and the ano-genital distance recorded. Biometric data are given in table 1. Mothers and newborns were kept in a closed barn for the first five days after delivery in stalls housing 3 to 4 mothers each. Mothers were allowed to grass and were also supplemented twice a day with food pellets for lactating sheep. Newborns remained with their mothers until 10 weeks of age, when they were weaned. After weaning, lambs were given free access to water, pasture and supplemented twice a day with hay and commercial pellet food. Food pellets based on dry matter (made of oat, corn, wheat, gluten feed, gluten meal, soybean meal, fish meal, sunflower meal and mineral salts) contained 18% protein, 11% crude fiber, 2% fat and 2450 Kcal/kg (Glovisor, Compañía Molinera El Globo, Chile). Beginning at 17 weeks of age, a blood sample was collected by venipuncture once a week and from 20 weeks of age twice weekly to determine progesterone concentrations and to define onset of puberty. Plasma progesterone concentrations higher than 1 ng/mL in one single or in two consecutive samples were considered to represent a previous ovulation and, therefore, onset of puberty (Recabarren et al., 2004).

All procedures were revised and approved by the local ethical committee on animal research.

### *GnRH agonist test*

Eight control and eight T-females, all of single birth, were selected for this study. The GnRH agonist test was conducted in all females at 5, 10 and 20 weeks of age. All procedures began at 14:00 hrs. The GnRH agonist test consisted of the intravenous administration of 10 µg/kg body weight of

leuprolide acetate (Lupron, Abbott Laboratory, Chile), through an previously inserted indwelling jugular vein catheter as described elsewhere (Recabarren et al., 1995). Blood samples were taken at 0, 3, 6, 9, 12, 18, 24, 30, 36, 42 and 48 hours after the leuprolide administration. Blood samples were received in heparinized tubes kept on ice and later spun at 1000 x g for 15 min at 4° C. Plasma was harvested and kept frozen at -20° C until later measurements of LH and estradiol by RIA.

#### *Hormone measurements*

Plasma LH concentrations were determined by RIA using ovine radio-iodinated LH (LER 1374-A), ovine antiserum CSU-204 and ovine LH standard oLH-S25 (provided by NIADDK, USA), in 200 uL duplicates, following procedures described elsewhere (Recabarren et al., 1996). The intra- and interassay coefficients of variation were 5 and 12%, respectively. The minimal detectable LH dose, defined as 90% of buffer control, was 0.1ng/mL.

Plasma progesterone concentrations were measured by RIA, using commercial kits (DPC, USA). DPC kits are routinely used to measure plasma progesterone in animals and have been validated for sheep plasma. The intra- and interassay coefficients of variation were 2 and 5%, respectively. The minimal detectable dose of progesterone, defined as 90% of control, was 0.1 ng/mL.

Plasma estradiol concentrations were measured using commercial kits (Estradiol ultrasensitive-DSL, Webster, TX, USA) validated for sheep. The intra- and interassay coefficients of variation were 3 and 7%, respectively. The minimal

detectable dose of estradiol, defined as 90% of control, was 5 pg/mL.

#### *Statistical analysis*

Plasma LH and estradiol concentrations present during the GnRH agonist test in each age and treatment group were analyzed by analysis of variance for repeated measures and comparisons of the means with the Newman-Keuls' test using the GB Stat v.5 program. Plasma LH and estradiol concentrations were transformed into area under the curve of response as the area of a polygon by the trapezoidal formula using a computer program based in Excell spreadsheet. These data were analyzed by analysis of variance for repeated measures with age as the repeated factor and comparison of the means with the Newman-Keuls' test using the GB Stat v.5 program. For all test, a P<0.05 was considered statistically significant. Data are given as mean ± standard error of the mean.

