Increasing ovulation rate and lambing rate in sheep by treatment with a steroid enzyme inhibitor*

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Summary. Treatment of ewes with a 3β -hydroxysteroid dehydrogenase (3β -HSD) inhibitor (Epostane) resulted in a significant increase in both ovulation rate and in the mean number of lambs per ewe lambing. The progestagen sponge plus 3β -HSD inhibitor treatment also caused a significant increase in oestrous cycle duration of approximately 1.5 days. Treatment of ewes with the 3β -HSD inhibitor caused a significant decrease in peripheral progesterone concentrations, which were reduced even further when 3β -HSD inhibitor treatment was given to ewes after insertion of a progestagen sponge. However, mean oestradiol concentrations were significantly higher in the two treatment groups, both at the end of the luteal phase and during the follicular phase of the oestrous cycle. These results demonstrate that ovulation rate and the production of lambs per ewe lambing can be significantly increased by 3β -HSD inhibitor treatment.

Introduction

In domestic animals, particularly sheep and cattle, the ability to control the number of eggs shed would lead to a significant improvement in reproductive efficiency. In sheep the exact mechanisms controlling ovulation rate are not known (see reviews by Webb & Gauld, 1985a, b; Bindon & Piper, 1984), although part of the control appears to involve a dynamic balance between the stimulatory effects of gonadotrophins upon the ovary and inhibition of gonadotrophin release by the feedback action of hormones from the ovary upon the hypothalamus–pituitary gland (Land & Carr, 1979). Differences in peripheral gonadotrophin concentrations have been found between breeds (see review by Bindon & Piper, 1984) and it has been demonstrated that follicle growth beyond 2.0 mm in diameter in ewes is dependent on adequate secretion of both LH and FSH by the pituitary gland (Dufour *et al.*, 1979; McNeilly *et al.*, 1986).

It is therefore suggested that perturbation of the hypothalamus–pituitary gland–ovarian feedback system should have a significant effect on ovulation rate and follicular growth. This hypothesis is supported by previous reports that both active and passive immunization against ovarian steroids can lead to a significant increase in ovulation rate (see reviews by Scaramuzzi & Hoskinson, 1984; Webb *et al.*, 1984) together with marked changes in the pattern of gonadotrophin release (Martensz *et al.*, 1979; Pathiraja *et al.*, 1984). Conversely, a physiological increase in the peripheral concentrations of oestradiol, by using subcutaneous implants, decreases ovulation rate (Webb & Gauld, 1985a) and also causes a reduction in the number of ‘active’ follicles (Webb & Gauld, 1986).

Treatment of animals with various competitive inhibitors of the enzyme, 3β -hydroxysteroid dehydrogenase (3β -HSD), has previously been shown to reduce steroid hormone production in different reproductive tissues of different species, including the rhesus monkey (Schane *et al.*, 1979), women (Pattison *et al.*, 1984a, b), sheep (Taylor *et al.*, 1982; Jenkin *et al.*, 1984) and rats (Snyder *et*

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et al., 1984). For example, treatment in sheep during the luteal phase of the oestrous cycle (Ashworth *et al.*, 1987) and also during late pregnancy (Ledger *et al.*, 1985) causes a significant reduction in progesterone concentrations. The aim of these studies was to investigate whether treatment with a 3 β -HSD inhibitor would increase ovulation rate by reducing ovarian steroid production.

Materials and Methods

Animals

Two experiments were carried out during the normal breeding season (November and December) in 44 Welsh Mountain ewes. For the period of the two studies the ewes were housed in sheds at Blythbank Farm, West Linton, Borders Region, and fed on hay and concentrates as dictated by normal husbandry practice.

Experimental designs

Experiment 1. There were 3 experimental groups: Group 1, treated with vehicle only ($N = 15$; mean $\pm$ s.e.m. bodyweight = 42.5 ± 1.0 kg); Group 2, treated with the 3 β -HSD inhibitor only ($N = 15$, mean $\pm$ s.e.m. bodyweight = 40.8 ± 1.1 kg); Group 3, treated with 3 β -HSD inhibitor together with a progestagen sponge ($N = 14$, mean $\pm$ s.e.m. bodyweight = 42.4 ± 1.1 kg).

