Aust. J. Biol. Sci., 1981, **34**, 569–75

Induction of Ovulation in Seasonally Anovular Ewes by the Introduction of Rams: Effects of Progesterone and Active Immunization against Androstenedione

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Abstract

The effects of progesterone priming and active immunization against androstenedione on ovulation, oestrus and premature luteal regression were observed in seasonally anovular Merino ewes which had been induced to ovulate by the introduction of rams. Ovulation was induced in 27 out of 36 ewes, and the response was not affected by either immunization or progesterone priming. The ovulation rate (mean number of ovulations per ewe ovulating) was higher in ewes immune to androstenedione, but there was no significant effect of this treatment on oestrus or luteal maintenance. Priming with progesterone prior to the introduction of rams prevented premature regression of the corpora lutea but had no effect on the other measures of reproductive function.

If active immunization were to be applied on a commercial scale, it would not interfere with the practice of mating ewes during the anoestrous season. The increase in ovulation rate in these ewes, with no increase in the proportion of ewes ovulating, indicates that the mechanism for the control of ovulation is separate from that controlling ovulation rate. The mechanism by which progesterone pretreatment prevents premature luteal regression awaits further investigation.

Introduction

In Western Australia over half of the commercial Merino flocks are joined with rams during the anoestrous season, that is, in late spring and early summer, even though few of the ewes are ovulating spontaneously at this time (Knight *et al.* 1975). This is possible because most of these ewes can be induced to ovulate and show oestrous cycles by the introduction of rams (Schinckel 1954*a*). The first ovulation is rarely accompanied by oestrus (Schinckel 1954*a*, 1954*b*) and in about half of the ewes luteal regression and a second ovulation are completed within 8 days (Oldham and Martin 1978; Martin 1979). The failure to show behavioural oestrus and to maintain luteal function is due to the absence of a progestational phase prior to the introduction of rams (Hunter *et al.* 1971; Oldham *et al.* 1980). The ovulation rate at the first ovulation induced by rams is higher than that in subsequent ovulations, and if progesterone pretreatment is used to prime the ewes so that they display oestrus at this time, there may be economic gains in reproductive performance (Cognie *et al.* 1980).

Active immunization against androstenedione has also been proposed as a method for increasing the ovulation rate of commercial flocks (Scaramuzzi *et al.* 1977). Merino ewes which are similarly immunized exhibit normal patterns of seasonal breeding (Martin *et al.* 1979), so should be capable of responding to the introduction

of rams, possibly with even further improvement in ovulation rate. However, immunization against androstenedione apparently produced antibodies to progesterone and induced progesterone secretion from ovaries without corpora lutea (Scaramuzzi *et al.* 1980). The effectiveness of progesterone pretreatment in inducing oestrus and preventing premature luteal regression may therefore be reduced in these ewes.

It was proposed to test whether seasonally anoestrous Merino ewes which were actively immunized against androstenedione would ovulate in response to the introduction of rams, and whether such ewes would exhibit oestrus and maintain their corpora lutea after progesterone pretreatment.

Materials and Methods

Animals

A flock of 6-year-old Merino ewes was divided into two groups for immunization against human serum albumin (HSA, $N = 42$) or androstenedione-7-HSA (A_4-HSA, $N = 30$). The ewes were kept together on lush pasture during the initial immunization procedures, and on lighter pasture with supplements of meadow hay and lupin grain from the start of isolation from rams until the conclusion of the experiment.

Vasectomized rams fitted with harnesses and marking crayons were with the ewes from the beginning of immunization until the flock entered anoestrus. The ewes were examined at 17-day intervals, and when less than 20% of the ewes were marked in such a period, the rams were removed (August, late winter). The ewes remained completely isolated from rams, i.e. without visual, auditory or olfactory contact, for 12 weeks until November (early summer) when the experiments began. At this time a sample of 10 ewes from each group underwent laparoscopy to confirm seasonal anovulation. A single corpus albicans was the only sign of ovarian activity in the 20 ewes.

