REPRODUCTION

Pituitary and testis responsiveness of young male sheep exposed to testosterone excess during fetal development

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Abstract

Prenatal exposure to excess testosterone induces reproductive disturbances in both female and male sheep. In females, it alters the hypothalamus–pituitary–ovarian axis. In males, prenatal testosterone excess reduces sperm count and motility. Focusing on males, this study tested whether pituitary LH responsiveness to GNRH is increased in prenatal testosterone-exposed males and whether testicular function is compromised in the testosterone-exposed males. Control males ($n=6$) and males born to ewes exposed to twice weekly injections of 30 mg testosterone propionate from days 30 to 90 and of 40 mg testosterone propionate from days 90 to 120 of gestation ($n=6$) were studied at 20 and 30 weeks of age. Pituitary and testicular responsiveness was tested by administering a GNRH analog (leuprolide acetate). To complement the analyses, the mRNA expression of LH receptor (LHR) and that of steroidogenic enzymes were determined in testicular tissue. Basal LH and testosterone concentrations were higher in the testosterone-exposed-males. While LH response to the GNRH analog was higher in the testosterone-exposed males than in the control males, testosterone responses did not differ between the treatment groups. The testosterone:LH ratio was higher in the control males than in the testosterone-exposed males of 30 weeks of age, suggestive of reduced Leydig cell sensitivity to LH in the testosterone-exposed males. The expression of LHR mRNA was lower in the testosterone-exposed males, but the mRNA expression of steroidogenic enzymes did not differ between the groups. These findings indicate that prenatal testosterone excess has opposing effects at the pituitary and testicular levels, namely increased pituitary sensitivity to GNRH at the level of pituitary and decreased sensitivity of the testes to LH.

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Introduction

Evidence is accumulating from both clinical and experimental studies that prenatal exposure to excess testosterone leads to a number of adverse consequences during adult life. In congenital adrenal hyperplasia (CAH), a classic model of prenatal androgen excess in humans, excess androgen production by the fetal adrenal gland leads to androgenization of female fetuses, leading to a series of consequences during postnatal life (Nimkarn et al. 2009). Males with CAH have also been found to manifest perturbations in gametogenesis and metabolic disorders (Cabrera et al. 2001, New 2001, Reisch et al. 2009, Falhammar et al. 2012).

Polycystic ovary syndrome (PCOS), an endocrine–metabolic disorder of high prevalence (10%) in women, is characterized by excessive production of androgens. The demonstration some years ago that pregnant PCOS women have higher circulating levels of androgen (Sir-Petermann et al. 2002) raises the possibility that the offspring of these women are likely to be exposed to excess androgen during fetal life. Prospective longitudinal studies to evaluate the effect of prenatal testosterone excess are difficult to perform in humans. Animal models of PCOS (Abbott et al. 1998) are a great resource in this regard. In female sheep exposed prenatally to excess testosterone, multiple neuroendocrine, ovarian, and behavioral disorders are evident from birth to adulthood (Padmanabhan et al. 2006).

Despite the significant advances in understanding the consequences of exposure to excess testosterone during development in females, few studies have addressed the corollary in males. Male offspring of PCOS mothers manifest metabolic disarrangements beginning at birth and culminating in altered lipid profile, insulin resistance and excess weight gain in

adulthood (Recabarren *et al*. 2008*b*). Adult male sheep exposed to excess testosterone manifest a significant reduction in the sperm count, scrotal circumference and sperm motility (Recabarren *et al*. 2008*a*), an increased number of Sertoli cells and an altered expression of FSH receptors and TGFB1 and TGFB3 (Rojas-García *et al*. 2010). To expand our understanding of the developmental consequences of testosterone excess in male sheep, we examined the response of the pituitary–gonadal axis following the administration of a GNRH analog, the expression patterns of LH receptor (LHR) and key steroidogenic enzymes P450c17,20 lyase, 17β-hydroxysteroid dehydrogenase (17β-HSD type 3 (17BHSD type 3)), P450 aromatase, P450scc, and 3β-HSD (3BHSD) in whole testicular tissue and the expression of P450c17, P450scc, 17BHSD type 3, and 3BHSD in Leydig cells cultured before and after an human chorionic gonadotrophin (hCG) challenge.

Results

Pituitary LH responsiveness

20 weeks (prepubertal)

Basal LH levels, mean of six samples immediately before GNRH analog administration, were higher in the testosterone-exposed males (1.35 ± 0.53 ng/ml) than in the control males (0.22 ± 0.03 ng/ml). GNRH analog administration induced an LH increase in both groups (Fig. 1) with a peak LH response occurring within the first 3 h. The acute and chronic areas under the curves (AUCs) of LH response in the testosterone-exposed males were twice as high as those in the control males (Fig. 2). *Post hoc* analyses revealed differences between the treatment groups at 1.5, 2.0, and 2.5 h after GNRH analog administration (Fig. 1). In the control males, LH levels returned to basal levels (≤ 1 ng/ml) 18 h after GNRH analog administration, while the testosterone-exposed males reached basal levels after 42 h (see inset, Fig. 1).

