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ORIGINAL ARTICLE

Altered testicular development as a consequence of increase number of sertoli cell in male lambs exposed prenatally to excess testosterone

Pedro P. Rojas-García · Mónica P. Recabarren · Teresa Sir-Petermann · Rodolfo Rey · Sergio Palma · Albert Carrasco · Carlos C. Perez-Marin · Vasantha Padmanabhan · Sergio E. Recabarren

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Abstract The reprograming effects of prenatal testosterone (T) treatment on postnatal reproductive parameters have been studied extensively in females of several species but similar studies in males are limited. We recently found that prenatal T treatment increases Sertoli cell number and reduced spermatogenesis in adult rams. If such disruptions are manifested early in life and involve changes in testicular paracrine environment remain to be explored. This study addresses the impact of prenatal T excess on testicular parameters in infant males, including Sertoli cell number and expression of critical genes [FSH receptor (FSHR), androgen receptor (AR), transforming growth factor beta 1 (TGFB1), 3 (TGFB3), transforming growth factor beta type 1 receptor, (TGFBR1), and anti-Müllerian hormone (AMH)] modulating testicular function. At 4 week of age, male lambs born to dams 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), had a higher number of Sertoli cells/testis ($P = 0.035$) than control males (C-males) born to dams treated with the vehicle. While no differences were observed in the expression of FSHR and TGFB3, testicular TGFBR1 expression was found to be lower in T-males ($P = 0.03$) compared to C-males. Expression level of AMH, TGFB1, and AR also tended to be lower in T-males. These findings provide evidence that impact of fetal exposure to T excess is evident early in postnatal life, mainly characterized by an increase in Sertoli cell number. This could explain the testicular dysfunction observed in adult rams.

Keywords Developmental programing · Ovine · Male reproduction · Testes

P. P. Rojas-García (✉) · M. P. Recabarren · S. Palma · A. Carrasco · S. E. Recabarren
Laboratory of Animal Physiology and Endocrinology, Faculty of Veterinary Sciences, University of Concepción, Av. Vicente Mendez 595, Chillán, Chile
e-mail: pedro.rojas@udec.cl

T. Sir-Petermann
Laboratory of Endocrinology and Metabolism, Department of Internal Medicine, Western Faculty of Medicine, University of Chile, Las Palmeras 299, Interior Quinta Normal, Casilla, 33052 Correo 33, Santiago, Chile

R. Rey
Center for Research in Endocrinology Children's Hospital "R. Gutiérrez" C1425EFD Buenos Aires, Argentina, and Department of Histology, Cell Biology, Embryology, University of Buenos Aires, C1121ABG Buenos Aires, Argentina

C. C. Perez-Marin
Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, 14014 Cordoba, Spain

V. Padmanabhan
Departments of Pediatrics and the Reproductive Sciences Program, University of Michigan, Ann Arbor, MI 48109, USA

Introduction

In contrast to the clear establishment of reproductive compromise in female sheep by prenatal exposure to excess testosterone (T), little attention has been given to consequences in the male. We have found that rams exposed during their fetal life to excess testosterone are characterized by higher number of Sertoli cells in the seminiferous tubules but reduced number of germ cells, resulting in low sperm count and motility [1, 2]. These males also showed higher testicular expression of the follicle-stimulating hormone

receptor (FSHR) and increased responsiveness of FSH to a GnRH analog [2]. However, it not known when, in the development trajectory of the testis, this alteration is initially observed. One possibility could be that number of Sertoli cells are elevated since the early postnatal life. In humans, concentrations of serum anti-Müllerian hormone (AMH), are elevated in infant boys born to mothers with the polycystic ovary syndrome (PCOS) a condition endocrinologically characterized by increased levels of androgens during pregnancy [3], and remained elevated until puberty. Because Sertoli cells are the source of AMH in the testis [4, 5], these findings suggest that the increased AMH levels in these boys may be the result of an increased number of Sertoli cells [6]. However, it is unclear if these testicular and endocrine disruptions observed in sons born to mothers with PCOS, are evidenced also early in life in infant rams. The ovine mother administered with testosterone during pregnancy has been used and validated as an animal model of PCOS [7–10].

