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Early prenatal androgen exposure reduces testes size and sperm concentration in sheep without altering neuroendocrine differentiation and masculine sexual behavior

C.M. Scully¹, C.T. Estill^{2,3}, R. Amodei⁴, A. McKune², K.P. Gribbin⁴, M. Meaker², F. Stormshak², and C.E. Roselli⁴

¹Department of Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, LA 70803

²Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR 97331-4501

³Department of College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331-4501

⁴Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, OR 97239-3098

Abstract

Prenatal androgens are largely responsible for growth and differentiation of the genital tract and testis and for organization of the control mechanisms regulating male reproductive physiology and behavior. The aim of the present study was to evaluate the impact of inappropriate exposure to excess testosterone (T) during the first trimester of fetal development on the reproductive function, sexual behavior and fertility potential of rams. We found that biweekly maternal T propionate (100 mg) treatment administered from day 30 to day 58 of gestation significantly decreased ($P < 0.05$) postpubertal scrotal circumference and sperm concentration. Prenatal T exposure did not alter ejaculate volume, sperm motility and morphology or testis morphology. There was, however, a trend for more T-exposed rams than controls to be classified as unsatisfactory potential breeders during breeding soundness exams. Postnatal serum T concentrations were not affected by prenatal T exposure, nor was the expression of key testicular genes essential for spermatogenesis and steroidogenesis. Basal serum LH did not differ between treatment groups, nor did pituitary responsiveness to GnRH. T-exposed rams, like control males, exhibited vigorous libido and were sexually attracted to estrous females. In summary, these results suggest that exposure to exogenous T during the first trimester of gestation can negatively impact spermatogenesis and compromise the reproductive fitness of rams.

Corresponding author: Charles E. Roselli, Ph.D., Department of Physiology and Pharmacology, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd. Portland, OR 97239-3098 USA. Phone: +1-503-494-8262; rosellie@ohsu.edu.

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Keywords

developmental programming; gestational hyperandrogenism; male reproduction; sexual behavior; testis; hypothalamus; ovine

1. Introduction

Dogma holds that mammalian embryos develop a female phenotype unless they are exposed to testosterone (T), which causes them to develop a male phenotype. Prenatal T also masculinizes brain functions that control sexually dimorphic gonadotropin secretion, sexual behaviors, and brain morphology. These effects are permanent and limited to critical periods early in life. Inappropriate androgen exposure during development has deleterious consequences in adult females. In humans, daughters born to hyperandrogenic mothers having polycystic ovarian syndrome (PCOS) or congenital adrenal hyperplasia (CAH) or girls born with classic CAH are at risk of having polycystic ovaries, impaired fertility, masculinized genitalia and behaviors [1-3]. Animal studies suggest that prenatal androgen excess produces reproductive dysfunctions in female offspring that culminate in infertility [4-8]. The reproductive defects associated with experimental gestational hyperandrogenism in females include, elevated serum androgen levels, reduced steroid negative feedback sensitivity, disrupted estrogen-positive feedback, increased LH secretion, and the development of multifollicular ovaries.

It has not been firmly established whether there is an altered male reproductive phenotype associated with prenatal androgen excess. However, sperm counts and fertility are reduced in men with classic CAH [3, 9] and Sertoli cell function may be altered in sons of women with PCOS [10]. In adult male rats and sheep excess prenatal T exposure reduces testes size, numbers of germ cells, sperm counts and motility and serum T concentrations [11-13]. The exact mechanisms for these effects are not understood, but have been associated with anatomical [14] or paracrine disruptions to the testis [15-17]. Prenatal androgen excess also affects the central control of reproduction in male sheep by altering FSH, LH and T responsiveness to a GnRH agonist [15, 18] or by altering hypothalamic release of GnRH [19, 20]. Finally, genital reflexes, copulatory behaviors and sexual partner preferences are altered in male rats and ferrets exposed to excess levels of T prenatally [21-24].