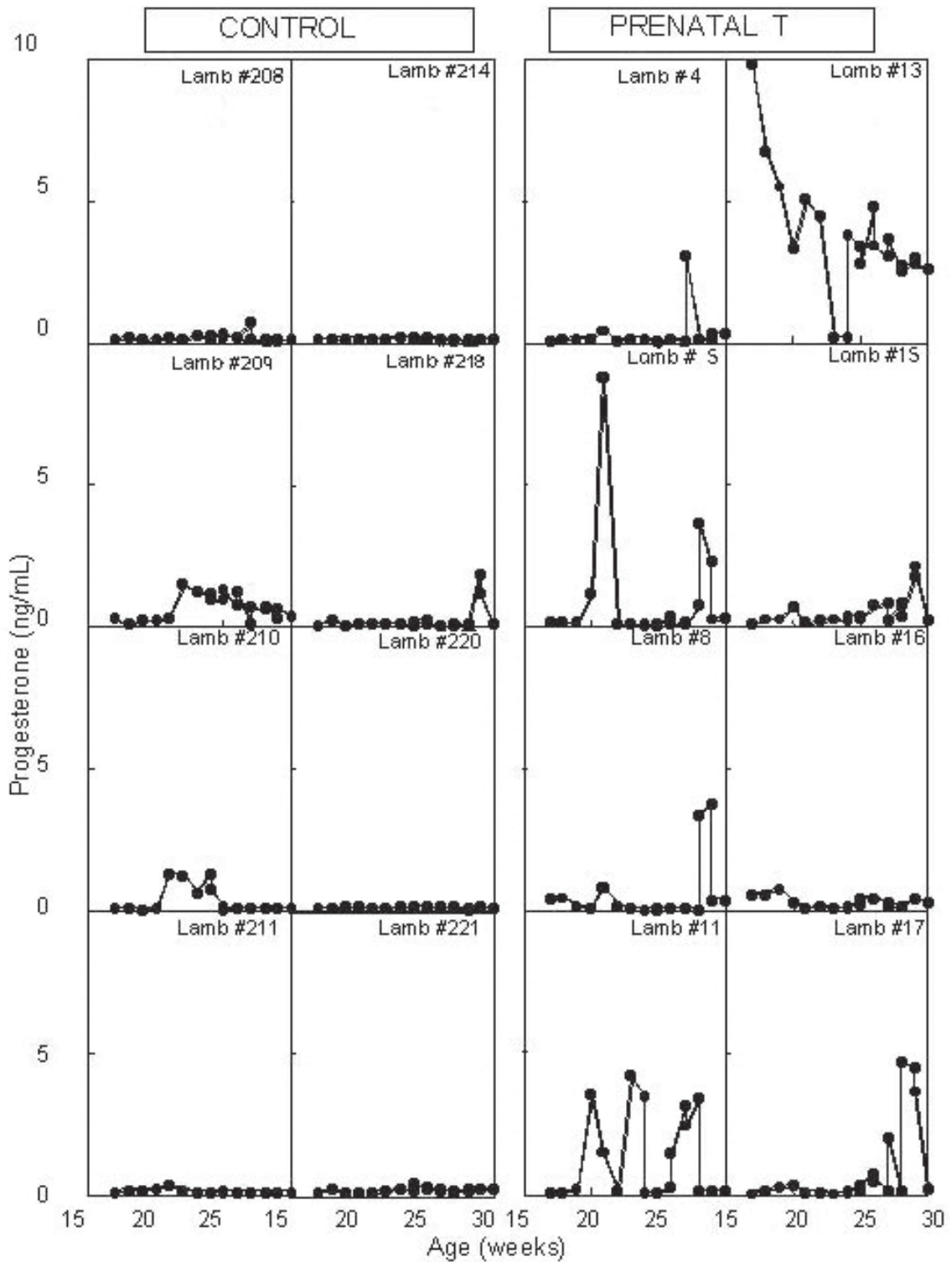
#### RESULTS

In the control group, one of eight sheep presented their first progesterone increase above 1 ng/mL at 22 weeks of age, another one at 24 weeks, and the last one at 29 weeks of age. Among the T-females, one of the eight sheep had progesterone levels above 1 ng/ml at 17 weeks when progesterone sampling began. Two T-female presented progesterone increases at 20 weeks of age, two at 27 weeks, one at 28 weeks, and another at 29 weeks. One T-female did not present progesterone increases until 30 weeks of age when progesterone sampling ended (Figure 1).

