Prenatal Testosterone Excess Reduces Sperm Count and Motility

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The reproductive system is extremely susceptible to insults from exposure to exogenous steroids during development. Excess prenatal testosterone exposure programs neuroendocrine, ovarian, and metabolic deficits in the female, features seen in women with polycystic ovary disease. The objective of this study was to determine whether prenatal testosterone excess also disrupts the male reproductive system, using sheep as a model system. The extent of reproductive disruption was tested by assessing sperm quantity and quality as well as Leydig cell responsiveness to human chorionic gonadotropin. Males born to mothers treated with 30 mg testosterone propionate twice weekly from d 30 to 90 and with 40 mg testosterone propionate from d 90 to 120 of pregnancy (T-males) showed a significant reduction ($P < 0.05$) in body weight, scrotal circumference, and sperm count compared with control males. Mean straight line velocity of sperms was also lower in T-males ($P < 0.05$). Circulating testosterone levels in response to the human chorionic gonadotropin did not differ between groups. These findings demonstrate that exposure to excess testosterone during fetal development has a negative impact on reproductive health of the male offspring, raising concerns relative to unintended human exposure to steroidal mimics in the environment.

A DEVELOPING FETUS is vulnerable to environmental insults. When an insult occurs at a gestational age critical for target organ differentiation, it leads to alterations in developmental trajectory of the organ, culminating in disruptions in adult phenotype, thus providing a basis for adult onset of diseases (1–6). Evidence exists to suggest that such insults may originate from changes in maternal environment due to restriction of the energy supply, tobacco smoking, alcohol consumption, stress, or exposure to environmental toxicants that act as steroid mimics (1–11). For example, nutritional insults have been shown to program hypertension, insulin resistance, type 2 diabetes, and obesity in adulthood (5, 6, 12).

The developing reproductive system is also extremely susceptible to insult by exogenous agents. Sex steroids and steroid mimics can cause irreversible effects on the fetus, some of which are not manifest until sexual maturity (4, 10, 11, 13–18). The effect of inappropriate steroid signaling using native steroids as model systems have been capitalized on extensively for understanding fetal origin of female infertility (13–16). For instance, exposure to excess testosterone (T) during fetal life has been shown to program reproductive neuroendocrine, ovarian, and metabolic deficits, features seen in women with polycystic ovarian syndrome (PCOS) (13–16). The impact of prenatal T excess on male reproductive development has not been so well studied. This is an important line of research to pursue, especially because both male and female fetuses of women with PCOS are getting exposed to elevated levels of androstenedione, T, and dehydroepiandrosterone sulfate (19). Furthermore, humans are exposed to several industrial pollutants that act as agonist or antagonist of native steroids (see review 20).

The objective of this study was to test the hypothesis that prenatal T excess disrupts reproductive function in the male, using sheep as a model system. Impact on reproductive function was tested by assessing sperm quantity and quality as well as T production after a human chorionic gonadotropin (hCG) challenge.

Abbreviations: CASA, Computer-assisted sperm analysis; hCG, human chorionic gonadotropin; PCOS, polycystic ovarian syndrome; T, testosterone; TP, T propionate; VSL, straight line velocity.

Materials and Methods

Breeding, prenatal T treatment, and animal maintenance

The study was undertaken in early March, during the natural breeding season at the Chillan Campus of the University of Concepcion, Chile (36° 36′ south latitude, 71° 30′ west longitude, 144 m above sea level). A group of adult Suffolk females was mated after synchronization of cycles with intravaginal progestogen pessaries for 7 d (Eazy Breed; Pharmacia and Upjohn, Auckland, New Zealand) followed by administration of prostaglandin $F_{2\alpha}$ (Genestren; Drug Pharma, Santiago, Chile). Once pregnancy was confirmed, pregnant sheep were allocated randomly to one of two treatments. One group of 20 pregnant sheep received twice-weekly im injections of 30 mg of T propionate (TP; Sigma, St. Louis, MO) in cottonseed oil, between 30 and 90 d of pregnancy and 40 mg T propionate from d 90 to 120 of pregnancy. Twenty other pregnant sheep served as controls and received vehicle twice weekly from d 30 to 120 of gestation. This regimen of TP administration was chosen to reflect the higher levels of T seen in women with PCOS during

their pregnancy (19). Twelve prenatal T-treated and 10 control male offspring were born from this breeding.

