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# Effect of active immunisation with follicular fluid on ovulation rates in Romney ewes

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## ABSTRACT

Active immunisation of Romney ewes with bovine follicular fluid (bFF) at monthly intervals for 4 months in the breeding season increased monthly mean ovulation rates by 8 to 100%, relative to non-immunised ewes, by increasing the frequency of double ovulations. The increases in ovulation rate were independent of the dose of bFF (from 1 to 10 ml) and whether an aqueous- or oil-based adjuvant was used. Immunisation with porcine FF (1 or 5 ml) did not increase ovulation rates significantly. Active immunisation with an androstenedione-based immunogen (Androvax), increased the mean monthly ovulation rates 31 to 63% by increasing the frequency of double and triple ovulations. Immunisation with bFF and Androvax together did not increase ovulation rates any more than immunisation with Androvax alone.

These results show that active immunisation with bFF can increase ovulation rates in sheep, though generally less effectively than by immunisation with an androstenedione-based immunogen.

**Keywords** Follicular fluid; androstenedione; immunisation; ovulation rate; sheep.

## INTRODUCTION

Inhibin is a complex glycoprotein produced by granulosa cells in ovarian follicles under the regulation of follicle stimulating hormone (FSH) and androgens (Henderson and Franchimont, 1983; Henderson *et al.*, 1984a). Inhibin acts on the pituitary to selectively suppress the secretion of FSH by reducing pituitary levels of mRNA coding for the b-subunit of FSH (Mercer *et al.*, 1987). In sheep, FSH has a major role in determining ovulation rate, which can be increased by raising endogenous FSH concentrations (Henderson *et al.*, 1988). Interfering with the normal regulation of FSH secretion can therefore provide a means of manipulating ovulation rate.

Active immunisation of ewes with partially purified inhibin increases the frequency of double and triple ovulations (Henderson *et al.*, 1984b). This is most likely a consequence of interference with the suppression of FSH secretion by endogenous inhibin. Inhibin therefore has the potential to be a useful agent for increasing twinning frequencies in sheep, and possibly other species such as the cow (Price *et al.*, 1987). Unfortunately, because of its complexity, inhibin is difficult and expensive to purify, even partially.

Thus to be commercially useful, a more convenient form of inhibin is required. Ovarian follicular fluid (FF) is rich in inhibin activity (Henderson *et al.*, 1984a), and is readily obtained in large amounts from slaughterhouse waste products. Recent studies have demonstrated that ovulation rates in sheep can be increased by repeated injections of FF (Henderson *et al.*, 1986). For practical purposes, however, an immunisation procedure would be more useful. The purpose of this study was to determine if active immunisation with FF could increase ovulation rates in sheep. In addition, the effectiveness of combined immunisation with FF and an androstenedione immunogen (known to be capable of increasing ovulation rate) was also studied.

## MATERIALS AND METHODS

### Preparation of Immunogens

Ovaries were obtained from cows and pigs slaughtered at a local abattoir. Antral follicles on the surface of the ovaries were punctured with a 20-gauge needle, and the FF aspirated. Debris and red blood cells were removed from pooled fluids by centrifugation at 1200 g for 15 min at 4°C, and

the supernatant was stored frozen at  $-20^{\circ}\text{C}$ . Several collections of FF were subsequently thawed, and the steroids removed by treatment with dextran T-70 (0.1%, Pharmacia Fine Chemicals AB, Uppsala, Sweden) coated charcoal (Norit A, 1% Fisher Scientific Co., Springfield, New Jersey, USA) as described previously (Henderson *et al.*, 1986). The FF was then lyophilised and stored at  $4^{\circ}\text{C}$  until needed.

Androvax (i.e., 6-hydroxyandrost-4-ene-3,17-dione-6 hemisuccinyl alacen), an androstenedione-based vaccine for increasing ovulation rates in sheep (McNatty *et al.*, 1988), was prepared at Wallaceville.

On the day before each immunisation, the FF and Androvax were formulated in a water-based adjuvant (aqueous DEAE-dextran, Pharmacia Fine Chemicals AB, Uppsala, Sweden) or in an oil-based adjuvant composed of Span 85 (sorbitan triolate; ICI Americas Inc, Washington, USA); Tween 85 (Polyoxyethylene 20 sorbitan triolate; Sigma Chemical Co., St Louis, Mo, USA); Marcol 52 mineral oil (Exxon Co., Houston, Texas, USA) in the ratio 1:1:8 v/v/v (STM) (Bokhout *et al.*, 1981).

