Am J Physiol Endocrinol Metab 299: E998–E1005, 2010.
First published September 21, 2010; doi:10.1152/ajpendo.00032.2010.

Prenatal testosterone excess alters Sertoli and germ cell number and testicular FSH receptor expression in rams

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Submitted 12 January 2010; accepted in final form 14 September 2010

Rojas-García PP, Recabarren MP, Sarabia L, Schön J, Gabler C, Einspanier R, Maliqueo M, Sir-Petermann T, Rey R, Recabarren SE. Prenatal testosterone excess alters Sertoli and germ cell number and testicular FSH receptor expression in rams. *Am J Physiol Endocrinol Metab* 299: E998–E1005, 2010. First published September 21, 2010; doi:10.1152/ajpendo.00032.2010.—Exposure to excess testosterone (T) during fetal life has a profound impact on the metabolic and reproductive functions in the female's postnatal life. However, less is known about the effects of excess testosterone in males. The aim of the present study was to evaluate the impact (consequences) of an excess of T during fetal development on mature male testis. The testicular evaluation was by histological analysis and by determination of mRNA expression of the FSH receptor (FSH-R), transforming growth factor-β type I receptor (TβR-I), and two members of the TGF-β superfamily, transforming growth factor-β3 (TGFβ3) and anti-Müllerian hormone (AMH) in males born to mothers receiving an excess of T during pregnancy. At 42 wk of age, postpubertal males born to mothers treated with 30 mg of T propionate twice weekly from day 30 to 90, followed by 40 mg of T propionate from day 90 to 120 of pregnancy (T males), showed higher concentrations of FSH in response to a GnRH analog, a higher number of Sertoli cells/seminiferous tubule cross-section, and a lower number of germ cells/tubules ($P < 0.05$) than control males (C males) born to mothers treated with the vehicle. The mRNA expression of FSH-R and of TβR-I was higher in T males compared with C males ($P < 0.05$). Moreover, in T males, AMH expression level correlated negatively with the expression level of TGFβ3. In C males, this latter correlation was not observed. These results suggest that prenatal exposure to an excess of T can negatively modify some histological and molecular characteristics of the mature testis.

fetal programming; follicle-stimulating hormone

EARLY EXPOSURE TO ANDROGEN EXCESS during fetal life is a known disruptor of several metabolic and reproductive parameters in females (33). Exposure to androgen excess can occur in fetuses of pregnant women with polycystic ovarian syndrome (PCOS) that show elevated levels of plasma testosterone (T) during the last third of pregnancy and in fetuses from females treated experimentally with T during pregnancy (12, 13, 36, 41, 42). The consequences of prenatal exposure to excess T in males have been under study only recently. We have shown that serum anti-Müllerian hormone (AMH) concentrations are increased in prepubertal sons born to mothers with PCOS, suggesting that these boys may show an increased Sertoli cell number or an altered function of Sertoli cells during infancy and childhood (39). Additionally, we have demonstrated that prenatal T excess reduces sperm count and motility in rams (38), suggesting alterations in spermatogenesis.

In humans, basal and poststimulated T concentrations after the administration of the gonadotropin-releasing hormone (GnRH) analog leuprolide acetate were similar between sons of women with PCOS and sons of control women (39). In our animal model of prenatal androgenization, we also observed that circulating T levels in response to human chorionic gonadotropin did not differ between males born to mothers treated with T during pregnancy and control males (38). Moreover, prenatally androgenized male rhesus monkeys do not exhibit elevated androgens during adulthood despite metabolic defects (5). Interestingly, in the above-mentioned models, Leydig cell function seems to remain unaffected by prenatal exposure to excess T. Therefore, these observations in humans and sheep suggest that the probable target of prenatal exposure to T could be the Sertoli cell, which in turn may generate alterations in spermatogenesis. The pathway to provoke sperm alterations because of this disturbance in the Sertoli cell is unknown.

Follicle-stimulating hormone (FSH) plays a central role in regulating Sertoli cell proliferation and AMH secretion (2, 27, 50). In the male rat, the peak of AMH expression coincides with Sertoli cell mitotic activity, which is under FSH control (14). AMH mRNA levels are increased in cultured Sertoli cells from human fetal testes after addition of cyclic AMP, the main second messenger implicated in FSH signaling of the FSH receptor (FSH-R) (46).

