Immunisation of ewes against oestradiol-17ß in an attempt to increase the yield of viable embryos with superovulation

A.W. Lishman*, M.C. van Deventer and D.G. Shaw
Department of Animal Science and Poultry Science, University of Natal, Pietermaritzburg, Republic of South Africa

Received 2 July 1996; accepted 20 September 1997

The effect of immunisation against oestradiol-17ß (E_2) on fertilisation rates and of human chorionic gonadotrophin (hCG) in reducing the proportion of unovulated follicles from superovulated ewes was examined. Forty South African Mutton Merino ewes were divided into four treatments in a 2 × 2 factorial arrangement with four replications (blocks). All ewes were synchronised with intravaginal progestagen sponges and superovulated with a combination of 700 IU of PMSG and 11 mg FSHp. Ewes were actively immunised against E_2 six weeks before superovulation. hCG (1500 IU) was administered intravenously 6 h after the onset of oestrus. Ewes were mated naturally. Recovered embryos were cultured for six days to assess their viability. At embryo recovery the mean anti-E_2 antibody titre (bound 40% of tracer E_2) was 217 ± 251. Immunisation had a positive effect ($p < 0.05$) on fertility, with 88.8 ± 2.8% of the recovered ova having been fertilised, as opposed to 79.3 ± 3.2% for control (no immunisation, no hCG) ewes. Immunisation had no significant effect on ovulation rate and did not improve the yield of viable embryos (immunised = 6.7 ± 1.1; control = 7.5 ± 1.3) per ewe. Embryos from immunised ewes appeared to possess a reduced viability. A significant ($p < 0.05$) interaction between immunisation and hCG treatment was observed, with hCG increasing ($p < 0.01$) the percentage of unovulated follicles in control, but not in immunised ewes. It is concluded that (a) immunisation of superovulated ewes against E_2 improves the fertility of these ewes, probably by negating the effects of high peri-ovulatory E_2 levels in these ewes, (b) immunisation against E_2 is not a feasible practice for increasing the yield of viable embryos of superovulated ewes and (c) hCG may have been administered too early after the onset of oestrus, particularly in control ewes.

Die invloed van immunisasie teen estradiol-17ß (E_2) op bevrugtingspersentasie en van menslike gonadotrofiese hormoon (mGH) op 'n verlaging in die persentasie ongeovuleerde follikels van superovuleerde ooie, is bestudeer. Veertig SAVM ooie is in vier behandelingsgroepe in 'n 2 × 2 faktoriale rangskikking met vier herhalings (blokke) verdeel. Al die ooie is met intravaginale progestageensponse gesinchroniseer en superovulasie is verwek met 'n kombinasie van 700IE DMSG en 11 mg FSHp. Ses weke voor superovulasie is die ooie aktief geïmmuniseer teen E_2. mGH (1500IE) is 6 h na aanvang van estrus binneaars toegedien. Natuurlike dekking is toegepas. Embrios wat herwin is, is vir ses dae gekweek om hul lewensvatbaarheid te meet. Die gemiddelde E_2-teenliggaamkonsentrasie (het 40% van merker E_2 gebind) by embriospoeling was 217 ± 251. Immunisasie het 'n positiewe invloed ($p < 0.05$) op bevrugting gehad met 88.8 ± 2.8% van die herwinde ova bevrug teenoor 79.3 ± 3.2% van die kontrole (geen immunisasie, geen mGH) ooie. Die ovulasietempo en die opbrengs van lewensvatbare embrios (geïmmuniseerde ooie = 6.7 ± 1.1; kontrole-ooie = 7.5 ± 1.3) is nie betekenisvol deur immunisasie verhoog nie. Embrios per ooi vanaf geïmmuniseerde ooie was klaarblyklik minder lewensvatbaar. 'n Betekenisvolle ($p < 0.05$) interaksie tussen immunisasie en mGH-toediening is waargeneem waar mGH die persentasie ongeovuleerde follikels by kontrole-, maar nie by geïmmuniseerde ooie verhoog het nie. Die gevolgtrekking is gemaak dat (a) immunisasie teen E_2 vrugbaarheid verhoog deur die negatiewe effekte van hoë E_2 peile rondom ovulasie teë te werk, (b) immunisasie teen E_2 nie 'n raadsame praktyk om opbrengs van lewensvatbare embrios van superovuleerde ooie te verhoog, is nie en (c) mGH is miskien te vroeg na aanvang van estrus toegedien, veral by kontrole-ooie.

Keywords: Immunisation, oestradiol-17ß, superovulated ewes, viable embryos

* To whom correspondence should be addressed

Introduction

Low fertilisation rates, or complete absence of fertilisation, continue to be major limiting factors in current superovulation procedures for embryo transfer in cattle and sheep (Armstrong, 1993). Fertilisation failure in superovulated animals has been attributed to abnormalities in oocyte maturation (Moor *et al.*, 1985), asynchrony between maturational events in the oocyte and follicle (de Loos *et al.*, 1991) and premature ovulations (Callesen *et al.*, 1987). Further compounding these problems are deficiencies in sperm transport in superovulated animals (cattle: Hawk, 1988; sheep: Evans & Armstrong, 1984; Hawk *et al.*, 1987). These deficiencies have been attributed to both oestrus synchronisation (prostaglandin F_2 and progestagen sponges) and superovulation treatments (Evans & Armstrong, 1984; Hawk *et al.*, 1987).