30 weeks (postpubertal)

As in prepubertal age, the testosterone-exposed males showed higher basal plasma LH levels than the control males (0.76 ± 0.33 ng/ml in the control males and 2.28 ± 0.48 ng/ml in the testosterone-exposed males). Both groups responded to the GNRH analog, although peak LH levels achieved were only half those achieved at 20 weeks of age. Despite this, the testosterone-exposed males had a higher LH response than the control males, specifically at 1.5, 2.0, and 2.5 h after GNRH analog administration (Fig. 1). This was reflected as a significant decrease in the AUC of LH response at this age relative to prepubertal AUC (Fig. 2). The mean acute and chronic AUCs between control males and testosterone-exposed males did not differ at this age. LH levels were below 1 ng/ml in the control males by 24 h and in the testosterone-exposed males after 30 h (see inset, Fig. 1).

Testicular responsiveness

20 weeks (prepubertal)

As with LH, basal plasma testosterone concentrations were higher in the testosterone-exposed males (3.02 ± 1.7 ng/ml) than in the control males (0.45 ± 0.27 ng/ml). The time-course of testosterone secretion after GNRH analog administration followed a parallel trajectory to that of LH secretion, with peak concentrations being observed within the first 3 h of the GNRH analog challenge, that is, during the acute phase. Neither the time-course of testosterone secretion nor testosterone concentrations achieved differed between the treatment groups (Fig. 1). The acute and chronic AUCs of testosterone were consistent with this pattern, with no difference being observed between the groups (Fig. 2).
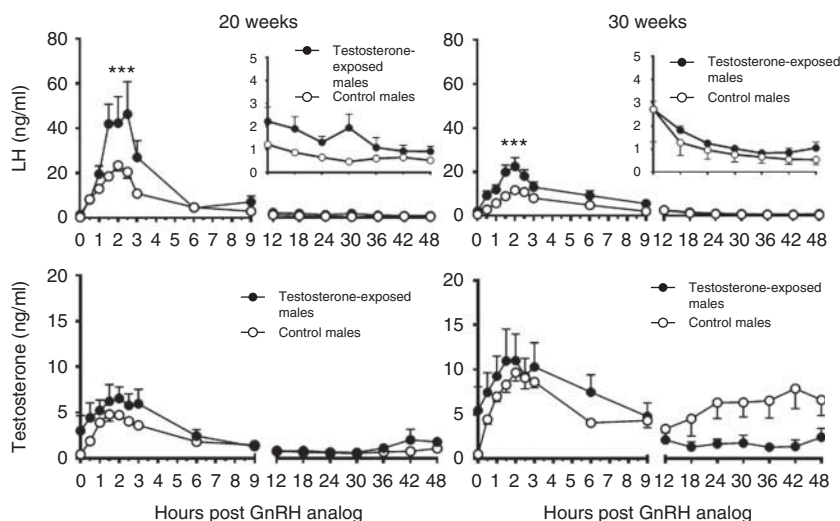

Figure 1 Plasma LH and testosterone responses to the GNRH analog challenge in the control males (open circles, $n=6$) and testosterone-exposed males (filled circles, $n=6$) at 20 and 30 weeks of age. Insets in the graphs of LH show the last 36 h after GNRH analog administration in ng/ml. Testosterone-exposed males were born to ewes receiving testosterone propionate from days 30 to 120 of gestation. Asterisks indicate a significant difference between control males and testosterone-exposed males.
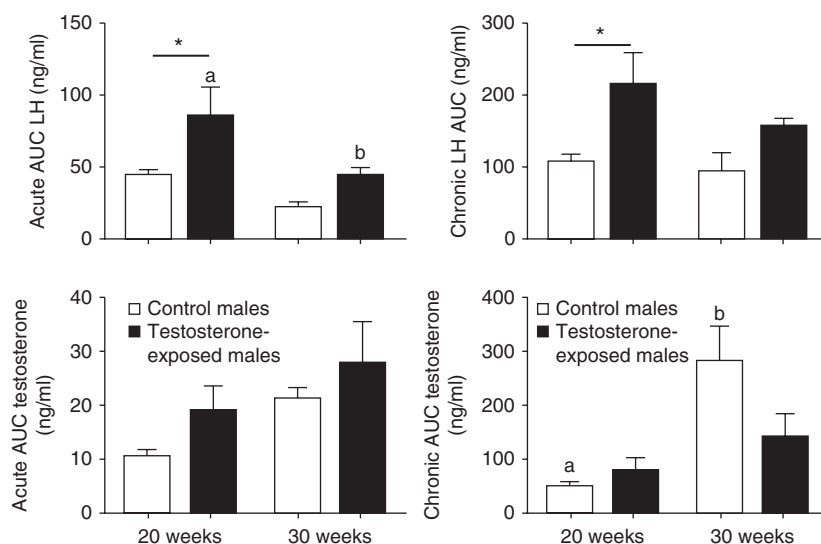
Figure 2 AUC of LH and testosterone responses following the GNRH analog test during the first 3 h (acute AUC, top left and bottom left) and during the entire 48-h period (chronic AUC, top right and bottom right) in the control males (white bars) and testosterone-exposed males (black bars). Asterisks indicate significant differences between control males and testosterone-exposed males. A significant age effect within each treatment group is indicated by different letters (a *versus* b).