Pregnant sheep were maintained under regular husbandry protocols at the sheep facility of the Faculty of Veterinary Sciences, University of Concepcion, Chillán, Chile. Lambs were born at the end of September, were left undisturbed with their mothers for 4 h after birth, and then weighed. Both groups of offspring were weaned at 8 wk of age. After weaning, male lambs were kept in a separate barn under natural photoperiod and given free access to water and pasture and supplemented twice a day with hay and commercial pelleted food for ruminants. Pelleted food made of oat, corn, wheat, gluten feed, gluten meal, soybean meal, fish meal, sunflower meal, and mineral salts contained (based on dry matter) 18% protein, 11% crude fiber, 2% fat, and 2450 kcal/kg (Glovigor; Compañía Molinera El Globo, Santiago, Chile). Body weight and blood samples were obtained at birth and continued at weekly intervals until 40 wk of age. All procedures were approved by the Ethical Committee in Animal Research of the Faculty of Veterinary Sciences of the University of Concepcion.

Scrotal measures and semen collection

Six control and six prenatal T-treated offspring were chosen for use in this study. Only singleton or one randomly chosen offspring of a given twin pair were used to ensure mother is the experimental unit. Other males were used in other metabolic studies or for tissue harvest. Scrotal circumference was measured at weekly intervals using a flexible tape beginning at 26 wk of age and continuing until 40 wk of age. Semen was collected on a weekly basis beginning at 26 wk of age using an electroejaculation procedure. All semen collection procedures began at 0800 h local time. A Bailey ejaculator (Nasco, Fort Atkinson, WI) designed for small ruminants was used. This has a rectal transducer 17.5 cm long and 2 cm diameter that delivers a fixed voltage of 6 V. Each male was stimulated with four cycles of 4 sec with a rest interval of 4 sec between each cycle. The ejaculate was received in a sterile graduated tube. From the ejaculate, the following parameters were determined: 1) ejaculate volume, 2) sperm count, and 3) motility characteristics. To facilitate these measures, the ejaculate was suspended in sperm analysis medium (21) in a 1:1 ratio. The composition of this medium was 2.65 mM calcium chloride, 0.49 mM magnesium chloride, 2.00 mM potassium chloride, 5.0 mM sodium bicarbonate, 0.28 mM sodium phosphate, 19.97 mM HEPES, 26.0 mM dl-lactic acid (60%) sodium salt, 5.55 glucose, 8.75 mM sucrose, 1.0 mg/ml polyvinyl alcohol, 1.0 mg/ml BSA, 75 μg/ml kanamycin, and miliQ water [290 mOsm/kg (pH 7.3)]. Sperm counts were determined in a 10-μl aliquot using a hemocytometer. A computer-assisted sperm analysis (CASA; HTM-IVOS version 12; Hamilton-Thorne, Beverly, MA) was carried out to compare the motility of spermatozoa from prenatal T-treated males with that of control males. In brief, semen samples (5.5 μl, ~10–20 $\times$ 10^6 spermatozoa/ml) were placed on slides (Conception Technologies, San Diego, CA) prewarmed to 37 C and covered with a 22 $\times$ 22-mm coverslip before immediate transfer to the CASA. Motility characteristics were determined by assessment of at least three randomly selected microscopic fields (>300 spermatozoa/sample) using factory CASA settings at an image sampling frequency of 60 Hz. Average path velocity (micrometer per segment); straight line velocity (VSL; micrometer per segment), curvilinear velocity (micrometer per segment), and linearity (percent) were estimated using procedures described previously (21).

