Plasma concentrations of FSH, LH and progesterone in sheep immunized against an androstenedione–protein conjugate

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Summary. A significant increase in the plasma concentrations of FSH ($P < 0.05$) and LH ($P < 0.001$) was observed during the luteal (Days 9–11) phase but not during the subsequent cloprostenol-induced follicular phase in androstenedione-immunized ewes compared to those in control ewes. Over the same time period the geometric mean (and 95% confidence limits) androstenedione antibody titres in the immunized ewes was 1/305 (1/158, 1/590) whereas they were not detectable in the controls. In the subsequent cycle, the ovulation rates were 1.6 ± 0.2 for the immunized ewes and 1.1 ± 0.1 for the control ewes ($P < 0.05$) and the luteal progesterone concentrations were significantly higher in the immunized ewes compared to the controls ($P < 0.01$). Collectively, these results suggest that active immunization against androstenedione leads to an increase in the plasma concentrations of both FSH and LH. The results are consistent with the hypothesis that FSH plays a central role in determining the ovulation rate in sheep.

Keywords: FSH; androstenedione immunization; sheep; ovulation rate

Introduction

It is known that active immunization of ewes against an androstenedione–protein conjugate leads to an increase in corpora lutea formation (i.e. ovulation rate) (Scaramuzzi *et al.*, 1977; Van Look *et al.*, 1978; Martin *et al.*, 1979; Gibb *et al.*, 1981; Smith *et al.*, 1981). However, the mechanisms by which the immunization procedure increases ovulation rate remain obscure (Smith, 1985). It is suggested that the antibodies directed against the androstenedione-bearing antigenic determinant bind to some of the free androstenedione to alter the steroid feedback relationships between the ovary and the brain (Scaramuzzi, 1979). Evidence in support of this notion is provided in several studies which show that androstenedione-immunized ewes have increased plasma concentrations of luteinizing hormone (LH), oestradiol-17 β , androstenedione and progesterone (Scaramuzzi *et al.*, 1977; Scaramuzzi, 1984; Scaramuzzi & Hoskinson, 1984). Paradoxically, the increase in plasma LH concentrations during the oestrous cycle seems to coincide with unchanged or significantly lower plasma concentrations of follicle stimulating hormone (FSH) in the immunized animals relative to the untreated controls (Martensz & Scaramuzzi, 1979; Scaramuzzi, 1984).

In Romney ewes, the reason why some experience twin ovulations while others have one is due, at least in part, to higher blood concentrations of FSH during the luteal phase for a 24-h interval within the 48 h before the onset of luteolysis (McNatty *et al.*, 1985). In contrast, Scaramuzzi & Radford (1983) suggested that ovulation rates in androstenedione-immunized ewes were independent of changes in plasma FSH concentration.

The aim of the present study was to examine in more detail the changes in FSH and LH secretion around the time of luteolysis in androstenedione-immunized and untreated control ewes to determine whether FSH is responsible for the increased ovulation rate in immunized ewes.

Materials and Methods

Animals, treatments and sampling procedures

This study was carried out during the normal breeding season. Fifty parous Romney ewes aged 2.5 years (55–65 kg) were grazed in the continual presence of vasectomized rams fitted with marking harnesses. Twenty-five of the ewes were vaccinated (s.c.) at a single site in the back of the neck with an androstenedione-6-hemisuccinate-bovine α -lactalbumin conjugate (3 mg/ewe) in an aqueous solution (1 ml) of diaminoethyl dextran (5% w/v) and revaccinated 30 days later. The molar ratio of steroid to protein in this conjugate was unknown. At 3 days after the second vaccination all 50 ewes were injected with cloprostenol (125 μ g s.c.; Coopers Animal Health, Upper Hutt, N.Z.) to induce oestrus. At 10 days after cloprostenol injection, 20 immunized and 19 control ewes were at Day 8 of the oestrous cycle (oestrus = Day 0): 14 ewes from each group were randomly selected for further study. These animals were penned indoors and each was fitted with an intrajugular cannula for hourly blood sampling. This intensive sampling (2.5 ml blood/collection), for LH and FSH measurement, began at 08:00 h on Day 9 of the oestrous cycle and continued for 120 h. At 72 h after the start of blood sampling all animals were injected again with cloprostenol (125 μ g s.c.) and an extra 5 ml blood sample was collected at that time to measure androstenedione antibody titre and specificity.