Cytokines have been shown as important mediators in Sertoli cell function (7). In fact, Sertoli cell adhesion constitutes the basis of the blood-testis barrier (BTB), which separates the seminiferous epithelium into basal and adluminal epithelia. The integrity of this barrier varies from assembled to disassembled to allow spermatogonia transfer to the seminiferous lumen to continue with the meiotic processes (34). The mechanisms by which these processes are attained are not fully understood, but evidence strongly suggests the participation of transforming growth factors-β (TGFβ). TGFβ1, -2, and -3 are members of the TGFβ superfamily that together with AMH belong to the cytokines. These ligands have been detected in

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testicular tissue (31) and have been implicated in reproductive performance in some species such as the rat, roe deer, and human, among others (9, 44, 47, 51), playing an important role in the testicular cellular function and spermatogenesis, mainly by paracrine actions. In particular, TGFβ3 has been established as an important cytokine in the regulation of tight junction dynamics in Sertoli cells, perturbing the BTB by diminishing the expression of proteins that constitute the tight junctions (24–26). We hypothesize that the deleterious effects exhibited by rams born to mothers exposed to T excess during pregnancy (38) may be explained by alterations in the expression of local regulatory factors such as FSH-R and/or cytokines, including increased expression of AMH and TGFβ3. Therefore, the aim of the present study was to evaluate the testis morphology in males exposed prenatally to an excess of T by analyzing the Sertoli cell and germ cell number and the mRNA expression of AMH, TGFβ3, FSH-R and TGFβ type I receptor (TβR-I).

MATERIALS AND METHODS

Animals, prenatal T treatment, and maintenance. Sixteen Suffolk-Down rams at 42 wk of age were included in this study. Rams at this age are adults and have attained full neuroendocrine and gonadal development (19). Nine rams (T males) were born to mothers that received twice weekly intramuscular injections of 30 mg of T propionate (TP; Sigma, St. Louis, MO) in cottonseed oil between days 30 and 90 of pregnancy, continuing with 40 mg of TP from day 90 to 120 of pregnancy, using a previously validated experimental procedure (38). This regimen of TP administration was chosen to reflect the pattern of secretion of T seen in women with PCOS during pregnancy, in which increasing levels of T during later parts of pregnancy are observed (41). Although fetal levels of T achieved with this dose of T administration remain to be determined, other studies using the 100-mg T dose have found T concentrations of female fetuses on days 65 and 90 of gestation to be in the range seen in 65-day-old control male fetuses (45). We expect the T levels in the current study to be approximately one-third of the 100-mg dose. Seven rams [control (C) males] were born to mothers that received only the vehicle twice weekly from days 30 to 120 of gestation and were identified as control rams. Four T males and five C males participated in a previously published study on sperm count (38). Mothers were fed with pasture and hay and supplemented with pelleted concentrated food according to feeding protocols for pregnant sheep. Rams were weaned at 8 wk of age, maintained under natural photoperiod, and given free access to water and pasture and supplemented twice a day with hay and commercial pelleted food for ruminants, as detailed elsewhere (38). Males born from singleton pregnancies were used for this study. All of them were born in mid-September by normal delivery and after full-length gestation. All procedures of management and methodologies were approved by the Ethics Committee in Animal Research of the Faculty of Veterinary Sciences of the University of Concepción and were performed according to the American Physiological Society's *Guiding Principles in the Care and Use of Animals* (2002).

FSH response to a GnRH analog. The FSH response to a GnRH analog (leuprolide acetate) was conducted in five C males and 5 T males at 40 wk of age. The males were moved to individual crates indoors and were provided with free access to pelleted food and water. To collect blood samples, an indwelling jugular vein catheter was placed under local anesthesia, as described previously (35). Blood samples were collected the day before the experiment at regular intervals to allow males to adapt to the blood collection procedures and to minimize stress. An intravenous dose of 10 μg leuprolide acetate/kg (Lupron; Abbott Laboratories) was administered to test pituitary responsiveness, as described previously (37). Blood samples were collected every 30 min for the first 3 h and then at 6, 9, 12, 18, 24, 30, 36, 42, and 48 h after the analog injection. Plasma was separated and kept frozen until FSH determination by radioimmunoassay (RIA).

Plasma levels of FSH were determined by RIA using ovine radioiodinated FSH (oFSH I-1 AFP 5679 C), ovine antiserum AFP C 5228113, and ovine FSH standard oFSHRP [provided by National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases (NIADDK)], in 200-μl duplicates. Intra- and interassay coefficients of variation were 8 and 13%, respectively. The minimal detectable FSH dose, defined as 90% of buffer control, was 0.1 ng/ml.

Basal T and LH. The day before surgical procedure, 3-ml blood samples were withdrawn from the jugular vein of each male to determine basal T and LH plasma levels. Blood samples were centrifuged at 1,000 *g* for 15 min, and plasma was stored at −20°C until hormone measurements. Basal circulating levels of T were measured by double-antibody RIA using a commercial kit (DSL, Webster, TX). The minimal detectable limit of the T assay was 0.5 ng/ml. Intra- and interassay coefficients of variation were 4 and 8%, respectively. The basal circulating levels of LH were determined by RIA using ovine radioiodinated LH (LER 1374-A), ovine antiserum CSU-204, and ovine LH standard oLH-S25 (provided by NIADDK) in 200-μl duplicates. Intra- and interassay coefficients of variation were 5 and 12%, respectively. The minimal detectable LH dose, defined as 90% of buffer control, was 0.1 ng/ml.