TABLE I

Biometric parameters in control and in androgenized pregnant female sheep at birth

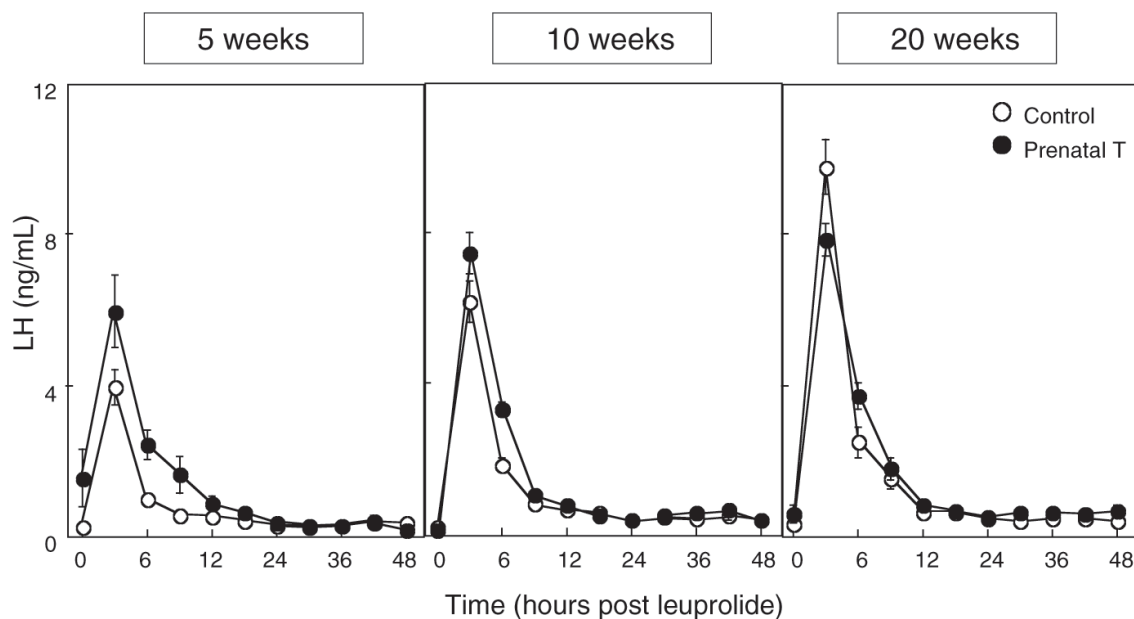
	Pregnancy (days)	N° newborn/ mother	Number of mother with twins	Placenta weight (kg)	Ano-genital distance (cm)
Control	147±1.8	1.5	9.0/20.0	0.44±0.03	1.5
Androgenized	147±1.5	1.6	11/20.0	0.57±0.05	15



**Figure 1.** Plasma concentrations of progesterone in control (left panel) and in androgenized female sheep (right panel). Blood samples were drawn from 17 weeks of age until 30 weeks of age. Three control females had increases in progesterone over 1 ng/ml before 30 weeks of age. Seven T-females had progesterone increases over 1 ng/ml before 30 weeks of age. One did not exhibit any increase in progesterone.

Figure 2 present the LH secretion profile in the control and T-females. The maximum LH level was observed at 3 hours post-analogue in both groups. Subsequently, plasma LH levels dropped until reaching basal levels 18 hours after leuprolide administration. In control sheep, the peak LH secretion in response to leuprolide increased significantly between 5, 10 and 20 weeks of

age, while in the T-females, the maximum LH concentrations did not differ statistically between the three ages. However, T-females of 5 and 10 weeks of age had peak LH concentrations in response to leuprolide greater than in the control group ( $P < 0.05$ ). For 30-week-old female sheep, the response to leuprolide is greater for the control group than for the T-females ( $P < 0.01$ ) (Table 2).