In addition to FSHR and the androgen receptor (AR), the transforming growth factor beta (TGFB) superfamily members namely transforming growth factor beta 1, 3 (TGFB1 and 3) and AMH, which are expressed in the testes, play a role in modulating testicular function and spermatogenesis [11–14]. AMH is a recognized marker of the Sertoli cell function [4, 5, 15], while TGFB1 and TGFB3 have been implicated in the regulation of tight junction in Sertoli cells, and consequently, in spermatogenesis. They appear to modify the blood testicular barrier (BTB) by diminishing the expression of tight-junction proteins [16–18]. Nevertheless, TGFBs although ubiquitously expressed in several tissues, may be accomplishing important tasks in the developing testis since they are highly expressed in immature testis, and its expression decreases during puberty when spermatogenesis begins. Similar pattern of expression applies to the TGFB receptor type 1 (TGFBR1) [19]. Previously, we found expression of TGFBR1 to be increased in adult rams born to mothers exposed to excess testosterone (T). Because these studies were conducted with adult male sheep, the developmental onset and progression of this disruption are not known. Therefore, the present study tested the hypothesis that fetal exposure to excess testosterone causes an early disruption in testicular cell population and in locally expressed mediators of testicular function.

Materials and methods

Prenatal testosterone (T) treatment

Twelve Suffolk-Down lambs of 4 weeks of age were included in this study. Six lambs were born to mothers that received twice weekly intramuscular injections of 30 mg of testosterone propionate (TP; Sigma, St Louis, USA) in cottonseed oil from days 30 and 90 of pregnancy, and 40 mg from days 90 to 120 of pregnancy, using a previously validated experimental protocol [1, 2]. These animals will be referred to from now on as T-males. Six lambs were born to mothers that received only the vehicle twice weekly from day 30 to 120 of gestation (C-males). Mothers were maintained on pasture and supplemented with hay and concentrated pelleted food to meet the increased needs of the pregnant sheep. Pelleted food consisted of oat, corn, wheat, gluten feed, gluten meal, soybean, meal, fish meal, sunflower meal, and mineral salts and contained (based on dry matter) 18 % protein, 11 % crude fiber, 2 % fat, and 2450 kcal/kg (Glovigor; Compañía Molinera El Globo, Santiago, Chile) [20]. Newborn lambs were weighed at birth. Eight hours after delivery the umbilical cord was cleaned and disinfected with 10 v/v of povidone-iodine [20]. Lambs were maintained with their mothers under natural photoperiod and with free access to water. Only males born from singleton pregnancies were used in this study. All lambs were born by normal delivery and after full length gestation, during mid-September (beginning of Spring in the Southern hemisphere). All procedures for management and experimental methodologies were approved by the Ethical Committee in Animal Research of the Faculty of Veterinary Sciences of the University of Concepción, and were performed according to the APS Guiding Principles of Care and Use of Animals (2002).

Basal plasma FSH, LH, and T determinations

Blood samples (3-ml) were obtained from the jugular vein of each male to determine plasma concentrations of FSH, LH, and T, just prior to surgery. Blood samples were centrifuged at 1,000 g for 15 min, and plasma separated and stored at −20 °C until hormone measurements. 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. The minimal detectable FSH dose, defined as 90 % of buffer control, was 0.1 ng/ml. Intra- and interassay coefficients of variation were 5 and 8 %, respectively. LH was measured by RIA using radioiodinated ovine LH (LER 1374-A provided by Dr. Leo Reichert Jr.), ovine antiserum CSU-204 (provided by Dr Gordon Niswender), and ovine LH standard oLH-S25 (NIADDK, USA) in 200-µl duplicates. The minimal detectable limit of LH assay was 0.1 ng/ml and the intra- and inter-assay coefficients of variation were 6 and 11 %, respectively. Circulating levels of T was measured by RIA using a commercial kit (DIAsource ImmunoAssays, Belgium). The minimal detectable

limit of the T assay was 0.07 ng/ml and the intra- and inter-assay coefficients of variation 5 and 9 %, respectively.

