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Plasma LH and FSH in Ewes that were either Fertile or Infertile after Long-term Grazing of Oestrogenic Pasture*

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Abstract

The levels of plasma LH and FSH were measured in serial blood samples taken at 15-min intervals for 6 h from ewes that had remained fertile after grazing oestrogenic pasture (clover-fertile ewes), from ewes that were permanently affected by clover disease (clover-infertile ewes) and from normal ewes. Two flocks of ewes from different locations were studied. In flock 1, tonic LH secretion (total area under the curve of LH concentration versus time, 1 area unit = 1 ng ml^{-1} × 1 h) was significantly ($P < 0{\cdot}05$) greater in clover-infertile ewes (10·4 area units) during anoestrus than in ewes that had remained fertile after prolonged grazing of oestrogenic clover (5·4 area units). Tonic LH and FSH secretions during the breeding season and FSH secretion during anoestrus were not significantly different. In flock 2, LH levels during the breeding season were significantly ($P < 0{\cdot}05$) elevated in clover-infertile ewes (10·9 area units) compared to normal ewes (5·4 area units) that had never grazed oestrogenic clover. LH secretion in clover-infertile ewes (7·8 area units) was intermediate to that found in infertile and control ewes. Concentrations of FSH, progesterone and ovarian vein oestradiol-17β (E_2) during the breeding season were similar in the three groups.

In another experiment, the positive feedback release of LH following administration of E_2 (12·5, 25 or 50 μg per ewe) was measured in anoestrous ewes of flock 2. Significantly ($P < 0{\cdot}01$) more clover-infertile ewes demonstrated a positive feedback effect than control ewes when given 12·5 μg E_2 but not when given higher doses.

The elevation of LH secretion in permanently affected clover-infertile ewes is consistent with the hypothesis that the hypothalamo–pituitary axis of these ewes is less responsive to the negative feedback effect of oestrogen. Furthermore, the patency of the positive feedback loop is consistent with the ability to ovulate.

Other keywords: Clover disease.

Introduction

Long-term grazing of oestrogenic clover pastures may cause permanent infertility in ewes (Bennetts *et al.* 1946; Barrett *et al.* 1965) although not all ewes grazing oestrogenic clover exhibit infertility. Infertile ewes have uterine endometrial hyperplasia and changes in cell type and function of the cervix (Adams 1976). In addition, there may be an associated neuroendocrine lesion. For example, Findlay *et al.* (1973) have reported that when infertile ewes which had previously grazed oestrogenic clover were ovariectomized, they were less likely to show elevated plasma LH levels, i.e. positive feedback (Goding *et al.* 1969), in response to oestradiol-17β (E_2) treatment than were normal ovariectomized ewes. This reduced responsiveness of the hypothalamo–pituitary axis to E_2 could be related to changes in the number or affinity

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of cytoplasmic E_2 receptors in the pituitary gland, or both (Tang and Adams 1978). If long-term grazing of oestrogenic clover does cause the hypothalamo–pituitary axis to become less responsive to E_2, then the basal plasma gonadotrophin levels should be higher than normal, due to a reduction in the negative feedback effect of E_2 (Scaramuzzi *et al.* 1971). The increased LH would stimulate the ovary to secrete more E_2 and eventually a new dynamic equilibrium would be set in which higher plasma levels of gonadotrophins and E_2 were produced. Elevation in gonadotrophin levels would stimulate follicular development, and in support of this Adams *et al.* (1979) have observed higher ovulation rates in ewes that became infertile after grazing oestrogenic clover.

In this study, the possibility that ewes having grazed oestrogenic clover had elevated LH and FSH secretion was examined. The E_2-induced positive feedback in intact anoestrous ewes following long-term grazing of oestrogenic clover was also studied.

Materials and Methods

Animals

Flock 1. Merino ewes (Bungaree strain, 5½yr) that had continually grazed oestrogenic clover at Kangaroo I., S.A. The ewes were allocated to two groups: group 1*a*, $\leqslant$ one completed pregnancy in 5 years (clover-infertile ewes) and group 1*b*, $\geqslant$ three completed pregnancies in 5 years (clover-fertile ewes).

Flock 2. Merino ewes (6–7 yr) that were grazing non-oestrogenic pastures at Werribee, Vic., throughout the study. These comprised the following:

Group 2*a*: clover-infertile ewes (Peppin strain) that had previously grazed oestrogenic clover and had a lambing rate (percentage of mated ewes which completed pregnancy) of less than 10% (Rizzoli and Moran 1977).

