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—Original—

Active Immunization Against 5α-androstane-3α, 17β-diol Increases Ovulation Rate in Merino Ewes

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Abstract. Cyclic Merino ewes were immunized three times against 5α–androstane-3α,17β-diol (3α-diol) at three week intervals (weeks 0,3 and 6). After the third immunization, estrous cycles were synchronized by a series of PG injections and intensive blood samples were obtained for gonadotropin analyses during the mid-luteal phase and during the early follicular phase. Ovulation rates were determined after each immunization and subsequent cloprostenol (PG)-induced luteolysis (weeks 3, 6, 8 and 11). After the second immunization, daily blood samples were obtained and concentrations of plasma progesterone (P) were measured. Immunization against 3α-diol significantly increased the ovulation rate after the second and third immunizations and at week 10 ($P<0.01$, $P<0.01$ and $P<0.05$ respectively). It also induced anovulation in several ewes. Plasma concentrations of P were significantly increased by 3α-diol immunization ($P<0.01$). Pulse frequency of LH and concentrations of FSH were also increased during the luteal phase ($P<0.01$ and $P<0.05$ respectively). There was a significant correlation between the mean concentrations of FSH during the luteal phase and the subsequent ovulation rates ($P<0.01$). These results indicate that immunization against 3α-diol increases ovulation rate in ewes and its effect was, at least partially, exerted by increased plasma concentrations of FSH.
Key words: Ovulation rate, Sheep, Immunization, 5α-reduced androgen
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Immunization against various steroid hormones has been shown to be an effective way to increase ovulation rates in ewes [1, 2]. In ewes immunized against estrogen, circulating levels of FSH are increased due to decreased negative feedback resulting in multiple follicular development [1]. In androstenedione (A_4) immunized ewes, however, the responsible mechanism is not well understood. In these ewes, levels of LH were increased while levels of FSH were either unchanged or decreased [1, 3–5]. Attempts to increase ovulation rate by increasing levels of LH have been unsuccessful [4, 6]. It has also been shown, that immunization does not increase ovarian sensitivity to gonadotropins [4]. Close examination of the size and distribution of follicles revealed that the immunization increases ovulation rate through decreasing the incidence of atresia in preovulatory follicles without changing the population of other follicles [1]. These results suggest the presence of a mechanism that suppresses development of preovulatory follicles and that its action is inhibited

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by immunization against A_4.

Non-aromatizable 5α-reduced androgens have been shown to inhibit folliculogenesis in rats [7–10]. We have found that immunization against A_4 also induces antibodies against 5α-reduced androgens, androsterone (3α-ol), dihydrotestosterone (DHT) and 3α-diol, comparable to that of A_4 [Tetsuka and Nancarrow, unpublished data]. Taken together these results suggest that the A_4-immunization may exert its effect through neutralization of 5α-reduced androgens. The aim of this study was to examine this possibility by using a 5α-reduced androgen as an immunogen. An antigen was selected with the criteria of minimizing cross-reaction with aromatizable androgens which are important for estrogen synthesis and maintenance of preovulatory follicles. Accordingly, 3α-diol was selected as the immunogen and the effects of immunization on the incidence of estrus, ovulation rate, and peripheral levels of P and gonadotropins were investigated in seasonally cyclic Merino ewes.

Materials and Methods

Experimental schedule

Four to 5 years old Merino ewes (body weight 42.0 ± 0.9 kg, mean ± s.e.m., n=20) were used. Estrus was synchronized by two injections of 125 mg $PGF_{2\alpha}$ (PG: Estrumate®, Coopers Animal Health Australia Ltd., North Ryde, NSW, Australia) given 10 days apart. After synchronization of estrus, ewes were assigned into two groups and injected intramuscularly and subcutaneously at multiple sites with approximately 0.8 mg of either 3α-diol-15-(carboxyethyl)thioether (CETE)-human serum albumin (HSA) (immunized n=10) or HSA (control n=10) in Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI, U.S.A.; Week 0). Booster immunizations were given 3 and 6 weeks after the primary immunization. Under this schedule, estrus was detected 4 to 16 days after each immunization. Antibody titers were monitored weekly for 12 weeks starting 1 week after the primary immunization.

