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Hypothalamic–Pituitary Function in Normal Ewes and Ewes which Grazed Oestrogenic Subterranean Clover for Several Years

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Abstract

Plasma luteinizing hormone (LH) concentrations were measured in normal Merino ewes and in Merino ewes with lowered fertility which had resulted from prolonged grazing of *Trifolium subterraneum* L. cv. Dinninup. During the anoestrous season, LH was measured at frequent intervals before and following administration of oestradiol-17β or gonadotrophin releasing hormone (GnRH), and around the time of spontaneous oestrus.

All ewes responded to GnRH treatment and there were no differences between the two groups in terms of the amount of LH released or the time to reach maximal plasma hormone concentration. Comparable proportions of ewes from both groups responded to oestradiol-17β treatment with LH levels showing initially a negative feedback response (i.e. suppression to approximately 80% of pretreatment levels) followed by positive feedback response. During the positive feedback phase, the quantity of LH which was released was comparable in both groups; however, the time interval over which this hormone was secreted was significantly shorter for clover-infertile ewes ($P < 0{\cdot}05$).

During the anoestrous season the mean LH level was lower in normal ewes than in clover-infertile ewes [$0{\cdot}45 \pm 0{\cdot}41$ ($n = 31$) versus $0{\cdot}82 \pm 0{\cdot}73$ ($n = 48$) ng/ml, $P < 0{\cdot}01$]. In the oestrous season the mean LH level for normal ewes rose to $1{\cdot}23 \pm 0{\cdot}65$ ng/ml ($n = 76$) but there was no change for the clover-infertile ewes ($0{\cdot}77 \pm 0{\cdot}59$ ng/ml, $n = 73$).

These experiments indicate that in the clover-infertile ewe LH concentration remains static throughout the breeding and non-breeding seasons but the results suggest that this altered endocrine status is not due to any differing level of sensitivity to negative feedback effects of oestradiol-17β as compared with normal ewes.

Introduction

In some regions of Western Australia grazing sheep can be exposed to a high intake of oestrogenic isoflavones originating from some of the cultivars of subterranean clover (*Trifolium subterraneum* L.). A consequence of this oestrogen-rich diet is the development of 'clover disease' which is reflected by poor reproductive performance in flocks so affected. A number of physiological changes and clinical manifestations of the condition have been described; including cystic hyperplastic endometritis (Bennetts *et al.* 1946), metaplasia resulting in cervical tissue looking more like uterine tissue (Adams 1976), variability in oestrous cycle length (Adams *et al.* 1975), production of large volumes of watery cervical mucus (Smith 1971) which can be associated with reduced sperm transport (Lightfoot *et al.* 1967), and alterations in luteal function (Adams *et al.* 1980).

The availability of synthetic gonadotrophin releasing hormone (GnRH) made it possible to test a limited number of ewes with a history of permanent clover infertility for possible alterations to normal hypothalamic–pituitary function (Findlay *et al.* 1973). These studies indicated that aged, ovariectomized, clover-affected ewes were capable of releasing lutenizing hormone (LH) in response to GnRH, but not in response to exogenous oestradiol-17β (E_2), and it was suggested that clover disease might be associated with some alteration in hypothalamic function.

In the experiments to be reported in this paper, LH responses to GnRH or E_2 were compared in a group of Merino ewes which had permanent clover-induced infertility and in a group of appropriate controls. In a further experiment LH secretion around the time of a spontaneous oestrus was monitored in detail in affected and control ewes.

Materials and Methods

The studies were conducted on Merino ewes (aged 8 years) which were used in the experiment of Rossiter and Marshall (1974). Clover-diseased ewes had grazed the potent oestrogenic Dinninup cultivar of subterranean clover for 4 years, and the lambing percentage in the year of study was 12·5% whereas the control ewes had grazed the very slightly oestrogenic Northam A cultivar during this period, and their corresponding lambing percentage was 84%. All ewes had grazed together on non-oestrogenic pasture for a year before the study. Three series of experiments were conducted in which plasma LH concentrations were measured in the non-treated and the oestrogen-treated animals.

Since analysis of the data required some definition of the start of an LH peak, this was defined as that point at which plasma LH increased by 50%, was further increased in one of the subsequent two plasma samples and then continued to increase beyond that point.

Series I: Administration of GnRH

These experiments were performed in November of 1973 and 1974 (i.e. during the anoestrous period). Twenty-six clover-diseased and six control ewes were studied and blood samples (10 ml) were collected by venipuncture from one jugular vein according to a standardized schedule: −30, −15, 0, 15, 30 min and at 30-min intervals thereafter to 180 min. At 0 min, each ewe received an intravenous injection of 20 μg GnRH (Hoechst, Frankfurt). Immediately after collection the blood samples were centrifuged and the plasma stored at −12°C until it was assayed for LH.

