1988

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Journal of Andrology, Vol. 9, No. 4, July/August 1988

Endocrine Parameters, Hormone Receptors, and Functions of the Testicular Interstitium and Seminiferous Epithelium in Estradiol-Immunized Ile-de-France Rams

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The testicular response of Ile-de-France rams actively immunized against estradiol (E_2) was evaluated during both the ovine nonbreeding season (spring) and breeding season (autumn). Plasma concentrations of LH, FSH and testosterone were elevated in E_2-immunized rams during both spring and autumn when compared with BSA-immunized controls. Testis weights were significantly elevated by E_2 immunization and were characterized by greater interstitial cell volume, including Leydig cells, blood and lymph vessels, greater seminiferous tubule length, and greater numbers of leptotene spermatocytes and round spermatids. Neither Sertoli cell number, Sertoli cell nuclear volume nor testicular FSH receptor number were affected by E_2 immunization, but testis weight, Sertoli cell nuclear area, FSH receptor number and LH receptor number were significantly greater in autumn than in spring. A positive effect of E_2 immunization on testicular LH receptors was evident in spring but not in autumn. Testicular androgen receptors were suppressed by E_2 immunization but were not affected by season. It was concluded that E_2 immunization results in moderate stimulation of the ovine testis to increase testosterone secretion and to enhance total daily spermatid production. This effect appears to result from a change in E_2 negative feedback and increased pituitary gonadotropin secretion.

Key words: gonadotropin secretion, Leydig cells, Sertoli cells, FSH receptors, androgen receptors, spermatogenesis, ovine breeding.

J Androl 1988; 9:278–283.

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Submitted for publication August 13, 1987; revised version received December, 1987; accepted for publication December 3, 1987.

In male sheep, estradiol (E_2) is a potent regulator of gonadotropin secretion (Riggs and Malven, 1974; D'Occhio et al, 1983). Active immunization of Suffolk rams against E_2 elicited a 2- or 3-fold increase in mean serum LH concentrations (Schanbacher, 1984). In crossbred rams passively immunized against E_2, mean LH plasma levels were more than double those in control rams due to more frequent episodic releases, increased baseline levels and increased peak height (Sanford, 1985). More recently, Schanbacher et al (1987) reported that in Ile-de-France rams actively immunized against E_2, mean FSH plasma levels doubled as did LH. In spring (nonbreeding season), this gonadotropin increase resulted in hyperactivation of the Leydig cells, as measured by cell volume and plasma testosterone (T) levels.

The aims of the following study were to assess how seminiferous tubules react to higher intakes of FSH and T and to determine if testicular functions during the ovine nonbreeding season (spring) can be increased to breeding season levels by E_2 immunization. We therefore analyzed the excised testis of Ile-de-France rams immunized by Schanbacher et al

(1987). Partial data from the same experimental animals were reported in the previously mentioned paper.

Materials and Methods

Animals and Immunization

Twenty 4-year-old Ile-de-France rams weighing approximately 80 kg were assigned to the following study. All animals were maintained under natural photoperiod at the I.N.R.A. research facility near Nouzilly and were fed alfalfa hay, wheat straw, and grain supplement as required for maintenance of body weight. Water, salt, and mineral supplements were provided *ad libitum*. Experimentation began for all 20 rams in October at the time of primary immunization, extending into the nonbreeding season for 10 rams and into the subsequent breeding season for the remaining 10 rams.

Rams were randomly assigned to one of two groups. Ten rams were actively immunized against the carrier protein, bovine serum albumin (BSA; 1 mg) while each of a second group of 10 rams was immunized with 1 mg of an E_2-6-CMO-BSA conjugate (E_2:BSA; Steraloids, Inc., Wilton, NH). Antigens were dissolved in 1 ml saline and then emulsified with 1 ml Freund's complete adjuvant as described previously (Schanbacher, 1984). Injections were made in 20 or more subcutaneous sites over the ribs. BSA and E_2:BSA rams were given the respective antigens in Freund's incomplete adjuvant as booster injections (1 mg each) in December and February for spring-castrated rams (N = 10) and in December, February, June, and August for autumn-castrated rams (N = 9; one ram was eliminated due to poor health).

Blood Sampling

BSA- and E_2:BSA-immunized rams were sampled via jugular venipuncture at 20-min. intervals for 8 hours either during April (ovine nonbreeding season) or during September (ovine breeding season). Plasma from these samples was stored frozen at −20 C for subsequent determination of FSH, LH and T.