Tissue collection. At 42 wk of age, the left testicle was removed from each ram for histology and total RNA isolation. In brief, rams were anesthetized with halothane, and the testicle was surgically removed and cut in small pieces ~0.5 cm^3 in size. One set of samples was left for histology, and another set was immersed in RNAlater (Ambion) and stored at −20°C for total RNA isolation.

Testicular histology and evaluation. For histology, samples ~0.5 cm^3 in size were cut with a sterile surgical blade and immersed in Bouin's fixative solution for 24 h at room temperature and then embedded in paraffin. Sections (5 μm) were stained using the hematoxylin-periodic acid Schiff method (29). The tissues were observed under a light microscope using a ×10 ocular lens and a ×40 objective and photographed with a digital camera (model DSC-P71; Sony). Images were processed and analyzed with the Image Tools 3.0 software (available online at http://ddsdx.uthscsa.edu/dig/itdesc.html).

For the morphometric analysis, six sections per animal were analyzed. Each section was separated from the next by 50 μm. Sampling was performed by counting ≥300 fully cross-sectional seminiferous tubules (lumen present and a difference of <20% between major and minor diameters) in blindly selected microscopic fields, using a ×40 objective and a ×10 ocular lens. Field selection was performed as follows: starting from the upper left microscope field of the section, the stage was moved manually by one point of the stage rule to the right edge and then one point downward. One point of the stage rule represents 500 μm in the described microscope observation conditions. Testicular histological analysis included the following parameters: diameter of the seminiferous tubule, diameter of the lumen of the seminiferous tubule, and height of the epithelium of the seminiferous tubule. The number of Sertoli cells, spermatogonia A1 and B1, primary spermatocytes, and elongating spermatids per tubule were also counted in cross-sections of tubules in stage 6 of the seminiferous epithelium cycle. The morphometric analysis and cell counting was performed by one observer who was not aware of the treatment of each animal.

Total RNA isolation and reverse transcription. Testicular tissue previously kept at −20°C in RNAlater was disrupted using a Lysing Matrix D (MP) and the FastPrep instrument (FP120, Bio 101; Thermo Electron). For total RNA isolation, the RNeasy Mini Kit (Qiagen) was used according to the manufacturer's instructions. Yield of total RNA was quantified photometrically at 260 nm using the Nanodrop 1000 spectrophotometer (Thermo Scientific). The quality of the RNA was verified after electrophoresis on formaldehyde containing 1% (wt/vol) agarose gel with ethidium bromide staining. The quality of the RNA

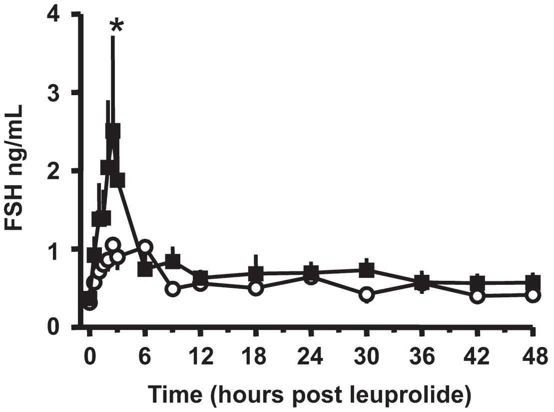

Fig. 1. Plasma FSH concentration in response to leuprolide acetate (gonadotropin-releasing hormone analog) in control (C) (○; $n = 5$) and testosterone (T) males (■; $n = 5$) at 40 wk of age. The T males were born to mothers treated twice weekly from day 30 to 90 of pregnancy with 30 mg and from day 90 to 120 of pregnancy with 40 mg of T propionate. *Significant differences at 2.5 h after a 2-way ANOVA ($P < 0.05$).

samples with low RNA concentration was verified using the Agilent 2100 Bioanalyzer and the RNA Nano Chips (Agilent, Waldbronn, Germany). To remove any DNA contamination, DNA digestion was performed before reverse transcription (RT) (16). DNase treatment was carried out in a total volume of 20 μl containing 1 μg of total RNA, 1 U DNase (Promega, Mannheim, Germany), and the 1× buffer used in the RT. This reaction mixture was incubated at 37°C for 30 min, heated at 75°C for 5 min to inactivate the DNase, and placed immediately on ice for 5 min. Forty microliters of premix containing 200 U Moloney-Murine Leukemia Virus reverse transcriptase (M-MLV RT; Promega), 2.5 μM of random hexameres (Amersham Biosciences), 0.666 mM of each dNTP (Amersham Biosciences), and 1× of the supplied RT buffer were added to each RNA sample. Samples without M-MLV enzyme were run at the same time to monitor the absence of any genomic DNA. The reverse transcription was performed at 25°C for 10 min and at 42°C for 1 h and then at 90°C for 2 min. The yielded cDNA was aliquoted in 20-μl volumes and stored at −20°C until analysis by real-time PCR.

