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Puberty arises with testicular alterations and defective AMH expression in rams prenatally exposed to testosterone

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ABSTRACT

The male gonadal tissue can be a sensitive target to the reprogramming effects of testosterone (T) during prenatal development. We have demonstrated that male lambs born to dams receiving T during pregnancy—a model system to the polycystic ovary syndrome (PCOS)—show a decreased number of germ cells early in life, and when adult, a reduced amount of sperm and ejaculate volume. These findings are a key to put attention to the male offspring of women bearing PCOS, as they are exposed to increased levels of androgen during pregnancy which can reprogram their reproductive outcome. A possible origin of these defects can be a disruption in the expression of the anti-Müllerian hormone (AMH), due to its critical role in gonadal function at many postnatal stages. Therefore, we addressed the impact of prenatal T excess on the expression of AMH and factors related to its expression like AP2, SOX9, FSHR, and AR in the testicular tissue through real-time PCR during the peripubertal age. We also analyzed the testicular morphology and quantified the number of Sertoli cells and germ cells to evaluate any further defect in the testicle. Experiments were performed in rams at 24 wk of age, hence, prior puberty. The experimental animals (T-males) consisted of rams born to mothers receiving 30 mg testosterone twice a wk from Day 30 to 90 of pregnancy and then increased to 40 mg until Day 120 of pregnancy. The control males (C-males) were born to mothers receiving the vehicle of the hormone. We found a significant increase in the expression of the mRNA of AMH and SOX9, but not of the AP2, FHSR nor AR, in the T-males. Moreover, T-males showed a dramatic decrease in the number of germ cells, together with a decrease in the weight of their testicles. The findings of the present study show that before puberty, T-males are manifesting clear signs of disruption in the gonadal functions probably due to an alteration in the expression pattern of the AMH gene. The precise way by which T reprograms the expression of AMH gene remains to be established.

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1. Introduction

The postnatal aftermath of a continuous prenatal exposure to androgens has gained broad interest in the light of the polycystic ovary syndrome (PCOS), a condition affecting a significant number of women during their reproductive age [1], hence the increased number of studies in animal models [2]. In this syndrome, hyperandrogenemia through androstenedione, testosterone (T) and dehydroepiandrosterone sulfate, are a persistent feature during pregnancy [3], even after the clinical managements performed to the patients to restore their fertility [4]. The latter is not only affecting the developing female fetus, as it has been a matter of major studies in this field, but also the males fetuses, leading them to the possibility to suffer of deleterious effects on their reproductive system. For instance, sons born to women with PCOS show increased levels of circulating anti-Müllerian hormone (AMH) [5]. In addition, it has been reported recently that AMH levels are increased in male relatives of women with PCOS [6], at ages when it should be decreased. AMH is a cytokine produced by the Sertoli cells that reflects the degree of maturity and functioning of the testis during prepubertal stages. Elevated levels of AMH predicts a still immature testis or a delay in growth and puberty [7], reflecting specifically Sertoli cell dysfunction [8,9].

The follicle-stimulating hormone (FSH) and testosterone play key roles in regulating AMH levels and hence, in the differentiation and function of Sertoli and germ cells. FSH has been found to upregulate AMH expression through a nonclassical cAMP-PKA pathway involving transcription factors AP2 [10] and SOX9 [11]. In fact, AP2, may also play a role in modulating testicular function [12]. At puberty, AMH start to decrease as a consequence of the prevailing inhibitory effect of testosterone over the stimulatory effect of FSH over AMH [13]. As in humans, animal models for the study of AMH also reveal its persistence in immature testis, as is the case in horses [14]. Mice lacking the FSH receptor (FSHR) and the androgen receptor (AR) are characterized by reduced Sertoli cell and germ cell number as well as impaired Sertoli cell activity and spermatogenesis [15,16]. In rams, there are reports stating that AMH levels are not an actual predictor of the number of Sertoli cells [17], however, more studies in this field are needed.