Rete Testis Fluid Samples

A few days after the autumn blood sampling, the rete testis from each of three rams was unilaterally cannulated according to the procedure described by Dacheux et al (1981) and rete testis fluid was collected for 2 days. Spermatozoa were separated by centrifugation (20 min at 1200 × g at 4 C) and daily fluid samples were stored at −20 C until assayed for androgen binding protein (ABP).

Testis Histology

At the end of the rete testis fluid collection period, all 10 rams were castrated under pentothal anesthesia. About 15 g of the noncannulated testis were immediately frozen and stored in liquid nitrogen until cytosol and membrane preparations could be prepared. One to 2 g from each testis were fixed in Bouin Hollande solution for histologic assessment of interstitial and tubular tissues, which was performed as previously described (Monet-Kuntz et al, 1987; Schanbacher et al, 1987).

Chemicals

Purified ovine FSH (YC 1115) and LH (YC 1051) were provided by Dr. Y. Combarnous (Nouzilly, France). They were iodinated with Na[^{125}I] (Radiochemical Centre, Amersham, U.K.) by the chloramine T method (Greenwood et al, 1963). 5α-dihydrotestosterone (1,2,4,5,6,7-^{3}H; DHT) (180 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.). Nonlabeled steroid was obtained from Steraloids (Wilton, NH).

Hormone Assays

Plasma FSH, LH and T were measured exactly as for the spring experiment (Schanbacher et al, 1987), except that T extracts had to be diluted 10-fold before assay. Assay sensitivities and intraassay coefficients of variation were FSH, 1 ng/ml HG-FSH (35 × NIH-FSH-S_3) and 7.5%; LH, 300 pg/ml LH-M-3 (1.8 × NIH-LH-S^1) and 8.9%; T, 2 ng/ml and 11.8% for a plasma pool containing 2.5 ng/ml.

Hormone Receptors Binding Assays

LH and FSH binding to testicular membranes was measured as previously described by Barenton and Pelletier (1983) with slight modifications. Membranes (0.5 mg protein/tube) were incubated with ^{125}I-radiolabeled LH or FSH (20,000 cpm) and nine different doses of unlabeled hormone ranging from 0.5 to 20 ng. An excess of 250 ng cold hormone was included for the determination of nonspecific binding. The data obtained from the competitive inhibition curves were plotted according to the method of Scatchard.

Androgen binding to testis cytosol was measured as described by Monet-Kuntz et al (1987), except that cytosol samples were treated twice instead of once with charcoal to remove endogenous steroids, and that double volumes of cytosol samples were used. Androgen binding to testis nuclei was measured as described by Monet-Kuntz and Terqui (1983).

Androgen Binding Protein Binding Assay

Rete testis fluid samples were stripped of steroid the same way as cytosol samples. They were used at a 1:15 dilution in Tris-HCl buffer (10 mM, pH 8.5), which was in the linear range of the ABP assay. Diluted samples were incubated with 2 nM of [^{3}H]5α-DHT, with or without a 100-fold excess of unlabeled steroid, for 16 h at 4 C. Separation of bound from free steroid was performed as described by Cailleau et al, (1984). Filter discs were solubilized with 1 ml Soluene-350 (Packard Instruments, U.S.A.) for 2 h at 20 C, and counted for radioactivity in 10 ml scintillation fluid. ABP concentration was calculated using the law of mass action, the dissociation constant for DHT-ABP complex being 1.7 nM under our conditions. The daily production of ABP was calculated from daily production of rete testis fluid.

Statistical Analysis

Student's *t*-test was used for histologic data and hormone level comparisons. For comparisons of testicular hormone receptor contents and ABP production, the nonparametric Mann-Whitney U test was used, since the normality of these variables has not been demonstrated.

Results

Plasma Gonadotropin Levels and Antibody Titers

In autumn, LH concentrations in E_2-immunized animals were twice that of the controls (1.6 ± 0.09 versus 0.64 ± 0.07 ng/ml; $P < 0.05$). FSH concentrations were also increased by E_2 immunization (7.2 ± 0.1 versus 5.1 ± 0.1 ng/ml; $P < 0.05$). At the time of the autumn assessment, E_2 binding titers were comparable to those reported previously (Schanbacher et al, 1987) for the same rams in the spring (ie, 30% and 40% of tracer bound at 1:1000 dilution).