Real-time PCR. In preliminary experiments, the expression of all investigated factors was examined by standard gradient PCR using a gradient thermocycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) to confirm the expected amplicon sizes as well as to estimate the optimal annealing temperature for each pair of primers. The following primers used for the real-time PCR were β-actin (178 bp, corresponding to bases 867–1,044 of Genbank accession no. U39357): forward 5′-CTC TTC CAG CCT TCC TTC CT-3′, reverse 5′-GGG CAG TGA TCT CTT TCT GC-3′; FSH-R (340 bp, corresponding to bases 164–503 of Genbank accession no. NM_174061): forward 5′-GAG AGC AAG GTG ACA GAG ATT CC-3′, reverse 5′-CCT TTT GGA GAG ACT GAA TCT T-3′; AMH [176 bp, (32)]: forward 5′-CTA TGA GCA GGC CTT CCT GG-3′, reverse 5′-CCT CCA GGT GCA GGA CCA CC-3′; TGFβ3 (290 bp, corresponding to bases 1,165–1,454 of Genbank accession no. NM_001101183): forward 5′-AGA ACT GCT GTG TGC GTC C-3′, reverse 5′-GCA GGA CTT CAC CAC CAT G-3′; and TβR-I (395 bp, corresponding to bases 479–873 of Genbank accession no. NM_174621.2): forward 5′-ATA ACC GCA CTG TCA TTC ACC-3′, reverse 5′-TGA CAC CAA CCA GAG CTG AG-3′. Real-time PCRs were performed in a Rotor-Gene RG-3000 thermocycler (Corbett Research, Mortlake, Australia). One microliter of cDNA was used as template for real-time PCR containing 0.2 μl of 50× SYBR Green and 5 μl of 2× SensiMix solution (Quantace, London, UK) and 0.4 μM of each primer in a final volume of 10 μl. The following real-time PCR protocol was applied: a denaturation step at 95°C for 10 min; a three-step amplification, including denaturation at 95°C for 15 s, the corresponding annealing temperature specific for each factor (see below) for 20 s, and extension at 72°C for 30 s; a melting curve program (50–99°C) with continuous fluorescence measurement; and a final cooling step to 40°C. Data acquisition was carried out at the end of each annealing and extension step. The number of cycles for all factors was 45, and the annealing temperature was 55, 54, 63, 61, and 63°C for β-actin, FSH-R, AMH, TGFβ3, and TβR-I, respectively. For mRNA quantification, a dilution series with known quantities of the specific PCR product was amplified simultaneously with the samples as a standard. Samples were measured in triplicates and standard curve in duplicates in the same run. The PCR products applied as standards were generated by conventional block RT-PCR and purified using the Invisorb Spin DNA Extraction Kit (Invitek, Berlin, Germany) as described by the manufacturer. Concentration of the purified PCR product was estimated in duplicate using the Nanodrop 1000 spectrophotometer.

The melting points of the amplified products served as confirmation of specific amplification. As negative controls, reactions containing no template (sterile RNase-DNase free water) or no reverse transcriptase were included to exclude any PCR products derived from contaminations or from genomic DNA. The content of each specific mRNA was normalized to a housekeeping gene. In previous steps during this study, the gene expression of the 18S ribosomal subunit and β-actin was tested to choose the suitable and reliable housekeeping gene as internal control for normalization of the genes under study. β-Actin expression resulted in a similar level between C and T males. Therefore, we chose to use β-actin as internal control. The data from real-time PCR are presented as a ratio between the specific mRNA gene and the β-actin expression.

Statistical analysis. Data are expressed as means or median ± SE or SD, as indicated in the figures and tables. The expression level of mRNA is given in relation to the expression level of the housekeeping gene β-actin. The data were analyzed using unpaired Student's *t*-test, Mann-Whitney nonparametric test, or two-way ANOVA whenever necessary. Pearson correlation analysis was used to evaluate the relationship among the variables of interest. A value of $P \leq 0.05$ was considered to be significant. All of the statistical evaluations were performed by using the GraphPad Prism 4.0 software.

RESULTS

FSH response to GnRH analog. Plasma concentrations of FSH before the GnRH analog were similar between groups.