FF in DEAE-dextran was prepared by dissolving lyophilised FF powder in 5% aqueous DEAE-dextran (w/v) to yield the equivalent of 1 to 10 ml FF in 1 to 4 ml of DEAE-dextran solution. FF in STM was prepared by dissolving lyophilised FF powder in distilled water and emulsifying with STM in the ratio 1:1.25 (aqueous:STM, v/v) to yield the equivalent of 5 ml FF in 4.5 ml emulsion. Androvax was prepared only in 5% aqueous DEAE-dextran (w/v) at a concentration of 5 mg/ml. All the formulations were stored at  $4^{\circ}\text{C}$  until administered to sheep the

next day.

### Sheep and their Treatment

Parous New Zealand Romney ewes aged 2.5 to 3.5 years and weighing an average of 51 kg were used in this study, which was undertaken over 2 consecutive breeding seasons. Different flocks of ewes, grazed on open pasture, were used in each of the 2 breeding seasons. The sheep were immunised at monthly intervals (March to June) during the breeding season. The FF (1-4 ml of DEAE-dextran solution or 4.5 ml STM emulsion) and Androvax (5 mg in 1 ml DEAE-dextran solution) immunisations were given subcutaneously into sites in the neck region or into the gracilar or axillary region. The number of ovulations (ovulation rate) was determined 1 month after each immunisation (April to July) by laparoscopic examination of the ovaries of each ewe, and counting the number of corpora lutea present.

### Data Analysis

Significant differences between the ovulation rates of non-immunised ewes, FF and Androvax immunised ewes were tested for by comparing the frequency of non-multiple (0 or 1) and multiple ( $\geq 2$ ) ovulations between the treatment groups, using either the Fisher exact test or  $\chi^2$  analysis. Frequency analysis was performed on each monthly set of data and on the combined data for each treatment group collected over each breeding season. The level of significance was set at  $P < 0.05$ .

TABLE 1 Effect of monthly immunisation with bovine (b) or porcine (p) FF on ovulation rate in Romney ewes

Treatment	N	April	Ovulation rates in:		
			May	June	July
Non-immunised	18	0.8 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1	0.9 $\pm$ 0.1
5 ml bFF	12	1.4 $\pm$ 0.2**	1.5 $\pm$ 0.2	1.7 $\pm$ 0.1	1.8 $\pm$ 0.4**
5 ml pFF	12	1.3 $\pm$ 0.2	1.5 $\pm$ 0.2	1.6 $\pm$ 0.2	1.2 $\pm$ 0.2

Values are mean  $\pm$  s.e.m. for N ewes.

\*\* indicates value differs from corresponding non-immunised value (Fisher exact test).

## RESULTS

**TABLE 2** Frequency table showing overall effect on ovulation rate of immunisation with bFF or pFF.

Treatment	Ovulation rate (OR)					Mean OR ± s.e.m.
	0	1	2	3	5	
Non-immunised	5	53	14	0	0	1.1±0.1
5 ml bFF	2	19	26	0	1	1.6±0.1***
5 ml pFF	1	31	12	4	0	1.4±0.1

\*\*\* Indicates value differs significantly from corresponding non-immunised value ( $\chi^2$  analysis).

Table 1 shows the effect of immunisation with 5 ml bovine (b) or 5 ml porcine (p) FF (given in DEAE-dextran) on monthly mean ovulation rates. A frequency table showing the overall distribution of ovulation rates is shown in Table 2. Immunisation with bFF increased mean ovulation rates significantly in April and July, when mean ovulation rates in the control ewes were at their lowest, and over all months there was a significant increase in the frequency of multiple ovulations. Immunisation with pFF also increased mean ovulation rates each month, but this failed to reach significance on any occasion, and overall there was no significant increase in the frequency of multiple ovulations. The effects of immunisation with bFF or pFF were independent of dose of FF. No significant differences in the mean ovulation rates each month, or in the overall frequency of multiple ovulations were obtained using 1, 5 or 10 ml bFF

**TABLE 3** Effect of monthly immunisation with 5 ml bFF and/or Androvax on ovulation rates in Romney ewes.

Treatment	Ovulation rates in:			
	April	May	June	July
Non-immunised	0.8±0.2	1.3±0.1	1.3±0.1	1.2±0.1
bFF (STM)	1.4±0.2*	1.5±0.1	1.5±0.1	1.3±0.1
bFF (DEAE-dextran)	1.2±0.2	1.5±0.1	1.6±0.1*	1.4 ±0.1
Androvax	1.3±0.2	1.9±0.2*	1.7±0.2*	1.6±0.2
bFF (DEAE-dextran) + Androvax	1.4±0.2*	1.8±0.2	1.7±0.1**	1.6±0.1*

Values are mean ± s.e.m. for 17-20 ewes/group.