hCG stimulation test

Alterations in adult reproductive function could be the result of endocrine disruptions programed developmentally and may involve a reduction in T production. To determine the gonadal T production, at 40 wk of age, after the last weekly semen collection, an hCG test was performed on all males. The hCG test (22) consisted of iv administration of 500 IU hCG dissolved in 2 ml saline. A jugular catheter was inserted under sterile conditions. Blood samples were collected (time 0), hCG was administered, and additional blood samples collected at 0.5-h intervals for the first 3 h of the test to determine acute response of T to hCG stimulus and then at 3- to 6-h intervals until 72 h to assess chronic effects. Plasma concentrations of T were measured by double-antibody RIA using a commercial kit (Diagnostic Systems Laboratories, Webster TX) (23). Minimal detectable limit of the T assay was 5 pg/ml. Intra- and interassay coefficient of variations were 4 and 8%, respectively.

Statistical analysis

Body weight, scrotal circumference, volume of ejaculate, sperm concentrations, and plasma concentrations of T were analyzed by ANOVA for repeated measures with treatment as the main factor and age as the repeated factor, except for plasma T concentrations in which sampling time was the repeated factor using the GB-Stat (Dynamic Mycrosystems Inc., Silver Spring, MD 20904) version 6.5 statistical program. For all analyses except sperm parameter, in which one T-male had no sperm in the ejaculate, there are six control (C-males) and six males born to mothers treated with 30 mg TP (T-males). Pairwise *post hoc* comparisons were made by the Newman-Keul's test. Because there were no within-group differences across time points studied, mean ratio of scrotal circumference to body weight and mean sperm motility were averaged across ages and compared using Student's t test. Results are shown as mean $\pm$ SEM.

Results

Body weight

Body weight from birth to 40 wk of age of C-males and T-males is presented in Fig. 1. Body weight increased from 5.3 $\pm$ 0.1 and 4.7 $\pm$ 0.4 kg at birth to 58.2 $\pm$ 1.1 and 50.3 $\pm$ 2.9 kg at 40 wk of age in C-males and T-males, respectively. Beginning at 8 wk of age, body weight of T-males was significantly less than C-males ($P < 0.05$). This difference persisted until 40 wk of age.

Scrotal circumference

Statistical analysis showed a high interaction between age and treatment in the scrotal circumference (Fig. 2A). Scrotal circumference of C-males was 32.0 $\pm$ 1.0 cm at 26 wk of age. Repeated-measures analyses found scrotal circumference increased significantly to 33.4 $\pm$ 0.9 cm at 28 wk ($P < 0.05$) and then continuing to increase progressively until 40 wk of age. Scrotal circumference of T-males at 26 wk of age was lower than the C-males (28.4 $\pm$ 2.5 cm; $P < 0.05$). Scrotal circumference of T-males was also lower than C-males at 40 wk of age ($P < 0.05$). The ratio of scrotal circumference to body weight did not differ statistically between treatment groups (control: 0.70 $\pm$ 0.06; T-male: 0.74 $\pm$ 0.07; $P = 0.56$).

Ejaculate volume

ANOVA showed a significant interaction between age and treatment ($P < 0.03$) in the ejaculate volume in each group

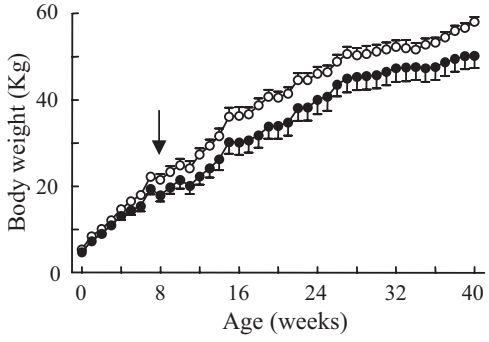

FIG. 1. Weekly body weight gain (mean $\pm$ SEM) in C-males (○, n = 6) and T-males (●, n = 6). T-males were treated twice weekly from d 30 to 90 of gestation with 30 mg and from d 90 to 120 of gestation with 40 mg TP. *Arrow* indicates the time point from which significant differences in body weight were evident between groups.