30 weeks (postpubertal)

Basal testosterone levels at this postpubertal age were also higher in the testosterone-exposed males (6.48 ± 2.98 ng/ml) than in the control males (0.53 ± 0.10 ng/ml). As was the case at 20 week, plasma testosterone concentration was elevated during the first 3 h after the GNRH analog challenge (Fig. 1). While there were no differences in the acute release of testosterone between the treatment groups (first 3 h), testosterone levels increased during later hours in the control males but not in the testosterone-exposed males (Fig. 2).

Testosterone sensitivity to LH

The testosterone:LH ratio serves as an indicator of Leydig cell sensitivity to LH. The testosterone:LH ratio was similar in the control males and testosterone-exposed males during the first 3 h of testing at both prepubertal and postpubertal ages. The cumulative testosterone: LH ratio (48 h) was significantly lower in the testosterone-exposed males than in the controls at 30 weeks of age. The age-related increase in acute and cumulative testosterone:LH ratios observed in the controls was not evident in the testosterone-exposed males (Fig. 3).

Testicular gene expression

Testicular LHR expression was lower in the testosterone-exposed males than in the control males (Fig. 4, $P=0.011$). In contrast, the expression levels of P450c17, which is located upstream in the biosynthetic pathway of steroid synthesis converting progesterone to 17-OH progesterone; 17BHSD type 3, whose role is to convert androstenedione to testosterone; and P450

aromatase, which is the last enzyme in this metabolic steroid cascade converting testosterone to estradiol, did not differ between the treatment groups (Fig. 4). There was no correlation between testicular LHR expression and AUC of testosterone in males at 30 weeks of age (Pearson's $r=0.263$, $P=0.615$). The mRNA expression of *CYP11A1* (*P450SCC*) and that of *CYP17A1* (*P450C17*) in isolated Leydig cells from the control males and testosterone-exposed males before GNRH analog administration were similar between the treatment groups at each time point studied (Fig. 5). The mRNA expression of *3BHSD* and *17BHSD* type 3 in Leydig cells was higher after hCG stimulation in both control males and testosterone-exposed males relative to pre hCG levels (Fig. 5). However, there was no significant prenatal testosterone treatment effect.

Discussion

Findings from the present study show that prenatal testosterone treatment increases pituitary responsiveness to GNRH while decreasing testicular sensitivity. The failure of the testicle to respond to increased LH release appears to be related to the downregulation of LHR and not changes in steroidogenic enzymes. The mechanisms underlying these functional differences and relevance to male fertility are discussed below.

Impact of prenatal testosterone excess on pituitary LH responsiveness to the GNRH analog challenge

It is well established that GNRH regulates the synthesis and release of LH (Stanislaus *et al.* 1998). Recently, we have found that the LH pulse amplitude of prenatal testosterone- and dihydrotestosterone- (DHT)-treated males is increased (Recabarren *et al.* 2012). In these
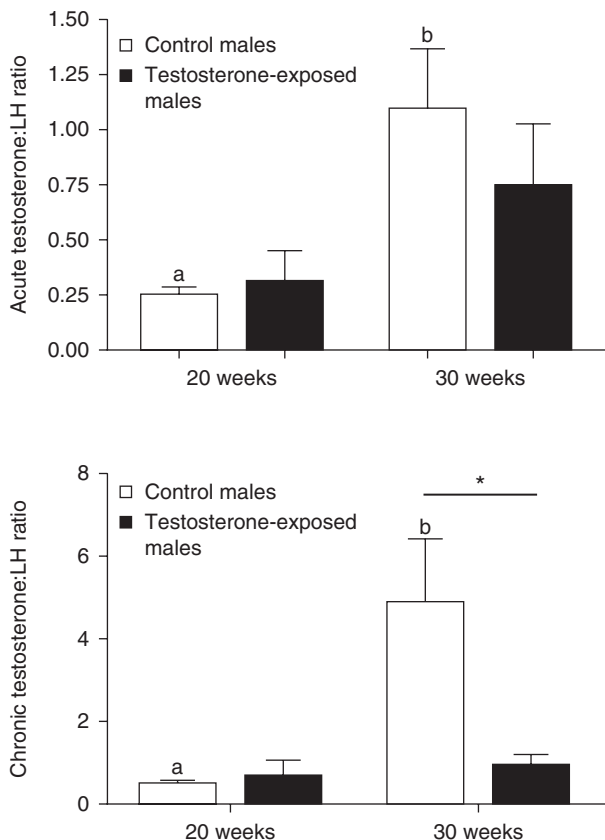
Figure 3 Ratio of testosterone:LH, as an index of gonadal sensitivity. Acute testosterone:LH ratio during the first 3 h (left) and chronic testosterone:LH ratio during the entire 48-h period (right) in the control males and testosterone-exposed males. A significant (white and black bars respectively) following GNRH analog administration. Asterisk indicates a significant difference between control males and testosterone-exposed males. A significant age effect within each treatment group is indicated by different letters (a *versus* b).