Previous studies in sheep evaluated the effects of excess prenatal androgen exposure for long durations on male reproduction. Lower doses and shorter durations of androgen exposure are known to produce females with varying degrees of genital virilization and alterations of the HPG axis [25]. Few studies have evaluated the effects of limiting the timing of exposure in males to the early part of the critical period when the gonads and genitalia differentiate and functional connections are established between the hypothalamus and pituitary [26, 27]. We reported that early exposure to exogenous T between days 30 and 60 of gestation (term = 147 days) acutely suppresses LH secretion and Leydig cell steroidogenesis in a manner consistent with enhanced negative feedback, but found that hypothalamic gene expression is altered even after exposure ended [12]. Early excess T also paradoxically reduced the volume of the ovine sexually dimorphic nucleus (oSDN) which is correlated with sexual

partner preferences in sheep [28]. The objective of the present study was to investigate whether early excess T exposure has enduring effects on the reproductive function, sexual behavior and potential fertility of adult rams. We hypothesized that early excess androgen exposure would disrupt the normal trajectory for development of the reproductive system leading to significant deficits in clinically based fertility parameters, impaired hormone responses and sexual behaviors in sexually mature rams. We also expected that prenatal T-treatment would alter the expression of specific testicular genes that regulate T synthesis and spermatogenesis. Our results showed that early prenatal androgen exposure significantly reduced testes size and sperm concentration in sheep without altering testicular cell numbers, neuroendocrine differentiation and masculine sexual behaviors.

2. Material and Methods

2.1. Experimental animals and treatments

Thirty Polypay ewes (*Ovis aries*) were bred at the sheep facility at Oregon State University. Animal husbandry and experimental protocols were conducted according to the principles and procedures specified by the Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Oregon State University.

Mature ewes were mated during the breeding season after they underwent estrous cycle synchronization using intravaginal progesterone treatments given by a controlled internal drug release (CIDR) device (Zoetis, New York, NY) followed by a prostaglandin F2 α injection as described previously [29]. Pregnant ewes were assigned randomly to control (n=14) and T propionate (TP) treatment (n=17) groups and received twice weekly i.m. injections of either corn oil vehicle or 100 mg TP (Sternaloids, Newport, RI) from day 30 to 58 of gestation. This androgen dose and regimen has been found previously to masculinize the external genitalia and affect the timing of puberty in female sheep [30] and produces concentrations of T in the fetus approximately equal to twice that of control male fetuses [31, 32].

Lambs were born between late February and early April. There were 23 control (C) lambs (male = 12; female = 11) and 26 TP-exposed (T) lambs (male = 15; female = 11). One T male died soon after birth and was not included in any analysis or summary data. The lambs were weighed at birth and periodically thereafter. Jugular blood samples were drawn for hormone measurements within the first 24 h after birth and then again at 9 mo. Crown-rump, ano-genital (AGD) and ano-umbilical (AUD) measurements were also recorded at birth and 1 month of age. Scrotal circumference was measured at birth, 1, 7, 9, 12 and 20 mo of age. Male offspring were studied from pregnancies where at least one male was present: 9 C pregnancies (2 single, 7 twin and 1 triplet litters) and 12 T pregnancies (6 single, 5 twin and 1 triplet litters). Of both the C and T pregnancies, one twin pair and the triplet set were males. C females were used as controls in the subsequent estrogen challenge and receptivity tests. T females were not studied after they were born. C and T-exposed lambs were raised together and weaned at 90 d of age after which they were separated by sex in non-adjointing pastures with ad libitum access to grass, water and mineral supplements. During the winter,

they were fed alfalfa hay supplemented with a barley-based concentrate. The sheep were regularly treated with anthelmintics to control internal parasite infestation.

2.2. GnRH challenge

Serum LH responses to GnRH administration were evaluated to determine whether excess prenatal exposure to T altered anterior pituitary sensitivity in T rams compared to C rams. The test was conducted in testes-intact rams at ~ 7 mo of age. Rams were given a bolus i.v. injection of GnRH (100 µg/2mL; Cystorelin, Merial Limited, Duluth, GA). Jugular vein blood samples were collected at 20-min intervals from 1 h before until 4 h after the injection. To evaluate whether there was a long-lasting effect, blood samples were collected every 15 min from hour 24 to hour 25 post injection. Blood samples were centrifuged, serum collected, and frozen and stored at -20°C pending hormone analysis.