Tissue collection

At 4 weeks of age, testes were removed from each lamb for histology and isolation of total RNA. In brief, lambs were anesthetized with halothane and the testes were surgically removed, cleaned of surrounding tissues, and weighed. One testis was cut into ~ 0.5 cm^3 pieces with a sterile surgical blade and processed for histological analysis, and the other testis was immersed in liquid nitrogen and then stored at –80 °C for subsequent RNA isolation.

Testicular histology and evaluation

For histology, tissue samples were immersed in Bouin's fixative solution for 24 h at room temperature and then rinsed, dehydrated, and embedded in paraffin. Sections (3 μm) were stained with hematoxylin-eosin and analyzed under a light microscope using a 10× ocular lens and a 40× objective, with a digital camera (model DSC-P71; Sony). Images were processed and analyzed with the NIS-Elements BR 3.00 software. For the morphometric analysis, 10 sections, 20 μm apart, were analyzed per animal using a 40× objective. From each section, 3 randomly selected microscopic fields were selected resulting in a total of 30 visual fields per testis for analyses. In all samples, the fields analyzed include sections from the tunica albuginea to the mediastino. Histological analysis focused on the following parameters, as previously described [21].

Diameter of the seminiferous tubule

A minimum of 45 seminiferous tubules were evaluated per animal. Cross sections, where perpendicular diameters of seminiferous tubule did not differ by more than 10 % between major and minor diameters, were used for diameter assessment. Both diameters were then averaged, for statistical analysis.

Volume of testicular compartments

Tissue area occupied by tubular and interstitial tissue in each of the selected field was determined using a 100× objective. Area of each compartment was expressed as percentage of total tissue area (sum of the areas of both compartments was set as 100 %). This information was used to express the relative volume of each compartment in the whole testicle.

Length of seminiferous tubules

This parameter was calculated by dividing the total volume occupied by the tubular tissue in the testis by the average area of seminiferous tubules.

Number of Sertoli and germ cells

Sertoli cell nucleoli and germ cell nuclei were counted in each visual field with a 100× objective. The number obtained for Sertoli cells and for germ cells was introduced in the Floderus' formula [22]. This allowed determination of the number of cells per testicular volume. Finally, this value was multiplied by the total volume of the seminiferous tubules to obtain the number of Sertoli and germ cells present in the testis [21, 23]. The total seminiferous tubule volume was calculated by dividing the percentage of the tubular compartment (obtained as described above) by 100 and then multiplying this by the testicular volume (testicular weight dividing by 1.038, [24]).

Total RNA isolation and reversed transcription

Testicular tissue was homogenized using the Precellys 24 instrument (bertin Technology, France). For total RNA isolation, the Invisorb Spin Tissue RNA Mini Kit (Invitek, Berlin, Germany) was used as per manufacturer's instructions. Total RNA yield was quantified photometrically at 260 nm using the BioPhotometer (Eppendorf, Germany). The quality of the RNA was verified after electrophoresis on formaldehyde containing 1 % (w/v) agarose gel with ethidium bromide staining. To remove any DNA contamination, DNA digestion was performed before reverse transcription (RT). DNase treatment was carried out in a total volume of 12 μl containing 3 μg of total RNA, and 3 U of DNase (Invitrogen, Brazil). The reaction mixture was first incubated at 37 °C for 30 min, followed by addition of 3 μl of 25 mM of EDTA. The sample mixture was heated for an additional 5 min at 75 °C and placed immediately on ice for 5 min. Fifteen microliters premix containing 200 U reverse transcriptase enzyme (SuperScript, Invitrogen, Brazil), 2.5 μM of random hexameres (Invitrogen, Brazil), 0.666 mM of each dNTP (Promega, USA) and 1× of the supplied RT buffer were added to each RNA sample. Samples without reverse transcriptase enzyme were processed in parallel to monitor the absence of any genomic DNA. The reverse transcription was performed at 25 °C for 10 min, 42 °C for 1 h, and then at 90 °C for 2 min. The cDNA obtained was aliquoted in 15 μl volumes and stored at –20 °C until analysis by real time PCR.

