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Effect of Ingestion of Oestrogenic Clover on Luteinizing Hormone Release in the Ovariectomized Ewe

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Abstract

Luteinizing hormone (LH) concentration was measured in the jugular plasma of six groups of ovariectomized ewes which were fed oestrogenic clover (*Trifolium subterranean* L. Gray cv. Yarloop) for 3 days. Ewes in groups B, D and F received an injection of oestradiol-17β (E_2) 24 h after first being offered clover, whilst ewes in groups A, C and E received no oestrogen treatment. Following the initial ingestion of clover, plasma LH concentrations for all groups fell within 3 h and then increased significantly. Each subsequent feeding of clover usually resulted in a temporary decrease and then an elevation of the LH concentrations. When ewes in groups B, D and F were injected with E_2, LH concentrations fell and remained low for approximately 9 h, then increased to a peak of $72 \cdot 3 \pm 10 \cdot 4$ ng/ml within 5 h. The LH concentrations remained elevated for a period of about 12 h. The present paper indicates that the ingestion of oestrogenic clover by ovariectomized ewes results in a depression and then a release of LH, and that this release does not appear to deplete the pituitary of LH or cause refractoriness to an oestrogenic stimulus.

Introduction

When ewes are grazed on highly oestrogenic clovers over the mating period, infertility results (Morley *et al.* 1964). This infertility has been demonstrated to be of a temporary nature since normal fertility returns 5 weeks after cessation of the intake of oestrogenic clover (Morley *et al.* 1966). This 'temporary infertility' should not be confused with the permanent and progressive infertility first described by Bennetts *et al.* (1946). In permanently affected flocks, ewes are normally joined some months after pastures have dried off and lost their oestrogenic potency. Ewes may show a decline in fertility after spending as little as 6 months on these pastures, and this decline becomes progressively more severe with each year of grazing. This disease is also characterized by maternal dystocia, post-natal mortality in lambs, uterine prolapse and cystic glandular hyperplasia of the endometrium.

The evidence available on the mechanism underlying 'temporary infertility' suggests that it involves a reduction in the percentage of ewes in oestrus (Engle *et al.* 1957; Coop and Clarke 1960; Lightfoot and Wroth 1974), a depression of fertilization rate, a disturbance of the rate of egg transport and a reduction in the number of sperm reaching the site of fertilization (Morley *et al.* 1964; Holst and Braden 1972; Lightfoot and Wroth 1974). There are conflicting reports as to whether ovulation rate is reduced (Ch'ang 1961; Morley *et al.* 1964, 1966; Lightfoot and Wroth 1974). The ingested phyto-oestrogens may also affect luteal function. Obst and Seamark (1970) reported that peripheral plasma progesterone levels fell 2–3 days earlier in ewes grazing the oestrogenic clover (*Trifolium subterranean* L. Gray cv. Yarloop) and

Lightfoot and Wroth (1974) observed that corpora lutea weighted less in clover-affected ewes.

Oestrogens have been shown to affect the release of luteinizing hormone (LH) from the pituitary gland in ovariectomized ewes. Following oestrogen administration, an initial depression, followed by a large release (*c.* 50 ng/ml) of LH occurred (Radford *et al.* 1969; Goding *et al.* 1970; Scaramuzzi *et al.* 1971; Hearnshaw 1972). The present paper examines the hypothesis that the ingestion of oestrogenic clover by ovariectomized ewes results in a release of LH, and further that this release might interfere with the large release of LH normally observed when ovariectomized ewes are treated with oestradiol-17β (E_2).

Table 1. Experimental schedule for ovariectomized ewes ingesting Yarloop clover and receiving E_2 injections

Prior to the commencement of observations on day 7, feed was removed from all ewes for 18 h (day 6). The clover offered to the ewes was *Trifolium subterranean* L. Gray cv. Yarloop

Group	No. of ewes	Feed offered on days:				E_2 injection on day 8
		1–3	4–5	6	7–9	
A	3	Clover	Clover	—	Clover	—
B	3	Clover	Clover	—	Clover	+
C	3	Hay	Clover	—	Clover	—
D	3	Hay	Clover	—	Clover	+
E	3	Hay	Hay	—	Clover	—
F	3	Hay	Hay	—	Clover	+

Materials and Methods

Eighteen Merino × Border Leicester ewes aged 5 years were ovariectomized at least 2 months prior to the experiment and were penned individually. The ewes were randomly allotted to six groups of three animals, and received Yarloop clover or chopped grass pasture hay for a total of 3, 5 or 8 days (Table 1). Half of the animals received 40 μg E_2 in saline intravenously at 0800 h on day 8.