Table 1. *Dimensional characteristics and cellular quantification of different testicular elements in Suffolk-Down C and T males at 42 wk of age*

Parameters	C Males ($n = 7$)	T Males ($n = 9$)
Seminiferous tubule diameter, μm	185.7 ± 17.6	176.7 ± 22.9
Seminiferous tubule lumen diameter, μm	72.6 ± 11.2	71.2 ± 12.6
Seminiferous tubule epithelium height, μm	56.6 ± 9.7	52.7 ± 11.1
Sertoli cells/tubule	12.4 (11.3–14.7)^{a}	15.1 (11.3–17.7)^{b}
Spermatogonia/tubule	12.38 ± 1.19^{a}	10.37 ± 2.57^{b}
Spermatocytes I/tubule	23.67 ± 2.77^{a}	18.00 ± 6.76^{b}
Spermatids/tubule	56.14 ± 10.76^{a}	36.96 ± 18.47^{b}
Spermatocytes I/spermatogonia	1.94 ± 0.15^{a}	1.67 ± 0.31^{b}
Spermatids/spermatocyte I	2.40 ± 0.35	1.97 ± 0.62

Data are shown as means ± SD. C, control; T, testosterone. The Sertoli cells/tubule parameter is shown as median and range (in parantheses). T males were born to mothers that received twice weekly intramuscular injections of 30 mg of T propionate between days 30 and 90 of pregnancy and 40 mg of T propionate between days 90 and 120 of pregnancy. Different superscripted letters indicate that means are significantly different at any given parameter ($P \leq 0.05$, unpaired Student's *t*-test).

After the GnRH analog challenge, maximum concentrations were achieved within the first 3 h in both groups. However, T males reached significantly higher concentrations of FSH, with a mean ± SE of 2.5 ± 1.2 ng/ml, compared with C males that reached 1.06 ± 0.17 ng/ml at 2.5 h (2-way ANOVA, $P < 0.05$; Fig. 1).

Basal T and LH concentrations. Basal concentrations of plasma T and LH previous to the tissue collection were similar between T and C males. For T, the mean concentration (± SD) was 1.49 ± 2.9 ng/ml in T males and 0.59 ± 0.21 ng/ml in C males. In the case of LH, the mean concentration (± SD) was 0.38 ± 0.35 ng/ml in T males and 0.34 ± 0.28 ng/ml in C males.

Histological findings. Spermatogenesis was qualitatively complete; i.e., elongated spermatids were observed within the seminiferous tubules, and interstitial tissue showed mature Leydig cells in C males and T males. Spermatogenic development was more heterogeneous in T males, with a lower number of advanced germ cells in most tubules (Table 1). The diameter of the seminiferous tubule, the diameter of the lumen of the seminiferous tubule, and the height of the seminiferous tubule epithelium were not significantly different between groups (Fig. 2 and Table 1). More Sertoli cells/seminiferous tubule cross-sections were found in the T males, but the number of germ cells/seminiferous tubule cross-sections was lower in this group (Table 1). The spermatocyte I/spermatogonia ratio was significantly lower in T males, indicating increased loss in the last spermatogonial mitosis and/or early meiosis. The spermatid/spermatocyte I ratio was similar between groups (Table 1).

mRNA expression of receptors and ligands. The mean mRNA expression of FSH-R in testicular tissue was significantly higher in T males compared with C males (Fig. 3*A*). In essence, T males showed an expression 33% higher than C males. The level of expression of FSH-R was not correlated with the Sertoli cells/tubule, suggesting that this higher expression was not originated by a higher number of Sertoli cells in T males. In contrast to FSH-R, the mean expression of AMH in both groups reached similar levels (Fig. 3*C*). A detailed observation of the AMH expression showed two T males with high levels of expression. However, the rest of the rams showed wide variation in the expression of AMH. The mean expression of the TGFβ3 cytokine was similar between C and T males, with expression levels being in a similar range (Fig. 3*D*). However, TβR-I expression was significantly higher in T males compared with C males (Fig. 3*B*).

Correlations between mRNA expression levels. In contrast to our hypothesis, AMH and TGFβ3 expression was similar between groups. Considering the great variability in the expression levels of AMH and TGFβ3 within each group, we matched the expression level of AMH with the expression of TGFβ3 and with the expression level of FSH-R and TβR-I as well (Table 2). In T males, AMH expression level correlated