Prenatal exposure to testosterone in animals has become a unique and helpful model to study the etiology and consequences of the PCOS in the offspring [18]. Similarly as in human population, studies in animals involving male offspring are lagging behind. Using a sheep model of PCOS in which pregnant ewes are treated with testosterone to create hyperandrogenemia, we have shown that newborn lambs and adult rams born to these ewes have an increased number of Sertoli cell per seminiferous tubules, suggesting AMH dysfunction [19,20]. An increased response of LH to a GnRH analog without the corresponding increase in the LH receptor in the testicular tissue, plus a reduced testosterone-to-LH ratio prior puberty, demonstrate additional defects in the reproductive axis [21]. The latter may underlie the alterations in the sperm characteristics that could be caused, in turn, by a potential persistence of AMH during the peripubertal period. Moreover, adult rams later show a reduction in germ cell number, sperm count, and motility [22]. In previous studies, we found an increase in FSH plasma levels and an increase of the expression of mRNA of FSHR but not of AMH in adult rams exposed prenatally to excess T [19]. However, we still do not have the information regarding testicular features either morphological or molecular before puberty arises, in essence, when AMH expression should be decreasing.

Therefore, our objectives were to study the morphological feature of the seminiferous tubule, the number of both Sertoli and germ cells, and key factors related to AMH synthesis before puberty, as elements that could explain the reproductive defects observed in adult rams prenatally exposed to testosterone. We hypothesize that AMH is dysregulated in the testicle and that the defects observed at 4 wk of age in this same animal model are maintained prior puberty.

2. Materials and methods

2.1. Animals

The study was conducted at the Chillan campus of the University of Concepción, Chile (36° 36′ south latitude, 71° 30′ west longitude, 144 m above the sea level). We analyzed male sheep at the age of 24 wk, which correspond approximately to a peripubertal age. We have previously studied this animal model at the age of 4 wk (neonatal age) and at 42 wk of age (adult). To obtain the animals for this study, female sheep were mated after estrous synchronization to concentrate pregnancies and births in a fixed period of time. Pregnant ewes 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, sun flower meal, and mineral salts. Based on dry matter, pellets contained 18% protein, 11% raw fiber, 2% fat, and 2,450 kcal/kg (Glovigor; Compañía Molinera El Globo) [23]. Pregnant dams were treated with testosterone propionate from Day 30 to 120 of pregnancy and control dams with the vehicle of the hormone. All lambs were born by normal delivery and at term, within a period of 2 wk during September (beginning of spring in the Southern hemisphere) and were weaned at 8 wk of age. Therefore, we studied experimental animals born to mothers treated with testosterone propionate during pregnancy (T-males group, $n = 12$) and control animals born to mothers receiving the vehicle of the hormone (C-males group, $n = 10$) [22].

2.2. Prenatal testosterone treatment

Pregnant ewes received testosterone propionate (TP; steraloids) dissolved in vegetable oil as vehicle twice weekly by intramuscular injections. Doses were 30 mg from Day 30 to 90 of pregnancy, and 40 mg from Day 97 to 120 of pregnancy, as previously described [19,22]. Injections were performed Mondays and Thursdays at 8 Am. There was veterinary supervision to the pregnant animals

at all times and the recommended guidance for care and use of laboratory animals was strictly followed [24].

2.3. Testicular tissue collection

For procuring tissues at 24 wk of age, the male sheep were anesthetized with isoflurane (Baxter, Healthcare Corporation), testicles were surgically removed, and cleaned of surrounding tissues like the epididymis and vas deferens before weighing [19,20]. One testicle was cut into ~0.5 cm^3 pieces with a sterile surgical blade and processed for histological analysis and the other testicle was cut and immersed in liquid nitrogen and then stored at −80°C for subsequent RNA isolation.