Real time PCR

In preliminary experiments, the expression of all relevant genes was examined by standard gradient PCR using a gradient thermocycler (Mastercycler gradient, Eppendorf AG, Hamburg, Germany) to confirm the expected amplicon sizes, as well as to determine the optimal annealing temperature for each pair of primers. The primer sequences, annealing temperature, length of PCR product used in the present study are indicated in Table 1.

Real time PCRs were performed in a Rotor-Gene RG-6000 thermocycler (Corbett Research, Mortlake, Australia). One microliter of cDNA (100 ng) was used as template for real time PCR reaction, which contained 0.2 µl of 50XSYBR Green, 5 µl of 2XSensiMix solution (Quantace Ltd, London, UK), and 0.4 µM of the 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 genes was 45. For mRNA quantification, a dilution series with known quantities of the specific PCR product was amplified along with the samples as a standard. Samples were measured in triplicate and standard curve in duplicate in each run. The PCR products applied as standards were generated by conventional block RT-PCR and purified using the E.Z.N.A Gel Purification Kit (Omega Bio-Tek, Inc., USA) as described by the manufacturer. Concentration of the purified PCR product was estimated in duplicate using the BioPhotometer. As negative controls, reactions containing no template (sterile RNase-DNase free water) or 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. As part of validation, the suitability of 18S ribosomal subunit and B-actin for use as internal control for normalization was tested. Because expression of 18-S ribosomal subunit did not differ between C- and T-male, 18S ribosomal subunit was chosen for use as the internal control. The results of real time PCR are presented as a ratio between the specific mRNA gene and the 18-S ribosomal subunit.

Statistical analysis

All data are expressed as mean ± SE or median, as indicated in the text or figures. Changes in morphometric parameters were compared between C and T-males by Students *t* test or the non-parametric Mann–Whitney test, as appropriate. The mRNA expression data were analyzed using Mann–Whitney non-parametric test. Based on previous findings [2], a one-tailed option for P value was used to analyze the number of Sertoli and germ cells, and the expression of FSHR. All analyses were performed using the GraphPad Prism 4.0 software. A P value of ≤ 0.05 was considered to be significant.

Results

Body and testicular weight

Body weight was similar between T-males and C-males at the time of birth (5.1 ± 0.41 vs. 4.6 ± 0.25 kg, for T- and

Table 1 Detailed sequence for primer used in real time PCR

Gene	Primers	Annealing (°C)	PCR product length (bp)	Reference
18S rRNA	F: 5′-TCA AGA ACG AAA GTC GGA GG-3′ R: 5′-GGA CAT CTA AGG GCA TCA CA-3′	60	493	Ambion, Wiesbaden
FSHR	F: 5′-GAG AGC AAG GTG ACA GAG ATT CC-3′ R: 5′-CCT TTT GGA GAG ACT GAA TCT T-3′	54	340	[2]
AR	F: 5′-GAG GAG CCA GCC CAG AAG-3′ R: 5′-AAG GAG TCA GGT TGG TTG TTG-3′	60	134	Genbank accession No. AF105713
AMH	F: 5′-CTA TGA GCA GGC CTT CCT GG-3′ R: 5′-CCT CCA GGT GCA GGA CCA CC-3′	63	176	[25]
TGFB1	F: 5′-CAT GAA CCC AAG GGG TAC C-3′ R: 5′-GCA CGA TCA TGT TGG ACA AC-3′	59	220	Genbank accession No. M36271
TGFB3	F: 5′-AGA ACT GCT GTG TGC GTC C-3′ R: 5′-GCA GGA CTT CAC CAC CAT G-3′	63	290	[2]
TGFBR1	F: 5′-ATA ACC GCA CTG TCA TTC ACC-3′ R: 5′-TGA CAC CAA CCA GAG CTG AG-3′	63	395	[2]