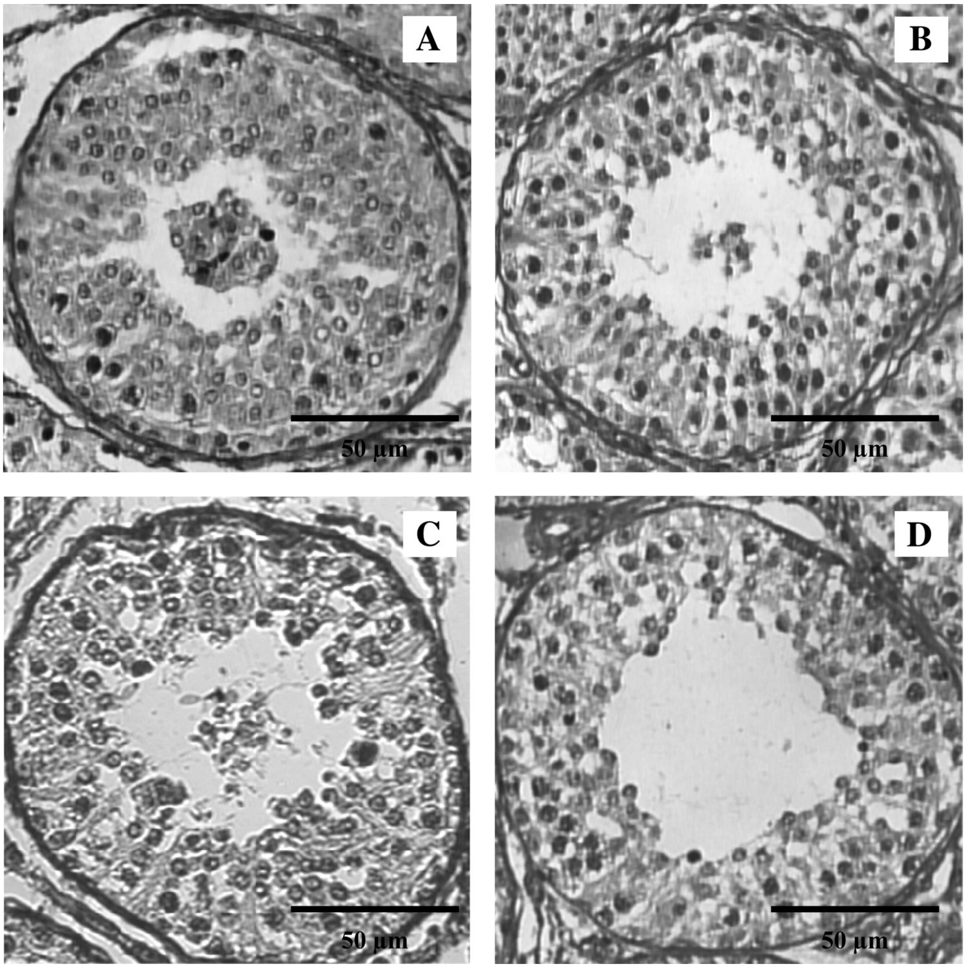

Fig. 2. Testicular tissue of C (*A* and *B*) and T male (*C* and *D*) sheep at 42 wk of age. The seminiferous tubule diameter, seminiferous tubule lumen diameter, and seminiferous epithelium height were not significantly different between groups. T males were born to mothers treated twice weekly from day 30 to 90 of pregnancy with 30 mg and from day 90 to 120 of pregnancy with 40 mg of T propionate. C males were the controls. The bar corresponds to 50 µm.