2.4. Testicular histology

Tissue samples procured for histology were immersed in Bouin's fixative solution for 24 h at room temperature and then rinsed, dehydrated, embedded in paraffin, and sectioned at 4 μm thickness. For the morphometric analysis, sections (5 per animal separated from the next by 400 μm) were stained with hematoxylin-eosin and analyzed under a light microscope (Leica DM 2000). Images were captured using a digital camera (Leica DFC 295) and analyzed with the LAS v3.8 software (Leica). Testicular analysis included the following parameters: numbers of gonocytes, spermatogonia, primary spermatocytes, elongating spermatids, and Sertoli cells per testis, according to Floderus [25]. In brief, we counted the number of Sertoli cells or of germinal cells observed per seminiferous tubule in each field. A field is an observable area (μm^2) delimited by the magnification of the sample in the microscope (40×). We took a picture every 10 fields but counted the cells only in those containing round or nearly round seminiferous tubules. We chose to count one tubule per field and the total number of fields counted represented the total area for each animal. To calculate the number of cells per testis, we divided the total number of cells by the total amount of area. The result was then divided by 4.8, which represents the sum of the width of the histological sample (4 μm) plus, the size of the nucleolus (1 μm), minus 2 times the size of the lost polar cap (10% of the size of the nucleolus). The given result expresses the number of cells per μm^3. Then we multiplied that result by the weight of the testis in cm^3, considering a density of 1 for the testis. The latter calculation gave us the number of cell per testis. From each of the round seminiferous tubule, we determined the tubular area and tubular diameter, lumen diameter and height of seminiferous epithelium.

2.5. RNA isolation and reverse transcription (RT)

Testicular tissue homogenization and total RNA isolation were carried out as described in Rojas-Garcia et al [19,20]. To remove any DNA contamination, DNA digestion was performed before reverse transcription. DNase treatment was carried out in a total volume of 12 μL containing 1 μg of total RNA, and 1 U of DNase (Invitrogen). The reaction mixture was first incubated at 37°C for 30 min, followed by addition of 1 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. Eighteen microliters of premix containing 200 U reverse transcriptase enzyme SuperScript (Invitrogen), 2.5 μM of random hexameres (Invitrogen), 0.666 mM of each dNTP (Promega), and 1x 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 volume and stored at −20°C until analysis by real-time PCR.

2.6. Real-time PCR

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). One microliter of cDNA (33 ng) was used as template for real-time PCR reaction, which contained 0.2 μL of 50x SYBR Green, 5 μL of 2x SensiMix solution (Quantace Ltd), 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 3-step amplification—including denaturation at 95°C for 15 s—the corresponding annealing temperature specific for each factor (Table 1) for 20 s, and extension at 72°C for 30 s, a melting curve program (50°C–99°C) with continuous fluorescence measurement, and a final cooling

Table 1
Detailed sequence, annealing temperature, and length of the primers for real-time PCR.

Gene	Primers	Annealing (°C)	Length (bp)	Reference
GADPH	F: 5′-GGC TGC CCA GAA CAT CAT CC-3′ R: 5′-CTC CAG GCG GCA GGT CAG A-3′	55	148	[26]
AP2	F: 5′-ATA GAG GAA CAG AAG TCA G-3′ R: 5′-AAG ATT CAG CCT TAT ATT GG-3′	56	114	Genbank accession no. NM_001009745
SOX9	F: 5′-ATG TAG TGT ATC ACT GAG TC-3′ R: 5′-AGA TTA AGG TCT GTC AGT GG-3′	56	199	[27]
AMH	F: 5′-CTA TGA GCA GGC CTT CCT GG-3′ R: 5′-CCT CCA GGT GCA GGA CCA CC-3′	63	176	[28]
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	[19]
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. AF 105713

step to 40°C. For mRNA quantification, a dilution series with known quantities of the specific PCR product was amplified along with the samples as a standard [19,20]. As negative controls, reactions containing no template (sterile RNase-DNase free water) or without reverse transcriptase were included to exclude any PCR products derived from contaminations with genomic DNA. The content of each specific mRNA was normalized to a housekeeping gene. The PCRs from the peripubertal male performed at different times and normalized to the housekeeping gene that did not change with treatment (GAPDH). The results of real-time PCR are presented as a ratio between the specific mRNA gene and the housekeeping gene.

2.7. Statistical analysis

The morphometric parameters and the expression of the mRNA by real-time PCR between C-males and T-males was compared through Students *t* test when variances were similar or the non-parametric Mann-Whitney test, when variances were statistically different according to the F test. A value of $P \leq 0.05$ was considered to be statistically significant. The data are expressed as mean ± SE or median, as indicated in the legends of the figures. All analyses were performed using the GraphPad Prism 6.0 software.