At the end of the intensive blood sampling regimen all animals were returned to pasture and bled (2.5 ml/collection) once every 1 or 2 days by venepuncture for 17 days for progesterone analysis. The ovulation rate was determined by laparoscopy 9 days after the second cloprostenol injection.

All heparinized blood samples for hormone assay were centrifuged at 4000 g at room temperature for 20 min within 30 min of collection and the plasma samples were stored at -20°C until assayed. The blood samples for antibody titre were allowed to clot overnight and the serum obtained the following day was also stored at -20°C until assayed.

Hormone assays

Progesterone. The radioimmunoassay (RIA) procedure was identical to that described by McNatty *et al.* (1981a). The antiserum (WA-26) was raised in an ovariectomized ewe against progesterone-11 α -hemisuccinate conjugated to bovine serum albumin and used at an initial dilution of 1:8000. Major cross-reacting steroids in the assay were 11 α -hydroxyprogesterone (120%), 11 β -hydroxyprogesterone (25%), 20 α -dihydroprogesterone (3.5%) and androstenedione (0.45%). The minimum detectable level of progesterone was 0.15 ng/ml. The intra- and interassay coefficients of variation were 10.6 and 14.0% respectively.

FSH. The RIA kit was that supplied by The National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Maryland, U.S.A. The ovine FSH (oFSH) for iodination was NIAMDD-oFSH-1-1, the oFSH reference preparation was NIAMDD-oFSH-RP-1 (biopotency 75 $\times$ NIH-FSH-S1) and the FSH antiserum was NIAMDD-anti-oFSH-1 (AFP-C5288113). The cross-reactions of other pituitary hormones at 50% displacement were very low: oLH (NIAMDD-23), <0.2%; ovine prolactin (AFP-4328-C), <0.001%; ovine growth hormone (AFP-5285-C), $\leq$ 0.02%. At a final FSH antiserum dilution of 1:80 000 this homologous assay had a working range of 0.01 to 5 ng per assay tube. Each plasma sample (0.1 ml) was assayed in duplicate. The intra- and interassay coefficients of variation were 6.3 and 9.6%.

LH. The RIA for LH was identical to that described by McNatty *et al.* (1981a). The LH antiserum was raised in a rabbit against NIH-LH-S11 and used at an initial dilution of 1:40 000. The antiserum exhibited low cross-reactivity reactions with NIH-P-S12 (0.09%), NIH-TSH-S8 (2.4%), NIH-GH-S11 (0.4%) and NIH-FSH-S10 (0.4%). Each plasma sample (0.2 ml) was assayed in duplicate. The minimum detectable level of LH was 0.3 ng/ml plasma. The intra- and interassay coefficients of variation were $\leq$ 10 and $\leq$ 13% respectively.

Antibody titres

The androstenedione antibody titres were measured as described by Gibb *et al.* (1981). The androstenedione antibody titre in this report is defined as that dilution of immune serum required to bind 50% of 33 pg [1,2,6,7(n)- ^3H]androstenedione (304 mCi/mg; The Radiochemical Centre, Amersham, U.K.). The specificity of the androstenedione antisera from the vaccinated ewes was tested as described by Gibb *et al.* (1982). All antisera were tested for their specificities at a dilution which bound 50% of the androstenedione tracer. The presence of steroid antibodies to oestradiol, oestrone, testosterone and progesterone were also examined as described by Martensz & Scaramuzzi (1979) on one pool of serum from the 14 immunized ewes and on another pool from the 14 control animals. The following radiolabelled steroids [1 α ,2 α (n)- ^3H]progesterone (160 mCi/mg), [1 α ,2 α (n)- ^3H]testosterone (208 mCi/mg), [2,4,5,7- ^3H]oestrone (361 mCi/mg) and [2,4,6,7- ^3H]oestradiol-17 β (337 mCi/mg) were also purchased from The Radiochemical Centre, Amersham, U.K.).