Prior to the commencement of the 9-day treatment period, blood samples were obtained by venipuncture from each ewe at hourly intervals for 6 h. On day 6 of the treatment period all ewes were denied access to food for 18 h in an attempt to ensure that the ewes were hungry and would begin eating the Yarloop clover immediately it was offered to them on day 7. On day 6, ewes were also fitted with an indwelling jugular catheter and jugular blood samples were then obtained at hourly intervals on days 7, 8 and 9. Blood samples were centrifuged immediately after collection and the plasma was stored for assay of LH concentration by the radioimmunoassay of Goding *et al.* (1969).

The Yarloop clover (*Trifolium subterranean* L. Gray cv. Yarloop) was grown as a pure stand on the State Research Farm, Werribee, and was cut daily and fed fresh in weighed aliquots. During the 3 days of blood sampling, each ewe was fed clover at approximately 0900 h on day 7, at 0400, 1300 and 1600 h on day 8 and at 1100 and 1600 h on day 9. Phyto-oestrogen content of a pooled sample from each cut of clover was estimated by the method of Francis and Millington (1965).

For statistical analysis, the data on LH concentration were grouped within five time periods:

Period 1. The period of 6 h prior to commencement of treatments.

Period 2. The period from 1 to 8 h after commencement of the final 3-day feeding schedule (day 7).

Period 3. The period from 9 to 18 h after commencement of the final 3-day feeding schedule (day 7).

Period 4. The period from 1 to 11 h after the time of the E_2 injection (day 8).

Period 5. The period from 12 to 26 h after the time of the E_2 injection (day 8).

These periods were selected such that at the end of periods 2 and 4 the mean LH concentrations were similar to the mean concentrations from period 1, and periods 3 and 5 were of sufficient length to include respectively the first LH release following the ingestion of clover on day 7 and the LH release following E_2 administration on day 8. Previous experiments had suggested that responses, if present, would occur within these periods. Minimal bias is introduced to the analysis by the selection of such periods (R. Jardine, personal communication).

The following comparisons and analyses of variance were performed on the LH concentration data:

(1) The effect of previously fed Yarloop clover given to ewes for 0, 2 or 5 days, on baseline levels of LH, and the effect of phyto-oestrogen intake on LH concentration. *Analysis 1*: Group A was combined with group B (previous ingestion of clover for 5 days), group C with D (previous ingestion of clover for 2 days) and group E with F (no previous ingestion of clover). Periods 1, 2 and 3 were compared.

(2) The effect on LH concentration, of superimposing an injection of 40 μg E_2 on ewes already ingesting Yarloop clover. *Analysis 2*: The six groups of ewes (A, C, E without E_2 treatment, B, D, F with E_2 treatment) were considered independently for periods 4 and 5 only.

Results

Phyto-oestrogen Intake

The proportion of leaf in the fresh clover was 48% by weight, and the dry matter percentages of leaves and stems were 19·5 and 9·3% respectively. Dried leaves contained by weight 0·7% formononetin, 3·3% genistein and 1·1% biochanin A, and the stem portion of the clover contained undetectable levels of the phyto-oestrogens.

Table 2. Mean phyto-oestrogen intake of ovariectomized ewes

Phyto-oestrogen intake was calculated from the leaf portion of the ration only; the stem portion contained undetectable levels of phyto-oestrogen

Treatment period	Groups	Mean phyto-oestrogen intake per ewe (g/day)		
		Formononetin	Genistein	Biochanin A
Days 1–6	A and B	1·6	7·59	2·50
	C and D	1·75	8·25	2·75
	E and F	—	—	—
Days 7–9	A and B	2·45	11·55	3·85
	C and D	2·45	11·55	3·85
	E and F	2·10	9·90	3·30

During days 7–9 of the treatment, each ewe in groups A, B, C and D consumed a total of approximately 10 kg of clover, whilst ewes of groups E and F consumed a total of approximately 9 kg of clover. These differences were not significantly different. Mean phyto-oestrogen intakes per day of ewes for days 1–5 and 7–9 of the experiment are given in Table 2.