3. Results

3.1. Body and testicular weight

Birth weight was similar between T- and C-males (3.82 ± 0.21 and 4.28 ± 0.14 Kg, respectively). At 24 wk of age, body weight of T-males (24.66 ± 1.26 Kg) was significantly reduced relative to the C-males (29.63 ± 1.85 Kg). T-males exhibited a lower testicular weight (52.47 ± 14.23 g) compared with testicular weight in C-males (81.09 ± 10.11 g), at 24 wk of age.

3.2. Histological findings

The number of Sertoli cells/testis displayed by the T-males (n = 12) was not significantly different ($P = 0.065$; Mann-Whitney signed rank test) to those from C-males (n = 10) at 24 wk of age. However, when the number of Sertoli cells was expressed in terms of tubular cross section, a significant difference was observed (Table 2).

The T-males showed a reduced number of germ cells per testis, including spermatogonia, spermatocytes, and round spermatids. The analysis of the number of cells per tubule backs up the differences observed per testis, where the reduction in spermatids was significantly reduced (Table 2). According to the histological sections, in some seminiferous tubules of the T-males, only Sertoli cells were evident with the absence of germinal epithelium. A representative photomicrography of one T-male shows a seminiferous tubule devoid of any germinal cells, which is a complete different feature from C-males, which show evidence of an active spermatogenesis process (Fig. 1).

The T-males exhibited a significant decrease in the dimensions of different elements of the seminiferous tubule's epithelia. The diameter as well as the height and the area of the tubule were reduced (Table 3). The diameter of the lumen was similar between the groups (Table 3).

3.3. mRNA expression of AMH and key regulators of AMH

The mRNA expression of AMH (Fig. 2) and of its transcription factor SOX9, was significantly higher in T-males compared with the corresponding controls, whereas for AP2, another transcription factor for AMH, the expression was not different between both groups. Eliminating an outlier point from the T-males did not change the statistically significant difference between the groups. FSHR expression was 0.056 ± 0.005 in C-males and 0.051 ± 0.003 in T-males. Androgen receptor expression was 0.068 ± 0.003 in C-males and 0.064 ± 0.008 in T-males. The expression of both the receptors was not significantly different.

4. Discussion

The present findings show that near puberty, rams born to dams treated with testosterone during pregnancy have morphological, cellular, and molecular disturbances that could anticipate the reproductive defects observed later in adulthood in this animal model. The defects include smaller testicles, obliterated seminiferous tubules, reduced number of germinal cells, and a trend for an increased number of Sertoli cells, plus reduced dimensions of the seminiferous tubule's epithelium. A concomitant altered AMH/SOX9 expression complement the above features, probably explaining the results observed.

Table 2
Seminiferous tubule's cellular counting (mean ± SE) in 24-wk-old Suffolk Down rams prenatally exposed to testosterone (T-males) or vehicle (C-males).

Cell type	Number of cell per testis			Number of cell per cross section		
	C-males	T-males	Value of *P*	C-males	T-males	Value of *P*
Sertoli cells	$8.37 \times 10^9 \pm 1.3 \times 10^9$	$2.64 \times 10^{10} \pm 7.6 \times 10^9$	0.065[a]	11.04 ± 1.4	18.27 ± 2.3	<0.05[b]
Spermatogonia	$2.36 \times 10^{10} \pm 5.4 \times 10^9$	$1.25 \times 10^{10} \pm 1.6 \times 10^9$	<0.05[a]	30.44 ± 5.3	15.72 ± 3.1	<0.05[b]
Spermatocyte	$2.84 \times 10^{10} \pm 5.4 \times 10^9$	$1.09 \times 10^{10} \pm 1.5 \times 10^9$	<0.01[a]	36.88 ± 5.3	14.19 ± 3.4	<0.01[b]
Spermatids	$4.79 \times 10^{10} \pm 1.3 \times 10^{10}$	$7.02 \times 10^9 \pm 2.6 \times 10^9$	<0.01[a]	61.96 ± 13.4	12.21 ± 5.2	<0.001[a]

T-males, n = 12.
C-males, n = 10.
[a] Mann-Whitney signed rank test.
[b] Unpaired Student *t* test.

