Effects of oestradiol implants on the ovulation rate of the ewe

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Summary. In a series of 5 experiments, ewes were treated with implants releasing oestradiol-17 β and the effects on ovulation rate were observed. Large doses of oestradiol-17 β (> 20 μ g/day) produced anovulation while smaller amounts only reduced the proportion of twin ovulations. Amounts of exogenous oestradiol comparable to ovarian production rate in the luteal phase (< 1 μ g/day) produced a significant ($P < 0.01$) suppression in ovulation rate. Treatment during the follicular phase of the oestrous cycle was most effective, but treatment during the luteal phase alone also appeared to suppress ovulation rate. Furthermore, in 2 of 3 experiments ewes treated with low amounts of oestradiol during the first half of the luteal phase were less likely to have multiple ovulations at the subsequent oestrous period. The results support the hypothesis that oestrogen is involved in the physiological control of ovulation rate in the ewe, but this action is probably not restricted to the assertion of dominance by a maturing follicle during the follicular phase.

Keywords: oestrogen; ovulation rate; sheep

Introduction

Oestrogen is believed to play an important role in determining the number of ova shed by the ewe (the ovulation rate). Immunization against oestrogen increases the ovulation rate in sheep (Scaramuzzi *et al.*, 1980) and monkeys (Zelevnik *et al.*, 1985). Furthermore, treatment with exogenous oestrogen decreases the ovulation rate in monkeys (Billiar *et al.*, 1985), sheep (Land, 1976), rabbits (Meuli *et al.*, 1987) and rats (Dierschke *et al.*, 1983). However, the manner by which oestrogen brings about these effects is unknown.

There have been few detailed studies on the role of oestradiol in the control of ovulation rate. Webb & Gauld (1985) found that oestradiol implants which caused only a small increase in circulating oestradiol concentrations could decrease ovulation rate, while Morley *et al.* (1963, 1966) showed that low amounts of diethylstilboestrol or plant oestrogens also reduced ovulation rate. Morley *et al.* (1966) suggested that the ovulation rate did not return to normal immediately after treatment with diethylstilboestrol ceased. The following studies were carried out to determine the minimum amount of oestradiol which could affect ovulation and the ovulation rate, to determine the stage of the oestrous cycle at which oestradiol was most effective, and to see whether oestradiol produced a prolonged action similar to that observed for diethylstilboestrol.

Materials and Methods

Implants were prepared using Silastic tubing (Dow Corning Corp., Midland, MI, USA) packed with crystalline oestradiol-17 β (Sigma, St Louis, MO, USA). Such implants release less than 1/1000 of their content of oestradiol-17 β

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over 24 h, and have a constant release rate for up to 1 year (Green & Foote, 1978). Implants were prepared in 601-285 tubing (i.d. 1.57 mm, o.d. 3.18 mm) or 601-335 tubing (i.d. 3.35 mm, o.d. 4.65 mm). The low amounts of oestradiol were very difficult to measure accurately in the peripheral plasma of treated ewes, and so the most reliable estimate of the amounts of oestradiol being used was the release rate *in vitro*. Release rates *in vitro* and *in vivo* are generally very similar (Green & Foote, 1978; N. R. Adams & M. R. Sanders, unpublished observations). Accordingly, the doses of oestradiol referred to in this paper were calculated from the release rate *in vitro*. Release rates of oestradiol-17 β were measured *in vitro* after preincubation for 3 days at 37°C in 0.01 M-Tris buffer (pH 7.4) containing 0.1 M-NaCl and 0.3% gelatin. Release rates were determined using a shaking waterbath, with aliquants taken at 1, 2, 4, 8 and 20 h. The samples were diluted in phosphate-buffered saline and 100 μ l were extracted in 1 ml diethyl ether. The increase in circulating oestradiol-17 β produced in ewes by the implants was examined in a plasma sample collected from 8 ovariectomized ewes bearing single 1-cm 601-285 tubing or 3-cm 601-335 tubing implants, 10 days after insertion. The plasma samples (2 ml) were extracted once with 6 volumes of diethyl ether. The extracts were assayed in a previously described radioimmunoassay (Atkinson *et al.*, 1986). Extraction recovery was 90% and the sensitivity of the assay was 2.73 pg/tube. The blank value in this assay for incubation medium was undetectable, and for ovariectomized ewe plasma was 7.4 pg/ml. The non-specific binding was 3.2% and the intra-assay coefficient of variation was 4.8%. All samples were analysed in one assay to avoid interassay variation. A log-logit transformation was applied to the standard curve from which the concentrations were read.