2.3. Hormone assays

Serum concentrations of LH were measured by a radioimmunoassay developed by Niswender et al. [33]. Assay sensitivity averaged 0.001 ng/mL (National Institutes of Health oLH-S-19). The average low and high point intra-assay variation was 4.7% and 8.4%, respectively; and the interassay co-efficient of variation were 6.4% and 6.6% using the same plasma pools. Serum concentration of T were measured by radioimmunoassay using previously published procedures [34]. Assay sensitivity averaged 5 pg/mL and the inter- and intra-assay variations were less than 8% and 6%, respectively.

2.4. Sexual behavior tests

Rams were given serving capacity tests and sexual partner preference tests to evaluate the effect of prenatal T exposure on libido and sexual preference. Serving capacity was tested on 3 consecutive days during the second breeding season beginning when rams were approximately 18 mo of age and repeated 3 times every two weeks (total = 9 tests). The test consisted of pairing each ram individually with 2 estrous ewes for 20 min. and recording the frequencies of precopulatory behaviors, mounting and ejaculation as described previously [35]. Two weeks later rams were given a series of sexual partner preference tests, each testing session separated by at least one week. Each preference test consisted of pairing the test ram with two estrous ewes and two unfamiliar rams for 10 min and recording the frequency of behaviors directed at each sex as described previously [36, 37]. The sex of the preferred stimulus animal was inferred from the mean frequencies of copulatory behaviors directed at either the male or female stimulus animals.

2.5. Breeding soundness exams (BSE)

At 22 mo of age, C rams (n = 12) and T rams (n = 12) were subjected to breeding soundness examinations (BSE) to evaluate and classify their potential fertility ability. The BSE was conducted according to the guidelines of the Society for Theriogenology (SFT) [38] and consisted of: 1) a physical examination, 2) inspection of the external genitalia and measurement of scrotal circumference, and 3) semen collection and evaluation. Scoring was performed by an examiner blinded to prenatal treatment of the ram. The physical examination assessed each ram's general health, structural soundness and body condition on

a score that ranged from 1 to 5 [39, 40]. The scrotum and testicles were examined and palpated to evaluate tone, symmetry and size. Scrotal circumference was measured using a ReliaBull scrotal tape (Lane Manufacturing, Denver, CO. USA). Erection, protrusion, and ejaculation was stimulated using a Lane Pulsator IV Electroejaculator (Lane Manufacturing, Denver, CO) with a preset program function using a rectal probe. The ejaculate was evaluated grossly for volume, color, and contamination. Sperm gross motility was assessed under a microscope using a 10× objective on a heated stage and scored on a 3-point score according to SFT guidelines. An additional semen sample was stained with eosin-nigrosin (live-dead stain) and the morphology of 100 sperm cells from each animal were examined under oil immersion at 1000× magnification to determine percentage of normal sperm. Morphological abnormalities were noted and morphologic defects were categorized according to SFT guidelines. Semen concentration and viability were determined using a NucleoCounter SP-100 according to the manufacturer's protocol (ChemoMetric A/S, Allerød, Denmark). Rams were classified as unsatisfactory, questionable, satisfactory, or exceptional based on the results of their BSE according to SFT guidelines.

2.6. Euthanasia and tissue collection

Two weeks after semen collection, jugular blood samples were taken, after which the 9 C and 11 T rams were randomly chosen and euthanized with a lethal dose (15 mg/kg) of sodium pentobarbital (Euthasol; Delmarva Laboratories, Inc. Midlothian, VA). The right and left testes were removed and cut sagittally. A 1-cm thick slice of tissue was removed lateral to the mediastinum testis at approximately the same location of the testis from each ram. The tissue was fixed in 10% formalin for 24 hours before transfer to 70% ethanol for paraffin wax embedding. Fresh testicular tissue was dissected into $6 \times 0.5 \text{ cm}^3$ samples, stabilized in RNALater solution (Ambion, Life Technologies, Carlsbad, CA. USA) and stored at -20°C until the time of RNA extraction.