Testis Weight

Testis weight was increased by E_2 immunization in both seasons. However, the testis of spring E_2-immunized rams remained smaller than in the controls in autumn (Fig. 1, upper panel). Seminiferous tubular volume closely paralleled that of testis weight (Fig. 1, middle panel). Interstitial tissue volume was increased by E_2 immunization in both seasons and was similar in E_2-immunized rams during the spring and in control rams during the autumn (Fig. 1, lower panel).

Interstitial Tissue

The total volume of blood and lymphatic vessels was increased by E_2 immunization in both seasons, and was similar in spring E_2-immunized and in autumn control rams (Table 1). The total volume occupied by Leydig cells showed the same pattern as the volume of the vessels. In spring, the effect of E_2 immunization was due to an increase in individual

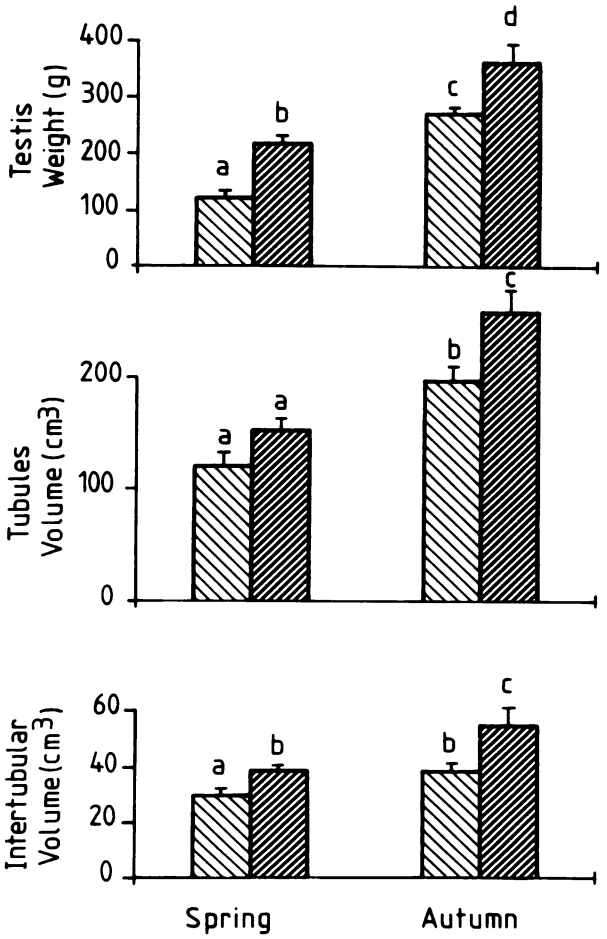

Fig. 1. Testicular weight, seminiferous tubular and interstitial tissue volumes in E_2-immunized rams, according to the season. Means (± SEM) without a common superscript differ ($P < 0.05$). Control, ▨ ; E_2-immunized, ▨ .

TABLE 1. Interstitial Tissue Characteristics in E_2-immunized Rams According to the Season*

	Spring		Autumn	
	BSA rams (5)	E_2:BSA rams (5)	BSA rams (4)	E_2:BSA rams (5)
Volume of blood and lymphatic vessels (cm³/testis)	10.4 ± 08†	13.5 ± 0.6‡	14.4 ± 2.2‡	22.5 ± 2.0§
Total volume of Leydig cells (cm³/testis)	2.62 ± 0.13†	3.66 ± 0.31‡	3.03 ± 0.22†‡	5.69 ± 1.0‡
Leydig cell number (10^9/testis)	13.1 ± 0.34‡	12.3 ± 0.6‡	7.53 ± 0.41†	13.3 ± 2.0‡
Area of peritubular Leydig cells (μm^2)	37.9 ± 2.2†	49.0 ± 1.7‡	65.2 ± 3.7§	65.0 ± 2.5§
LH receptors (pmole/testis)	10.1 ± 3.4†	20.2 ± 2.2‡	32.0 ± 1.6§	70.8 ± 8.2‖
Plasma testosterone (ng/ml)	2.5 ± 0.3†	23.7 ± 4.9§	14.4 ± 0.84‡	42.8 ± 7.9‖

*( ): number of animals.
†/‡/§/‖ Means (± SEM) without a common superscript differ ($P < 0.05$).