Statistical procedures

As hourly plasma concentrations of FSH are unlikely to be independent from one another (Akbar *et al.*, 1974), 24-h mean values were obtained for each ewe during the follicular and/or luteal phase in an attempt to reduce the level

Fig. 1. Hourly mean $\pm$ s.e.m. changes in the plasma concentrations of FSH (a) and LH (b) in untreated ($\bullet$ — $\bullet$; $N = 14$ ewes) and androstenedione immunized ($\circ$ — $\circ$; $N = 14$ ewes) before and after an injection (s.c.) of cloprostenol at 72 h. All animals were on Day 9 of the oestrous cycle when blood sampling began at 0 h. Only the upper or lower s.e.m. (shaded areas) are shown to improve clarity.

of dependence of one sample value on another. Thereafter a two-way analysis of variance (ANOVA) was performed to test the effects of treatment (immunization vs control) and time of sampling. For LH, for which the hourly concentrations are likely to be independent from one another (Akbar *et al.*, 1974), a two-way ANOVA was performed on the hourly LH values for each ewe (i.e. immunization vs control and time) during the follicular and/or luteal phase.

The plasma progesterone concentrations on any sampling day and ovulation rate comparisons between immunized and control sheep were made using an unpaired two-tailed Student's *t* test.

Results

The mean ($\pm$ s.e.m.) plasma FSH and LH concentrations in the immunized and control ewes over the 120 consecutive hours of blood sampling are shown in Fig. 1. During the luteal phase $>90\%$ of the hourly mean FSH and LH values in the immunized animals were higher than in the controls. When the 24 h mean FSH values for each ewe over the 72 h before cloprostenol injection (Table 1) were analysed by two-way ANOVA (immunization vs control and time of sampling), there was a significant effect of immunization ($P < 0.05$) but no effect of time and no immunization $\times$ time interaction. When the 24-h mean FSH values over the 72 h before cloprostenol injection were compared by unpaired two-tail Student's *t* test (see Table 1), none was significantly higher than the control value. When the hourly mean LH values for each ewe over the 72 h before cloprostenol injection were analysed there was a significant effect of immunization ($P < 0.001$) but no effect of time and no immunization $\times$ time interaction. After cloprostenol injection, the mean FSH values in both groups declined to basal levels some 16–36 h later before increasing once more to luteal phase values. In contrast to FSH, the mean LH values in both groups increased about 1.5-fold after cloprostenol injection for some 32–40 h before an even more dramatic increase corresponding with the expected onset of the preovulatory LH surge. During the cloprostenol-induced follicular phase, about 50% of the hourly mean FSH and LH values in the immunized animals were similar to, or lower than those in controls. For the FSH values after cloprostenol treatment, no significant effects were noted with respect to the 24 h means (Fig. 1, Table 1). For LH there were no treatment effects after cloprostenol with respect to the hourly mean values but there was a significant effect of time ($P < 0.025$).

At the time of cloprostenol injection, the geometric mean (and 95% confidence limits) androstenedione titre in the immunized animals was 1/305 (1/158, 1/590). In the control animals, the androstenedione titres were not measurable. The specificities of the androstenedione antisera in all vaccinated ewes are shown in Table 2. All steroid cross-reactions were $<1\%$ except for 3 ewes for which the cross-reactions with dehydroepiandrosterone were 1.6, 1.8 and 2.3% respectively. The binding of exogenous steroids to the pooled sera from the control or androstenedione immunized ewes is shown in Table 3. The pooled sera from the controls bound only testosterone at a low titre whereas that from the immunized ewes bound mainly androstenedione with some low level of binding to testosterone and oestrone.