studies, it was unclear whether the increased LH pulse amplitude of LH was the result of increased GNRH input or increased pituitary sensitivity to GNRH. Other studies have found that pituitary responsiveness is increased in female offspring born to gestational testosterone-treated animals (Manikkam *et al.* 2008). Findings of increased LH release following GNRH analog administration in this study indicate that pituitary responsiveness to GNRH is also increased in the males (this study) as was the case with the females (Manikkam *et al.* 2008). Together, these findings support a role for increased pituitary responsiveness to GNRH as a contributing factor in the observed increase in the LH pulse amplitude of endogenous LH pulses of the testosterone-males (Recabarren *et al.* 2012). Whether the mechanism mediating this increase relates to an increase in LH synthesis or an increased GNRH receptor expression remains to be investigated. Evidence to date has supported the hypothesis that GNRH has the ability to upregulate its own receptor (Katt *et al.* 1985, Turzillo *et al.* 1995*a*, 1995*b*, Sakurai *et al.* 1997) and that the

amount of LH released is a function of the GNRH receptor number (Evans *et al.* 1997) and LH content (Clarke *et al.* 1987). It should be recognized that these findings do not exclude any additional contribution stemming from increased GNRH input. Interestingly, a recent study has provided evidence in support of the effect of testosterone at the level of the pituitary. The authors found that pituitary glands of adult prenatal testosterone-treated sheep (both sexes) were 40% heavier than those of the controls. While no differences in the density of LH- or ERα-immunoreactive cells were found in the pituitary of the control and prenatal testosterone-treated sheep, the percentage of cells co-localizing LHβ and ERα was lower in the prenatal testosterone-treated females. This is suggestive of reduced estradiol feedback and consequent hypersecretion of LH (Robinson *et al.* 2011), a possibility that remains to be explored in the testosterone-exposed males.

The magnitude of response to the GNRH analog at 30 weeks was half of that observed at 20 weeks in both control males and testosterone-exposed males. This probably reflects the negative feedback exerted by testosterone secreted by the maturing testis. With increasing testosterone being produced by the male as it gets older, the pituitary gland gets exposed to higher testosterone negative feedback (Olster & Foster 1986). Earlier studies of Tilbrook *et al.* (1991) in rams have shown that the feedback of testosterone is exerted predominantly at the level of the hypothalamus. This could have led to reduced GNRH priming at the pituitary level, consequent reduction in LH production and release by the pituitary gland.

Effect of prenatal testosterone excess on gonadal testosterone responsiveness to the GNRH analog challenge

The findings from this study substantiate the role of LH in stimulating gonadal testosterone response. The LH response resulting from the GNRH analog challenge was translated into testosterone increase at the gonadal level in both groups of animals. The increase in acute testosterone response at 30 weeks (postpubertal) compared with the response at 20 weeks (prepubertal) in both treatment groups probably reflects the increasing sensitivity of the maturing testis to LH. In male lambs, the response to endogenous LH increases as they mature (Foster *et al.* 1978). Paradoxically, the increased LH release that occurred in the testosterone-exposed males following the GNRH analog test was not reflected as an increase in acute testosterone release at both developmental time points. Furthermore, cumulative testosterone secretion over the 48-h test period was reduced in the testosterone-exposed males at 30 weeks than in the control males. Overall, these findings support reduced testicular sensitivity of the exposed males to LH stimulation. This is substantiated by the fact that the
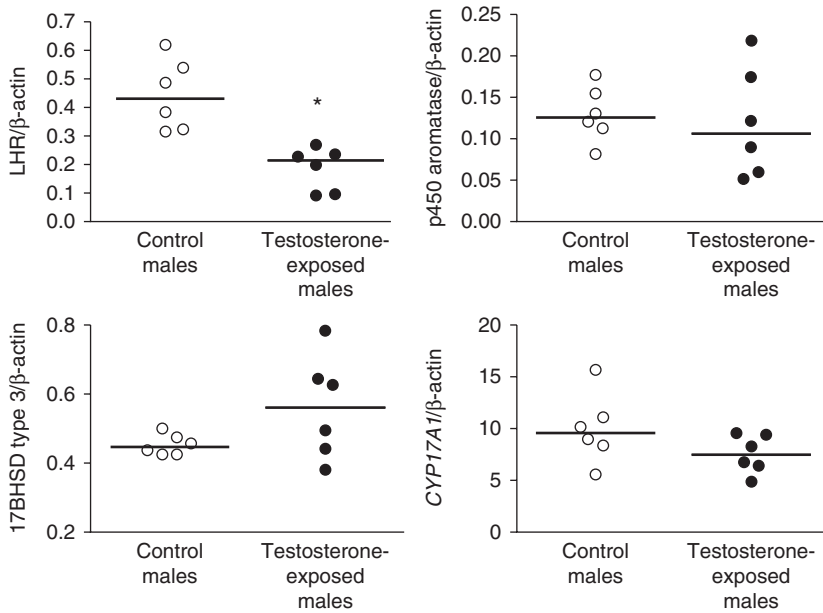
Figure 4 mRNA expression of LHR and steroidogenic enzymes in the testis of the control males (open circles) and testosterone-exposed males (filled circles) at 40 weeks of age. The asterisk indicates significant differences between the groups.