Mature Merino ewes aged 3–5 years and weighing 55–65 kg were grazed on dry, non-oestrogenic pasture in groups of 35–50 per treatment. The studies were carried out over a 4-year period during the breeding season (January to April), a time when the ovulation rate of the flock normally varies between 1.15 and 1.45. Ewes were not given implants more than once in a season except in Exp. 4. Oestrus was synchronized with intravaginal sponges containing 60 mg medroxyprogesterone acetate (Upjohn, Rydalmere, NSW, Australia), and implants were placed subcutaneously in ewes tranquilized with acetylpromazine (Apex Labs., St Marys, NSW, Australia). Implants were equilibrated for 2 days in the buffer described above before insertion. Sponges were removed after 12 days. The number of corpora lutea on the ovaries was counted by laparoscopy performed under local anaesthesia using lignocaine (Troy Labs., Smithfield, NSW, Australia) in ewes tranquilized with acetylpromazine.

In Exp. 1 the induction of anovulation was examined. Ewes were given 3 cm or 12 cm of 601-335 tubing implants, 0.6 cm of 601-285 tubing implants, or blank implants at the time vaginal sponges were inserted. The number of ewes studied, and the estimated release rates of the implants, are given in Table 1. Implants were removed at the time of laparoscopy 7 days after sponge removal. The ewes were then resynchronized with vaginal sponges 28 days later for 12 days and re-examined by laparoscopy 4 days after the removal of the second sponge.

In Exp. 2 the stage of the oestrous cycle most affected by oestradiol was examined. All ewes received progestagen sponges for 11 days to synchronize their oestrous cycles. Ewes (49 per group) were given 1-cm 601-335 tubing implants (estimated to release 7.5 μ g oestradiol/24 h) for 2 days immediately before (luteal) and after (follicular) sponge removal, or for the full 4-day period (luteal + follicular). Other ewes received empty implants for 4 days. The ewes were examined by laparoscopy 7 days after sponge removal, and again 24 days later, after resynchronization.

Experiments 3–5 examined the minimum amount of oestradiol necessary to reduce the frequency of twin ovulations, using different lengths (between 0.2 and 1.6 cm) of 601-285 tubing. Between 35 and 40 ewes were examined in each treatment group. Implants were placed in the ewes at the time vaginal sponges were inserted, and were removed at the time sponges were removed (Exp. 5) or at the time of laparoscopy 10 days later (Exps 3 and 4). Laparoscopy was performed 7 days after sponge removal in Exp. 5. In Exps 3 and 4, sponges were placed in the ewes again at 7 and 10 days after the initial withdrawal for a further 12 days, and the ewes re-examined by laparoscopy 9 days later.

Data were analysed statistically by comparison with the χ^2 distribution after correction for continuity. When aspects of independent experiments examined the same phenomenon, χ^2 values were added. Unless otherwise stated, ewes which did not have a corpus luteum were not included in the analysis. Ovulation rates, when given, are expressed as the proportion of ewes with twins in the ewes which ovulated.

Results

The release rate of oestradiol-17 β from 601-335 tubing was estimated *in vitro* to be 7.5 ± 0.7 μ g/cm/24 h, and from 601-285 tubing to be 1.9 ± 0.5 μ g/cm/24 h. The 3-cm 601-335 implants increased plasma oestradiol concentrations by 3.1 pg/ml, while the 601-285 implants did not produce a detectable increase in this assay.

The number of ewes which failed to ovulate was increased by the large dose of oestradiol in Exp. 1 (90 μ g/day; Table 1), but amounts <23 μ g/day did not cause a significant increase in the level of anovulation (Tables 1–3) in the period immediately after sponge withdrawal.