The mean $\pm$ s.e.m. ovulation rates in the treated and control ewes during the oestrous cycle following the hourly blood sampling regimen were 1.6 ± 0.2 and 1.1 ± 0.1 respectively ($P < 0.05$). The ovulation rates in individual animals from the treated and the control groups ranged from 1 to 3 and 1 to 2 respectively.

Table 1. The 24-h mean $\pm$ s.e.m. FSH concentrations before and after cloprostenol (PG) injection at 72 h†

Treatment	FSH conc. (ng/ml)‡				
	1–24 h	25–48 h	49–72 h	73–96 h	97–120 h
Control (N = 14)	2.6 ± 0.2	2.3 ± 0.1	2.2 ± 0.1	2.0 ± 0.2	2.0 ± 0.2
Immunized (N = 14)	2.8 ± 0.2	2.7 ± 0.2	2.6 ± 0.1	2.1 ± 0.1	2.1 ± 0.2

†The PG was administered immediately after the blood sample at 72 h.

‡Values are the 24 h means from each ewe sampled hourly at 1–24, 25–48, 49–72, 73–96 and 97–120 h. All ewes were in the 9th day of the oestrous cycle when blood sampling began. N = number of animals.

Table 2. Specificity of antisera from ewes (N = 14) vaccinated (s.c.) with an androstenedione-6-hemisuccinate-bovine α -lactalbumin conjugate

Steroid	% cross-reaction at 50% displacement of [1,2,6,7(n)- ³ H]androstenedione
Androstenedione	100
Testosterone	0.04 (<0.001, 0.10)
5 α -Dihydrotestosterone	0.03 (<0.001, 0.16)
5 β -Dihydrotestosterone	0.08 (<0.001, 0.66)
Dehydroepiandrosterone	0.26 (<0.001, 2.33)
Oestrone	0.03 (<0.001, 0.09)
Oestradiol	<0.001

Values are relative to androstenedione and are given as medians (and ranges).

Table 3. Steroid antibody titres* in control and androstenedione-immunized sheep

Treatment	Tritiated steroid				
	Progesterone	Androstenedione	Testosterone	Oestrone	Oestradiol-17 β
Control	NB	NS	1:3	NB	NB
Androstenedione immunized	NB	1:220	1:13	1:6	NB

*Defined as the dilution of plasma which binds 50% of 26–33 pg tritiated steroid. NB = no binding (i.e. <50% binding at a dilution of 1:1).

Fig. 2. Mean $\pm$ s.e.m. (vertical bars) plasma progesterone concentrations from the 3rd day after cloprostenol injection in control (○—○; N = 14 ewes) or androstenedione-immunized (△—△; N = 14 ewes) ewes. * P < 0.05; ** P < 0.01.

The mean plasma progesterone values over a 17-day period starting from Day 3 after the second cloprostenol injection are shown in Fig. 2. From Days 3 to 16, the progesterone values in the immunized animals were significantly higher than in the controls (P < 0.05). Moreover, in both groups the progesterone values increased and then decreased over the same time frame. Oestrus occurred in all ewes on Days 18, 19 or 20.

Discussion

These results show that, as a consequence of an increase in androstenedione antibody titre after active immunization with an androstenedione-protein conjugate, there is a significant overall increase in the mean plasma concentrations of both FSH and LH during the luteal phase of the oestrous cycle. An increase of ~8–25% in plasma FSH concentrations for 24 h within the 48 h before the onset of luteolysis is known to be sufficient to increase the ovulation rate of Romney ewes from 1 to 2 (McNatty *et al.*, 1985). In the present study the overall mean FSH concentration in the immunized animals was 13.8% higher than in the controls during the 72-h interval immediately preceding a cloprostenol-induced follicular phase and the subsequent mean ovulation rates in the two groups were 1.6 and 1.1 respectively. The present study therefore supports the notion that increased plasma FSH concentrations may contribute, at least in part, to the increase in ovulation rate in androstenedione-immunized ewes.