AUC-testosterone-testosterone:AUC-LH ratio was lower in 30-week-old testosterone-exposed males than in the control males.

While testicular responsiveness to the GNRH analog was reduced in prenatal testosterone-treated males, basal testosterone release was high compared with that in the control males. This suggests that prenatal testosterone-treated males may have increased GNRH pulse frequency similar to prenatal testosterone-treated females and thus subject to repetitive pituitary and gonadal stimulation. If so, further stimulation with endogenous LH released in response to the GNRH stimulus may not lead to increased testosterone production.

There are several explanations for the reduced testosterone response to GNRH analog stimulation. One possibility is that the LH released in response to GNRH analog testing may be of qualitatively different nature compared with that released in the controls and not recognized by the RIA. Proper posttranscriptional processing of the LH molecule (Ciccone & Kaiser 2009) and the resulting polysaccharide moiety is important in conferring biological activity to the LH molecule (Bergendah & Veldhuis 2001). Several studies have shown that changes in the mix of LH isoforms may contribute to differences in the response of the female gonad, but less is known about the response in the Leydig cells of the male gonad (Castro-Fernández *et al.* 2000, Perera-Marín *et al.* 2007, Olivares *et al.* 2009). In a rat model of diet-induced obesity, LH bioactivity was reduced, rendering the male hypogonadal (Olivares *et al.* 2010). More acidic forms are predominant in eugonadal men (Bergendah & Veldhuis 2001). The altered pattern of endogenous GNRH release (assessed from LH pulsatility) during post-development trajectory
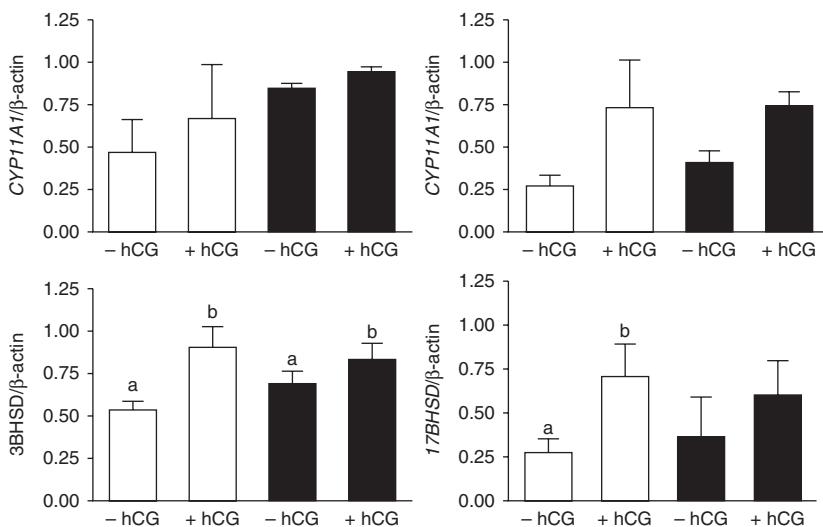

Figure 5 mRNA expression of steroidogenic enzymes in Leydig cells from the control males (white bars) and testosterone-exposed males (black bars) without hCG stimulation (−hCG) and 24 h after stimulation with hCG (+hCG). Significant difference within groups before and after hCG stimulation is shown by different letters (a *versus* b).

in the testosterone-exposed males (Recabarren *et al.* 2012) may have contributed to the production of less acidic fast-clearing LH that does not provide sustained support in the testosterone-exposed males as in the control males. This premise is consistent with our earlier finding that hCG stimulation of the control males and testosterone-exposed males elicits similar testosterone responses at 40 weeks of age (Recabarren *et al.* 2008a).