**Figure 2.** Mean ( $\pm$ sem) plasma LH concentrations (ng/ml) in response to leuprolide at 5, 10 and 20 weeks of age control (○-○) and T-females (●-●). T-females were born to mothers exposed to testosterone from day 30-90 of pregnancy.

TABLE II

Peak LH secretion after GnRH agonist leuprolide in control and in T-females.

CONTROL FEMALES

5 WEEKS	10 WEEKS	20 WEEKS
3.94 $\pm$ 0.48 a	6.18 $\pm$ 0.55b	9.78 $\pm$ 0.73c

T- FEMALES

5 WEEKS	10 WEEKS	20 WEEKS
5.95 $\pm$ 0.96 x	7.46 $\pm$ 0.52 x	7.85 $\pm$ 0.44x

a,b  $P < 0.01$  a,c  $P < 0.01$  b,c  $P < 0.01$

a,x  $P < 0.05$  b,x  $P < 0.05$  c,x  $P < 0.01$

Anova for repeated measures and post-hoc Newman-Keuls test.

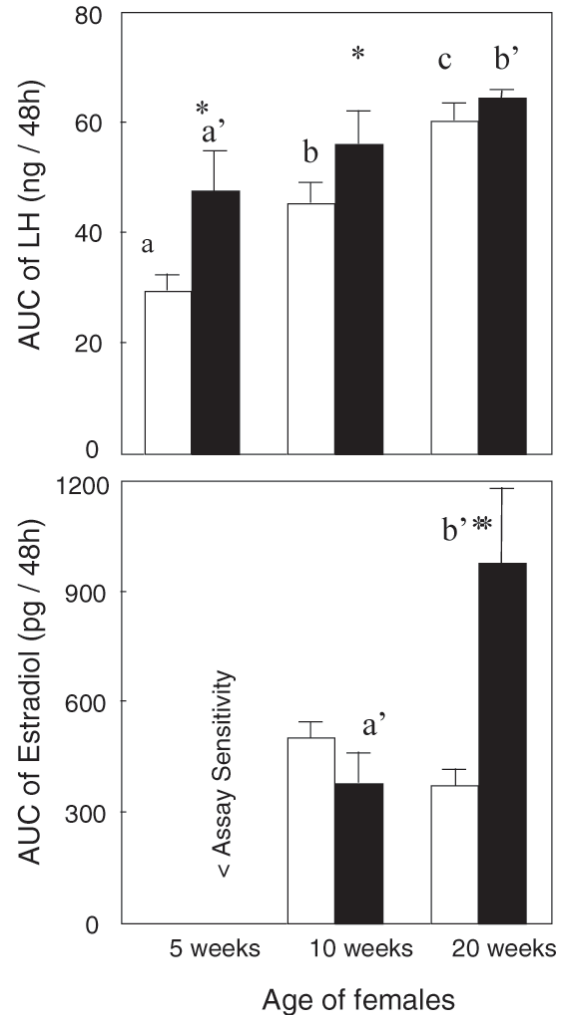
The areas under curve of LH secretion in the control and androgenized females are presented in Figure 3. The area under the LH curve increased significantly in the control group from  $29.54 \pm 3$  at 5 weeks to  $60.3 \pm 3.1$  ng/ml/48hr at 20 weeks of age ( $P < 0.01$ ). In the T-females, the areas under the curve of 5-week-old and 10-week-old sheep were not different ( $47.9 \pm 7$  vs.  $56.3 \pm 5.7$  ng/ml/48h). The area under the LH curve in the 20-week-old T-female is greater than the area presented by the 5-week-old T-females ( $64.54 \pm 0.9$  vs.  $47.9 \pm 7$ ,  $P < 0.05$ ). When both groups are compared by ages, the area under the curve is greater in the 5-week-old and 10-week-old T-females compared with the control group of the same age ( $P < 0.01$  and  $P < 0.05$  respectively).