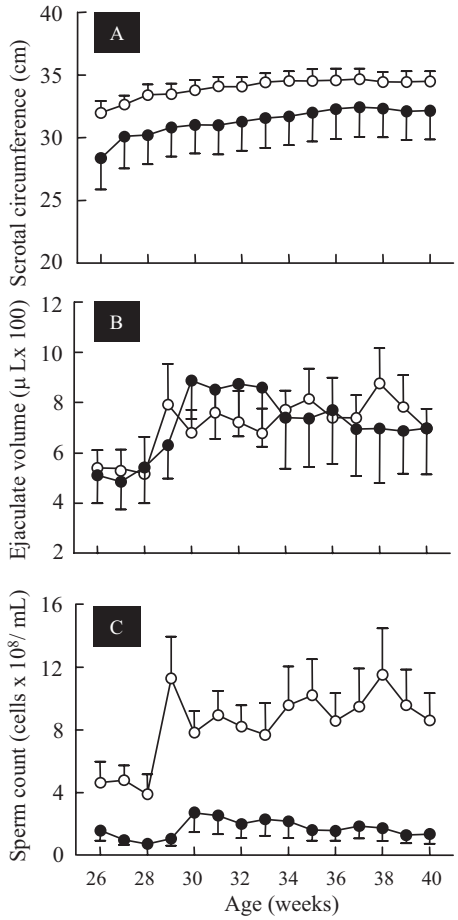

FIG. 2. Testicular and spermatic parameters (mean ± SEM) in C-males (○, n = 6) and T-males (●, n = 6). A, Scrotal circumference. B, Ejaculate volume. C, Sperm count. One T-male did not show sperms in the ejaculate and was omitted from the statistical analysis of the sperm count. T-males were treated twice weekly from d 30 to 90 of gestation with 30 mg and from d 90 to 120 of gestation with 40 mg TP.

(Fig. 2B). Ejaculate volume was 540 ± 71.6 μl in C-males and 510 ± 110 μl in T-males at 26 wk of age. Highest volume of ejaculate (876 ± 139 μl) was obtained at 38 wk of age in C-males and at 30 wk of age in T-males (888 ± 154 μl), plateauing thereafter.

Sperm concentration

A significant age ($P < 0.001$), treatment ($P < 0.01$), and age × treatment interaction ($P < 0.0003$) was evident with sperm concentrations. Sperm concentration was lower in T-males than C-males at every age studied (Fig. 2C). One T-male did not show cells in any of the weekly ejaculate and was excluded from the statistical analysis of sperm parameters resulting in an n of six controls and five T-males for these measures. Mean sperm concentration in T-males (195 ± 69.2 million) were significantly lower ($P < 0.05$) at 26 wk compared with C-males (462.5 ± 133.4 $P < 0.05$). Sperm concentrations increased from 26 to 29 wk of age in C-males and plateaued thereafter. At 40 wk of age, sperm concentrations averaged 859.2 ± 171.7 million in C-males. In contrast, sperm concentrations remained low throughout the study period in T-males, averaging 160.4 ± 67.0 million/ml at 40 wk of age ($P < 0.01$), an 80% reduction from that of the C-males.

TABLE 1. Mean ± SEM of 14 weekly sperm motility parameters from control male sheep (n = 6) and T-males (n = 5) obtained by CASA

Parameter	C-males	T-males
Average path velocity (μm/segment)	115 ± 2	118 ± 7
Straight line velocity (μm/segment)	103.6 ± 2	89.5 ± 6.9[a]
Curvilinear velocity (μm/segment)	170 ± 3	168.3 ± 11
Linearity (%)	51.9 ± 2	53 ± 1.8

T-males were prenatally treated from d 30 to 90 of pregnancy with 30 mg and from d 90 to 120 of pregnancy with 40 mg TP twice weekly.
[a] $P < 0.05$ *vs.* control males.