The oestrous cycles of all ewes had previously been synchronized using a 12-day intravaginal treatment with progestagen sponges containing 60 mg medroxyprogesterone acetate (Veramix: Upjohn Ltd, Crawley, Sussex, U.K.). Blood samples were collected from all ewes by jugular venepuncture at 12-h intervals from Day 8 (oestrus = Day 0) of the treatment cycle until oestrus. Serum was stored at -20°C until assayed. After blood sample collection, ewes were injected subcutaneously twice daily, either with vehicle (2 ml ethanol/ethyl oleate, Group 1), or 3 β -HSD inhibitor (50 mg/injection; Groups 2 and 3) from Day 10 to the morning of Day 15 of the treatment cycle. A previous dose-response study (Ashworth *et al.*, 1987) demonstrated that a single 50 mg subcutaneous injection with the 3 β -HSD inhibitor (Epostane; Sterling-Winthrop, Guildford, Surrey, U.K.) could reduce circulating progesterone concentrations by about 70% over a 12-h period. To ensure that the low progesterone concentrations did not allow the premature occurrence of preovulatory LH surges, progestagen sponges were positioned in the vagina of Group 3 ewes from Day 8 to Day 14 of the treatment cycle. Oestrus was checked by running the ewes with raddled vasectomized rams and the ovaries of all ewes were observed at mid-ventral laparoscopy between Days 3 and 7 of the following oestrous cycle to ascertain ovulation rate and ovarian morphological structures.

Experiment 2. Ewes were reallocated into two groups: Group 1 ($N = 22$) receiving vehicle (in Exp. 1 the animals were in the control group plus 7 ewes from 3 β -HSD inhibitor only group; mean ovulation rate = 1.50 ± 0.13) and Group 2 ($N = 22$) receiving 3 β -HSD inhibitor (in Exp. 1 the animals were in the 3 β -HSD inhibitor plus sponge group plus 8 ewes from the 3 β -HSD inhibitor only group; mean ovulation rate = 1.77 ± 0.13). The animals were not randomized before reallocation because it has already been demonstrated (Ashworth *et al.*, 1987) that the effect of the 3 β -HSD inhibitor is of short duration, about 12 h compared to a 16-day oestrous cycle, and therefore the chances of carryover were considered to be minimal. Ewes in Groups 1 and 2 were treated twice daily between Days 10 and 15 as described for Exp. 1. At the oestrus after treatment ewes were hand-mated with intact rams. All ewes underwent mid-ventral laparoscopy between Days 4 and 9 of the following cycle.

Hormone assays

Oestradiol. Concentrations of oestradiol in peripheral plasma were determined in duplicate by radioimmunoassay incorporating an affinity chromatography extraction procedure as described previously (Webb *et al.*, 1985). The assay results were computed by the A.B.R.O. Radioimmunoassay Program package based on the method of Rodbard & Lewald (1970). The minimum detectable value was 1.01 pg/ml. The peripheral concentration of the 3 β -HSD inhibitor (W. E. Jones, Sterling-Winthrop, personal communication) was <40 ng/ml and the cross-reaction in the assay at this concentration was undetectable. Moreover, negligible cross-reaction ($<0.001\%$) was observed at 50% displacement of the tracer when tested *in vitro*. There was no difference in peripheral oestradiol concentrations between control ewes and those treated with the progestagen sponges, indicating a lack of cross-reactivity with the circulating medroxyprogesterone acetate. The inter-assay ($n = 10$ assays) coefficients of variation using three standard quality control samples with mean oestradiol concentrations of 7.9 ± 0.4 , 14.2 ± 0.5 and 25.9 ± 0.8 pg/ml were 14.8, 11.3 and 9.5% respectively, and the intra-assay coefficient of variation was 10.5%.