In Exp. 2, oestradiol significantly reduced the ovulation rate if given only during the follicular phase of the oestrous cycle (Table 2). However, the degree of suppression of ovulation rate in the ewes given oestradiol only during the luteal phase approached statistical significance ($P = 0.065$) when compared to the controls.

Table 1. Number of ewes with 0, 1 or 2 ovulations after treatment with oestradiol-17 β (Exp.1)

Dose of oestradiol ($\mu\text{g/day}$)	No. of ewes	Ovulations			% Not ovulating	Ovulation rate (OR)	OR at 3rd cycle
		0	1	2			
0	39	3	29	7	8	1.19	1.17
1.2	37	8	28	1	22	1.03	1.26
22.4	40	5	34	1	13	1.03	1.29
89.5	11	9	2	0	82*	1.00*	1.30

*Different from control, $P < 0.05$.**Table 2.** Effect of 7.5 $\mu\text{g/day}$ oestradiol at different stages of the oestrous cycle on the number of ewes with 0, 1 or 2 ovulations (Exp. 2)

Stage	Ovulations			Ovulation rate (OR)	OR at next cycle
	0	1	2		
Control	3	26	20	1.43	1.25
Luteal	5	33	11	1.25	1.37
Follicular	7	37	6	1.14*	1.31
Luteal + follicular	3	39	6	1.13*	1.32

*Different from control, $P < 0.05$.**Table 3.** Number of ewes with 0, 1 or 2 ovulations after treatment with low doses of oestradiol-17 β

Exp.	Dose of oestradiol ($\mu\text{g/day}$)	Ovulations			Ovulation rate (OR)	OR at next cycle
		0	1	2		
3	0	4	26	10	1.28	1.33
	1.55	6	34	3	1.08	1.14*
	3.10	9	30	2	1.06*	1.18
4	0	3	19	14	1.42	1.41
	0.39	2	30	3	1.09*	1.10*
	0.78	3	25	8	1.24	1.32
	1.55	2	26	8	1.24	1.37
5	0	2	28	12	1.30	—
	0.58	2	35	6	1.15	—

*Different from controls, $P < 0.05$.

In studies to determine the minimal effective dose of oestradiol (Exps 3 and 4), relatively low doses of oestradiol (between 0.39 and 3.1 $\mu\text{g/24 h}$) produced statistically significant depressions in ovulation rate (Table 3). The degree of suppression appeared variable, possibly because of the discrete nature of the data. However, it is clear from the results presented in Table 3 that implants releasing $< 1 \mu\text{g}$ oestradiol/day reduced the twinning rate of ewes. Combined analysis of the 3 treatment groups in which $< 1 \mu\text{g}$ oestradiol/day was used (Table 3) gave an additive χ^2_3 value of 11.56 ($P < 0.01$).

When the ovulation rate of the subsequent oestrous cycle was examined, continued depression of the ovulation rate was observed in ewes receiving low doses of oestradiol in Exps 3 and 4 (Table 3). No carry-over effect was observed in Exps 1 or 2 (Tables 1 & 2).

Discussion

These results show that administration of $< 1 \mu\text{g}$ oestradiol-17 β per day can reduce the ovulation rate of Merino ewes. It is also probable that ovarian production of oestradiol was suppressed by the exogenous hormone, so that differences between the treated and control groups were even less than the amount administered. Such low amounts of oestradiol are comparable with estimates of the ovarian production rates during the luteal phase of 0.3–2 μg per day (Cox *et al.*, 1971; Baird & Scaramuzzi, 1976) and well below the 5–8 $\mu\text{g}/24 \text{ h}$ produced at oestrus. These results, together with the increase in ovulation rate produced by immunization against oestrogen (Scaramuzzi *et al.*, 1980), indicate that oestrogen is involved in the control of ovulation rate. Immunization against oestrogen or aromatizable androgen is accompanied by increased concentrations of LH, but not necessarily FSH, in peripheral plasma (Scaramuzzi & Radford, 1983). Implants similar to those used in the present work can decrease the frequency of LH pulses during the luteal phase (Atkinson *et al.*, 1989). However, the dynamic relationship of oestradiol and inhibin from the ovary with LH and FSH from the pituitary has made it very difficult to determine whether ovulation rate is controlled primarily by hypothalamic drive, or whether feedback from the ovary plays the major role. The present work demonstrates the extreme sensitivity of the control mechanism to oestrogen, and defines some of the characteristics of its action. Further progress awaits a sensitive assay which can integrate the pulsatile secretion of oestrogen to produce an indication of its overall activity in the animal.