Reduced expression of LHR mRNA of the testosterone-exposed males compared with the expression in the control males may be responsible for the reduced testicular response. However, diminished LHR mRNA expression did not correlate with the AUC of testosterone response to GNRH analog testing. Whether LHR protein changes correlate with testosterone response remains to be determined. The lack of difference in the mRNA levels of key steroidogenic enzymes indicates that the decreased cumulative testosterone response of the testosterone-exposed males may not be the function of steroidogenic enzymes being limiting. Furthermore, although *3BHSD* and *17BHSD* type 3 mRNA expression in Leydig cells in a suspension culture increased in response to hCG stimulation, there was no prenatal treatment effect, confirming observations in testicular mRNA expression. These findings at the level of mRNA need to be interpreted with caution since changes may have occurred at the protein level.

These findings provide evidence that exposure to excess testosterone during fetal development not only disrupts testicular differentiation, leading to a reduced number of sperm cells and compromised Sertoli cell function (Recabarren *et al.* 2008a, 2008b, Rojas-García *et al.* 2010, 2013), but also disrupts the interface of the pituitary–gonadal axis. How these findings are enchained remains to be investigated.

While we did not analyze spermatogenesis in this study, we have investigated this extensively in previous studies. These studies showed no differences between control males and testosterone-exposed males (Rojas-García *et al.* 2013) at 4 weeks of age. In contrast, spermatogenesis was found to be impaired with a lower number of spermatogonia, spermatocytes I, and spermatides per tubule in adult prenatal testosterone-treated males (Rojas-García *et al.* 2010).

Considering that adult reproductive function is the culmination of proper developmental ontogeny of the hypothalamo-pituitary–gonad axis, any perturbation in this developmental sequence would lead to perturbation in fertility. In the present study, a derangement was observed in the relationship between the pituitary gland and the testicle in peripubertal males because of the prenatal exposure to testosterone. This could jeopardize the future fertility of the testosterone-exposed. Since excess of testosterone may be seen as an endocrine disruptor as well as an environmental disruptor (Skakkebaek *et al.* 2001), these findings in our animal model

could, although with caution, be extended to human reproductive health, in particular, to sons of pregnant PCOS women. In support of this premise, placentas from PCOS mothers were found to have lower aromatase and higher 3β-HSD1 activities, which could lead to increased androgen levels during pregnancy (Maliqueo *et al.* 2013). The studies of Veiga-Lopez *et al.* (2011) in sheep have found male and female sheep fetuses whose mothers were administered testosterone to have elevated plasma testosterone levels compared with their control counterparts.

Materials and Methods

General management, breeding, and prenatal testosterone exposure

This study began in late March/early April during the natural breeding season at the Chillán Campus of the University of Concepcion, Chile (36° 36′ south latitude, 71° 30′ west longitude, 144 m above the sea level). Adult Suffolk Down females were mated as described previously (Recabarren *et al.* 2008a). After synchronization of estrous and controlled mating, pregnant sheep were allocated randomly to one of two treatments. One group of pregnant sheep received twice weekly i.m. injections of 30 mg testosterone propionate (T; Steraloids, Newport, RI, USA) dissolved in cottonseed oil as a vehicle, between 30 and 90 days of pregnancy, followed by injections of 40 mg testosterone from days 90 to 120 of pregnancy. Another group served as a control and received an oil vehicle twice weekly from days 30 to 120 of gestation. This regimen of testosterone administration was chosen to reflect the higher levels of testosterone observed in women with PCOS during their pregnancies and has been used in previous studies (Sir-Petermann *et al.* 2002, Recabarren *et al.* 2008a).

Ewes were maintained on pasture and supplemented with pellet food to meet the energy requirements of pregnant sheep. Lambs were born in mid-September by normal delivery, after full-length gestation (mean of 147 days), and were weaned at 8 weeks of age. They were 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 (Recabarren *et al.* 2008a). Only males born from singleton pregnancies were used in this study. All management and experimental methodologies were approved by the Ethical Committee in Animal Research of the Faculty of Veterinary Sciences of the University of Concepción.

Pituitary/gonadal responsiveness

GNRH analog testing for assessing pituitary and gonadal responsiveness was undertaken with the control males ($n=6$, born to ewes receiving the vehicle) and testosterone-exposed males ($n=6$, born to ewes receiving testosterone during pregnancy) at 20 and 30 weeks of age. These ages were chosen because they encompass the peripubertal window with, 20 weeks representing the prepubertal period and 30 weeks the postpubertal period of sexual development. Puberty is defined as the age at which the presence of sperm

cells in the ejaculate is first observed. In an earlier study, we found first sperm cells appearing in the ejaculate at 26 weeks of age with a steady presence of sperm cells being evident at 30 weeks of age (Recabarren *et al*. 2008*a*). A similar definition for puberty in male sheep has been employed by other investigators (Olster & Foster 1986, Wheaton *et al*. 2003), except that in Olster & Foster (1986)'s study, the presence of sperm cells was observed at 16–18 weeks of age, while in our flock, this was observed at 26 weeks of age. This difference may be related to the body conditions of lambs.