Sperm motility

The intrasubject variability of VSL over time ranged between 7 and 10% in C-males and 9 and 13% in T-males. There were no differences in VSL across time points within each group. Mean VSL of T-males averaged across time points was lower ($P < 0.05$) in T-males (89.5 ± 6.9 μm/segment) compared with C-males (103.6 ± 2.0). There were no differences in other three parameters of sperm motility studied (Table 1).

T response to hCG challenge

Basal T concentrations at time 0 were similar in C-males and T-males (Fig. 3). After administration of hCG, plasma T concentrations increased in parallel in both groups, reaching a maximum at 2–2.5 h after the hCG challenge. Circulating T concentrations decreased 6 h after hCG in both groups. Thereafter T concentrations again began to increase. At 72 h, plasma T concentrations were higher than seen before the hCG challenge ($P < 0.01$). There were no differences in T concentrations at any time point between C-males and T-males.

Discussion

Our findings provide unequivocal evidence that inappropriate exposure to excess steroids during critical stages of development, in addition to having detrimental effects on the female offspring (13, 14, 16), negatively impacts the reproductive development of the male offspring. The negative impact of exposure to excess T from d 30 to 120 of pregnancy

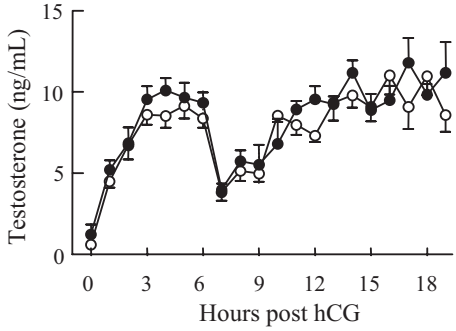

FIG. 3. Plasma T concentrations (mean ± SEM) in response to 500 IU hCG in control (○, n = 6) and T-males (●, n = 6). Time 0 depicts time of hCG injection. Blood samples were taken every 0.5 h for the first 3 h, then every 3 h, and finally every 6 h. T-males were treated twice weekly from d 30 to 90 of gestation with 30 mg and from d 90 to 120 of gestation with 40 mg TP.

was manifested as reduced scrotal circumference and reduced sperm count and motility.

Considering that androgens play a role not only in genital tract differentiation, phenotypic virilization, and maintenance of secondary male characteristics but also in initiation and maintenance of spermatogenesis (24), the reduced sperm count seen in T-males is likely the result of increased androgen signaling during development. Whereas we did not determine the amount of T reaching the male fetus, earlier studies found that injection of 100 mg T to pregnant ewes at 30 d gestation doubled circulating T levels from 0.3 to 0.6 ng/ml (25). Therefore, the male fetuses in this study are estimated to have been exposed to about 0.4 ng/ml T.

The underlying mechanisms mediating disrupted spermatogenesis are unclear. Because regulation of cellular growth is essential for maintenance of spermatogenesis in the adult sheep, the reduced testicular size is consistent with growth reduction and consequent detriment to spermatogenesis. A possibility to consider is that the reduced testicular growth of prenatal T-treated male offspring is secondary to the overall reduction in growth rate of these animals. Consistent with this premise, we found no statistically significant differences in the ratio of scrotal circumference to body weight between C- and T-males. Earlier studies in cattle and sheep found a positive correlation between testis size and active spermatogenesis (26–28). It remains to be investigated which of the several endocrine and paracrine factors that are involved in maintenance and control of testis cell function and differentiation are involved in programming of reduced spermatogenesis. Brooks *et al.* (29) found blockade of the LH secretion with a GnRH agonist, which reduces circulating T, resulted in a reduction in testis mass and number of Sertoli cells at birth. Similarly, male infant monkeys born to mothers treated with a GnRH analog also had reduced testicular weight (30).