Effect of Ingestion of Oestrogenic Clover on Plasma LH Concentration

Previous ingestion of oestrogenic clover by ovariectomized ewes for 0, 2 or 5 days did not significantly modify the baseline plasma levels of LH or the LH response to the subsequent intake of clover on days 7–9. Changes in the plasma LH concentration during the 24 h following the ingestion of oestrogenic clover occurred whether or not the ewes had been previously eating clover. Thus there were no significant differences

between the LH concentrations for periods 1, 2 and 3 for all groups of ewes (Tables 3 and 4). Therefore, the data were pooled into two groups—data for those ewes ingesting clover and receiving an E_2 injection (groups B, D and F) and data for those ewes ingesting clover but not receiving E_2 (groups A, C and E).

Table 3. Effect of ingestion of oestrogenic clover on plasma LH concentrations in ovariectomized ewes

Each value is the mean ± s.e. for three sheep for each period (period 1 is 6 h, period 2 is 8 h, period 3 is 10 h)

Group	Plasma LH concentration (ng/ml) in period:		
	1	2	3
A	9·3±1·8	8·9±0·9	15·1±2·6
B	9·6±1·4	12·1±0·8	19·8±3·0
C	8·9±0·1	11·9±1·3	20·5±3·0
D	8·4±0·9	10·4±0·8	17·5±2·1
E	7·0±1·6	7·5±1·8	14·2±3·8
F	7·7±3·3	7·6±2·6	10·2±4·3

Table 4. Effect of ingestion of oestrogenic clover on the plasma LH concentration in ewes which had been previously ingesting clover for 0, 2 or 5 days

Summary of analysis of variance for ewes from groups A–F for periods 1, 2 and 3. Error 1 is the between animal within treatment variation. Error 2 is the period by between animal within treatment variation

Source of variance	d.f.	Mean square	Variance ratio
I Previous clover treatment (0 *v.* 2 *v.* 5 days)	2	83·0	2·79 n.s.
II Period of experiment (1 *v.* 2 *v.* 3)	2	308·56	42·97**
I×II	4	11·47	1·6 n.s.
Error 1	15	29·80	4·15**
Error 2	30	7·18	—

** $P < 0·01$. n.s. Not significant.

Following the initial ingestion of clover on day 7, the plasma LH concentrations for groups B, D and F and groups A, C and E declined in 3 h to 7·1±0·8 and 7·1±0·5 ng/ml respectively, then rose again to control levels (8·6±1·1 and 8·4±0·8 ng/ml respectively). The LH concentrations then increased significantly to 19·5±4·0 and 23±4·8 ng/ml respectively. Each subsequent feeding of clover usually resulted in a temporary decrease and then an elevation of LH concentrations (Figs 1*a* and 1*b*). LH concentrations in ovariectomized ewes fed non-oestrogenic rations did not show these consistent patterns (Hearnshaw 1972). The analysis of variance of the first three periods for all ewes indicated that the LH concentrations in period 3 were significantly greater ($P < 0·01$) than those in periods 1 and 2. The analysis did not demonstrate any difference between periods 1 and 2 (Table 4). However, the fall in LH levels following ingestion of oestrogenic clover (period 2) occurred in all animals and appeared to be biologically significant (Figs 1*a* and 1*b*).

When ewes in groups B, D and F were injected with 40 μg E_2, LH concentrations fell to $5{\cdot}0 \pm 0{\cdot}2$ ng/ml in 3 h, and remained low for approximately 9 h (period 4). LH concentrations then increased to a peak of $72{\cdot}3 \pm 10{\cdot}4$ ng/ml in 5 h, and they remained elevated for a total of about 12 h (period 5). The duration and magnitude of this LH response were similar to responses obtained when ovariectomized ewes not fed oestrogenic clover were injected with E_2 (Hearnshaw 1972). Fig. 1*a* shows the mean LH concentrations ($\pm$s.e.) for the ewes ingesting oestrogenic clover and receiving an E_2 injection (groups B, D and F). The analysis of variance of this data (Table 5) showed a significant difference ($P < 0{\cdot}01$) between periods 4 and 5, and between the oestrogen (groups B, D and F) and no oestrogen (groups A, C and E) treatment groups. The interaction between treatment and periods was significant. Those ewes receiving the E_2 injection had significantly lower LH concentrations in period 4, and significantly higher LH concentrations in period 5 than did those ewes not receiving E_2.