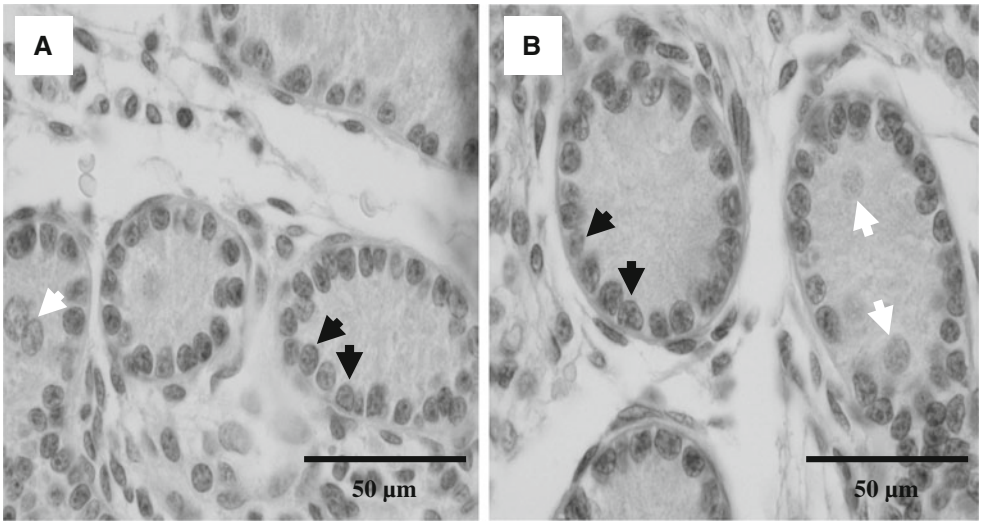

Fig. 1 Histology of testes from C-male **a** and T-male **b** lambs at 4 weeks of age. *Arrows* indicate Sertoli cells (*dark*) and gonocite (*white*) in the seminiferous tubules. The *bar* corresponds to 50 µm

C-males, respectively). At 4 weeks of age, body weight was 11.85 ± 0.86 kg in T-males and 9.5 ± 0.69 kg in C-males. Testicular weight was also similar between T-males (2.03 ± 0.26 g) and C-males (1.55 ± 0.11 g) at 4 weeks of age.

Circulating concentrations of FSH, LH, and T

Plasma FSH levels were similar between C- and T-males. Mean plasma levels were 1.73 ± 1.01 ng/ml in C-males and 1.42 ± 0.82 ng/ml in T-males. Plasma LH levels were similar between the two groups. Mean plasma LH levels were 0.46 ± 0.05 ng/ml in T-males and 1.04 ± 0.35 ng/ml in C-males. Plasma T concentrations were below the detection limit of the assay in 2 of 6 C-males and in 4 of 6 T-males. The mean T concentrations in those with detectable T levels were in the low prepubertal range for both groups (0.115 ± 0.015 ng/mL in C-males and 0.25 ± 0.10 ng/ml in T-males).

Histological findings

Seminiferous tubules without lumen were observed in C-males and T-males (Fig. 1). Sertoli cells were located close to the basal membrane with oval or rounded shapes and had one or two nucleoli. Germ cells were large, with a clear cytoplasm and a large nucleus with one nucleolus, and were mainly located toward the center of the seminiferous tubules. The Sertoli cell number/testis was higher in T-males compared with C-males (Fig. 2, $P = 0.035$). No significant differences were observed between T-males and C-males in seminiferous tubule volume, diameter, and length, number of germ cells, germ cell/Sertoli cell ratio, and interstitial tissue volume (Fig. 2).

mRNA expression of AR, FSHR, and TGFBs family members

The expression of TGFBR1 in testicular tissue was significantly lower in T-males compared to C-males ($P = 0.03$, Fig. 3). Conversely, no significant differences between groups were observed in the mRNA expression levels of FSHR, and TGFB3. The mRNA expression of AR, AMH, and TGFB1 was similar between both groups (Fig. 3).