TABLE 2. Sertoli Cell Characteristics in E_2-immunized Rams According to the Season*

	Spring		Autumn	
	BSA rams (5)	E_2:BSA rams (5)	BSA rams (4)	E_2:BSA rams (5)
Sertoli cell number (10^8/testis)	31.4 ± 3†	36.8 ± 3.3†	30.3 ± 4.5‡	35.5 ± 2.5‡
Nuclear area of Sertoli cells at stage 8 (μm^2)	44.4 ± 1.9†	46.5 ± 1.4†	68.8 ± 1.9‡	68.0 ± 2.2‡
FSH receptors (pmole/testis)	20.2 ± 3.7†	25.9 ± 3.4†	160 ± 16‡	212 ± 18‡
Androgen receptors (pmole/testis)	117 ± 11‡	37.1 ± 13†	125 ± 12‡	82.3 ± 18†‡
ABP in rete testis fluid (pmole/day)	—	—	475 ± 111† (N = 3)	446 ± 206† (N = 3)

*( ): number of animals.
†,‡ Means (± SEM) without a common superscript differ ($P < 0.05$).

Leydig cell volume rather than an increase in Leydig cell number. The opposite was true in the autumn.

The testicular content of LH receptors was increased by immunization in both seasons. In spring, the number of LH receptors per Leydig cell was doubled by treatment (1.6 versus 0.77 pmole/10^8 cells) whereas in autumn LH receptor number was not increased significantly (5.3 versus 4.25). Plasma T concentrations were markedly increased by E_2 immunization. In the spring, immunized animals secreted even more T than the autumn controls.

Somatic Cells in Seminiferous Tubules

The number of Sertoli cells and the nuclear area of these cells exhibited large seasonal variations but were not affected by E_2 immunization (Table 2). Testicular content of FSH receptors also was not affected by immunization; in the spring, the E_2-immunized rams still had 8 times less receptors than the control rams in autumn.

The binding of androgens to testicular cytosol was reduced by E_2 immunization, especially in the nonbreeding season. To investigate whether this was due to a decrease in the total receptor content in target cells or to an enhanced occupancy of receptors in the nuclei, androgen binding to nuclei was measured in the testis of spring-castrated rams. Binding was as low in E_2-immunized rams as in control rams (19 versus 13 pmole/testis). The amount of ABP secreted daily into the rete testis fluid was not affected by immunization, at least in autumn-collected rams.

Germ Cells

Seminiferous tubules were longer in E_2-immunized animals than in controls. In the spring, treated rams had the same total tubule length as control rams in autumn. Seminiferous tubule diameter was not affected by immunization (Table 3). The daily production of A_1 spermatogonia was not modified by E_2 immunization in the spring (5.10 ± 0.5 versus 4.00 ± 0.2) or in the autumn (4.43 ± 0.23 versus 3.87 ± 0.75 × 10^7 cells per testis, mean ± s.e.m.). The daily production of leptotene spermatocytes was significantly increased by E_2 immunization only in the autumn. The daily production of round spermatids exhibited the same pattern as leptotene spermatocytes. When expressed per Sertoli cell, the daily production of round spermatids was unaffected by E_2 immunization in spring (6.75 versus 6.44) but was increased in autumn (14.3 versus 11.4 round spermatids per Sertoli cell per day $P < 0.05$).

TABLE 3. Seminiferous Tubular Dimensions and Germ Cell Daily Production in E_2-immunized Rams According to the Season*

	Spring		Autumn	
	BSA rams (5)	E_2:BSA rams (5)	BSA rams (4)	E_2:BSA rams (5)
Seminiferous tubule length (m/testis)	2712 ± 167†	3350 ± 209‡	3433 ± 486‡	4287 ± 266§
Seminiferous tubule diameter (μ)	199 ± 6.6†	201 ± 6.0†	216 ± 7.0†‡	224 ± 6.8‡
Daily production of leptotene spermatocytes (10^9/testis)	0.53 ± 0.06†	0.78 ± 0.12†‡	1.08 ± 0.07‡	1.85 ± 0.1§
Daily production of round spermatids (10^9/testis)	1.95 ± 0.14†	2.44 ± 0.26†‡	3.44 ± 0.55‡	5.02 ± 0.39§

*( ): number of animals.
†,‡,§ Means (± SEM) without a common superscript differ ($P < 0.05$).