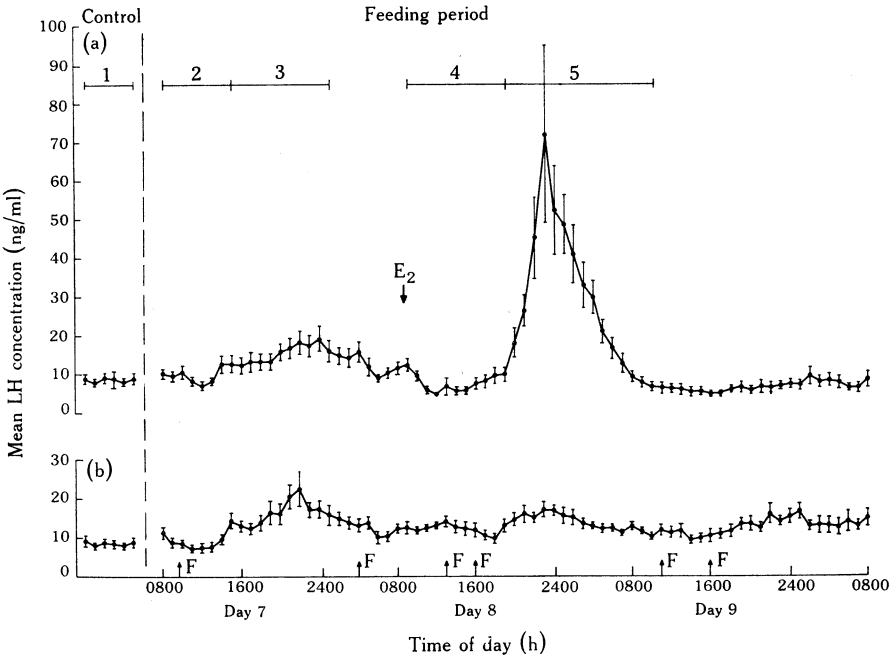

Fig. 1. Effect of ingestion of oestrogenic clover on plasma LH concentrations in ovariectomized ewes. (*a*) Ewes receiving an E_2 injection (groups B, D and F; $n = 9$). (*b*) Ewes not receiving an E_2 injection (groups A, C and E; $n = 9$). Fresh clover was added (F) to feed troughs as available clover was eaten. Periods 1–5 are indicated above the graphs. Standard errors are shown by vertical bars.

Discussion

The data indicate that the ingestion of oestrogenic clover can evoke increases in LH secretion in ovariectomized ewes. It is known that the degree of oestrogenicity of a pasture for grazing sheep is more closely related with the levels of formononetin than with those of genistein or biochanin A (Morley *et al.* 1968). It is therefore of interest to note that the clover fed to the ewes had a formononetin content of 0·7% of the leaf dry matter, a level considered only to be of moderate oestrogenic potency.

Despite this, the amount of phyto-oestrogen ingested by the ewes was sufficient to evoke both the typical 'negative' and 'positive' responses of LH which occur following steroidal oestrogen stimulation (Scaramuzzi *et al.* 1971).

The changes that occurred in the concentration of LH during the 24 h following the ingestion of oestrogenic clover did so whether or not the ewes had been previously eating clover. The fact that all ewes were fasted for 18 h prior to the blood sampling period may have contributed to the lack of residual oestrogenic effects of the previously ingested clover. However, the hypothalamo–pituitary axis may have become refractory if the ewes had continued to ingest the oestrogenic clover for a greater length of time before being sampled for LH. Since ovariectomized ewes ingesting oestrogenic clover exhibited a normal surge release of LH following oestradiol administration, it appeared that the phyto-oestrogens did not interfere with the mechanism responsible for this LH release and therefore probably did not cause any saturation or refractoriness of hypothalamic receptor sites.

The data suggest that LH concentrations of ovariectomized ewes may be sensitive enough to the intake of phyto-oestrogens to use this measurement of LH as a monitor of pasture oestrogenicity.

Table 5. Effect of ingestion of oestrogenic clover and E_2 injection on plasma LH concentrations in ewes which had been previously ingesting clover for 0, 2 or 5 days

Summary of analysis of variance for ewes from groups A–F for periods 4 and 5. Error 1 is the between animal within treatment variation Error 2 is the period by between animal within treatment variation

Source of variance	d.f.	Mean square	Variance ratio
I Previous clover treatment (0 *v.* 2 *v.* 5 days)	2	18·46	0·42 n.s.
II Oestradiol (0 *v.* 40 μg)	1	648·55	14·91**
III Period (4 *v.* 5)	1	1811·92	59·35**
I × II	2	4·44	0·10 n.s.
I × III	2	9·62	0·32 n.s.
II × III	1	1388·80	45·49**
I × II × III	2	3·39	0·11 n.s.
Error 1	12	43·49	—
Error 2	12	30·53	—

** $P < 0·01$. n.s. Not significant.

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