The results of the present study are at variance with the results of Martensz & Scaramuzzi (1979) and Pathiraja *et al.* (1984) who showed that the plasma FSH concentrations in androstenedione-immunized ewes were lower or unchanged relative to those in control ewes. In the present study the circulating antibody titres and specificities were comparable to those reported by Martensz & Scaramuzzi (1979). However, there were major differences between the two studies in the blood-sampling frequency, RIA methodology and the number of animals sampled over each time frame. In the study of Martensz & Scaramuzzi (1979) ≤ 3 vaccinated and ≤ 3 control animals were sampled over a 6-h time frame whereas in the present study the animals were sampled hourly for 120 h and there were 14 animals in each group. In addition, in the present study an homologous sheep FSH RIA was used whereas in the earlier study a heterologous assay was performed using a rat FSH preparation as the iodination standard. In the present study, as with that by Pathiraja *et al.* (1984), no appreciable cross-reactions were noted with respect to the androstenedione antibodies. In the study of Pathiraja *et al.* (1984) ewes with intravaginal progesterone sponges were treated with antiserum to androstenedione on Days 10, 11 or 12, with the sponges being withdrawn on Day 14. However, monitoring of the plasma FSH concentrations was followed only for the first 18 h after antiserum administration. As changes in plasma FSH concentrations before and after progesterone withdrawal influence the ovulation rate in sheep (see Henderson *et al.*, 1986) the role of FSH with respect to its long-term changes after passive administration of androstenedione antiserum remains obscure.

With the exception of the FSH data, the present results which show higher plasma concentrations of LH and progesterone in the immunized compared to control ewes are consistent with those of the earlier studies of Scaramuzzi *et al.* (1977), Pathiraja *et al.* (1984) and Scaramuzzi (1984). With regard to LH, there is some uncertainty as to its role in influencing ovulation rates. For example, the evidence from anoestrous ewes which were induced to ovulate with exogenous LH showed that the ovulation rate remained at 1 despite plasma LH concentrations that varied between 0.7 and 8 times those observed during the follicular and/or luteal phases of seasonally breeding ewes (McNatty *et al.*, 1981b). Also, the patterns of LH secretion during the follicular phase have not been found to differ between animals with single or twin ovulations (Scaramuzzi & Radford, 1983).

Individual corpora lutea are significantly heavier in androstenedione-immunized ewes than in control animals (Scaramuzzi, 1984) but the cellular composition in the former is not known. LH is known to influence the cellular morphology of the corpora lutea of sheep and also the level of progesterone production (Niswender *et al.*, 1985). Collectively, therefore, the elevated luteal phase concentrations of LH (and possibly FSH) may play an important role in enhancing the luteal progesterone output in the androstenedione-immunized animals.

In summary, the results of the present study suggest that immunization against androstenedione leads to a significant increase in the plasma concentrations of both FSH and LH. Moreover, these findings are consistent with the hypothesis that FSH plays an important role in determining the ovulation rate in sheep (McNatty *et al.*, 1985).