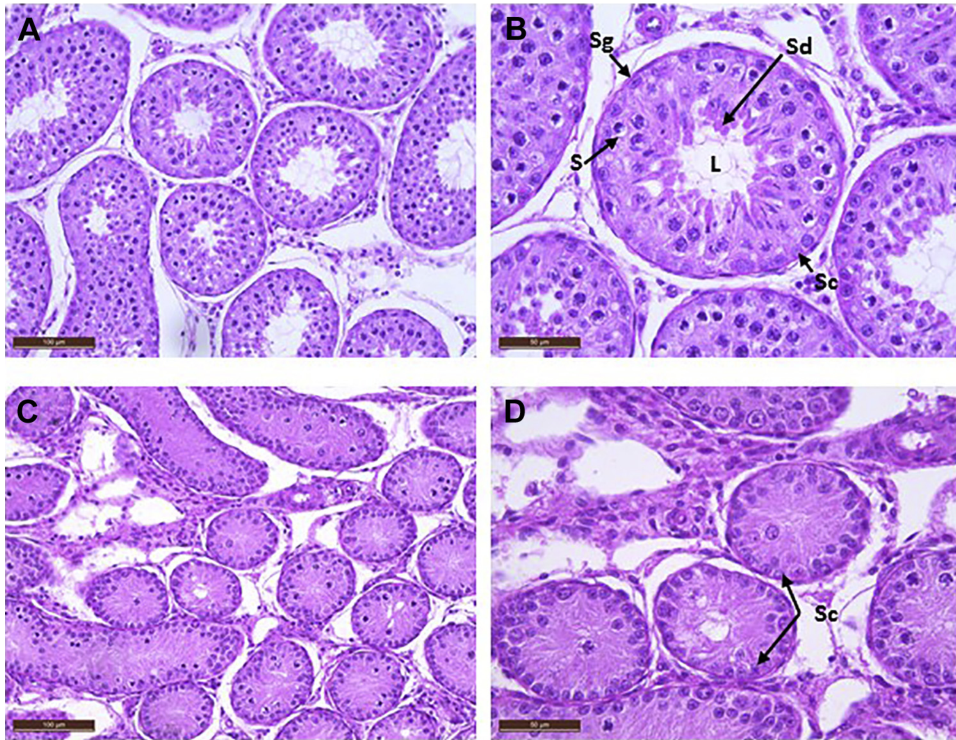

Fig. 1. Histological features in the seminiferous tubule of peripubertal male sheep. C-males (A and B) showed symmetrical seminiferous tubules and an active gametogenesis process. T-males (C and D) showed small seminiferous tubules, obliterated lumen, and altered gametogenesis. Sg (spermatogonium); S (primary spermatocyte); Sc (Sertoli cell); SD (elongated spermatids); L (Lumen). Bar (100 μm left panel and 50 μm right panel).

AMH is a known marker of Sertoli cell function, and its expression increased in T-males in the present study. This increase could be the consequence of an increased number of Sertoli cells or an increase in the unitary cellular production of AMH [10,29]. Our results show a significant increase in the number of Sertoli cells per cross section but not per testis, therefore, the increased expression of AMH and its upstream factor SOX9 in testicular tissue of T-males may represent an increased transcriptional activity of the Sertoli cells. The increased expression of AMH, which eventually could lead to an increase in AMH plasma levels, is suggesting a failure in Sertoli cell functioning [30]. It could be the result of an augmented activity of the FSHR, acting either through AP2, or through SOX9 in a response to increased PKA activity [31]. In the present study, only SOX9 and not AP2 showed an increased level of mRNA expression, suggesting a preference for the activation of the former. This activation happened with normal levels of expression of the FSHR, which acts via SOX9 to promote AMH transcription. We did not measure FSH plasma levels in the present study; however, previously we have shown that adult T-males have increased the secretion of plasma FSH after a treatment with a GnRH analog at 40 wk of age and also increased pituitary sensitivity at 20 and 30 wk [19]. If the pathway is not through FSH, SOX9 might be increased because of other stimulus. Other roles for SOX9 have been proposed in the postnatal testis, in which this factor could act to maintain the integrity of the seminiferous tubule, avoiding a male-to-female fate that could eventually occur even after sexual differentiation [32].

Table 3
Epithelial dimensions in seminiferous tubules from 24-wk-old ram prenatally exposed to testosterone (T-males) or vehicle (C-males).