Following the second immunization (week 3), daily blood samples (5 ml) were obtained via the jugular vein for five weeks for P analysis.

At week 8, estrus cycles were synchronized in all ewes by a single injection of 125 mg PG. Then 5 ewes from each group were penned individually in a barn for intensive bleeding for gonadotropin analysis. Fourteen days after the PG injection (week 10), blood samples (5 ml) were collected via the jugular cannulae every 15 min. for 8 h starting at 0900 h (mid-luteal phase). The following morning at 0900 h, 125 mg PG was given to each ewe and luteolysis was induced. Twenty-four hours later, 10 min. interval bleeding was started and continued for 6 h (early follicular phase).

All blood samples were collected in heparinized glass tubes and kept on ice. After centrifugation at 2600 g for 15 min. at 4 C, plasma was harvested and stored at –20 C until assay.

The numbers of corpora lutea (ovulation rate) were determined by a laparoscopy for three consecutive cycles (weeks 3, 6, and 8) and 1 week after the intensive bleeding and PG injection (week 11).

Progesterone assay

Concentrations of plasma P were determined by radioimmunoassay. Antiserum (SIROSERA 9817: kindly provided by Dr. R.I. Cox, CSIRO, Prospect) was raised in sheep against P-7-(O-carboxymethyl)oxime-HSA and used at a final dilution of 1:33,000 (B/T 70%). At this dilution, this antiserum cross-reacts with 11β-hydroxy-progesterone (18.5%), 5α-pregnane-3,20-dione (7.1%), pregnenolone (3.7%) and corticosterone (0.9%).

Plasma samples (0.1–0.5 ml) were extracted twice for 10 min. with 4 vol. of hexane. The organic phase was taken and evaporated to dryness and the residue dissolved in assay buffer. All samples were assayed in duplicate. A portion of assay buffer containing sample (0.1 ml) was incubated with 0.1 ml antiserum and 0.1 ml labeled solution containing 22,200 dpm of [1,2,6,7-^{3}H]-P (Amersham Australia Pty Ltd., North Ryde, NSW, Australia) overnight. Free and bound steroids were separated by adding 0.5 ml of dextran charcoal suspension into each tube. After 15 min. incubation at 4 C, the tubes were centrifuged (2600 g for 15 min.) and supernatants were taken for counting.

Recovery rates of P after extraction were 71.2 ± 0.4% (mean ± sem) for control (N=217) and 61.8 ± 0.3% for immunized plasma (N=261). Sensitivity of the assay was 10 pg per tube. The intra- and interassay coefficients of variation were 8.2 and 11.8% respectively.

Titration of antibodies

Antibody titers were determined by estimating the plasma dilution that binds 50% of 50 pg tritiated steroids added [11]. Tritiated A_4, testosterone (T), DHT, 3α-diol and estradiol-17β (E_2) were supplied by Amersham Australia while tritiated 3α-ol was supplied by New England Nuclear (Boston, Mass., U.S.A.). While antibody titers for 3α-diol were measured in 3 assays, those for the other steroids were measured in a single assay. The intra- and interassay coefficients of variation for 3α-diol were 14.2 and 11.0% respectively and the intraassay coefficients of variation for the other steroids were less than 15%.

Gonadotropin assays

FSH and LH were measured by double antibody radioimmunoassay [5, 12]. To avoid interassay variation each gonadotropin was measured in a single assay.