Our finding of lack of difference in Leydig cell response of C-males and T-males to hCG, in the face of reduced sperm count in the T-males, suggests that the critical period for Leydig cell insult may differ from that of germ cells. Because responsiveness to hCG was tested only at 40 wk of age, there is also the possibility that the effects of prenatal T treatment on T release may have been manifested at an earlier time point. Our recent studies in males treated prenatally with 60 mg TP twice weekly from d 30 to 90 of fetal life found that pituitary LH released in response to a pharmacologic GnRH agonist challenge was reduced at 20 and 30 wk of age (23) and that this was reflected not as a reduced but rather an increased T response at 20 wk of age. If the same holds true for the T-males used in this study, which received a lower dose of T for a longer period during their fetal life, remains to be determined. This, however, appears unlikely because basal T levels (before hCG challenge) were similar in C- and T-males. Interestingly, circulating T levels in prenatal T-treated male rhesus monkeys determined at about 11 yr of age were also similar to that of controls (31). In contrast, in females, prenatal T treatment reduces sensitivity to estradiol-negative feedback, culminating in increased LH release (32) and consequent ovarian disruption (33–35).

It is unclear how much of the effects of prenatal T on spermatogenesis was due to its androgenic effect as opposed to effects via aromatization to estrogen. Whereas mRNA encoding aromatase have not been detected at any stage of testis development by Quirke *et al.* (36), aromatization is possible via placental aromatase activity. During normal development, the source of T is the fetal Leydig cells, which stimulates Sertoli cells and peritubular cells to provide a supporting environment for sperm cell differentiation. In general, sperm cell count is positively correlated to Sertoli cell counts (37). Whether the reduced sperm count of T-males is a reflection of reduced Sertoli cell count remains to be ascertained. Alternatively, decreased concentration of sperm in the ejaculate may be the result of occlusion of seminiferous tubule lumen and consequent block of sperm transport.

The findings from this study are likely to be of clinical relevance. Because testicular cancer incidence is 20-fold higher in men with abnormal semen analysis (38) and about 6–8% of adult men have subnormal sperm counts (39, 40), the detrimental effects of prenatal steroid excess on reduced sperm count is of concern. It appears that disorders of sperm production, for the most part, originate during fetal life (41, 42). In this regard, it is of interest that men with congenital adrenal hyperplasia, who are exposed to excess adrenal-derived testosterone during development, have low sperm counts and reduced fertility (43, 44).

It is also of clinical interest to relate findings from this study to the reproductive phenotype of sons of PCOS women, who are exposed to higher levels of T during gestation (19). Whereas absence of differences in circulating T levels between control and T-males evidenced in this study parallel lack of differences in circulating concentrations of T that we recently found in sons of control and PCOS women (45), the reduction in sperm count seen in prenatal T-treated male sheep (this study) was not evidenced in sons of PCOS women (45). In addition, the postnatal growth trajectories of prenatal T-treated sheep differed from that of sons of PCOS women; sons of women with PCOS had higher body weights from early infancy onward (46) as opposed to the prenatal T-treated male sheep that had lower body weights. Similarly, whereas infants of PCOS mothers showed insulin resistance from early infancy (46), no differences in insulin sensitivity was found in prenatal T-treated male sheep (47). Differences in phenotypes of prenatal T-treated male sheep and sons of PCOS women, in addition to the obvious species difference, may originate from changes in level and duration of T exposure during fetal life and/or metabolic status of the mother or offspring during pre- and postnatal development.

In summary, our results demonstrate that excess T during fetal development have detrimental effects on sperm concentration and motility and brings to the forefront the threat posed by exposure to excess steroids, native or environmental, on reproductive health of the male offspring (10, 11, 48, 49).

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