Measurement	C-males	T-males	Value of P
Diameter (μm)	162.8 ± 4.0	121.4 ± 9.579	<0.01[a]
Diameter of the lumen (μm)	66.9 ± 2.8	49.2 ± 8.2	0.12[a]
Epithelium height (μm)	52.1 ± 1.1	31.5 ± 5.02	<0.01[a]
Area (μm^2)	21,063 ± 1,037	12,453 ± 1,804	<0.01[b]

T-males, n = 12.
C-males, n = 10.
[a] Mann-Whitney signed rank test.
[b] Unpaired Student *t* test.

As mentioned, testosterone and the androgen receptor are key elements in the process of spermatogenesis, but the precise mechanism by which they promote this process is still not clear [33]. In this study, as during 4 wk of age [20], we did not find a difference in the expression of the AR. We should remark that the expression we measured represents that from the whole testis, therefore we cannot reliably conclude that the lack of difference between groups discards the role of the AR in this model. In fact, for the expression of AMH to be reduced leading to Sertoli cells maturation [34], the expression of the AR in Sertoli cells is critical. In a mouse model constructed to lack the expression of the AR, specifically in the Sertoli cells, the

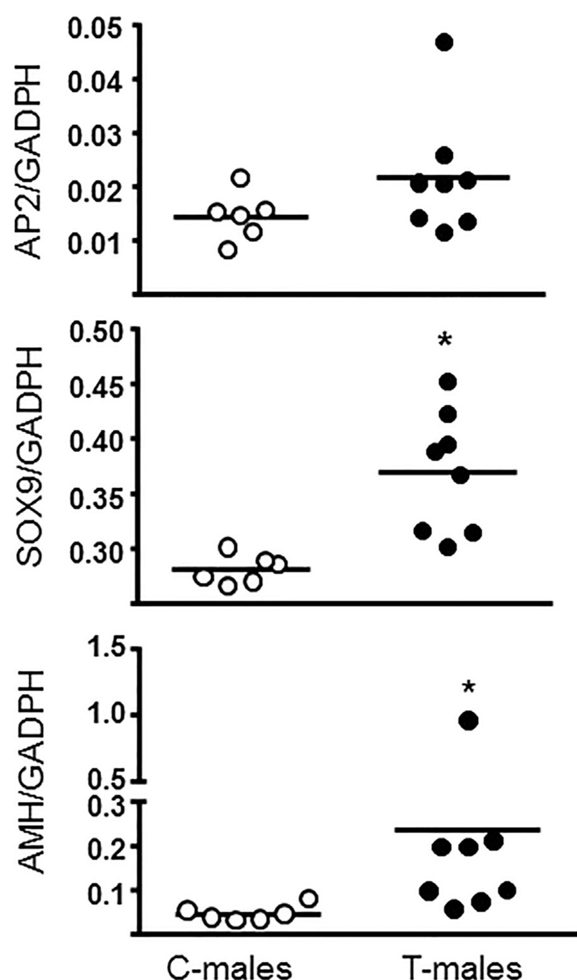

Fig. 2. Expression of the mRNA of AMH and its transcription factors in the testis of 24-wk-old ram prenatally exposed to testosterone (T-males) or the vehicle of the hormone (C-males). From top to bottom are AP2, SOX9, and AMH. The expression is against the constitutive gene GADPH. The units are arbitrary. Each symbol represents an animal. Horizontal line denotes the mean of the group and the asterisk denotes significant difference between T and C-males ($P \leq 0.05$. Mann-Whitney test). AMH, anti-Müllerian hormone.

expression of AMH increased and that of spermatogenesis is largely decreased [35]. Moreover, considering that testosterone has proven to act through both classical and nonclassical signaling pathways in the Sertoli cells [36] and that germ cells do not express the AR [37], it increases the possibilities of testosterone to contribute in the process of spermatogenesis through other mechanisms and through other cell types. In the present study, we point out to the AMH/SOX9 in the Sertoli cells as a possible cause to this feature. Nonetheless, it is known that for AMH to decrease in a normally developed testis at puberty, testosterone must increase [30,38] and seems that this requirement may not be happening in the T-males. We have not measured the local production of testosterone but we have demonstrated that the basal levels of T in T-males are high during this postnatal age, but the sensitivity of the gonad to LH is reduced [21]. This response could add an additional element to explain the over expression of AMH. High levels of AMH are found in the Sertoli cells of adult patients with Sertoli-cell-only syndrome and androgen insensitivity syndrome [39,40] a feature that could resemble our findings. It has been shown, recently, that germ cells can also express AMH and AMHR2 in the rat, opening a new path by which spermatogenesis can be controlled [41].