2.7. Testis histology and morphometry

Serial 5- μm thick tissue sections were cut from paraffin blocks and mounted on positively-charged slides. Tissue sections were stained with hematoxylin-eosin and analyzed digitally photographed with a light microscope (Leica DM 2000, Leica Microsystem, Inc., Buffalo Grove, IL). Seminiferous tubules were evaluated for spermatogenesis using the Yoshida scoring method [41], which gives a score ranging from 1 (a total absence of cells within the seminiferous tubule) to 12 (many late spermatids and/or spermatozoa). Leydig cell density and morphology were scored on a 3-point scale according to the method of Donovan et al [40]. Adjacent sections were processed for vimentin immunohistochemistry using mouse monoclonal anti-vimentin antibody Vim 2B4 (Abcam, Cambridge, MA) according to the manufacturer's protocol. Vimentin-stained sections were used to evaluate Sertoli cell numbers and seminiferous tubule morphology. Ten seminiferous tubules were selected randomly and digitally photographed at 200× magnification. Sertoli cells were counted using the multi-point tool in Image J software (National Institutes of Health, Bethesda, MD). Sertoli cells were counted if: a) there was an outline of a cell stained or b) a circular shape of stain was apparent. Histological evaluations and measurements were performed by a single observer blinded to the treatment groups.

2.8. Quantitative reverse transcription real time polymerase chain reaction (qtRT-PCR)

Total RNA was extracted using an RNAqueous-4PCR kit (Invitrogen, Carlsbad, CA. USA) following the manufacturer's instructions. RNA sample concentration and purity were evaluated as described previously [12]. RNA was reverse transcribed to cDNA using Oligo dT primers with the First Strand Superscript III Kit (Invitrogen, Carlsbad, CA. USA). Real time PCR reactions were run in triplicate for each sample using PowerSYBR Green Master Mix (Invitrogen, Carlsbad, CA. USA). Primer sets for the ovine genes evaluated have been validated and published previously [12]. All reactions were run on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Life Technologies). Quantification of gene expression was performed by the relative standard curve method, normalized against the reference gene GAPDH and reported as the fold difference relative to the mean expression level in pooled tissues from C rams.

2.9. Statistical Analysis

Treatment effects on physical measurements, testis morphometric parameters, sperm counts, and serum T data were analyzed using analysis of covariance with number of male fetuses per litter as the covariate. Normality of data was tested using the D'Agostino-Pearson normality test. Data not normally distributed were analyzed with a Mann-Whitney U test. Data represented by percent were arcsine transformed before analysis. Behavioral data were analyzed by 2-way ANOVA followed by Bonferroni posttests. Statistical analysis of qtRT-PCR was performed using the Student's t-test. Analyses were performed using GraphPad Prism version 5 (GraphPad Prism software, LaJolla, CA) or GB Stat version 10 (Dynamic Microsystems, Inc., Silver Spring, MD). Values are reported as mean $\pm$ SEM; $P < 0.05$ was considered significant for all comparisons.

3. Results

3.1. Physical measurements and serum testosterone

Prenatal T exposure did not affect body weight at birth (C rams = 3.68 ± 0.25 kg; T rams = 3.98 ± 0.32 kg) or linear growth i.e., crown-rump length (C rams = 51.9 ± 1.3 cm; T rams = 53.0 ± 1.2 ; $P > 0.05$ cm) or the anogenital distance/anal umbilicus distance (AGD/AUD) ratio (C rams = 0.83 ± 0.03 ; T rams = 0.87 ± 0.01 ; $P > 0.05$) in newborn ram lambs. To assure that effective masculinizing doses of T were reaching the fetus, female offspring of TP treated mothers were examined and shown to have developed a pseudo-penis, empty scrotum and displayed a male-typical AGD/AUD ratio (0.90 ± 0.002). Scrotal circumferences did not differ between male treatment groups at birth (C rams = 9.71 ± 1.6 cm; T rams = 7.09 ± 0.3 cm), 1 mo of age (C rams = 9.18 ± 0.5 cm; T rams = 9.67 ± 0.4 cm) and 7 mo of age (C rams = 30.5 ± 0.4 cm; T rams = 28.6 ± 0.8 cm) of age. However, scrotal circumference was significantly smaller in T rams than in C rams at 9 mo of age in November (T rams = 29.9 ± 1.0 cm; C rams = 32.9 ± 0.6 cm; $P < 0.05$) and 12 mo of age in February (T rams = 27.6 ± 0.3 cm; C rams = 29.2 ± 0.5 cm; $P < 0.05$) of the first breeding season. The treatment effect on scrotal circumference persisted into the second breeding season and was recorded during the BSE (T rams = 30.5 ± 0.6 cm; C rams = 32.3 ± 0.6 cm; $P < 0.05$).