Group 2*b*: fertile ewes (Murray strain) that had previously grazed oestrogenic pasture until 2 years prior to the present study and had greater than 90% lambing rate (clover-fertile ewes).

Group 2*c*: fertile ewes (Peppin strain) that had never grazed on oestrogenic pastures.

Experimental Procedure

In the first three experiments, plasma LH and FSH concentrations were measured in serial blood samples. Jugular venous cannulae were inserted on the day before samples were taken. Blood samples were collected in heparinized syringes at 15-min intervals for 6 h, centrifuged immediately, and the plasma stored at −20°C until assay.

Experiment 1. Ewes from groups 1*a* ($n = 5$) and 1*b* ($n = 4$) were sampled during the anoestrous season (October).

Experiment 2. Ewes from groups 1*a* ($n = 4$) and 1*b* ($n = 5$) were given intramuscular injections of 100 μg cloprostenol (Estrumate, I.C.I. Australia, Ltd.) to induce luteal regression (Baird and Scaramuzzi 1975) during the breeding season (February). Blood samples were collected from these ewes 13 days after treatment.

Experiment 3. Ewes from flock 2 (9 or 10 ewes per subgroup) were treated as in experiment 2 and blood samples taken at 15-min intervals for 6 h, 12 days after cloprostenol treatment. Immediately following the sampling period, all ewes in groups 2*a* and 2*c* and six ewes in group 2*b* underwent laparotomy after anaesthesia which had been induced with thiopentone/pentobarbitone and maintained with halothane. For measurement of plasma E_2, blood samples (10 ml) were collected into heparinized syringes by needle (23 gauge) puncture of each ovarian vein. The level of E_2 in each ewe was taken as the average concentration in venous plasma collected from both ovaries. Plasma progesterone concentrations were measured in the last jugular venous sample taken during the serial blood-sampling period. One ewe from group 2*b* did not have a corpus luteum at

the time of laparotomy and its peripheral plasma progesterone concentration was 0·2 ng ml^{-1}. This animal was excluded from the trial.

Experiment 4. To examine the positive feedback effect of E_2 on LH secretion during the anoestrous season, ewes from flock 2 were treated with subcutaneous progesterone implants (Sil-Estrus, Abbott Laboratories Ltd) for 14 days. Twelve hours after implant removal, E_2 (12·5, 25 or 50 μg, vehicle 1·0 ml peanut oil) was injected intramuscularly and jugular venous blood samples were collected by venepuncture at 2-h intervals from 8 to 24 h and 28, 32 and 36 h after the injection.

Hormone Assays

For each sheep, all plasma samples were measured in the same assay. Samples were distributed between assays so that each treatment group was equally represented.

Plasma LH levels in experiments 1–4 were measured by a solid-phase radioimmunoassay (Goding *et al.* 1969) and in experiment 4 by a double antibody method using the same antiserum, raised in a horse against bovine LH (Snook 1968) and used at a final dilution of 1 in 10^6. The double antibody method used a goat anti-horse serum raised by Dr T. Stelmasiak, Department of Agriculture, Vic., as a source of second antibody (final dilution 1 in 250). Assay procedure was similar to that described by Lee *et al.* (1976). Purified ovine LH (Papkoff G3222B, biopotency 2·7 × NIH-LH-S1) donated by Dr H. Papkoff, Hormone Research Laboratory, University of California, was used in the preparation of tracer by the chloramine-T method (Greenwood *et al.* 1963). NIH-LH-S18 (biopotency 1·03 × NIH-LH-S1) was used for the standard. The between-assay coefficients of variation (c.v.) of two plasma pools were 16% (seven assays) at 7·0 ng ml^{-1} and 20·0% at 1·8 ng ml^{-1} and the within-assay c.v. (Burger *et al.* 1972) was less than 20% between 0·7 ng ml^{-1} and 25·4 ng ml^{-1}.

Plasma FSH was measured using a heterologous double antibody radioimmunoassay with an antiserum (Butt M94) raised in a rabbit against human FSH (McNeilly *et al.* 1976) and NIH-FSH-S6 (biopotency 1·24 × NIH-FSH-S1) as standard and Papkoff FSH (G4-150C; biopotency 54 × NIH-FSH-S1) for preparation of tracer (Salamonsen *et al.* 1973). This assay has been previously described by Bremner *et al.* (1980). The within-assay c.v. was less than 20% over the range 30–800 ng ml^{-1} (eight assays). The between-assay c.v. of five assays for three plasma pools were 5·9% at 418 ng ml^{-1}, 16·4% at 234 ng ml^{-1} and 25·8% at 81·2 ng ml^{-1}.