The FSH used for iodination was NIAMDD-oFSH-1-1 and 12,000 cpm was added per tube. The FSH used for standards and quality control was NIAMDD-oFSH-RP-1. The antiserum used was NIAMDD-anti-oFSH-1 at a final dilution of 1:160,000 (B/T 30%). At this dilution this antiserum crossreacted with ovine thyroid-stimulating hormone (oTSH: 2%), oLH (<0.2%), ovine prolactin (oPRL: <0.01) and ovine growth hormone (oGH: <0.01). A portion of plasma samples (0.2 ml) was assayed in duplicate. Sensitivity of the assay was 0.25 ng per ml, and the intra-assay coefficient of variation was 13.8%.

The LH used for iodination was purified oLH and 12,000 cpm was added per tube. The LH used for standards and quality controls was NIH-oLH-S20. The antiserum used was R115 [12] and used at a final dilution of 1:1,000,000 (B/T 30%). At this dilution this antiserum crossreacted with oFSH (0.5%), oPRL (<0.1%) and oTSH (18%). Sensitivity of assay was 0.18 ng per ml, and the intra-assay coefficient of variation was 4.6%.

Statistical analysis

Anti-3α-diol titers were analyzed by a paired t-test following log-transformation. The relationships between levels of the antibodies, gonadotropins and ovulation rates were analyzed by linear regression. Concentrations of P in control and immunized groups were compared by an unpaired t-test following log-transformation. Ovulation rates in control and immunized ewes were compared by Fisher's exact test. Amplitude, nadir and pulse frequency of LH were determined by the Munro pulse analysis program (Elsevier-Biosoft, Cambridge, U.K.). The number of pulses per hour was subjected to square root transformation, while pulse amplitude and nadir were transformed to logarithms before two-way analysis of variance (ANOVA) followed by unpaired t-test. FSH concentrations in the two groups were compared by 2-way repeated measures ANOVA.

Results

Incidence of estrus and ovulation rate

After the second and third immunization, the numbers of immunized ewes returning to estrus decreased consistently and only 50% of ewes remained cyclic after the third immunization.There was no difference in the duration of the estrous cycle between control (17.5 ± 1.4 days, n=24) and

Table 1. Number of corpora lutea observed in control and 5α-androstane-3α, 17β-diol-immunized ewes at weeks 3, 6, 8 and 11

Group	Week 3	Week 6	Week 8	Week 11
Control (n=10)	1.40 ± 0.52 (4/10)	1.22 ± 0.44[a] (2/9)	1.13 ± 0.35[a] (1/8)*	1.40 ± 0.52[a] (4/10)
Immunized (n=9)**	1.33 ± 0.50 (3/9)	2.60 ± 0.55[b] (5/5)	2.75 ± 0.50[b] (4/4)	2.13 ± 0.64[c] (7/8)

* One ewe was given a second PG injection 14 days after the previous oestrus and therefore was excluded from the data.

** Laparoscopy was not performed in one ewe due to heavy fat cover and this ewe was excluded from the data.

Values are mean ± s.d. of ovulated ewes and (number of ewes with multiple CL/number of ewes ovulated). [a,b] $P<0.01$; [a,c] $P<0.05$.

Table 2. Reciprocal antibody titres in 5α-androstane-3α, 17β-diol-immunized ewes 7 days after the first, second and third immunization*

Steroid	Reciprocal antibody titre		
	1 st	2 nd	3rd
3α-diol	7	557	2960
	(6–9)	(244–2153)	(1357–8246)
3α-ol	NB	215	647
		(48–308)	(226–1002)
DHT	22	50	118
	(12–35)	(18–110)	(42–189)
A_4	NB	12	24
		(<5–56)	(9–51)
T	5	25	50
	(<5–8)	(7–63)	(15–102)
P	NB	27	40
		(<5–51)	(11–74)
E_2	NB	7	8
		(<5–9)	(<5–34)

* Values are given as medians and (ranges). NB= no binding (i.e. <30% binding at a dilution of 1:5).