Progesterone. Concentration of progesterone in peripheral plasma was determined in duplicate using the radioimmunoassay described by Corrie *et al.* (1981) and modified by Ashworth (1985). The minimum detectable value was 0.15 ± 0.03 ng/ml (8 assays). It has been demonstrated that there is negligible cross-reaction with the 3 β -HSD inhibitor as $<0.001\%$ cross-reaction was observed at 50% displacement of the progesterone tracer *in vitro* and the injection of 50 mg inhibitor into 5 ovariectomized ewes daily, for 3 days, had no effect on the basal progesterone concentrations (Ashworth *et al.*, 1987). Likewise, medroxyprogesterone acetate also had negligible cross-reaction with the assay as

progesterone concentrations actually decreased after progestagen sponge treatment. The interassay coefficients of variation ($n = 8$ assays) in 3 quality control samples with mean progesterone concentrations of 2.10 ± 0.06 , 3.01 ± 0.16 and 7.7 ± 0.33 ng/ml was 7.8, 15.2 and 12.2% respectively and the intra-assay coefficient of variation was 8.8%.

Statistical analyses

Unless otherwise indicated, significant differences between groups were analysed using analysis of variance and incorporating Scheffe's multiple comparison test (Scheffe, 1959). The hormone data were transformed to natural logarithms before analysis.

Results

Ovulation rate, ovarian morphology and oestrous cycle duration

Experiment 1. Treatment with the 3β -HSD inhibitor alone caused a significant increase ($P < 0.01$) in ovulation rate (Table 1). The increase in ovulation rate in the ewes treated with the progestagen sponges together with the 3β -HSD inhibitor was less marked. These ewes did, however, have a significant increase ($P < 0.01$) in oestrous cycle duration of about 1.5 days (Table 1), so that the interval between the end of treatment and ovulation was greater. There was no significant difference in oestrous cycle length between the control ewes and the ewes receiving the 3β -HSD inhibitor only (Table 1). The increase in ovulation rate after inhibitor alone was due to more ewes showing double and triple ovulations, whereas in both the control group and the group treated with the 3β -HSD inhibitor plus the progestagen sponge none of the ewes had more than 2 ovulations.

Experiment 2. As in Exp. 1 treatment again induced an increase in ovulation rate (Table 2), which reached significance ($P < 0.05$) when omitting the 2 ewes that did not ovulate. In this study 8 other animals also failed to exhibit oestrus. Again the increase in ovulation rate was due to more ewes showing double and triple ovulations. The increase in ovulation rate was also accompanied by changes in gross ovarian morphology, which was more pronounced in Exp. 2 compared with Exp. 1 (see Tables 1 & 2). Large follicles (estimated at 6–8 mm in diameter) were present at the time of laparoscopy, together with ovarian follicular cysts. As in Exp. 1, there was no difference in oestrous cycle length between the two groups.

Table 1. Effect of treatment on ovulation rate, oestrous cycle duration and gross ovarian morphology in sheep

	Control	3β -HSD inhibitor only	3β -HSD inhibitor plus progestagen sponge
No. of ewes	15	15	13†
Mean ovulation rate ($\pm$ s.e.m.)	1.27 ± 0.12^a	2.00 ± 0.17^b	1.62 ± 0.14^{ab}
No. of corpora lutea $\begin{cases} 1 \\ 2 \\ 3 \end{cases}$	$\begin{matrix} 11 \\ 4 \\ 0 \end{matrix}$	$\begin{matrix} 3 \\ 9 \\ 3 \end{matrix}$	$\begin{matrix} 5 \\ 8 \\ 0 \end{matrix}$
No. of ewes with large follicles‡	1	2	4
No. of ewes with cystic follicles	0	2*	0
Duration of oestrous cycle (days)	$16.80 \pm 0.14^*$	$16.46 \pm 0.17^*$	18.15 ± 0.19^y
Range for the group (days) over which oestrus was exhibited	16–18	15–17	17–19

Values with different superscripts are significantly different ($P < 0.01$).

†Data from Ewe 42 not included because of a 20-day oestrous cycle, due to an extended luteal phase (see Fig. 2).

‡Well vascularized, 6–8 mm in diameter, similar to preovulatory follicles.

*One ewe had 2 cystic follicles.