Peripheral plasma progesterone was measured by the method of Hossain *et al.* (1979). All the samples were measured in one assay and were within the range of 0·6–10·4 ng ml^{-1} (c.v. <20%).

Ovarian venous plasma E_2 was measured by radioimmunoassay using an antiserum (9R-727) raised in a Welsh Mountain ewe (Scaramuzzi *et al.* 1975); there was detectable cross-reaction with oestrone (11·0%) and oestriol (0·5%). Plasma samples (0·5–1·0 ml) were extracted with hexane–diethyl ether (4:1 v/v). The extracts were then applied to celite columns (Abraham *et al.* 1970) and E_2 eluted with ethyl acetate–isooctane (2:3 v/v). The assay was conducted in a similar manner to that of Baird *et al.* (1974). All the samples were measured initially in one assay, and some very high and very low samples were reassayed. Values obtained for buffer extract and wether plasma were 2 pg per tube. The intra-assay c.v. was 20%, from 7 to 45 pg per tube and sample values were corrected for procedural losses.

Analysis of Results

The plasma LH and FSH secretion (area units) for the 6-h period was estimated for each ewe as the area under the curve formed by joining the plasma concentration of the hormone (ng ml^{-1}) at each 15-min interval by a straight line (1 area unit = 1 ng ml^{-1} × 1 h) (Hooley *et al.* 1974). The mean secretion of each group was compared to that of controls by Student's *t*-test. For analysis of LH secretion by the pituitary gland, it has been accepted that pulsatile secretory episodes are reflected in transitory elevations of the hormone levels in plasma (Lincoln 1976; Martensz *et al.* 1976). In the present study, a secretory episode was identified when a sample value exceeded the level of the previous sample by at least two standard deviations of the assay value of the previous sample. The frequency of secretory episodes (pulses) and the maximum plasma level of LH during each secretory episode (pulse height) were analysed by χ^2 test of independence and Student's *t*-test respectively. In experiment 4, the number of ewes that showed positive feedback of E_2 on LH secretion (plasma levels >10 ng ml^{-1} for 6 h or >20 ng ml^{-1} for 4 h after E_2 injections) in each group were compared by χ^2 analysis.

Results

Experiments 1 and 2

In flock 1, the infertile ewes had significantly ($P < 0{\cdot}05$) higher plasma LH levels than the fertile ewes during anoestrus (Table 1). Although a similar trend was evident during the breeding season, the difference between the two groups was not significant (Table 1). During anoestrus, secretory episodes of LH were more frequent in the

Table 1. LH and FSH secretion (mean ± s.d.) in flock 1 ewes during the anoestrous or breeding season

Group	No. of ewes	Season	Total area under curve (area units)	
			LH	FSH
1*a* (infertile)	5	Anoestrous	10·4±1·6[A]	188±71
1*b* (fertile)	4	Anoestrous	5·4±1·3	306±114
1*a* (infertile)	4	Breeding	10·7±7·3	141±84
1*b* (fertile)	5	Breeding	6·7±3·2	142±62

[A] Significantly ($P < 0{\cdot}05$) different from fertile anoestrous ewes by Student's *t*-test.

infertile ewes (one per 5 h) than in the controls (one per 24 h; only one pulsatile discharge recorded in four ewes). Secretory patterns were not as well defined in the breeding season; some of the infertile ewes had elevated basal LH levels but there was little evidence of clearly defined secretory episodes and a statistical evaluation was not attempted.

Table 2. LH and FSH secretions, peripheral plasma progesterone levels and ovarian venous plasma levels of E_2 (mean ± s.d.) in flock 2 ewes during the breeding season

Group:	2*a*	2*b*	2*c*
Description	Clover-infertile	Clover-fertile	Control
No. of ewes	10	9	10
LH			
Total area under curve (area units)	10·9±4·1[A]	7·8±4·6	5·4±4·9
Pulse frequency	1 per 2·5 h	1 per 2·8 h	1 per 6·0 h
Pulse height (ng ml^{-1})	3·0±1·6	2·2±1·2	2·4±1·0
FSH			
Total area under curve (area units)	384±91	439±112	413±111
Progesterone (ng ml^{-1})	5·2±2·9	4·8±3·3	5·4±2·7
E_2 (pg ml^{-1})	159±142	54±29[B]	123±101

[A] Significantly ($P < 0{\cdot}05$) greater than control by Student's *t*-test.
[B] Ovarian venous samples taken from only six ewes.

Plasma FSH secretion did not differ between fertile and infertile ewes in anoestrus or during the breeding season (Table 1).