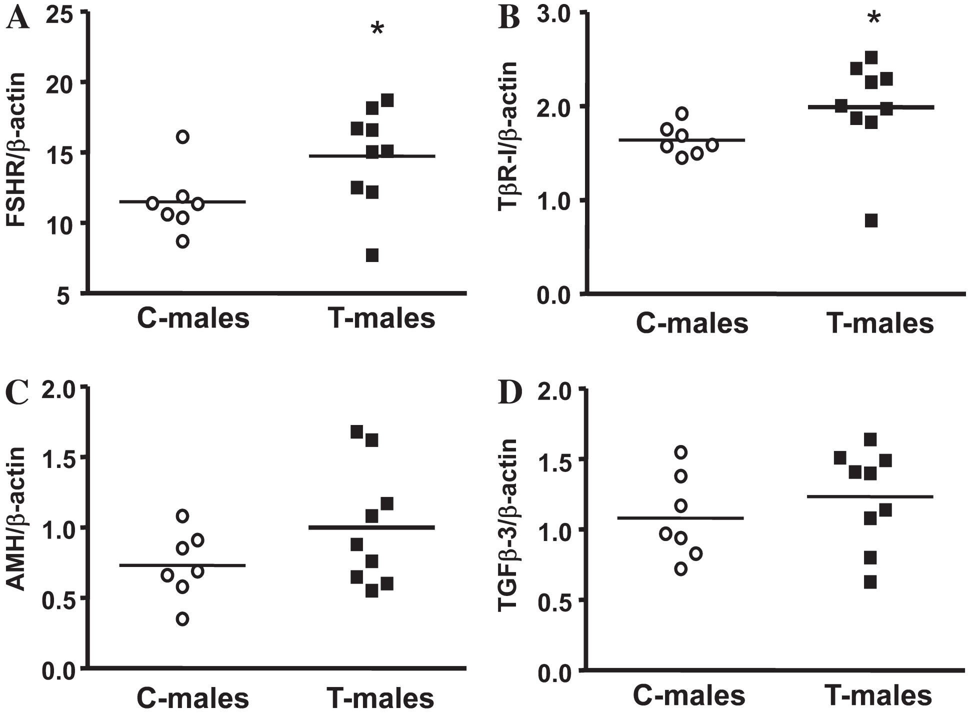

Fig. 3. Expression of the FSH receptor (FSH-R; *A*), transforming growth factor-β (TGFβ) type I receptor (TβR-I; *B*), anti-Müllerian hormone (AMH; *C*), and TGFβ3 (*D*) mRNA in testicular tissue of C (○; n = 7) and T male sheep (■; n = 9) at 42 wk of age. Expression was related to the housekeeping gene β-actin. The T males were born to mothers treated twice weekly from day 30 to 90 of pregnancy with 30 mg and from day 90 to 120 of pregnancy with 40 mg of T propionate. The horizontal line refers to the median. *Significant difference between groups ($P \leq 0.05$).

negatively with the expression level of TGFβ3. In other words, those T males showing higher levels of AMH expression showed at the same time lower levels of TGFβ3 mRNA expression. Moreover, TGFβ3 was correlated positively with FSH-R and TβR-I. In addition, the FSH-R expression level was correlated positively with the expression level of TβR-I (Table 2). None of these relationships were observed in the C males.

DISCUSSION

Results in this study show that rams prenatally exposed to an excess of testosterone exhibit higher concentrations of FSH in response to a GnRH analog and an increase in the number of Sertoli cells/seminiferous tubules but a subtle reduction in the number of germ cells compared with control rams. Additionally, T males showed an increase in the expression of FSH-R and TβR-I and a negative correlation between the expression of AMH and TGFβ3. We have shown recently that the exposure to an excess of testosterone during gestation induces an impairment in reproductive parameters that is revealed by decreased scrotal circumference and reduced sperm count and motility (38). The results of the present study suggest that a potential origin of these latter abnormalities may arise from the testicle itself, probably in its paracrine environment.

Sertoli and germ cell number. The higher quantity of Sertoli cells in T males may be due to a prolonged or enhanced proliferation of the Sertoli cells during fetal development that is triggered by the prenatal testosterone excess. In the male sheep fetus, the peak of Sertoli cell proliferation occurs during late gestation, with mitotic division being more numerous before birth than afterward (15). It has been shown that androgens are important for the number of Sertoli cells as revealed in *Tfm* mice, which lack the androgen receptor. In these mice, the Sertoli cell number is reduced (28). It has been suggested that this effect of testosterone on the fetal gonad may be through peritubular myoid cells, which do express androgen receptors during fetal development (49). A second possibility for this high number of Sertoli cells is an increased proliferation of Sertoli cells during postnatal prepubertal development. In rams, proliferation of Sertoli cells stops at 25–40 days of postnatal life (30), and this proliferation depends on FSH levels. In fact, immunization against FSH reduces the number of Sertoli cells (21). The higher levels of FSH observed in five T males after the GnRH analog is suggestive of an increase in FSH stimulation of Sertoli cell proliferation, which is consistent with the histological findings and elevated FSH-R expression in T male testes.

The reduction in the germ cell number observed in T males could be a consequence of increased apoptosis during spermat-

Table 2. *Pearson correlation between the expression level of the mRNA of different factors in testicular tissue from T males*

Factors	TGFβ3	FSH-R	TβR-I
AMH	$r = -0.7651$, $P = 0.02$*	NS	NS
TGFβ3		$r = 0.6777$, $P = 0.05$*	$r = 0.6689$, $P = 0.05$*
TβR-I		$r = 0.9550$, $P < 0.0001$*	

TGFβ3, transforming growth factor-β3; FSH-R, FSH receptor; TβR-I, TGFβ type I receptor; AMH, anti-Müllerian hormone; NS, not significant. T males were born to mothers that received twice weekly intramuscular injections of 30 mg of T propionate between days 30 and 90 of pregnancy and 40 mg of T propionate between days 90 and 120 of pregnancy. In C males, all correlations were NS. Pearson's r and P; *significant in Pearson correlation.

ogenesis (3, 4), because cell death has been estimated to result in the loss of up to 75% of the potential numbers of spermatozoa (10, 17). Another possibility to explain the decrease in the number of germ cells may be lower levels of plasma testosterone. In fact, testosterone acts as a survival factor preventing germ cell death (43). However, both the basal testosterone and LH levels were similar between the groups. Therefore, the underlying mechanisms implicated in the reduced germ cell number remain to be elucidated. Despite a different proportion of the cellular elements within the seminiferous tubule between groups, the other three-dimensional characteristics studied appeared to be similar, indicating that no cellular stage during spermatogenesis was lost. Interstitial cell population (Leydig cells) may be implicated in the previously observed difference (38), a possibility that has not yet been explored in our studies.