Serum T concentrations measured between 20 to 30 h after birth were low and not significantly different ($P>0.05$) between newborn C (61 ± 20 pg/mL) and T (25 ± 3.0 pg/mL) ram lambs. There was no difference in serum T concentrations between C (3.70 ± 1.31 ng/mL) and T (3.91 ± 0.99 ng/mL) rams at 9 mo of age.

3.2. Neuroendocrine feedback: LH response to GnRH

Injection of GnRH caused a significant increase in LH secretion in both C and T males (Fig. 1). No differences were apparent in the area under the response curve, peak LH response or time to peak.

3.3. Male-typical sexual behavior and partner preference

All of the C rams ($n=12$) and 12 of 14 T rams mounted and ejaculated by the fifth serving capacity test. The 2 T rams that did not mount after 5 tests failed to mate females throughout all 9 tests. Fig. 2A shows the mean behavioral frequencies from the final serving capacity test. There was no difference in any performance measure of male sexual behavior between groups and on average both groups achieved a similar number of ejaculations during testing. The latency to mount, a measure of motivation, did not differ between C rams (31.0 ± 5.9 sec) and T rams (23.6 ± 6.6 sec). Prenatal T exposure did not alter sexual partner preference (Fig. 2B). Both C and T rams courted and mounted estrous ewes significantly ($P<0.05$) more than stimulus rams although, T rams exhibited significantly ($P<0.05$) lower levels of pre-courtship behavior. Neither cohort ejaculated with stimulus rams.

3.4. Breeding soundness exam

Adult rams were given breeding soundness exams (BSE) using SFT Guidelines to evaluate their breeding potential [42]. The exam included a thorough physical, body condition score (1, very thin to 5, very fat), scrotal circumference, electroejaculation, and microscopic semen evaluation. All vital signs (heart rate, respiration rate and body temperature) were within normal ranges and did not differ between treatment cohorts (data not shown). Ram weights (C rams = 84.5 ± 3.0 kg; T rams = 87.0 ± 3.5 kg) and body condition scores (C rams = 3.3 ± 0.2 ; T rams = 3.2 ± 0.2) did not differ between treatment. No abnormalities were observed on physical, scrotal examinations or evaluation of the prepuce and penis of the rams in either group although, as noted above, scrotal circumference was significantly ($P<0.05$) smaller in T rams than controls. Ejaculate volume (C rams = 1.1 ± 0.2 mL; T rams = 1.5 ± 0.2 mL), gross motility (C rams = 2.8 ± 0.2 ; T rams = 2.4 ± 0.3), percent morphologically normal sperm (C = $83.0 \pm 2.5\%$; T = $82.4 \pm 3.1\%$) and percent sperm viability (C rams = $68.7 \pm 5.7\%$; T rams = $56.9 \pm 6.4\%$) did not differ between groups. However, total sperm per ejaculate was reduced by 32% in T rams (2536 ± 431 mil/mL) compared to C rams (3694 ± 440 mil/mL; $P=0.03$). Based on SFT Guidelines for breeding soundness, 75% (9/12) of the C rams were found to be satisfactory potential breeders, whereas only 41.7% (5/12) of the treated rams were found to be satisfactory potential breeders ($P = 0.1$, χ^2 test).