Discussion

Immunoneutralization of circulating E_2 resulted in elevated plasma FSH and LH concentrations during the breeding season (reported herein) as well as during the nonbreeding season in the same rams (Schanbacher et al, 1987). The result was increased testis weight. In contrast, testis weight of adult rabbits (Nieschlag et al, 1975) and adult rams (Sanford, 1987) was not modified by E_2 immunization. In the latter study, the author suggested that the immunization period (8 weeks) may not have been sufficient for increments in spermatogenic activity to be manifested as increased testis weight.

The increase in testis weight was due to a stimulation of both interstitial and tubular compartments. Within the interstitium, blood and lymphatic vessels had a larger volume in E_2-immunized rams than in controls. Similarly, Nieschlag et al (1975) observed a higher percentage volume of blood vessels in the E_2-immunized rabbit testis. These effects are probably due to higher LH levels, since hCG injections have been shown to cause increased testicular lymph flow and similar changes in blood flow and the volume of blood within the testis (Setchell and Sharpe, 1981). Such an effect of LH could be mediated by a vasoactive secretory product of the Leydig cells, since selective elimination of Leydig cells from the testis abolishes the action of hCG on the permeability of testicular blood vessels (Setchell and Rommerts, 1985).

At moderate latitudes, mean plasma levels of LH in the ram are maximal in summer and decrease during autumn (Sanford et al, 1974). Chronic treatment with large doses of hCG increases the number of Leydig cells in the testis of adult rats (Christensen and Peacock, 1980) and, therefore, seasonal variation in mean LH levels could explain the fall decline in Leydig cell number in control rams and the lack of such decreases in E_2-immunized animals.

A 2-fold increase in plasma LH combined with a 2-fold increase in the total content of testicular LH receptors resulted in a 9-fold increase in plasma T during the nonbreeding season and a 3-fold increase during the breeding season. Several authors have reported elevated levels of T in E_2-immunized rams (Sanford, 1985) and rats (Nishihara and Takahashi, 1983). In these same Ile-de-France-immunized rams, Schanbacher et al (1987) verified that the metabolic clearance rate of T was not affected by immunization, so that increases in the levels of plasma T in E_2-immunized rams reflect increased Leydig cell T production. In a thorough study of hormone variations in E_2-immunized rams, Sanford (1985) reported that moderate increases in LH peak frequency and height were accompanied by supraphysiologic levels of T and therefore suggested that this disproportionate increase could be attributed to the supression of a direct inhibitory effect of E_2 on Leydig cell steroidogenesis. Papadopoulos et al (1986) tentatively identified an ovine venous plasma factor that suppresses T production by rat Leydig cells. Due to its absence in E_2-immunized rams, the authors hypothesized that immunization against E_2 suppresses an LH receptor binding inhibitor secreted by the testis (personal communication). From our results, it cannot be ruled out that such intratesticular effects of E_2 immunization amount to more than a peripheral effect on gonadotropin release.

Within the tubular compartment, the only effect of E_2 immunization on somatic cell functions was a large decrease in the total number of androgen receptors. The observation that the largest drop in androgen receptors (−68%) occurred in spring, at which time the highest increase in plasma T was observed (+ 850%), strongly suggests that a down regulation of androgen receptors took place. Such a phenomenon has not been seen in any testicular androgen target cell, but T has been shown to up and down-regulate sequentially rat ventral prostate androgen receptor activity in an age-dependent manner (for review, see Pinsky et al, 1983).

In the spring, although Sertoli cells received twice the normal level of FSH and 9 times the normal level of T, Sertoli cell nuclear area was not increased and spermatid daily production was only modestly increased. Based on these observations, E_2 immunization does not appear to be a simple solution for overcoming seasonal depression in daily sperm production.

From the findings presented above, it is clear that E_2 immunization of mature rams results in a modest stimulation of Leydig cells. This effect is uncertain in prepubertal males since mean plasma T is only slightly increased (lamb: Jenkins et al, 1986; calf: D'Occhio et al, 1987) or unchanged (lamb: Land et al, 1981) following E_2 immunization. Therefore, susceptibility of gonadotropin sensitivity to E_2 negative feedback may either be age-dependent or differentially important across species.

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