Series II: Administration of Oestradiol-17β

Fifteen clover-diseased and 12 control ewes were studied and two of the clover-diseased ewes were studied on each of two separate occasions. These experiments were carried out during the period of seasonal anoestrus in November 1974 and September 1975. At least 1 day prior to the start of each experiment, ewes were brought into an enclosed shed and one jugular vein was cannulated using Silastic tubing (Dow Corning, Houston, Texas). Ewes were then run in group pens with free access to feed and water. Blood samples were taken at intervals of 2 h for up to 38 h. Each ewe received an intramuscular injection of 40 μg E_2 in peanut oil 8 h after the start of blood collection. Plasma was collected as described for the series I experiments and later assayed for LH.

Series III: Spontaneous Secretion of LH around the Time of Oestrus

An initial series of experiments was conducted in the breeding season (May) in which 13 clover-diseased and 11 control ewes were used. Three ewes of each type had been used previously in series II experiments. The ewes had been selected from the flock so that their expected date of oestrus occurred during the study period. All ewes had one jugular vein cannulated and were then placed with fresh, harnessed vasectomized rams whereupon blood sampling was commenced. A sample of 10 ml blood was collected from each ewe at intervals of 4 h. Ewes were observed continually for oestrous behaviour, and when any ewe had stood for a ram the sampling interval was reduced to 2 h and continued for a further 36 h. The plasma samples were later assayed for LH.

A similar experimental program was followed using a second group of animals (11 clover-diseased and 11 controls) although in this case, ewes were not run continually with rams. Instead, cotton dental swabs were introduced into the vagina and changed every 12 h. After removal, the swab was weighed and oestrus was assumed to have begun during the 12 h interval in which the weight of mucus collected was more than 0·79 g (Smith and Allison 1971). A 10-ml blood sample was collected from these ewes every 2 h commencing 24 h before the predicted time for the onset of oestrus and continuing over a period of at least 72 h. Plasma samples were later assayed for LH.

Hormonal Assays

All plasma samples which were collected in these experiments were assayed for LH by using the solid-phase radioimmunoassay described by Goding *et al.* (1969), in which a purified LH preparation with a biological potency of 2×NIH-LH-S1 (Papkoff *et al.* 1965) was used. The antiserum used in the assay was an anti-ovine LH serum which was provided by Dr W. Hansel, Cornell University, New York State. Within an experiment, plasma samples from both control and treated animals were assayed in the same assay and more than 90% of the values were in the range 0·2–2·5 ng/ml. The limit of sensitivity of the assay was <0·2 ng/ml. Between-assay coefficients of variation were as follows: 0·2–1·0 ng/ml, ±32%; 1·0–2·5 ng/ml, ±40%; 2·5–5·0 ng/ml, ±61%; 5·0–10·0, ±24·8%. Within-assay variation for two pools of plasma was ±17·8% at 1·8 ng/ml and ±11·5% at 7·0 ng/ml (15 aliquots measured).

Table 1. LH levels and the characteristics of preovulatory-type LH surges which were induced by oestradiol-17β (E_2) given to anoestrous normal and clover-infertile Merino ewes (series II)

* $P < 0·05$. ** $P < 0·01$. Differences of significance tested by Student's *t*-test

Parameter	Normal ewes	Clover-infertile ewes
No. of ewes treated	12	17
No. of ewes having an LH peak	8	14
Mean ±s.d. LH concn (ng/ml) before treatment with E_2	0·45±0·41	0·84±0·73**
No. of samples	31	48
Mean±s.d. LH concn (ng/ml) between administration of E_2 and start of surge	0·36±0·29	0·65±0·56*
No. of samples	58	73
Interval (h) from administration of E_2 to start of LH surge	12·0±5·35	10·31±7·26
Interval (h) from administration of E_2 to maximal LH level	19·0±6·5	14·76±8·17
Duration of LH surge (h)	17·1±6·86	11·85±3·14*
Total LH released (area units)[A]	188·5±57·8 (8)	237·3±84·2 (11)

[A] Calculated only for those ewes which showed a complete LH peak. Number of ewes shown in brackets.

Results

Series I

All ewes responded to GnRH treatment and there was no significant difference in mean LH levels between the two groups of animals during the pretreatment period (clover-diseased ewes: 4·0±10·5; controls: 4·6±9·6 ng/ml). Mean LH response as calculated from the area under the response curve (Hooley *et al.* 1974) was 153±104 area units for clover-diseased ewes and 74±58 area units for controls. Differences were not significant. There was no difference in the time taken to reach peak LH concentration following GnRH stimulation (control: 95±22 min; clover-diseased: 96±36 min).