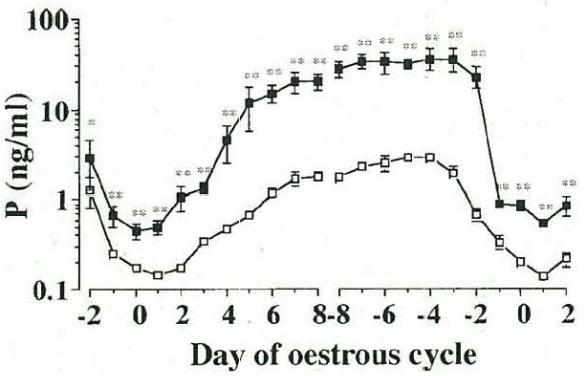

Fig. 1. Concentrations of plasma progesterone in control (open square: n=8) and 5α-androstane-3α, 17β-diol-immunized (black square: n=5) ewes. Values are mean ± s.e.m. (vertical bars). One control and 5 immunized ewes failed to show cyclic patterns of progesterone and were excluded from the data. One control ewe was given a cloprostenol injection 14 days after the previous estrus and was also excluded from the data. *P<0.05; **P<0.01 (unpaired t-test).

immunized (17.3 ± 1.4 days, n=19) groups and all cyclic ewes had normal length estrous cycles throughout the experimental period.

In one immunized ewe, the ovulation rate could not be observed due to a heavy fat cover and this ewe was excluded from the data. The ovulation rate was not affected by the primary immunization. After the second immunization, 4 ewes became anovulatory with persistent CL while the remaining 5 ewes had either 2 or 3 CL (Table 1). The ovulation rate in the immunized group doubled and was significantly higher than in the control group (P<0.01). After the third immunization, one ewe became anovulatory and all 4 remaining ewes had 2–3 CL. The ovulation rate was significantly higher in the immunized group than that in the control group (P<0.01). After the cloprostenol-induced luteolysis at week 10, newly formed CL were observed in 8 out of 9 immunized ewes, of which 7 ewes had 2 or 3 CL. Newly formed CL were observed in all control ewes, of which 4 ewes had 2 CL. The ovulation rate was significantly higher in the immunized group than in the control group (P<0.05). Throughout the experimental period, no control ewe had more than two CL and the ovulation rate never exceeded 1.4 in the control group.

Antibody titers

The antibody titer in each immunized ewe

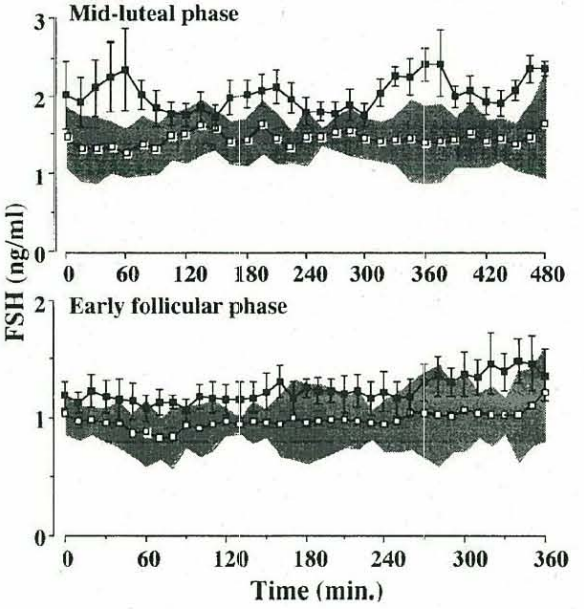

Fig. 2. Plasma concentrations of FSH (mean ± s.e.m.) in control (open square: n=5) and 5α-androstane-3α,17β-diol-immunized (black square: n=5) ewes at mid-luteal (14 days after cloprostenol injection) and follicular (24–30 h after cloprostenol injection) phases. Plasma samples were collected every 15 min for the luteal phase and every 10 min for the follicular phase. Shaded areas indicate 95% confidence limits of the control group.