Table 2. Effect of 3 β -HSD inhibitor on ovulation rate, oestrous cycle duration and gross ovarian morphology in sheep

	Vehicle only	3 β -HSD inhibitor
No. of ewes	22	22
Mean ovulation rate ($\pm$ s.e.m.)	1.27 $\pm$ 0.10	1.75 $\pm$ 0.16*
Distribution of corpora lutea $\left\{ \begin{array}{l} 0 \\ 1 \\ 2 \\ 3 \end{array} \right.$	$\left\{ \begin{array}{l} 0 \\ 16 \\ 6 \\ 0 \end{array} \right.$	$\left\{ \begin{array}{l} 2 \\ 8 \\ 9 \\ 3 \end{array} \right.$
No. of ewes with large follicles	0	3
No. of ewes with cystic follicles	0	9†
Duration of oestrous cycle (days)	16.86 $\pm$ 0.14	17.00 $\pm$ 0.18‡

*Significantly different from controls ($P < 0.05$; Student's t test). Two ewes did not ovulate, but if these animals are included the mean ovulation rate = 1.59 $\pm$ 0.18.

†One ewe had 2 cystic follicles and one ewe had 4 cystic follicles.

‡8 ewes failed to exhibit oestrus.

Table 3. Effect of treatment on the number and distribution of lambs per ewe

	Vehicle only	3 β -HSD inhibitor
No. of ewes lambing†	21	11
Mean no. of lambs per ewe lambing ($\pm$ s.e.m.)	1.19 $\pm$ 0.09	1.55 $\pm$ 0.16*
Distribution of lambs $\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$	$\left\{ \begin{array}{l} 17 \\ 4 \end{array} \right.$	$\left\{ \begin{array}{l} 5 \\ 6‡ \end{array} \right.$

*Significantly different from vehicle only group ($P < 0.05$, Student's t test).

†Includes only those ewes that were pregnant to the mating after the treatment cycle.

‡Two lambs from the treatment group, both from sets of twins, were born dead.

Number of lambs

Experiment 2. In those ewes that became pregnant to the mating directly after treatment there was a significant increase ($P < 0.05$) in the mean number of lambs per ewe lambing (Table 3). This was due to a higher proportion of ewes having twin lambs, with no ewes giving birth to triplets.

Peripheral progesterone and oestradiol concentrations

Experiment 1. Treatment with the 3 β -HSD inhibitor caused a significant reduction ($P < 0.01$) in the peripheral concentrations of progesterone (Figs 1a & 1b). In addition, the progestagen sponge treatment significantly reduced progesterone concentrations further ($P < 0.01$, t test), even before 3 β -HSD inhibitor treatment ($P < 0.05$, t test). Three of the animals showed a marked transient increase in progesterone concentration after the last injection with inhibitor, with progesterone concentration reaching luteal-phase levels. This was particularly evident in Ewe 42 (Fig. 2) in which luteal-phase progesterone concentrations were maintained for at least 2 days after the cessation of 3 β -HSD treatment. This animal also had an oestrous cycle length of 20 days, 1 day longer than any of the other ewes in the treatment group and therefore has been dealt with separately. Oestradiol concentration in this ewe also increased significantly during the follicular phase of the cycle (Fig. 2).

Fig. 1. Mean ($\pm$ s.e.m.) progesterone concentration during Exp. 1 (a) Concentrations chronologically aligned in relation to the time of treatment. The range for each group over which oestrus was exhibited is indicated by the open bar containing the identifications of the group. (b) Concentrations chronologically aligned in relation to the time of oestrus.

Fig. 2. Progesterone (●—●) and oestradiol (▲—▲) concentrations during the oestrous cycle of Ewe 42 treated with 3β -HSD inhibitor plus a progestagen sponge. The results for this animal are not included in Table 1 or Figs 1 and 3.

There appeared to be no increase in the length of the follicular phase between the groups (Fig. 1b), although this becomes difficult to assess due to the lowering of the progesterone concentrations by the treatment.

As expected, all 3 groups showed a significant increase ($P < 0.01$) in mean oestradiol concentration during the follicular phase of the oestrous cycle (Figs 3a & 3b). Unexpectedly, however, the ewes treated with the 3β -HSD inhibitor also had significantly higher oestradiol concentrations ($P < 0.01$) than did control animals both during the follicular phase of the oestrous cycle and during treatment on Day 14 of the oestrous cycle (Fig. 3a).