Discussion

The findings from the present study demonstrate that exposure to excess T during fetal life increases the number of Sertoli cells in the infant lamb testis. In concert with our previous findings in adult T-male rams, which manifested increased Sertoli cell number and impaired spermatogenesis [2], these data support the hypothesis that reprogramming during fetal development by prenatal T excess results in an early alteration in the cell population of the seminiferous tubules. Such early perturbations, together with the lower expression of the TGFBR1 observed in T-males may contribute toward the reduction in the number of germ cells [2], decreased scrotal circumference and a reduced sperm count and motility [1] found in adult T-males.

Our finding that testes of newborn T-males have a tendency to be heavier compared to C-males is in agreement with results reported by Bormann et al. [26], using a different window of T treatment (days 30–90 of gestation). Considering that Sertoli cells of the seminiferous tubules are the most conspicuous component of the testis in fetal and prepubertal periods of life, the increased number of

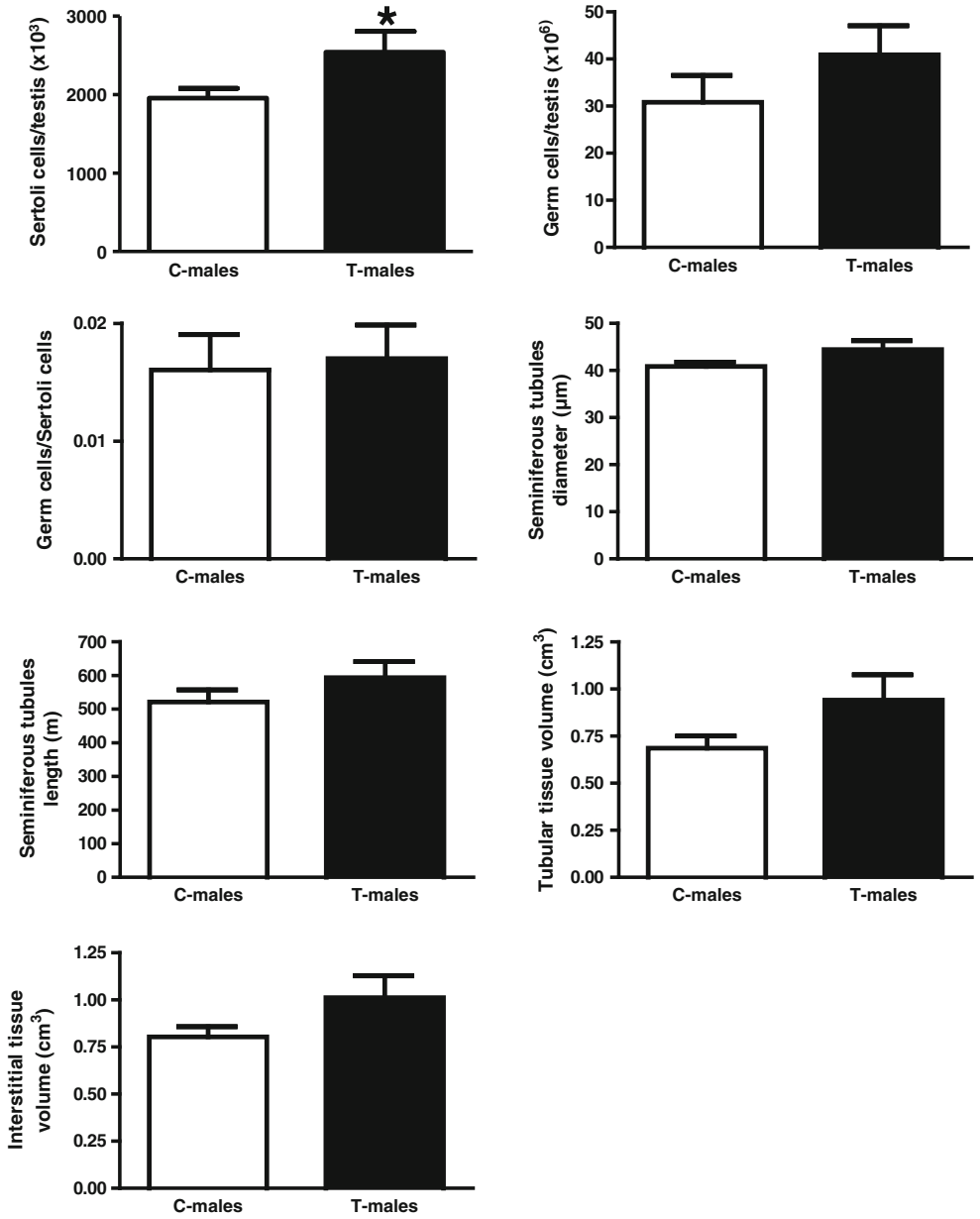

Fig. 2 Mean (±SE) dimensional characteristics and cellular quantification of different testicular elements in Suffolk-Down C-males and T-males at 4 weeks of age. T-males were born to mothers that received twice weekly intramuscular injections of 30 mg of testosterone propionate between days 30 and 90 of pregnancy and 40 mg TP between days 90 and 120 of pregnancy. C-males are corresponding controls. *Asterisk* indicates statistically significant difference ($P \leq 0.05$, unpaired Student's t test)

Sertoli cells supports increased testis weight. In mammals, increased or decreased testis volume before pubertal onset is directly associated with increased or decreased Sertoli cell numbers, respectively [21, 27–29]. Although our experimental design did not address the mechanisms contributing to increase Sertoli cell number, there is compelling evidence that androgens induce Sertoli cell proliferation in early stages of life [30–32]. As such, the increase in Sertoli cell number appears to be the direct reflection of the prenatal testosterone treatment. The proliferative effect of androgen on fetal Sertoli cells is likely mediated through the peritubular myoid cells which express AR during fetal development [33], since Sertoli cells do not express the AR until postnatal life [34–38].

The findings from this study, which document early increase in Sertoli cell number with no change in germ cell number during early postnatal life as opposed to reduced germ cell number during adulthood [2] suggests that Sertoli cell impairment is the initial target of prenatal T excess and that the loss of germ cell observed in adult T-males may