We confirmed the suggestion of Morley *et al.* (1966) that ovulation rate did not always return to normal immediately after cessation of treatment with oestrogen. It is most unlikely that the reduced ovulation rate observed in the subsequent oestrous cycle was due to a persistence of oestradiol in the animal, because the amount of oestradiol released by the implants was very low, and clearance of oestradiol is very rapid in the ewe (Challis *et al.*, 1973) with a half-life $< 3 \text{ min}$. It is therefore inconceivable that effective amounts of exogenous oestrogen could have remained in the ewes at the time of luteolysis many days later. It is probable instead that the reduced ovulation rate was due to the presence of oestrogen during the first part of the luteal phase. In Exps 3 and 4, in which continued suppression was observed, the implants were removed at the time of the initial laparoscopy, which was about Day 7 of the subsequent oestrous cycle. On the other hand, no subsequent depression in ovulation rate was observed in Exps 1 and 2 in which the implants were not present during the relevant cycle. In Exp. 2 implants were removed before the new oestrous cycle began, while in Exp. 1 ewes were not examined until the second oestrous cycle after removal of the implants.

Our findings are consistent with previous observations on sheep exposed to non-steroidal oestrogens. Anovulation and a reduction in the incidence of multiple ovulations have been produced by diethylstilboestrol and isoflavone phyto-oestrogens (Morley *et al.*, 1966) and by coumestan phyto-oestrogens (Kelly *et al.*, 1976). Smith *et al.* (1979) found that ewes consuming as little as 50 mg coumestans per day had a reduced ovulation rate. The relative activities observed by Braden *et al.* (1967) indicate that this is equivalent to daily injection with 0.4 μg oestradiol, a value similar to the amounts used in the present study.

Although exogenous oestradiol appeared most effective when given during the follicular phase, it also had some effect when given during the luteal phase. Furthermore, as described above, oestradiol given during the early luteal phase reduced the ovulation rate. These results indicate that it is unlikely that the exogenous oestrogen reduced the ovulation rate solely by supplementing the negative feedback from the dominant follicle on the gonadotrophins during the follicular phase. Rather, oestradiol may influence the ovulation rate by more than one mechanism. Dierschke *et al.*

(1983) showed that oestradiol may have a direct action on the ovary of the rat. Such a local action has been dismissed in the ewe because multiple ovulation is a random event with regard to the ovary from which the second ovulation occurs, and it was believed that significant amounts of oestrogen were secreted only during the follicular phase. However, if the amount of oestrogen secreted in the luteal phase can affect ovulation rate, both ovaries may be exposed to roughly equivalent amounts of oestrogen. Therefore, the possibility that oestrogen may influence ovulation rate by a direct effect on the ovary in the ewe cannot be ruled out.

Supra-physiological amounts of oestradiol rendered the ewes anovulatory (Table 1), as has been observed for monkeys (Billiar *et al.*, 1985). Lesser amounts of oestradiol did not prevent ovulation, but reduced the incidence of multiple ovulations. Such a result is not possible if the ovulation rate followed a Poisson distribution, as would be required if follicles matured at random, and then were selected by passing through a fixed 'gateway' (Baird, 1987). If oestradiol had simply suppressed follicular maturation, for example by decreasing the width of the 'gateway' through which maturing follicles can avoid atresia and so go on to ovulate, the decreased number of twins should have been accompanied by an increased number of zero ovulations. There are a number of possible explanations as to why this did not occur, but the most likely is that oestradiol interacted with a product of the maturing follicle to produce suppression of other follicles. Such an interaction has been described, for example, between oestradiol and inhibin in the suppressions of FSH (Martin *et al.*, 1988). Suppression could not occur in the absence of a follicle, and so zero ovulations would not ensue.

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