Immunization

The antigens used were human serum albumin (HSA) for the control group and androst-4-ene-3,17-dione-7-carboxyethyl-thioether-HSA (A_4-HSA). In February 1979 the ewes received their primary immunization, consisting of 1·2 mg antigen dissolved in 1·5 ml saline, and mixed with 1·5 ml DEAE-dextran adjuvant. This was injected at eight sites: two 0·9-ml aliquots intramuscularly, and six 0·2-ml aliquots subcutaneously. Each ewe also received 0·5 ml Pertussis vaccine (Commonwealth Serum Laboratories, Melbourne) as an additional adjuvant.

The first booster immunization given 12 days later was similar to the primary injection except that the dose of antigen was reduced to 0·5 mg per ewe. The second booster was given in November, 3 weeks before the introduction of rams. Each ewe received 0·5 mg antigen, which was dissolved in 1 ml saline, homogenized with 1 ml Freund's complete adjuvant (Commonwealth Serum Laboratories, Melbourne) and again homogenized with 1 ml of 1% (v/v) Tween-80 (Cox and Wilson 1976). The mixture was injected into eight sites as before and all ewes received a further injection of Pertussis.

Titres were estimated in plasma from blood sampled 12 days after the second booster immunization. The plasma was diluted $1:10^2$, $1:10^3$, $1:10^4$ or $1:10^5$ and 0·1 ml was added to tubes containing 22 pg of 1,2,6,7-H^3 androstenedione in 0·2 ml buffer [0·1 M phosphate, 0·14 M NaCl, 0·1% (w/v) azide, 0·1% (w/v) gelatin, pH 7·5]. The tubes were incubated overnight at 4°C before the addition of 0·5 ml dextran-coated charcoal [0·25% (w/v) Norit A charcoal, 0·025% (w/v) dextran T-70, 0·1% (w/v) gelatin, 0·14 M NaCl, 0·01 M phosphate, pH 7·5]. The tubes were incubated for 10 min at 4°C, then centrifuged at 1500*g* for 10 min at 4°C. An aliquot (0·5 ml) of the supernatant was counted in 4 ml of scintillation cocktail containing toluene, Triton X-100 and 0·4% (w/v) Omnifluor. The Omnifluor and radiochemical were obtained from New England Nuclear, and were used without any checks for purity.

The titre was estimated by the dilution at which 50% of the labelled steroid was bound by the plasma.

Experimental Design and Treatments

The ewes were allocated to treatments in a 2 × 2 × 2 factorial design, with approximately equal numbers of ewes in each subgroup. The treatments were progesterone pretreatment, immunization against androstenedione and the introduction of rams. Progesterone was administered by silicone implants (Silestrus, Abbott Laboratories, Sydney) placed subcutaneously in the axillae. They were removed after 10 days and 48 h prior to the introduction of rams.

The flock was divided across progesterone and immunization treatments, and one group was maintained in complete isolation from rams. The remaining group was introduced to 20 vasectomized Merino rams which were wearing harnesses and marking crayons. The rams remained with the ewes for 30 days, until 4 January 1980, and daily checks were made for ewes in oestrus.

Ovulation and Premature Luteal Regression

The ewes underwent laparoscopy (Oldham *et al.* 1976) 4 and 11 days after the introduction of rams. On each occasion the number of corpora haemorraghia or corpora lutea and their positions on the ovaries were noted, and their ages were estimated (Oldham and Martin 1978; Oldham and Lindsay 1980).

Premature regression of corpora lutea was detected by the presence of younger (1–2 days) corpora lutea on the contralateral ovary or in new positions on the ovary ipsilateral to the ram-induced ovulation. On most occasions, corpora albicantia were observed in the positions previously occupied by the ram-induced corpora lutea (Oldham and Martin 1978; Oldham and Lindsay 1980). There was no assessment of luteal function through the analysis of progesterone secretion.

The ovulation rate (mean number of ovulations per ewe ovulating), the proportions of ewes ovulating, and the proportions then showing premature luteal regression were calculated. Differences between groups of these variables, and for oestrus, were tested by χ^2 analysis.

Results

Immunization

When diluted 1 : 1000, the plasma from the 30 ewes immunized against A_4-HSA bound $49 \pm 4 \cdot 5\%$ (mean ± s.e.) of the labelled androstenedione. The range was 11–100% at this dilution. Two ewes had titres approaching 1 : 10000 and only one had a titre of less than 1 : 100. The non-specific binding in the assay system was always less than 7%.