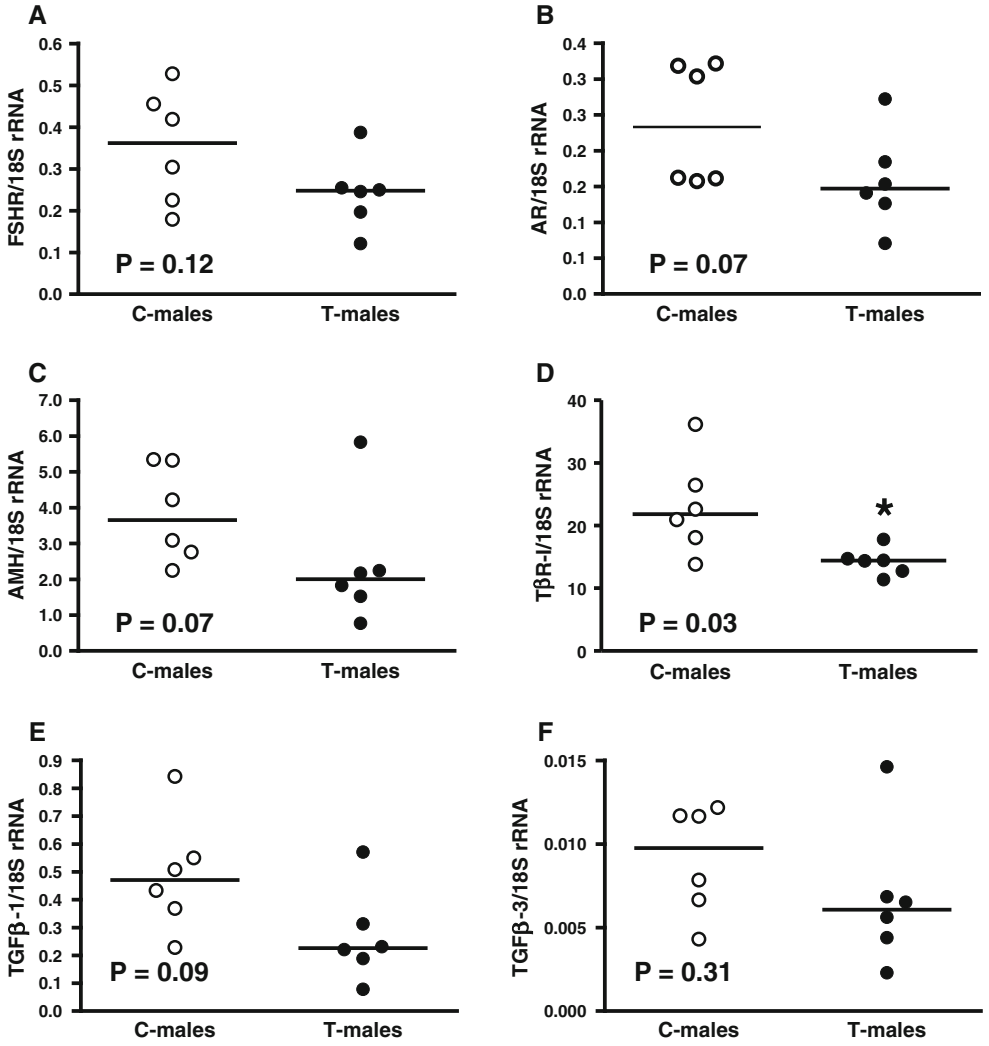

Fig. 3 mRNA expression of FSHR **a**, AR **b**, AMH **c**, TGFBR1 **d**, TGFB-1 **e**, and TGFB-3 **f** in testicular tissue of C-males ($n = 6$) and T-males ($n = 6$) at 4 week of age. 18S rRNA was used as internal control. *P* values shown are from non-parametric Mann–Whitney test. The *horizontal line* refers to the median. *Asterisk* indicates significant difference between groups

arise near puberty, when spermatogenesis begins. Evidence to date indicates that spermatid number in adults is proportional to the numbers of Sertoli cells produced during perinatal development [39]. The finding of increased Sertoli number in the face of decreased sperm count [1] in adult prenatal T-treated sheep disagrees with this premise and strongly supports disrupted Sertoli cell function as a contributing factor. To what extent the early perturbation in Sertoli cell number and function contribute to later disruption in spermatogenesis remains to be determined.

Lack of change in expression of the FSHR in the infant testes as opposed to increased FSHR expression in postpubertal males [2] suggests later development of this disruption. Evidence exists to indicate that FSHR expression in the Sertoli cell membrane in fetal, newborn, and adult testis differs depending on the time point studied [30, 38]. For example, the most prominent FSH-dependent testicular growth in rams occurs between postnatal days 60 and 90, when an increase in the number of FSH binding sites is evident in the testis [40], in essence one or two months later from our study.