Table 3. Characteristics of LH pulse profiles in control and 5α-androstane-3α, 17β-diol-immunized ewes at the mid-luteal (14 days after cloprostenol injection) and early follicular (24–30 h after cloprostenol injection) phase

Group	No. of pulses/h	Pulse interval (min)	Pulse amplitude (ng/ml)	Nadir (ng/ml)
Luteal phase				
Cont. (n=5)	0.20 ± 0.05	≥ 190.5	0.74 ± 0.18	0.24 ± 0.03
Imm. (n=5)	0.48 ± 0.11*	57.9 ± 5.5	1.42 ± 0.27	0.47 ± 0.13
Follicular phase				
Cont. (n=5)	0.90 ± 0.16	≥ 143.7	0.63 ± 0.09	0.47 ± 0.04
Imm. (n=5)	1.23 ± 0.15*	50.3 ± 2.2	1.33 ± 0.54	0.94 ± 0.17*
ANOVA				
Treatment (A)	P=0.001	–	P=0.030	P=0.006
Phase (B)	P<0.001	–	P=0.493	P=0.001
A × B	P=0.819	–	P=0.684	P=0.811

Pulse intervals were estimated ≥240 min for the luteal, and ≥180 min for the follicular phases when a profile contained only one LH peak. Samples were collected every 15 min. for 8 h (luteal phase) and every 10 min. for 6 h (follicular phase).
* Different from control value within the phase (P<0.05).

consistently increased over the previous week for 7 weeks after the primary immunization and maintained plateau levels of 1:2500–1:3000 throughout the rest of the sampling period. Only a small number of ewes were examined and no correlation was found between 3α-diol antibody titers and ovulation rate or incidence of estrus.

Antibody titers against 3α-diol and other steroids at one week after each immunization are shown in Table 2. After the second immunization, antibody titers against all steroids tested were detected. Antibodies were mainly formed against 5α-reduced androgens and levels of antibodies against A_4, T, P and E_2 were relatively low. After the third immunization, median antibody titers against 3α-ol and DHT were 22% and 4% of that of 3α-diol. Median antibody titers against A_4, T, P and E_2 were less than 2% of that of 3α-diol.

Progesterone

Mean plasma concentrations of P in the control and immunized cyclic ewes after the second immunization are shown in Fig. 1. Because of the experimental schedule, all ewes were given the third immunization at day 7 to 14 of the estrous cycle. Both control and immunized ewes showed similar and typical P profiles. Concentrations of P in immunized ewes during the follicular (0.5 ng/ml) and luteal (~35 ng/ml) phases were significantly higher than the respective concentrations in control ewes (0.2 ng/ml and ~3 ng/ml; P<0.01). One control and 5 immunized ewes failed to show cyclicity. In these ewes, concentrations of P remained elevated throughout the sampling period (2–5 ng/ml in control, and 10–70 ng/ml in immunized ewes). After the PG injection at week 8, concentrations of P decreased to the follicular phase levels in all ewes.

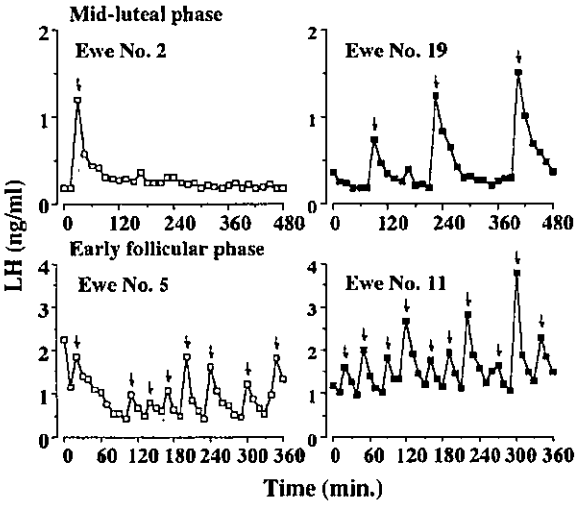

Fig. 3. Representative LH pulse profiles in control (open square) and 5α-androstane-3α, 17β-diol-immunized (black square) ewes at the mid-luteal (14 days after cloprostenol injection) and the early follicular (24–30 h after cloprostenol injection) phase. Plasma samples were collected every 15 min. for the luteal phase and every 10 min. for the follicular phase. Arrows indicate peaks detected by Munro pulse analysis (see text).