Table 1. Proportion of seasonally anovular ewes immunized against androstenedione and pretreated with progesterone, which ovulated following the introduction of rams

Immunization	Pretreatment	Proportion of ewes which ovulated		
		Rams absent	Rams present	Total
HSA	No progesterone	0/11	8/12	8/23
	Progesterone	1/10	7/9	8/19
Total		1/21	15/21	16/42
A_4-HSA	No progesterone	0/8	7/8	7/16
	Progesterone	0/7	5/7	5/14
Total		0/15	12/15	12/30
Total (A_4 + HSA)		1/36	27/36	28/72

Ovulation and Ovulation Rate

The proportion of ewes which ovulated in the period up to 4 days after the introduction of rams is given in Table 1. The rams induced ovulation in 27 out of

36 ewes (*v.* 1 out of 36 in the isolated group, $P < 0{\cdot}001$) and, although the proportion of ewes ovulating was 80% in the A_4-HSA group and 71% in the HSA group, the difference was not statistically significant. Pretreatment with progesterone had no effect on the response to the rams, nor did it stimulate ovulation in the absence of rams.

There was a highly significant effect of immunization against A_4-HSA on ovulation rate (Table 2), increasing it from 1·00 to 1·83 ($P < 0{\cdot}005$). All of the ewes which ovulated in the HSA group (15 out of 15) had single ovulations. There was no significant effect of progesterone on these responses.

In the ewes which ovulated in the A_4-HSA group, the number of ovulations induced by the introduction of rams was significantly correlated with the amount of labelled steroid bound by the plasma ($r = 0{\cdot}67$, $P < 0{\cdot}05$).

Table 2. Effect of immunization against androstenedione and progesterone pretreatment on premature luteal regression, oestrus in ewes ovulating and ovulation rate after the introduction of rams

Immunization	Observation after pretreatment		
	No progesterone	Progesterone	Total[A]
	Proportion showing premature luteal regression		
HSA	6/8	1/7	7/15
A_4-HSA	7/7	1/5	8/12
Total	13/15	2/12	15/27
	Proportion in oestrus		
HSA	1/8	2/7	3/15
A_4-HSA	0/7	0/5	0/12
Total	1/15	2/12	3/27
	Ovulation rate[B]		
HSA	1·00(8)	1·00(7)	1·00
A_4-HSA	1·57(7)	2·20(5)	1·83
Mean	1·27	1·50	1·37

[A] Mean values are given for ovulation rate.
[B] Mean number of ovulations per ewe ovulating. Sample number is given in parentheses.

Premature Luteal Regression

Of the 27 ewes ovulating in response to contact with rams, there was premature luteal regression in 15 (56%), with a second ovulation in eight (30%) and a return to ovulation in six (21%). There was a highly significant depression in the incidence of premature luteal regression in the ewes pretreated with progesterone (Table 2, $P < 0{\cdot}005$) but no significant effect due to immunization against A_4-HSA.

Oestrus

Only three ewes were well marked (Table 2) by the rams in the first 4 days (when 27 ewes were ovulating) and all were in the HSA group. Neither immunization nor progesterone pretreatment significantly affected the expression of behavioural oestrus at the first ovulation. No oestrous ewes were detected between the fourth and

eighteenth days after the introduction of rams, i.e. during the period of re-ovulation in ewes undergoing premature luteal regression. Of the 13 ewes which maintained their corpora lutea beyond day 11, seven were marked between days 19 and 21 and the remaining six became anoestrous. No further ewes were marked between days 22 and 24, but four of the eight ewes which had ovulated twice by day 11 entered oestrus between days 25 and 29. The remainder of the flock were not marked at all during the 30 days following the introduction of rams.

Discussion

Active immunization against androstenedione did not affect the proportion of seasonally anovular ewes which ovulated in response to the introduction of rams but did increase the ovulation rate of the ewes which did respond. The magnitude of the response in ovulation rate, about 80%, is similar to that we have seen in Merino ewes during the normal breeding season (Martin *et al.* 1979), indicating that if this technique were to be introduced on a commercial basis, gains in ovulation rate could be made whether the ewes were mated in spring or autumn. Since all the ewes in the control (HSA) group had single ovulations, there could not have been an increase in ovulation rate in response to the introduction of rams. This result does not agree with the results of previous work in both this laboratory and in France (Cognie *et al.* 1980), and may have been due to other factors which affect ovulation rate, such as nutrition (Lindsay 1976). The failure to induce multiple ovulations in the control ewes and the rapid return to anoestrus in both groups also prevented us from testing for an interaction between immunization and the ram effect on ovulation rate.