The FSH receptor. The importance of FSH-R in normal testicular development is crucial. The absence of expression of this receptor accounts for several alterations in the testicle, such as a decrease in the levels of circulating testosterone and a reduction in the weight of the testis, in the diameter of the seminiferous tubule, and in the number of Sertoli cells (1, 20, 22). In mutant mice, the absence of FSH-R (FSH-R$^{-/-}$ mice) causes a reduction in the diameter of the seminiferous tubule, which reveals a decreased thickness of the epithelium and a smaller lumen (11). In the T males from this study the findings were different, since an increase in the expression of FSH-R was observed, whereas no difference in the morphometry of the seminiferous tubule was detected. Interestingly, although we found an increased level of expression of FSH-R in T males, this was not correlated with the number of Sertoli cells. Thus, the increased expression of FSH-R is not a consequence of the higher number of Sertoli cells but maybe the consequence of an upregulation of the FSH-R by the increased levels of plasma FSH (40).

Other studies, in which an overexpression of FSH-R has been observed, show the presence of immature Sertoli cells still in the stage of proliferation (8), which suggests a sustained prepubertal stage. The increase in FSH-R expression shown here and the sperm abnormalities observed previously in our T males suggest that one plausible hypothesis to explain sperm deficits is a disruption in the molecular environment of the testicle. One possibility to explain the mentioned abnormalities relies on the FSH signaling pathway; however, further studies may clarify this hypothesis.

A target of FSH is AMH. The positive effect of FSH on testicular AMH production is due to both the proliferation of Sertoli cells and the increase in AMH's transcriptional activity in the Sertoli cell (27). In ovarian granulosa cells of PCOS women, i.e., in a cellular homolog of the testicular Sertoli cells, there is increased FSH-R and AMH expression (6). Despite the fact that the AMH expression levels were not significantly different between both groups, there were important correlations that suggest a hidden alteration in cytokine expression in the T males. The AMH expression level correlated negatively with TGFβ3. TGFβ3 is a key factor for the normal function of the BTB. This cytokine can be expressed mainly (but not exclusively) by the Sertoli cells (18). This cytokine has been strongly related to the Sertoli cell tight junction dynamics both in vivo and in vitro (24, 26, 48). The BTB must be disassembled (opened) and then reassembled (closed) to allow migration of preleptotene spermatocytes from the basal to the adluminal compartments of the seminiferous tubule (26). High levels of TGFβ3 are observed when BTB is disassembled (opened), and low levels of TGFβ3 are observed when BTB is reassembled (closed) (23, 24). It has been shown by in vitro studies that TGFβ3 may inhibit the expression of proteins associated with tight junctions (23), limiting the Sertoli-Sertoli adhesion. In our T males, this inverse correlation between TGFβ3 and AMH may reveal that those rams showing high TGFβ3 and low AMH expression additionally show a disassembled BTB. By contrast, those rams showing low TGFβ3 and high AMH expression may show a reassembled BTB.

An additional sign of altered cytokine environment in T males comes from the increased expression of TβR-I. TGFβs signal through type I and type II receptors (serine/threonine kinase receptors) that form heteromeric complexes. Initially, TGFβ3 binds to type II receptor, and afterwards type I is recruited (34). The increased expression of TβR-I suggests an increased signaling of TGFβ3, which is supported by a positive correlation between TGFβ3 expression and TβR-I expression. Therefore, the consequence of an increased expression of this receptor may be a more permeable BTB.

The present study suggests that testosterone excess during fetal development acts as an endocrine disruptor, generating in adult life an apparently altered local molecular and cellular environment consisting of an increase in the number of Sertoli cells and in the expression of the FSH-R and TβR-I and a reduction in germ cells. Local factors that are key components in the process of spermatogenesis and in the functional characteristics of Sertoli cells (BTB for example) are apparently deregulated due to prenatal exposure to excess testosterone. The impact of these abnormalities and other potential altered factors on the fertility of these animals remains to be investigated.

ACKNOWLEDGMENTS

We are grateful to Dr. Victor Alfaro for support in the surgeries, Dr. José F. Cox for the facilities of the Reproductive Biotechnology Laboratory of the University of Concepción, and Dr. Daniel Sandoval for the preliminary analysis of tissue samples (from the University of Concepción); to Christoph Holder for technical support; to Dr. Soroush Sharbati-Tehrani and Jutta Scholven for critical advice in the methodology of real-time PCR (from the Freie Universität Berlin, Germany); and to Dr. Vasantha Padmanabhan for helpful advice in preparation of the manuscript.

GRANTS

This work was supported by Research Project Grants Fondecyt 1050915 and DFG GZ 444 113/7/0-1.

DISCLOSURES

The authors have nothing to declare.

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