Discussion

Treatment with the 3β -HSD inhibitor induced a significant increase both in the number of eggs ovulated and in the number of lambs born per ewe lambing. This appears to be the first demonstration of an enzyme inhibitor treatment causing an increase in ovulation rate. The improvement in ovulation rate was due to an increase in the proportion of ewes showing twin and triple ovulations rather than to an occasional ewe showing multiple ovulations. This is a major advantage as the response to gonadotrophin treatment is very variable between animals and can lead to excessive superovulation (see Hunter, 1980), with increased prenatal loss (Hanrahan & Quirke, 1985) and reduced lamb birthweight (Black, 1983). The smaller increase in ovulation rate in the ewes treated with inhibitor plus progestagen sponges was probably related to the significant increase in oestrous cycle length in this group of ewes. At the dose given, the 3β -HSD inhibitor only lowers progesterone concentrations for about 12 h (Ashworth *et al.*, 1987), hence the lengthening of the oestrous cycle and the interval from final treatment until the preovulatory gonadotrophin surge presumably reduced the effect of the inhibitor.

The increase in ovulation rate was also accompanied by changes in gross ovarian morphology (Tables 1 & 2), as indicated by the presence of large follicles and cystic follicles at laparoscopy, 3–9 days after oestrus. The presence of cystic follicles, especially in Exp. 2 suggests that the treatment interfered with the hormonal feedback equilibrium between the hypothalamus–pituitary gland and

Fig. 3. Mean ($\pm$ s.e.m.) oestradiol concentrations during Exp. 1. (a) Data chronologically aligned in relation to the time of treatment. The range of oestrus for each group is indicated by the open bar containing the group treatment. (b) Data chronologically aligned in relation to the time of oestrus.

the ovaries. This is supported by the observation that, in Exp. 2 (Table 2), 2 ewes were anovulatory and a further 8 ewes failed to exhibit oestrus. As the ewes had been synchronized with a progestagen sponge treatment two oestrous cycles previously, the animals were perhaps not closely synchronized and the inhibitor was probably given later in the follicular phase. The inhibitor may therefore be interfering at the level of hypothalamus with the positive feedback effects of oestradiol, necessary to trigger both oestrous behaviour and the preovulatory gonadotrophin surge (see review by Baird & McNeilly, 1981). This conclusion is supported by the results from Exp. 1, indicating that peripheral oestradiol concentrations are not limiting and, in fact, were significantly increased by the treatment (Figs 3a & 3b). A similar effect has been demonstrated after immunization against ovarian steroids

(Fairclough *et al.*, 1976; Scaramuzzi *et al.*, 1981) when there is an optimum range of antibody titre (Webb *et al.*, 1984); too high a titre led to abnormal follicular growth coupled with blocked oestrus and failure to ovulate. However, these effects of steroid antisera are probably caused by a lack of steroid feedback rather than by direct antibody action on the hypothalamus–pituitary gland.

One further possible cause of the increase in the number of ewes not showing oestrus, not ovulating and having cystic follicles in Exp. 2 may be due to the repeated treatment of the ewes. However, at the dose given the effect of the inhibitor on progesterone concentrations lasts for less than 24 h (Ashworth *et al.*, 1987) and in Exp. 1, 2 ewes in the 3β -HSD inhibitor only group (Table 1), not previously treated, also had cystic follicles. Furthermore, ewes not previously treated with the 3β -HSD inhibitor also showed cystic follicles and a few did not ovulate despite there being an overall increase in ovulation rate (R. Webb, unpublished observation). Obviously, further studies are required to eliminate the possibility of any residual effect of repeated treatment with the 3β -HSD inhibitor.

Ovulation rate can be significantly reduced by oestradiol treatment (Webb & Gauld, 1985a). By contrast, the increase in ovulation rate in these studies was associated with a significant increase in follicular oestradiol concentrations, despite a significant decrease in progesterone production by the corpus luteum. Steroid precursor for oestradiol production by the follicles is clearly not limiting, and the enhancement of circulating oestradiol concentrations may occur because there is increased passage of steroid precursors via the Δ^5 enzyme pathway. Studies of women during the first trimester of pregnancy, with two different 3β -HSD inhibitors, have demonstrated an increase in pregnenolone and dehydroepiandrosterone concentrations (Van Der Spuy *et al.*, 1983). The exact mechanism by which the effect on follicular growth and the increased ovulation rate is achieved is not known and further studies are currently in progress, but as discussed previously one possibility is that the treatment interferes with the hormonal feedback equilibrium between the hypothalamus–pituitary gland and the ovaries. A direct effect at the ovarian level cannot be discounted, however, especially as there was also an increase in peripheral oestradiol concentrations, follicular growth and ovulation in Group 3, which had been treated with inhibitor plus progestagen sponges.