Very few ewes maintained regular oestrous cycles after the first or second ovulation, despite the continued presence of the ram. Flocks of anoestrous Merino ewes stimulated by the introduction of vasectomized rams will finally return to anoestrus within the same season and the rate of return increases as the summer solstice approaches (Oldham and Cognie 1980). Our experiment ran through the summer solstice when the anoestrous season is deepest for Merinos (see fig. 1, Martin *et al.* 1979) and this may have been the cause of the rapid re-entry into anoestrus.

Immunization against androstenedione probably increased the number of pre-ovulatory follicles available for ovulation. This is indicated by the increases in ovulation rate observed in our current study and has previously been demonstrated in similarly immunized ewes (Scaramuzzi *et al.* 1980). Despite this, the proportion of ewes failing to ovulate in response to the introduction of rams was not lowered by the immunization treatment. This indicates that failure to ovulate is not entirely due to an inability of the ovary to respond to ovulatory stimuli but is due to a failure of the central nervous system to begin and to sustain the sequence of events which lead to ovulation. Furthermore, the ability of a seasonally anovular ewe to ovulate and the subsequent number of ovulations must be controlled by two separate systems—increases in ovulation rate are not simply due to a greater stimulus to ovulate.

Immunization against A_4-HSA did not affect the proportion of ewes experiencing premature luteal regression, nor the ability of progesterone priming to prevent this regression. This effect of progesterone is not understood, but is perhaps related to the fact that it delays the ram-induced ovulatory surge of LH (Martin *et al.* 1980*a*)

and may allow follicles to reach the correct stage of development prior to ovulation. The rapid surge of LH induced in unprimed ewes (Oldham *et al.* 1978; Martin *et al.* 1980*a*) may be likened to the surge of LH resulting from a single injection of LHRH, which induces non-functional corpora lutea in anoestrous ewes (Crighton *et al.* 1973). It is not known whether the prematurely regressing corpora lutea seen in the present study were functional, since progesterone production was not measured. This is an important area for future studies.

The low incidence of behavioural oestrus after progesterone pretreatment contradicts the reports by Hunter *et al.* (1971) and Oldham *et al.* (1980), but these differences were probably due to time of withdrawal of progesterone. Both these workers gave the last injection, or removed the intravaginal sponges, at the same time as introducing the rams whereas we removed our implants 48 h earlier. 48 h is the optimal period between the end of progesterone treatment and an injection of oestrogen for the induction of oestrus in ovariectomized ewes (Moore and Robinson 1957). We assumed that the oestrogen levels would begin to rise as soon as the frequency of LH pulses rose, i.e. within 10 min of introducing the rams (Martin *et al.* 1980*b*), since each LH pulse produces oestradiol in the anoestrous ewe (Scaramuzzi and Baird 1976). The absence of behavioural oestrus in these circumstances raises two interesting possibilities: first, the progesterone-induced sensitivity to oestrogen may be diminishing more rapidly in the seasonally anovular ewes than in the ovariectomized ewes; second, since withdrawal of progesterone at 48 h affects the response in oestrus but not premature luteal regression, the mechanism of action is different for these two effects. Further studies are required to verify these possibilities.

In conclusion, immunization against androstenedione does not interfere with ovulation induced in anoestrous ewes by the introduction of rams. Furthermore, the progesterone priming which is probably necessary if the ewes are to display oestrous behaviour has no effect on the increase in ovulation rate due to the immunization procedure but does reduce the frequency of short cycles. Both of these findings are valuable if these techniques are to be introduced into commercial flock management in Australia.

Acknowledgments

This project received financial support from the Australian Meat Research Committee.

The antigens and adjuvant systems were synthesized, tested and provided by Drs R. I. Cox and R. M. Hoskinson, Division of Animal Production, CSIRO, whose help in designing the immunization protocol was invaluable. The technical assistance of D. Suckling, R. Armstrong and D. Turner was greatly appreciated, and special thanks go to R. H. King for laparoscopic work, and to Professor R. J. Moir for help with preparation of the manuscript.

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Manuscript received 2 March 1981, accepted 19 June 1981