3.5. Testes morphology

Fig. 3 shows representative digital images of the seminiferous tubules. Panels A and C are hematoxylin-eosin-stained sections used to evaluate spermatogenesis and Leydig cell density

in C and T rams, respectively. Panels B and D are vimentin-stained sections used to measure tubule diameter and Leydig cell number in C and T rams, respectively. There was no treatment effect on the mean seminiferous tubule diameter (C rams = $195.0 \pm 6.0 \mu\text{m}$; T rams = $194.0 \pm 9.0 \mu\text{m}$), seminiferous tubule circumference (C rams = $612 \pm 19.9 \mu\text{m}$; T rams = $613 \pm 28.3 \mu\text{m}$) or Sertoli cells counts (C rams = 25.2 ± 0.9 per tubule; T rams = 25.6 ± 1.5 per tubule). Spermatogenesis as scored using the Yoshida method did not differ between treatment groups (C rams = 11.9 ± 0.12 ; T rams = 11.9 ± 0.12). In addition, Leydig cells exhibited normal morphology and equivalent densities by Donovan scoring for both treatment groups (C rams = 1.1 ± 0.19 ; T rams = 1.1 ± 0.44).

3.6. Serum T concentrations and testis gene expression in adult rams

Serum T did not differ between adult C rams ($2.4 \pm 0.8 \text{ ng/mL}$) and rams prenatally exposed to T ($4.6 \pm 1.8 \text{ ng/mL}$). Prenatal T exposure had no effect on the expression of genes specific for Leydig cell steroidogenesis (*LHR*, *STAR*, *CYP 17*), Sertoli cell function (*INHBA*, *AMH*) or the germ cell marker (*POU5F1*) (Table 1).

4. Discussion

We found that excess exposure to exogenous T in early gestation (GD30 to 60) reduced scrotal circumference and sperm concentration in adult rams, but did not alter ejaculate volume, sperm motility and viability or testis morphology. Although not significant, there was a trend for more T rams than controls to be classified as unsatisfactory potential breeders. Serum T concentrations were not affected by prenatal androgen exposure, nor was the expression of key testicular genes essential for spermatogenesis and steroidogenesis. Prenatal T treatment did not alter baseline serum LH concentrations nor did it affect pituitary LH responsiveness to a GnRH challenge. Both T-exposed and C males exhibited vigorous libidos and were sexually attracted to estrous females. Taken together, these results suggest that exposure to excess exogenous T during an early period of development can impact testicular development and spermatogenesis and compromise the future reproductive potential of rams.

Our findings that scrotal circumference and sperm count were reduced in T-exposed males compared to C males indicate that the treatment used had an effect on the developing testis and agree in part with results reported by Recabarren et al. [11] using a longer treatment window (days 30 to 120 of gestation). These investigators reported that extended T exposure not only reduced scrotal circumference and sperm count but also reduced sperm motility, decreased number of germ cells and increased Sertoli cell number [11, 15]. Similarly, in humans, sons born to hyperandrogenized mothers with PCOS were found to have an increase in anti-Mullerian hormone levels indicative of an increase in Sertoli cell number or function [10]. In sheep, when prenatal androgen exposure was reduced to 60 days (days 30 to 90 of gestation), dihydrotestosterone, but not T, reduced testis weights, number of seminiferous tubule germ cell layers, and sperm motility [14]. In rats, prenatal T exposure decreased testis weights, Sertoli cell numbers, spermatogenesis and sperm motility in a dose- and duration-dependent manner [13]. By comparison to these studies, the testicular effects observed in the present study after only 30 days of exposure were less disruptive to

sperm production and motility and did not alter the expression of key testicular genes in adults. This could be due to methodological differences in assessments of germinal epithelial morphology and sperm motility, but is more likely the consequence of the timing, duration and/or level of androgen exposure. It appears that longer treatments and more potent androgens reprogram testicular development to a greater extent and result in more severe defects in testicular structure and function in adulthood.