Gonadotropins

FSH: Mean concentrations of FSH in the control and immunized groups during the luteal and follicular phases are shown in Fig. 2. During the luteal phase, concentrations of FSH in the immunized group were significantly higher than those in the control group ($P<0.05$). During the follicular phase, mean concentrations of FSH in the immunized group were consistently higher than those in the control group although no significant difference was found between the two groups ($P=0.11$). There was a significant correlation between mean plasma concentrations of FSH at the luteal phase and the ovulation rate in the following cycle ($r=0.92$, $P<0.001$). Mean concentrations of FSH in ewes with 2 and 3 ovulations were respectively 35 and 88% higher than in ewes with 1 ovulation.

LH: Parameters of LH pulse profiles are summarized in Table 3. While representative LH pulse profiles in the control and immunized ewes during the luteal and follicular phases are shown in Fig. 3. Immunization against 3α-diol significantly increased pulse frequency of LH ($P=0.001$). The mean pulse amplitude in the immunized group was significantly greater than in the control group during both luteal and follicular phases ($P<0.05$). Mean nadir levels of LH pulses during the follicular phase were significantly higher than those during the luteal phase in both control and immunized groups ($P<0.005$). Mean nadir levels during both luteal and follicular phases were twice as high in immunized ewes as in controls ($P<0.01$).

Discussion

This experiment demonstrated that 3α-diol immunization effectively increases the ovulation rate in Merino ewes. Immunization against various steroids, such as P [13], A_4 and T [1, 2], dehydroepiandrosterone [14], oestrone and E_2 [1, 2] has been shown to increase the ovulation rate in ewes. However, to our knowledge, this is the first time to demonstrate that immunization against a 5α-reduced androgen increases the ovulation rate in ewes.

In A_4-immunized ewes, antibodies against both non-aromatizable 5α-reduced androgens and aromatizable androgens were formed [Tetsuka and Nancarrow, unpublished data]. The hypothesis investigated in the present experiment is that the ovulation rate may be increased in A_4-immunized ewes by neutralization of 5α-reduced androgens, the steroids implicated as inhibitory factors in follicular development [7–10].

The main effects of 3α-diol immunization were 1) an increased ovulation rate that was associated with an increased incidence of acyclicity; 2) an increased pulse frequency of LH at the luteal phase and an increased pulse amplitude and nadir at the luteal and follicular phases; 3) increased plasma concentrations of FSH during the luteal phase, and 4) increased plasma P concentrations during both luteal and follicular phases. The first two effects are common to A_4-immunized ewes [1]. Increased P concentration was also a common feature in the A_4-immunized ewes, however the increase was confined to the luteal phase [5, 15]. Plasma concentrations of FSH were generally unchanged or even decreased by A_4-immunization [1, 3–5] with one notable exception [15], where immunization against A_4 increased plasma concentrations of FSH during the luteal phase.

Do 3α-diol immunization and A_4-immunization increase the ovulation rate by a similar mechanism? Since increased pulse frequency of LH during the luteal phase is associated with twin ovulation [16] and is a universal feature in A_4-immunized ewes, several attempts have been made to correlate this index to ovulation rate. However, attempts to increase the ovulation rate by increasing LH pulse frequency during the luteal phase have not been successful [4, 6]. The current consensus is that exogenous LH does not increase the ovulation rate [6]. Thus, it was assumed that the increased LH pulse frequency observed in the 3α-diol-immunized ewes was not the cause of the increased ovulation rate.