The estradiol secretion profiles in 10-week-old and 20-week-old sheep from both groups are presented in Figure 4. The secretion profiles for 5-week-old females are not presented, since measured levels were below the RIA sensitivity level (5pg/mL). In 10-week-old and 20-week-old females, the secretion pattern differed between the control and T-females. In the control group, estradiol secretion was lower at 20 weeks of age than at 10 weeks of age, the first significant increase above the basal concentrations being observed at 24 hours post-leuprolide. In contrast, in the T-females, the response increased between 10 and 20 weeks of age. In 20-week-old T-females, the response was faster and greater than the response presented by 10-week-old T-females. The first significant increase of estradiol appeared at 24 hours in the 20-week-old T-females and at 30 hours for 10-week-old T-females. Additionally, estradiol secretion is significantly greater in the 20-week-old T-females than in the control group of the same age. This difference is observed in the area under the curve of estradiol secretion that is presented in Figure 3.

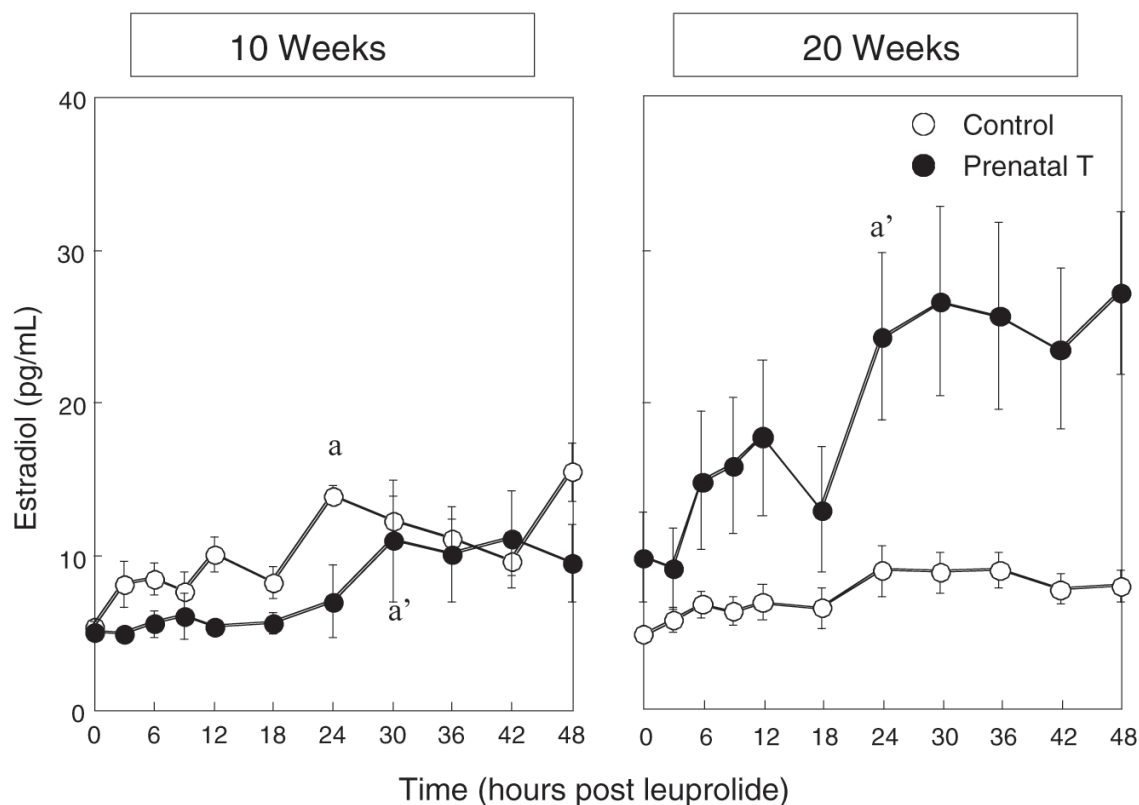
DISCUSSION

Results from the present study show that prenatal exposure to androgens during a limited time span of sheep pregnancy is associated in the offspring with a