The exact targets affected by inappropriate exposure to excess T during development have not been well characterized. Recent studies have shown that exposing developing male lamb fetuses to excess androgen prior to midgestation inhibits pituitary gonadotropin secretion and suppresses expression of testicular enzymes catalyzing T production. Using the same treatment paradigm as the current study, we found previously that excess T suppressed LH levels and androgen synthesis enzyme expression in the testis as early as day 60 of gestation [12]. This effect was reversed two weeks after treatment stopped suggesting that the testicular disruption was shortlived. Connolly et al. [16] reported that longer exposure to T from day 30 to 90 of gestation has similar reversible effects on LH secretion, Leydig cell distribution and function, but permanently up-regulated gene markers for Sertoli cells (*inhibin A*) and down-regulated markers for germ cells (*POU5F1*). These latter changes observed only after longer T exposure. A significant increase in the expression of the Sertoli marker antimüllerian hormone (AMH) was also observed in testis of fetal and peripubertal T males [43, 44]. These data suggest that as a consequence of excess prenatal exposure to T, a functional discrepancy between Sertoli cells and sperm production is initiated during fetal life and completed before puberty. Thus, it appears that when gonadotropin support to the developing testis is reduced sufficiently, gestational hyperandrogenization differentially influences Leydig cells, Sertoli cells and germ cells during development in a time and/or dose dependent manner that can lead to altered adult testicular function.

Our finding that basal and GnRH-stimulated levels of LH did not differ between T males and C males indicates that early prenatal exposure to excess androgens did not alter subsequent pituitary gonadotroph sensitivity. These results agree with the study of Wilson and Tarttelin [20] who also found no differences in the LH responses to GnRH either before or after castration and concluded that prenatal androgenization from day 20 to 65 of gestation does not affect pituitary sensitivity or hypothalamic steroid negative feedback responsiveness. However, these same authors also provided evidence that basal GnRH secretion may be impaired in the T-exposed males because, compared to controls, both LH and T levels are suppressed in lambs from 4 to 30 weeks of age [45]. In an earlier study, we found that early prenatal T exposure reduced the expression of GnRH and estrogen receptors and increased kisspeptin expression in the hypothalamus two weeks after treatment ended [12]. These enduring effects indicate that the trajectory of hypothalamic development is disrupted by early excess T exposure and may underlie deficits in adult GnRH secretion. In contrast, Recabarren et al. [19] found that animals exposed to exogenous T or DHT from days 30 to 120 of gestation exhibited higher mean LH secretion and higher LH pulse amplitude compared to C males, suggesting that excess androgen exposure increases pituitary responsiveness and/or GnRH release. A follow up study by this group showed that GnRH-stimulated LH concentrations were higher in T males than in controls at both 20 and

30 weeks of age supporting the conclusion that excess prenatal T exposure increases pituitary sensitivity to GnRH in this model of prenatally hyperandrogenized rams [46].

These results agree with studies in females that found prenatal hyperandrogenization amplifies GnRH-induced LH secretion by inducing developmental changes in gene expression of pituitary GnRH and estrogen receptors and paracrine modulators of LH and FSH synthesis [47]. Studies in females have also found prenatal hyperandrogenization reduces the numbers of neurokinin B, dynorphin and progesterone receptor-positive neurons in the arcuate n./median eminence but not the number of kisspeptin neurons, suggesting a possible mechanism by which hypothalamic sensitivity to steroid is reduced [48]. Whether a similar central mechanism underlies changes in gonadotropin secretion in hyperandrogenized males observed by Recabarren et al. remains to be tested. Nonetheless, it is apparent that the male hypothalamus and pituitary, like the testis, are also vulnerable to excess prenatal androgen exposure and the magnitude of reprogramming depends on the timing and extent of the exposure.