Concentrations of FSH, in contrast to LH, were mostly unchanged or even decreased in A_4-immunized ewes [1, 3–5]. As the ovarian sensitivity to exogenous gonadotropins, in terms of cAMP production, was not affected by A_4-immunization [4], Campbell *et al.* [5] concluded that FSH was not responsible for the increased ovulation rate in A_4-immunized ewes. In this experiment, however, concentrations of FSH were significantly increased by 3α-diol immunization during the mid-luteal phase, a result similar to that reported in A_4-immunized ewes by McNatty *et al.* [15], who concluded that the increased FSH concentrations were at least partially responsible

for the increased ovulation rate in A_4-immunized ewes. It has been well documented that treatments with exogenous FSH [17–19] or treatments that increase endogenous FSH levels such as immunization against inhibin [21, 22] or E_2 [1, 22], all increase ovulation rates in ewes. In the present study, a significant correlation was found between mean plasma concentrations of FSH during the mid-luteal phase and the number of CL formed in the following cycle. Mean concentrations of FSH in ewes with 2 and 3 ovulations were well in excess of that required to increase the ovulation rate from 1 to 2 [17]. Therefore, increased concentrations of FSH, in response to 3α-diol-immunization are likely to be at least partially responsible for the increased ovulation rate in these ewes. If this is the case, the ovulation rate was increased by immunization against 3α-diol through a mechanism similar to that of immunization against E_2 rather than A_4.

What factor is responsible for the increased concentrations of FSH in 3α-diol-immunized ewes? The increased plasma FSH concentration, despite the presence of multiple ovulatory follicles in the 3α-diol-immunized ewes, indicates that negative feedback was impaired in these ewes. In A_4-immunized ewes, concentrations of free E_2 were decreased due to antibodies binding to A_4 and E_2 [23]. However, the increased number of preovulatory follicles resulted in higher concentrations of inhibin which compensated for the reduced negative feedback of E_2, and thus secretion of FSH remained unchanged or slightly decreased [24]. Peripheral plasma concentrations of E_2 and inhibin were not determined in this study. However, it was assumed that the secretion of these feedback hormones was increased by immunization against 3α-diol as the number of ovulatory follicles was nearly doubled in these ewes. The immunization induced a low antibody titer against E_2 that was comparable to that reported in A_4 immunized ewes [23]. Although it was shown that very low levels of E_2 antibody significantly reduced concentrations of free E_2 in A_4-immunized ewes [23], it is doubtful if this was sufficient to suppress negative feedback allowing a rise in FSH. In ewes passively immunized against E_2, Mann *et al.* [22] reported that an antibody titer higher than 1:400 was required to increase levels of FSH up to 140% of the pre-treated value, a level comparable to that observed in the present study. The relationship between ovulation rate and E_2 antibody titer in ewes passively immunized against E_2 [2] also indicates that the E_2 titer found in the present experiment might be much too low to increase the ovulation rate.

It is possible that other factors such as 5α-reduced androgens may be involved in the regulation of FSH secretion. It has been shown that DHT decreases pituitary gonadotropin secretion in the rat [25] and the pony mare [26]. In ewes, the negative effect of DHT on FSH secretion was examined *in vitro* and shown to be either negligible [27] or discernible only at pharmaceutical concentrations [28]. However, it might be possible that locally produced 5α-reduced androgens amplify the feedback effects brought about by E_2 or inhibin. A further study is necessary to clarify this possibility.

An unexpectedly high proportions of ewes immunized against 3α-diol became acyclic. Similar results were reported in ewes immunized against other steroids [1]. The mechanism underlying this failure of ovulation is not clear. However, in the present experiment 4 out of 5 anovulatory ewes did ovulate after PG injection at week 10. Therefore, impaired prostaglandin synthesis by the endometrium, due to inappropriate P and/or E_2 levels, might be responsible for the ovulation failure in the immunized ewes.

In conclusion, immunization against 3α-diol increases the ovulation rate in Merino ewes. This is at least partially due to the increased concentrations of FSH. The mechanism responsible for the increase in FSH was not clearly identified in this study.

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