masculinization of the genitalia, a tendency to an early onset of puberty, greater pituitary gland sensitivity to leuprolide and high ovary sensitivity to endogenous LH stimulation in comparison with control



**Figure 3.** Mean ( $\pm$ sem) area under the curve (AUC) of LH response (upper panel) and estradiol response (lower panel) to leuprolide in control (open columns) and in T-females. AUC of LH increased significantly in control females (a, b, c  $P < 0.05$ ) while in androgenized females area was higher in females of 20 (a', b') weeks compared to 5 weeks of age. AUC of LH in androgenized females of 5 and 10 weeks was higher than in control females of the same age ( $*P < 0.05$ ). AUC of estradiol was higher in T-females of 20 weeks than in T-females of 10 weeks of age or in control females of the same age ( $*P < 0.05$ ).



**Figure 4.** Mean ( $\pm$ sem) plasma estradiol concentrations (pg/ml) in response to leuprolide in control (○-○) and in T-females (●-●) of 5, 10 and 20 weeks of age.

females. Studies in female sheep exposed to testosterone in uterus with a scheme of treatment similar to the present study but with a higher dose (100 mg testosterone propionate biweekly), showed that the preovulatory-like surge of LH was not present in either gonadectomized males or females when stimulated with a preovulatory dose of estradiol. On the other hand, females exposed to testosterone in uterus displayed an increase in tonic LH secretion at an age similar to that of males (Kosut et al., 1997). This early increase in tonic LH secretion is defined as a neuroendocrine puberty, since ovaries are absent. These phenomena are attributed to the masculinization of the hypothalamic GnRH pulse generator (Wood et al., 1991; Wood et al., 1995; Herbosa et al., 1996). In normal control females, the tonic increase in LH secretion begins at 30 weeks of age in contrast to the earlier initiation of tonic LH secretion, which is usually displayed at 10

weeks of age in T-females (Wood et al., 1991; Wood et al., 1995). However, in other studies of prenatally androgenized females with intact ovaries, females unexpectedly did not show the early increase in tonic LH secretion leading to puberty but rather puberty was achieved at the same time as controls (Sharma et al., 2002; Birch et al., 2003). Our results provide additional support to the findings of Sharma and colleagues (2002), since we observed that the onset of puberty in two androgenized females was almost coincident with two control females. However, the total number of females showing progesterogenic cycles was greater in androgenized than in control females. Additionally, in the study of Sharma and colleagues (2002) the duration of the breeding season and the number of cycles that occurred during the first breeding season were similar between control and prenatally androgenized sheep. In contrast, prenatal exposure to androgens

compromised the positive feedback effects of estradiol. This does not seem to be our case because the estradiol positive feedback should have provoked progestogenic cycles. It is therefore possible that the lower dose of testosterone used in our study did not completely compromise the estradiol positive feedback and ovulation did occur in these sheep.