Asterisks indicate values differing significantly from corresponding non-immunised values ( $\chi^2$  analysis).

or 1 or 5 ml pFF.

The following breeding season, in an effort to improve upon the increase in ovulation rate obtained by immunisation with bFF given in DEAE-dextran, ewes were immunised with bFF in the oil-based STM adjuvant. In addition, ewes were immunised with bFF and Androvax together to determine if the combined treatment would more effectively increase ovulation rate than each treatment individually. The results are shown in Tables 3 and 4. The mean ovulation rates were increased by 8 to 75% following bFF immunisation and there was a significant increase in the overall frequency of multiple ovulations. There was no significant difference between ewes immunised with bFF in STM or DEAE-dextran. Immunisation with Androvax increased monthly

**TABLE 4** Frequency table showing overall effect on ovulation rate of immunisation with 5 ml bFF and/or Androvax.

Treatment	Ovulation rate (OR)				Mean OR ± s.e.m.
	0	1	2	3	
Non-immunised	10	51	17	1	1.1±0.1
bFF (STM)	4	41	34	1	1.4±0.1**
bFF (DEAE-dextran)	3	44	31	2	1.4±0.1*
Androvax	3	34	31	10	1.6±0.1***
bFF (DEAE-dextran) + Androvax	3	29	36	7	1.6±0.1***

Asterisks indicate values differing significantly from corresponding non-immunised value ( $\chi^2$  analysis).

mean ovulation rates by 33 to 63% and significantly increased the overall frequency of multiple ovulations. However, immunisation with bFF and Androvax together was no more effective in increasing ovulation rates than immunisation with Androvax alone.

## DISCUSSION

Active immunisation of sheep with bFF increased mean ovulation rates by 8 to 100%, relative to non-immunised ewes, by increasing the frequency of double ovulations. The increases in mean ovulation rates were independent of the dose of FF administered (in the range 1 to 10 ml bFF), or whether an aqueous (DEAE-dextran) or oil (STM) based adjuvant was used. It is unlikely, however, that immunisation with bFF will be commercially useful as a means of increasing ovulation rates in sheep, and hence their fecundity. The increases in mean ovulation rates in response to bFF immunisation were very variable, and generally lower than those obtained with Androvax. There was also no advantage in immunising with Androvax and bFF together compared to immunising with Androvax alone.

Interestingly the greatest increase in mean ovulation rates, in response to immunisation with bFF or Androvax, was generally observed early in the breeding season (April) when mean ovulation rates of the non-immunised ewes were at their lowest. At this time 22 and 40% of the non-immunised ewes in each breeding season were still anovulatory whereas only 0 to 8% and 10 to 15% of the bFF or Androvax immunised ewes were anovulatory in each of the 2 breeding seasons respectively. Thus immunisation with bFF may advance the onset of the breeding season in some ewes, as has previously been demonstrated following immunisation with androstenedione (Gibb *et al.*, 1982).

The immunogen in FF responsible for the increase in ovulation rates is assumed to be inhibin, as active immunisation with semi-pure inhibin derived from follicular fluid will increase ovulation rates in sheep (Henderson *et al.*, 1984b, Cummins *et al.*, 1986). However, the possibility that other components may also be responsible

cannot be excluded. Indeed some factors in the FF may actually be aggravating the positive ovulatory response to the immunogen(s) involved. More consistent and higher increases in mean ovulation rates may be obtained using highly purified forms of inhibin. The use of the a subunit of inhibin as an immunogen, prepared by recombinant DNA methods, may offer some potential in this regard (Forage *et al.*, 1987).

The mechanism by which Androvax (androstenedione) immunisation increases ovulation rates in sheep is uncertain, but increased FSH secretion may be involved (McNatty *et al.*, 1988). Androgens can stimulate ovarian inhibin production *in vitro* (Henderson and Franchimont, 1983). Androvax immunisation may therefore cause a reduction in ovarian inhibin production. Thus, the consequences of both bFF and Androvax immunisation may be mediated through effectively reducing the negative feedback effects of inhibin on pituitary FSH secretion. Such a common mechanism would be consistent with the observation that immunisation with bFF and Androvax together was no more effective in increasing ovulation rates than immunisation with Androvax alone.

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