There is evidence from studies of rats and ferrets [21-24] that perinatal exposure to excess androgen paradoxically impairs masculinization and decreases the display of male-typical behaviors. The current study indicates that T males are completely masculinized since they exhibited robust male-typical sexual behaviors and were strongly attracted to females. Collectively, these results do not support the idea that prenatal hyperandrogenization disrupts masculinization in sheep. A possible explanation for these results may be that after male fetuses are exposed to T from days 30 to 58 of gestation, they are able to rapidly reestablish hormone homeostasis and fully restore T concentrations to physiological levels before the window for brain sexual differentiation closes around day 90 of gestation [28]. In contrast, in rats and ferrets exposure to excess T occurred throughout the critical period and most likely disrupted the formation of relevant brain circuits or epigenetic marks resulting in permanent effects. Thus, we predict that longer term exposure to excess prenatal T would disrupt behavioral masculinization in association with the altered hormone responses discussed above.

In summary, our findings support the hypothesis that excess androgen exposure disrupts the normal trajectory for development of the reproductive system and alters its function in adult male sheep. However, it appears that limiting exposure to the first half of the critical period of sexual differentiation has a small impact overall that manifests as significantly smaller testes and reduced sperm counts and generally lower breeding potential. These results contrast with reports of significantly increased numbers of Sertoli cells, more significant impairment of spermatogenesis and reprogramming of central control mechanisms that occur when T exposure occurred throughout a more extended period of gestation [19, 44]. The important implication is that the mechanisms mediating reproductive development and brain masculinization exhibit some amount of plasticity, which can be disrupted when exposure to exogenous T is elevated for longer extended periods of gestation and ultimately imperil adult fertility.

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Highlights

- Excess prenatal androgen exposure reduces testis size, sperm concentration and breeding potential in rams.
- Gestational hyperandrogenized rams exhibit male-typical sexual behavior and gonadotropin secretion.
- Developmental mechanisms mediating reproductive development and brain masculinization exhibit resilience to early disruption.

Figure 1. LH secretion in response to an i.v. injection of GnRH (100 μ g/animal) in C and T rams (n= 8 rams per group). Blood samples were collected every 20 min from 1 h before to 4 h after GnRH administration and continuing between 24 to 25 h after injections. Values are the mean $\pm$ SEM

Figure 2.

(A) Frequency of male reproductive behaviors exhibited in serving capacity tests. Rams were placed with two sexually receptive ewes and behaviors recorded for 20 min on nine separate occasions. Data represent the mean $\pm$ SEM frequencies on the final test ($n = 12$ C rams and 12 T rams per group). Two T rams that failed to mount ewes were excluded from the calculations. (B) Sexual partner preference tests. Each ram was given three preference tests at approximately 19 mo of age. The precopulatory behaviors (Pre; consisting of the combined frequencies of anogenital sniffs, foreleg kicks, nudges, flehmen and vocalizations), mounts (Mnts) and ejaculations (Ejac) directed at either estrous female stimulus animals (Female-directed) or male stimulus animals (Male-directed) were recorded. Data represent the mean $\pm$ SEM frequencies on the final preference test ($n = 12$ C rams and 14 T rams per group). In comparisons of female- and male-directed behaviors, bars with the same superscripts differ significantly ($P < 0.05$). Significant differences in pre-copulatory ($P < 0.05$) behaviors between treatment cohorts are indicated with an asterisk.

Figure 3. Representative digital images of seminiferous tubule sections. (A and C) Sections stained with hematoxylin-eosin and used to evaluate spermatogenesis and Leydig cell density in C and T rams, respectively. (B and D) Sections exhibiting vimentin immunoreactivity and used to measure tubule diameter and Leydig cell number in C and T rams, respectively. Magnification = 200× bar = 75 μ m.

Table 1
Effect of prenatal androgen exposure on testis gene expression in adult rams

Gene	Relative Fold Expression		
	Control	T	P-Value
<i>AR</i>	1 ± 0.21	0.98 ± 0.11	0.9404
<i>AMH</i>	1 ± 0.08	1.18 ± 0.10	0.1833
<i>CYP 17</i>	1 ± 0.26	0.88 ± 0.20	0.7040
<i>INH3A</i>	1 ± 0.08	1.06 ± 0.03	0.5117
<i>LHR</i>	1 ± 0.33	0.64 ± 0.18	0.3473
<i>StAR</i>	1 ± 0.34	1.18 ± 0.18	0.7312