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References

- Akbar, A.M., Nett, T.M. & Niswender, G.D. (1974) Metabolic clearance and secretion rates of gonadotrophins at different stages of the estrous cycle in ewes. *Endocrinology* **94**, 1318–1324.
- Gibb, M., Thurley, D.C. & McNatty, K.P. (1981) Active immunization of Romney ewes with an androstenedione-protein conjugate. *N.Z. J. agric. Res.* **24**, 5–9.
- Gibb, M., Hudson, N., Kieboom, L. & McNatty, K.P. (1982) Long-term active immunization of Romney ewes with an androstenedione-protein conjugate—effects on oestrus, ovulation and lambing. *N.Z. J. exp. Agric.* **10**, 125–128.
- Henderson, K.M., Ellen, R.L., Savage, L.C., Ball, K. & McNatty, K.P. (1986) Effect on ovulation rate of increasing or decreasing ovarian exposure to follicle stimulating hormone during the preovulatory period in ewes. *N.Z. Soc. Anim. Prod.* **46**, 157–160.
- Martensz, N.D. & Scaramuzzi, R.J. (1979) Plasma concentrations of luteinizing hormone, follicle stimulating hormone and progesterone during the breeding season in ewes immunized against androstenedione or testosterone. *J. Endocr.* **81**, 249–259.
- Martin, G.B., Scaramuzzi, R.J., Cox, R.I. & Gherardi, P.B. (1979) Effects of active immunization against androstenedione or oestrone on oestrus, ovulation and lambing in Merino ewes. *Aust. J. exp. Agric. Anim. Husb.* **19**, 641–768.
- McNatty, K.P., Gibb, M., Dobson, C., Thurley, D.C. & Findlay, J.K. (1981a) Changes in the concentrations of gonadotrophic and steroidal hormones in the antral fluid of ovarian follicles throughout the oestrous cycle of the sheep. *Aust. J. biol. Sci.* **34**, 67–80.
- McNatty, K.P., Gibb, M., Dobson, C. & Thurley, D.C. (1981b) Evidence that changes in luteinizing hormone secretion regulate the growth of the preovulatory follicle in the ewe. *J. Endocr.* **90**, 375–389.
- McNatty, K.P., Hudson, N., Gibb, M., Ball, K., Henderson, K.M., Heath, D.A., Lun, S. & Kieboom, L.E. (1985) FSH influences follicle viability, oestradiol biosynthesis and ovulation rate in Romney ewes. *J. Reprod. Fert.* **75**, 121–131.
- Niswender, G.D., Schwall, R.H., Fitz, T.A., Farin, C.E. & Sawyer, H.R. (1985) Regulation of luteal function in domestic ruminants: new concepts. *Recent Progr. Horm. Res.* **41**, 101–151.
- Pathiraja, N., Carr, W.R., Fordyce, M., Forster, J., Land, R.B. & Morris, B.A. (1984) Concentration of gonadotrophins in the plasma of sheep given gonadal steroid antisera to raise ovulation rate. *J. Reprod. Fert.* **72**, 93–100.
- Scaramuzzi, R.J. (1979) Antibodies to androgens and ovulation in the ewe. *J. Steroid Biochem.* **11**, 957–961.
- Scaramuzzi, R.J. (1984) Changes in pituitary-ovarian functions in ewes immune to steroid hormones. *Proc. Aust. Soc. Anim. Prod.* **15**, 191–194.
- Scaramuzzi, R.J. & Hoskinson, R.M. (1984) Active immunization against steroid hormones for increasing fecundity. In *Immunological Aspects of Reproduction in Mammals*, pp. 445–474. Ed. D. B. Crighton. Butterworths, London.
- Scaramuzzi, R.J. & Radford, H.M. (1983) Factors regulating ovulation rate in the ewe. *J. Reprod. Fert.* **69**, 353–367.
- Scaramuzzi, R.J., Davidson, W.G. & Van Look, P.F.A. (1977) Increasing ovulation rate in sheep by active immunization against an ovarian steroid androstenedione. *Nature, Lond.* **269**, 817–818.
- Smith, J.F. (1985) Immunisation of ewes against steroids: a review. *Proc. N.Z. Soc. Anim. Prod.* **45**, 171–179.
- Smith, J.F., Cox, R.I., McGowan, L.T., Wilson, P.A. & Hoskinson, R.M. (1981) Increasing the ovulation rate in ewes by immunization. *Proc. N.Z. Soc. Anim. Prod.* **41**, 193–197.
- Van Look, P.F.A., Clarke, I.J., Davidson, W.G. & Scaramuzzi, R.J. (1978) Ovulation and lambing rates in ewes actively immunized against androstenedione. *J. Reprod. Fert.* **53**, 129–130.

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