Altered TGFB signaling in Sertoli cells may be a contributing factor in subsequent impairment in testicular function evidenced as decrease in sperm number [2]. TGFB regulates several testicular functions like spermatogenesis, steroidogenesis, extracellular matrix synthesis [12, 16, 41, 42]. Recent studies have emphasized the effects of TGFB*s* in maintaining the integrity of the blood-testis barrier [16, 43–46]. The reduced TGFBR1 expression found in T-males at 4 weeks of age suggests a potential

impact of prenatal T treatment on the postnatal development of the blood–testis barrier.

The low plasma LH levels in T-males and lack of difference between treatment groups are consistent with findings from an early study, which involved 60 mg of TP treatment from days 30 to 90 of pregnancy [47]. The similarity in plasma levels of FSH, LH, and T in C- and T-males at the time of the study support the hypothesis that the higher number of Sertoli cells was the result of direct effect of the prenatally T excess on the testis and a change in endogenous circulating levels of T may not be a contributing factor. Since LH and T are released in a pulsatile manner, the absence of changes in these hormones should be viewed with caution, as they come from a single time point measurement.

In summary, our findings support the hypothesis that prenatal exposure to excess testosterone impairs the developmental ontogeny of the testis by having an impact on the number of Sertoli cells and the expression of key mediators of testicular differentiation. The findings of Sertoli cell disruption, evident as early as 4 weeks of age, are supportive of a role for impaired Sertoli cell function in reprograming of testicular differentiation.

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

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