Series II

The results of this series of experiments are shown in Table 1. A preovulatory-type LH response (both negative and positive feedback) to E_2 treatment was observed in 14 out of 17 ewes with clover infertility and 8 out of 12 normal ewes. Mean plasma LH levels were significantly higher in clover-infertile ewes during the interval before E_2 injection and from this point to the start of the LH peak (see Table 1). In both groups, E_2 depressed LH values by a similar extent (around 80% of pre-injection levels). There were no differences between groups in the time interval between E_2 treatment and either the start of the LH peak or the point when peak plasma LH levels were attained (Table 1). The total quantity of LH measured during the preovulatory-type surge was similar in both groups; however, the interval of time over which this was secreted was significantly shorter for the clover-diseased ewes (Table 1).

Table 2. Oestrous behaviour, LH levels (mean ± s.d.) and the characteristics of spontaneous preovulatory LH surges in normal and clover-infertile ewes (series III)
* $0 \cdot 1 > P > 0 \cdot 05$ (Student's *t*-test)

Parameter	Normal ewes	Clover-infertile ewes
No. of ewes	22	24
No. of ewes coming into oestrus	11	16
No. of ewes showing an LH peak	8	9
Mean ± s.d. LH concn (ng/ml) in 24 h before start of LH surge	1·23 ± 0·65	0·77 ± 0·59*
No. of samples	76	73
Duration of LH surge (h)	19·5 ± 5·95	18·47 ± 7·22
Total LH released (area units)	354·8 ± 145·6	269 ± 171·66

Series III

The results obtained in these experiments are shown in Table 2. Of the 24 clover-diseased ewes, 16 came into oestrus. A preovulatory peak of plasma LH was detected in eight of these and in one other ewe which did not come into oestrus. In the control ewes, 11 came into oestrus with seven of these and one other having a preovulatory LH peak. In these experiments differences in mean levels of plasma LH during the 24 h preceding the start of the preovulatory peak were not significant and neither were there differences in the duration or magnitude of the LH peak.

Discussion

LH responses to GnRH in normal and clover-infertile ewes were similar to those described by Findlay *et al.* (1973). Whereas these investigators were unable to trigger LH secretion by administering E_2 (given intravenously in ethanol–saline) to clover-infertile ewes we were able to elicit a preovulatory-type LH surge, using the same dose of E_2, in most of both clover-infertile and normal ewes in the series II experiment. No obvious explanation for this discrepancy is available; however, it is possible that our attempts were more successful because the ewes were younger and were entire, whereas Findlay *et al.* (1973) studied responses in older ovariectomized ewes. Modes of administration and differences in injection vehicle may also have been

important. It is not clear why the surge of LH which was induced by E_2 treatment was secreted over a shorter period in the clover-diseased ewes (Table 1). Possibly the higher LH levels measured during the pretreatment period and during the response phase, when negative feedback would be operative (Scaramuzzi *et al.* 1971), reflect a greater degree of priming of the pituitary by endogenous luteinizing hormone releasing factor (Aiyer *et al.* 1974). Because the total quantity of LH released was similar in both groups, this observation infers that in the clover-diseased ewe either LH enters the peripheral circulation at a faster rate or the metabolic clearance rate for LH is greater.

The most interesting information to come from the series II experiments conducted during anoestrus was the significant differences in basal levels of LH both before E_2 was given and during the phase of the response when negative feedback would be operative (Scaramuzzi *et al.* 1971). No differences were observed in series I experiments, possibly because too few samples were taken for an accurate assessment. No differences in basal LH levels and in the duration of the spontaneous preovulatory LH surge were observed in the series III experiments which were conducted during the breeding season. This suggestion of an effect of season upon differing degrees of feedback regulation was also evident in the experiments reported by Rodgers *et al.* (1980). Consideration of these data and those of Rodgers *et al.* (1980) indicates that the basis for this apparent seasonal effect is the failure of the clover-diseased ewes to raise their basal LH levels during the breeding season, or to lower it during anoestrus.

These observations suggest an important deviation in the clover-diseased ewe from normal neuroendocrine function. It has been established that in the ewe, the breeding season is associated with enhanced pituitary activity as reflected by frequent and pulsatile LH release and an overall increase in the concentration of LH in the peripheral circulation (Baird *et al.* 1976; Legan *et al.* 1977). With the onset of the anoestrous season, this frequency with which LH pulses are secreted from the pituitary decreases and there is a concomitant fall in the peripheral plasma LH concentration (Scaramuzzi and Baird 1977; Legan and Karsch 1979). This change in the pattern of LH secretion has been attributed to an increase in sensitivity of the hypothalamic–pituitary axis to negative feedback effects of E_2 (Legan and Karsch 1979).

In the above experiments the degree of suppression of LH following administration of E_2 to normal and clover-infertile ewes during anoestrus was comparable for both types of animal. This suggests that differences between normal and clover-diseased ewes in mean LH concentration, which we observed within either the breeding season or the non-breeding season, were not due to differences in sensitivity to negative feedback effects of E_2. Furthermore, in the clover-diseased ewes plasma LH concentrations did not change as they passed from the breeding to the non-breeding season, suggesting that in such sheep, sensitivity to negative-feedback effects of oestrogen does not alter according to season.

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