One or two days before GNRH analog testing, indwelling catheters were placed in both jugular veins under local anesthesia as described previously (Recabarren *et al*. 1995). Blood sampling was performed to acclimatize the males to the procedures and to review the patency of the catheters. On the day of the experiment, males were placed in experimental crates. Pasture and water were available at all time points. All studies began at 1400 h local time.

GNRH analog testing involved the administration of leuprolide acetate (Lupron Abbott, 10 μg/kg body weight) via one of the indwelling catheters and collection of blood samples through the second catheter at 0, 0.5, 1, 1.5, 2.0, 2.5, and 3 h for determining the acute LH and testosterone responses and at 6, 9, 12, 18, 24, 30, 36, 42, and 48 h for assessing the long-lasting effect of the GNRH analog on the LH and testosterone release. Blood (2 ml) was collected in tubes containing 10 μl of heparin (10 U/μl) and centrifuged at 1000 *g* for 4 min at 4 °C degrees and plasma was harvested. Plasma samples were stored at −20 °C degrees until later RIA for LH and testosterone.

Testicular gene expression studies

To avoid transient changes stemming from GNRH analog testing in testicular gene expression, these studies were undertaken at 40 weeks of age. The left testicle was removed from each male for RNA isolation. In brief, rams were anesthetized with halothane, and their testicles were surgically removed and cut into small pieces of 0.5 cm^3 in size. Some of these pieces were left immersed in RNAlater (Ambion, Carlsbad, CA, USA) and stored at −20 °C for total RNA

isolation. Other pieces were also used for the *in vitro* culture of Leydig cells.

For RNA isolation, testicular tissue was homogenized using Lysing Matrix D (MP) and the FastPrep instrument (FP120, Bio 101; Thermo Electron, Osterode, Germany) and total RNA was isolated using the RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions. The yield of total RNA was quantified photometrically at 260 nm using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Schweter, Germany). The quality of the RNA samples was assessed after electrophoresis on formaldehyde containing 1% (wt/vol) agarose gel with ethidium bromide staining. The quality of the RNA samples with a low RNA concentration was further verified using the Agilent 2100 Bioanalyzer and the RNA Nano Chips (Agilent, Waldbronn, Germany). To eliminate DNA contamination, the samples were subjected to DNase treatment prior to RT. A 1μl sample of total RNA was incubated with 1 U DNase (Promega) in a total volume of 20 μl of 1× buffer used in the RT at 37 °C for 30 min, heated at 75 °C for 5 min to inactivate the DNase, and then placed immediately on ice for 5 min. A 40 μl volume of a premix containing 200 U Moloney-murine leukemia virus reverse transcriptase (M-MLV RT; Promega), 2.5 μM of random hexamers (Amersham Biosciences), 0.666 mM of each dNTP (Amersham Biosciences), and 1X of the supplied RT buffer were added to each RNA sample. Samples without the M-MLV enzyme were run at the same time to monitor the absence of any genomic DNA. RT was performed at 25 °C for 10 min and at 42 °C for 1 h and then at 90 °C for 2 min. The cDNA was aliquoted in 20 μl volumes (16 ng/μl cDNA) and stored at −20 °C until analysis by real-time PCR.

Real-time PCR

In preliminary experiments, the expression of all genes 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. Real-time PCRs were performed in a Rotor-Gene RG-3000 thermocycler (Corbett Research, Mortlake, NSW, Australia).

Table 1 Sequence for primers used in the real-time PCR.

Genes	Primers	Annealing (°C)	PCR product length (bp)	References
β-Actin	F: 5′-CTC TTC CAG CCT TCC TTC CT-3′ R: 5′-GGG CAG TGA TCT CTT TCT GC-3′	55	178	Rojas-García *et al*. (2010)
LHR	F: 5′-GAT AGA AGC TAA TGC CCT TGA CAA C-3′ R: 5′-CCA GAA TGA AAT TAA ATT CAG AGG AG-3′	60	197	GenBank accession no. L36329
P450c17,20	F: 5′-TGG CGG TCA CCA ACA TAA TC-3′ R: 5′-CAT TCA CCT TGG CTT GCA TC-3′	60	300	GenBank accession no. AF251388
P450 aromatase	F: 5′-TCG TGG TTA AAA TCC AGG GG-3′ R: 5′-TCA TTG CCT CTT CAA CCT GG-3′	60	341	GenBank accession no. AJ012153
CYP11A1	F: 5′-AGAGAATCCACTTTCGCCACATC-3′ R: 5′-GGTCTTTCTTCCAGGTTCCTGAC-3′	55	237	Vanselow *et al*. (2004)
3BHSD	F: 5′-TGTTGGTGGAGGAGAAGGATCTG-3′ R: 5′-TGGGTACCTTTCACATTGACGTTC-3′	57	253	Vanselow *et al*. (2004)
17BHSD type 3	F: 5′-TGG GAG AAT GGG CAG TGA TC-3′ R: 5′-TGT TAA GGA AAT GGC TTG GG-3′	60	297	GenBank accession no. NM_001076439

F, forward; R, reverse.