In previous work, we have reported an increased number of Sertoli cells per cross section of seminiferous tubule at adulthood [19] and also at 4 wk of age in this same animal model [20]. Even though it is not a common or conventional way to express cell counting, it is still reliable. There are reports that have expressed the number of Sertoli cells per transverse tubular section, which can back up our model [42,43]. In the present work, it is possible to observe a significantly increased number of Sertoli cells in the T-males when expressed per cross section and a trend to be higher when expressing it per testis. An increased number of Sertoli cells represents an overactive process of proliferation of the Sertoli cells [44]. In the ram, the final number of Sertoli cells is established around postnatal wk 11 [45], therefore, by the time of our experiments, that number was already established. An increased number of Sertoli cells in T-males may indicate that factors promoting its proliferation must have been upregulated. One of these factors is FSH, which has a strong impact on the proliferation and differentiation of the Sertoli cells [33]. During the proliferative stage, the FSH receptor increases in number and decreases when the definite number of Sertoli cells has been reached [46]. In our studies, FSH receptor expression showed no difference between groups, probably because the proliferative window of the Sertoli cells has ended. Surprisingly, similar feature occurred in the T-males at 4 wk of age when the proliferative process must have been active [20]. Therefore, it is possible to suspect that other factors may enhance the proliferation of Sertoli cells apart from the FSH-FSH receptor mechanism if it is the case in our studies. It is also possible to suggest that the Sertoli cell proliferation in T-males must have begun soon after birth [47] as in preliminary studies we have observed that the number of Sertoli cell is not different between T- and C-males at 120 d of gestation (data not published). The apparently increased number of Sertoli cells observed is not consistent with the spermatogenesis process observed, as the number of germ cells at different stages of spermatogenesis was significantly reduced. Therefore, we cannot rule out an alteration of the spermatogenesis due to an emerging deterioration of Sertoli cells functioning/number triggered by a reprogramming effect during gestation under the influence of extra T levels. Similar findings show that prenatal T excess may induce dysfunctional Sertoli cells that are incapable of supporting germ cell proliferation and maturation through an impairment in lactate production, as lactate produced by Sertoli cell is the principal source of energy for germ cell metabolism [48].

The testicular weight was reduced in T-males at 24 wk of age, which can predict a poor outcome in terms of spermatogenesis. Reports of correlations between testicular size and sperm production confirm that both features are correlated, in rams [49]. The testicular weight and the scrotal circumference are also good predictors of

spermatogenesis success in rams [50]. The reduction in both body and testis weight evident at 24 wk of age in T-males may account for the reduced scrotal circumference previously reported in adult T-male rams [22]. The T-males also showed epithelial measurements that could explain a reduced testicular weight as a reduced diameter of the lumen and reduced area similar to what is observed during the sexual regression [51] and in a rodent model of prenatal T exposure [52]. In this latter article, exposed rats studied before and after puberty showed a reduced number of round spermatids and spermatocytes, but also a decreased number of Sertoli cells per tubule's cross section. This difference may in part be explained by the particular overlap between the neonatal period and puberty that occurs in the rat, which have an impact on the characteristics of the Sertoli cells proliferation and differentiation [44].

To what extent altered Sertoli cell function serves as a causal link between prenatal T excess and the compromised adult testicular function remains to be investigated [53].

In summary, these findings provide evidence of permanent consequences in the male gonadal function due to fetal exposure to excess testosterone. The mechanism by which the reprogramming effect of T is carried out is still not clear but it can affect the reproductive performance of the exposed male fetus. In the present experiments, we treated the dams with supraphysiological levels of T, lower than those used by other groups [54], therefore, a maybe subtle exposure can still have a great impact of the reproductive outcome of the exposed offspring. The effects of the prenatal exposure in these terms are more clearly observed on spermatogenesis and through the expression activity of AMH/SOX9.

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