The 3β -HSD inhibitor significantly reduced peripheral progesterone concentration (Figs 1a & 1b). After progestagen sponge treatment progesterone concentrations were significantly reduced even further and this was effective before the start of the inhibitor treatment. A similar observation, after progestagen treatment, has been noted in women (Johansson, 1971) and is due to a direct action of progestagen on the 3β -HSD enzyme system in the corpus luteum (Shinada *et al.*, 1978). This can be overcome by gonadotrophin treatment (Johansson, 1971). Whether the effect is due to an additive action directly on the 3β -HSD enzyme system or via gonadotrophin secretion needs to be investigated.

One further observation was the increase in oestrous cycle duration in the ewes given the 3β -HSD inhibitor together with progestagen sponge treatment (Table 1; Figs 1a, 1b & 2). This is probably due to a delay in the regression of the corpus luteum. The transient increase in peripheral progesterone concentrations in 3 of the ewes observed in Group 3 (Exp. 1) on Day 15 of the cycle (Fig. 1a) and particularly in Ewe 42 (Fig. 2), immediately after sponge withdrawal and the cessation of inhibitor treatment, suggests an effect on corpus luteum function rather than any residual effect from the progestagen sponge. Furthermore, no residual effect from progestagen sponge treatment was noted in the pretreatment synchronization cycle, during which oestrus occurred about 48 h after sponge removal, compared with the treatment cycle in which the interval from sponge removal to oestrus was at least 4 days.

The extension of the luteal phase is presumably not due to an increase in luteotrophic support, because the level of steroid negative feedback would have been maintained by the presence of the progestagen sponge together with the higher circulating values of oestradiol-17 β (Figs 3a & 3b). One further possible explanation could be a lack of progesterone priming of the uterus thereby interfering with prostaglandin release and consequently luteolysis (Baird *et al.*, 1976; Louis *et al.*, 1977). However, a lack of progesterone priming as an explanation for the increased oestrous cycle

length is doubtful as the ewes had been treated with progestagen sponges until Day 14. Furthermore, luteolysis appeared to occur normally in the ewes treated with inhibitor only (Fig. 1a & 1b) and the competitive inhibition of 3β -HSD activity is ineffective in bringing about structural luteolysis (Jenkin *et al.*, 1984). It is possible that 3β -HSD enzyme activity plays a central role in the luteolytic mechanism and that the synergistic action of the inhibitor together with the synthetic progestagen perturbed this action.

Previous reports have demonstrated a close temporal association between peripheral progesterone and oxytocin concentrations (Webb *et al.*, 1981; Sheldrick & Flint, 1981), although Rice *et al.* (1986) have suggested that the release of progesterone from subcellular particles is not affected by treatments which stimulate the release of oxytocin from luteal granules. Oxytocin appears to be important for CL regression because immunization against oxytocin prolongs the luteal phase (Sheldrick *et al.*, 1980; Schams *et al.*, 1983). Furthermore, Flint & Sheldrick (1985) have demonstrated that continuous infusion of oxytocin prevents luteal regression and delays the onset of behavioural oestrus in cyclic ewes, possibly by down regulating the uterine oxytocin receptor. If during luteolysis there is a positive feedback between oxytocin and endometrial PGF-2 α (Flint & Sheldrick, 1982, 1983, 1985), then perturbation of this mechanism by the treatment given to ewes in Group 3 (Exp. 1) may have affected the timing of luteolysis.

In conclusion, these studies demonstrate that ovulation rate and production of lambs per ewe lambing can be significantly improved by 3β -HSD inhibitor treatment. Whether this approach will be of commercial significance depends on finding both a suitable dose to induce an increase in ovulation rate, without the detrimental side effects of blocking oestrus and ovulation, and also finding a simple delivery system.

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