Leuprolide has been used in human and animal studies. In boys and girls, leuprolide acetate stimulates gonadotropin and gonadal steroid secretion during puberty in both sexes (Potau et al., 1999). The results from the present study indicate that the GnRH agonist stimulated both the pituitary gland and the ovary in both groups. The sensitivity of the pituitary gland to the leuprolide challenge (measured as AUC of LH) differed between the control and T-females. In control females, the sensitivity is low at 5 weeks of age and increased as the female matured. This change in sensitivity may represent a combination of factors including decrease in negative estrogen feedback on the pituitary gland and an increase in the number of GnRH receptors as a consequence of tonic GnRH release. (Viscarra et al., 1997; Turzillo et al., 1998). On the other hand, the LH release in response to leuprolide in androgenized females did not change between females of five and ten weeks of age. However, at both ages, the LH release was higher than in control females. Female sheep exposed prenatally to testosterone (Kosut et al., 1997) or DHT (Masek et al., 1999) initiate their increase in tonic LH secretion between seven and ten weeks of age due to a decrease in negative estradiol feedback. The reduction in negative feedback may have increased GnRH tonic secretion, and this increase in GnRH secretion in turn should have primed the pituitary gland to the GnRH agonist stimulation. This may explain the difference between both groups. The pituitary gland of T-females may be secreting large amounts of LH, since its secretion does not increase further between 5 and 20 weeks of age as it does in control females. In keeping with this, the AUC of LH secretion is similar between 5-week-old T-females when compared to 10-week-old and 20-week-old control females, suggesting that pituitary secretion in T-females matures earlier and became stabilized at 20 weeks of age.

Estradiol secretion was observed in both groups of female at 10 and 20 weeks of age after leuprolide, however it was not possible to determine estradiol secretion in 5-week-old females. The exaggerated LH response in T-females of 5 weeks of age did not correspond with estradiol secretion, suggesting that the ovary was not fully competent to respond to the endogenous LH stimulation or the bioactivity of LH was not comparable with the immunoactivity measured by RIA at that early age. However, at 20 weeks of age, a high response was observed in T-females. In androgenized adult female monkeys, a high response to recombinant HCG in testosterone and 17 $\alpha$  hydroxyprogesterone was also observed after 24 hours of stimulation. The exaggerated thecal cell steroid response to recombinant hCG in the prenatally androgenized female monkeys was attributed to a heightened 17 $\alpha$ -hydroxylase activity of cytochrome P450c17a, a key enzyme in ovarian thecal cell steroid production (Eisner et al., 2002). Recent evidence has also shown that in addition to 17 $\alpha$ -hydroxylase, increased T production in PCOS theca cells does not result from deregulation of "androgenic" 17 $\alpha$ HSD activity or altered expression of AKRs that may express 17 $\alpha$ HSD activity but rather the increased synthesis of T precursors is the primary factor driving enhanced T secretion in PCOS (Nelson et al., 2001). In PCO women, during the early follicular phase the administration of leuprolide led to an increased androgen secretion and 17-OHP compared to normal women. There was also a high response in normal women with ovaries classified by ultrasound as polycystic but not corresponding to women with PCO, because other endocrinological and metabolic features were absent (Chang et al., 2000). Authors attributed this high ovarian steroid secretion as an abnormality in the 17-hydroxylase and C-17,20 lyase in the ovarian delta 4 pathway, comparable to that observed in PCO. It is then possible to speculate that the ovary of T-females in our study also present such abnormalities with an overproduction of androstenedione and testosterone, which is later converted to estradiol. On the other hand, the exaggerated ovarian response could be a consequence of alterations in the ovarian development and folliculogenesis in



androgenized lambs. Such alterations may include an inhibition in apoptosis of granulosa cells similar to what has been proposed to occur in fetuses from ewes nutritionally restricted during pregnancy (Borwick et al., 1997). These fetus showed 50% more oocytes than fetus from adequately fed mothers, a finding that was interpreted as a delay in the normal process of oogonal degradation by phagocytosis of oocytes (Borwick et al., 1997). Further work is needed to clarify the underlying disturbances leading to an exaggerated response in the ovary of androgenized female sheep.

In summary, the present study shows that the pituitary gland and the ovary of androgenized female sheep are altered from an early age in prepubertal development. The exaggerated estradiol secretion at 20 weeks of age suggest that abnormalities in the secretory pathway are already established and may contribute to subsequent infertility problems in the mature stage, as suggested in adolescents with PCOS.

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