The primer sequences, annealing temperature, and length of the PCR products used in the present study are given in Table 1.

A 1μl volume of cDNA (16 ng) was used as a 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 gene (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. 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 the standard curves 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. The concentration of the purified PCR products was estimated in duplicates 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 an internal control for the normalization of the genes under study. The expression of β-actin resulted in a similar level between control males and testosterone-exposed males. Therefore, we chose to use β-actin as the internal control. The data from real-time PCR are presented as a ratio of the specific mRNA gene to β-actin expression.

Leydig cell gene responsiveness to hCG

Leydig cells were isolated from six testosterone-exposed males and three control males and subjected to an hCG (Chorulon, Intervet, Chile) challenge. Approximately 50 g of testis tissue were subjected to enzymatic dispersion with 0.25 mg/ml of collagenase (type I, Gibco) at 37 °C for 15 min in a shaking bath to isolate interstitial cells. Interstitial cells were then filtered through gauze to remove fragments of seminiferous tubules. Leydig cells were purified from the crude interstitial fraction on a discontinuous gradient of Percoll prepared in HEPES buffer, pH 7.4, as described by Anakwe et al. (1985). Briefly, 20 ml of a 90% Percoll (Sigma) solution were layered under 20 ml of a 40% Percoll solution in a 50 ml conical tube. Interstitial cells were layered on top of the gradient in M199 and centrifuged for 15 min at 800 *g*. The Leydig cell fraction at the interface of the two Percoll solutions was aspirated off, diluted with M199, and collected by centrifugation at 600 *g* for 10 min. The cell pellet was resuspended; the process was repeated until no cell aggregates were visible. The isolated Leydig cells were collected by centrifugation, washed two times with M199, and then suspended in 5 ml M199. Leydig cell number was established using a hemocytometer and cell viability was assessed by Trypan Blue exclusion. Leydig cell purity was confirmed by a histochemical reaction for 3BHSD and immunocytochemistry for P450c17 enzyme using a polyclonal rabbit anti-bovine antibody (Peterson et al. 2001) donated by Dr Alan J Conley (University of California School of Veterinary Medicine, Davis, USA).

Purified Leydig cells (1×10^6) were maintained in a suspension culture at 34 °C under 5% CO_2–95% air for 12 h. Cell suspensions were washed and then incubated with or without 1 IU/ml of hCG for 24 h (Cheng et al. 2003). Leydig cells were then washed twice with PBS. For total RNA isolation, cells were lysed using the RNA extraction kit (Invitek) according to the manufacturer's instructions. All procedures were performed in duplicates. For the determination of RNA yield and its quality, DNase treatment (carried out in a 5 μl volume), RT, and real-time PCR were carried out using the procedures described above. RT was performed with 250 ng of total RNA in a final volume of 20 μl.

Hormone measurements

Plasma LH concentrations were determined by RIA using ovine radio-iodinated LH (LER 1374-A, provided by Dr Leo E Reichert, Jr), ovine antiserum CSU-204 (provided by Dr Gordon Niswender), and ovine LH standard oLH-S25 (provided by NIADDK, Bethesda, MA, USA) in 200 μl duplicates, following the previously described procedures (Recabarren et al. 1996). The minimal detectable LH dose, defined as 90% of the buffer control, was 0.1 ng/ml and the intra- and interassay coefficients of variation (CVs) were 5 and 12% respectively. Plasma testosterone concentrations were determined by RIA using commercial kits validated for ovine plasma (DSL, Webster, TX, USA). The minimal detectable limit of testosterone assay corresponding to 90% of the buffer control was 0.05 ng/ml and the intra- and interassay CVs were 3 and 7% respectively.

Statistical analysis

Results from the GNRH analog test at each age were analyzed by ANOVA for repeated measures and comparisons of the means with the Newman–Keuls test using the GB Stat v.5 program. LH and testosterone responses during the first 3 h of the test and during the entire experiment (48 h) were converted to AUC using the trapezoidal formula. These data were analyzed by ANOVA for repeated measures with treatment (control *versus* testosterone) as the main factor and age (20 and 30 weeks of age) as the repeated-measures factor, using the GB Stat version 6.5 statistical program. Pairwise *post hoc* comparisons were made with the Dunnet test using the GB Stat v.5 program.

Pearson's correlation was made between total AUC of testosterone in males of 30 weeks of age and LHR mRNA expression to characterize the association between both variables.

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 or the Mann–Whitney *U* nonparametric test and a two-way ANOVA, whenever necessary, using the GraphPad Prism 4.0 Software (GraphPad, San Diego, CA, USA). For all tests, $P < 0.05$ was considered statistically significant. Data are given as mean or median ±s.e.m. or s.d. as indicated in the figures.

Declaration 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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