Experiment 3

At laparotomy 12 days after cloprostenol treatment, 29 out of 30 ewes had at least one functional (peripheral plasma progesterone concentration > 1 ng ml^{-1}) corpus luteum. The remaining ewe was eliminated from the experiment.

Ewes that were infertile after grazing oestrogenic clover had significantly ($P < 0{\cdot}05$) greater LH secretion during dioestrus compared with control ewes of normal fertility (Table 2). Furthermore, ewes that had grazed clover but remained fertile had LH secretion that was intermediate to that of control and clover-infertile ewes (Table 2). Elevated plasma LH secretion at this time was reflected in the greater frequency of pulsatile discharges, but not in the height of the discharges (Table 2). FSH secretion, peripheral plasma progesterone, and ovarian venous E_2 levels in the three groups were not significantly different. There was wide variation in the levels of E_2 measured.

Experiment 4

Ewes that had previously grazed oestrogenic clover showed a significantly ($P < 0{\cdot}01$; $\chi^2 = 16{\cdot}25$, 2 d.f.) greater propensity to discharge LH in response to 12·5 μg E_2 than did control ewes (Table 3). This trend was not apparent when the ewes were given 25 or 50 μg of E_2.

Table 3. Proportion of ewes showing a positive feedback effect to E_2 injections given during anoestrus (Expt 4)

Responses are defined in Materials and Methods

E_2 (μg per ewe)	Proportion of ewes responding		
	Group 2*a*	Group 2*b*	Group 2*c*
12·5	5/6	6/6	2/6[B]
25·0	6/7	5/6	4/6
50·0	—[A]	6/6	5/5

[A] Group 2*a* ewes not treated with this dose.
[B] Significantly ($P < 0{\cdot}05$) fewer ewes responded than in groups 2*a* or 2*b* treated at this dose.

Discussion

Ewes that had grazed oestrogenic clover but remained fertile had only slightly elevated LH secretion, whereas those with severe infertility had significantly elevated LH secretion. Earlier findings (Hearnshaw *et al.* 1977) demonstrated that ingestion of oestrogenic clover resulted in acute increases in LH in the ovariectomized ewe. This study has shown that LH levels can be elevated in clover-infertile ewes, even after the ewes have not grazed oestrogenic pasture for some years.

Elevation of LH secretion is consistent with the hypothesis that the hypothalamo–pituitary axis in clover-infertile ewes is less sensitive to feedback regulation by oestrogen (Tang and Adams 1978). Changes in responsiveness to oestrogen, in terms of negative feedback on LH, also occur in ovariectomized ewes, with changing photoperiod (Legan *et al.* 1977) and during the *post partum* period (P. J. Wright *et al.*, unpublished data). FSH secretions were not significantly altered in the clover-infertile ewes. This is consistent with the hypothesis that the release mechanisms

for LH and FSH are under differential control (Findlay and Cumming 1977; Radford *et al.* 1978).

Elevated LH secretion might be expected to increase plasma E_2 levels since pulsatile LH release stimulates ovarian E_2 secretion (Baird *et al.* 1976; Baird 1978). In the present study, no significant differences were detected in E_2 levels in single samples taken from the ovarian vein of infertile or normal ewes. However, the sampling procedure was not adequate to account for variation in secretion rates of E_2. It will be necessary to determine E_2 secretion rates before this point may be clarified.

When the positive feedback effects of E_2 on LH secretion were monitored during anoestrus, the clover-infertile ewes were more responsive to a low dose of E_2 (12·5 μg) than normal ewes. These results disagree with those previously reported by Findlay *et al.* (1973) who used ovariectomized ewes in which the LH secretion responses to oestrogen were different from those in entire ewes (Brown *et al.* 1972; Karsch *et al.* 1977). Furthermore, the present results were corroborated in a study by W. A. Chamley (personal communication) who also demonstrated that clover-infertile ewes were more sensitive to the positive feedback effects of E_2, releasing LH earlier than normal ewes after treatment in anoestrus. The sampling intervals employed in the present study (experiment 4) did not allow this assessment to be made.

In conclusion, the present results demonstrate a hypothalamo–pituitary lesion in clover-infertile ewes. Radford (1979) concluded that the E_2 negative feedback effect occurred principally at the hypothalamus in sheep. A reduction in the negative feedback sensitivity to E_2 and normal or increased positive feedback sensitivity in clover-infertile ewes could be due to changes in oestrogen receptors in the pituitary (Tang and Adams 1978; Rodgers 1979) and hypothalamus (Rodgers 1979), respectively.

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