Evans & Armstrong (1984) observed that sperm transport was adversely affected to a greater extent in ewes superovulated with PMSG than with FSH and proposed that the high peri-ovulatory E_2 blood levels in superovulated ewes were responsible for the deficiencies in sperm transport. This was based on the demonstration that peri-ovulatory E_2 blood levels in goats were more elevated, and remained so, for a longer period after superovulation with PMSG than with FSH (Armstrong *et al.*, 1983). This observation has been supported by research with sheep (Whyman & Moore, 1980; Jabbour & Evans, 1991; Bainbridge *et al.*, 1995).

The hypothesis proposed by Evans & Armstrong (1984) has been supported by the observations of Croker *et al.* (1975) and Hawk & Conley (1975) which showed that administration of exogenous oestrogen to ewes adversely

affected sperm transport. There is thus at least one reason to control peri-ovulatory E_2 levels in superovulated animals, since these levels may be detrimental to sperm transport and consequent fertility. Reducing the effects of abnormally high E_2 levels in superovulated ewes may, therefore, improve the fertility.

High E_2 levels around the time of oestrus and ovulation may not only reduce fertilisation rates, but according to Armstrong (1993) they may also increase post-fertilisation embryo losses. Work with rabbits (Stone *et al.*, 1977) and sheep (Armstrong, 1993) has suggested that the excessive levels of E_2 in superovulated animals may result in defective oviductal and uterine environments that are inconsistent with normal embryo development.

A further reason for limiting the impact of high E_2 levels is the demonstration that active immunisation against E_2 doubled the total ovarian response (ovulations plus large unovulated follicles) in PMSG-superovulated ewes (Boland *et al.*, 1985). The low ovulation rate was attributed to a possible inadequate, E_2-induced, release of LH following immunisation (Boland *et al.*, 1985). Therefore, the ideal E_2 antibody level would be one that did not totally eliminate the positive feedback effect of E_2 on the pre-ovulatory LH release. In this way most of the mature follicles could be expected to ovulate. A single immunisation regime is unlikely to elicit a low antibody response in all animals. This is because of the substantial variation amongst individuals in antibody responses (Hurn & Chantler, 1980). Consideration should thus be given to the advisability of including an exogenous source of LH in the superovulatory protocol.

This experiment was designed to determine whether (a) active immunisation against E_2 prior to superovulating ewes would increase fertilisation rates and thereby result in more viable embryos and (b) hCG, as an exogenous source of LH, could reduce the proportion of unovulated follicles in ewes immunised against E_2.

Materials and methods

Management of experimental animals and treatments

The experiment was conducted during the breeding season (March). Forty mature South African Mutton Merino ewes were randomly divided among the four treatments in a 2 × 2 factorial arrangement with four randomised complete blocks (replicates). The experiment was conducted over a four-week period with one week separating each replicate. The ewes were penned throughout the course of the study and received a commercial complete balanced diet, *ad libitum*.

An immunisation protocol similar to that used by Boland *et al.* (1985) was employed because it had been shown to elicit a low antibody response. Immunity to E_2 was induced by active immunisation of the ewes (IMM group) against 17ß-oestradiol-6-(O-carboxy-methyl)oxime conjugated to BSA (Sigma). The immunogen had approximately 30 mol of E_2 per mol of bovine serum albumin (BSA). A single subcutaneous injection on the inside of one hind leg was given to each ewe six weeks before planned superovulation. The injection consisted of 1 mg of immunogen dissolved in 2 ml of 5% (w/v) diethylaminoethyl dextran (DEAE-dextran, pH 7.5, Sigma). The control group was not immunised (nIMM).

All ewes were treated with 60 mg medroxyprogesterone acetate sponges (Repromap) for 13 days. Superovulation was induced with a combination of 700 IU PMSG (Ovastim) and 11 mg FSHp (Sigma) as twice-daily injections over 3 days in a decreasing dose regime of 2.5, 2.5, 2.0, 2.0, 1.0, 1.0 mg (Jabbour & Evans, 1991). The first injection of FSH and the single dose of PMSG were administered 48 h before sponge withdrawal.

Treatment with hCG (1 500 IU hCG, Sigma) intravenously, 6 h after the onset of oestrus (Barry *et al.*, 1988) was included in an attempt to ensure ovulation in ewes that developed high antibody titres to E_2. It was also hoped that hCG treatment would reduce the proportion of unovulated follicles. At sponge withdrawal, the vasectomized rams that had been running with the ewes, were replaced by a pool of fertility-tested rams. Each ram, fitted with a harness containing a coloured crayon, was used for 4 h, at a ram to ewe ratio of 1:10, before being replaced by a fresh ram. Accurate recording of the oestrous response was ensured by 24-h observation.

Embryo recovery and culture

Ewes were slaughtered approximately 60 h after the onset of oestrus and the reproductive tracts excised. After determining the ovulation rate and total ovarian response (number of ovulations + number of large unovulated follicles > 5 mm in diameter) the oviducts were flushed with M-199 containing 1% foetal calf serum and 0.1% gentamycin. All fertilised embryos were co-cultured with ovine oviductal epithelial to assess their viability cells, as described by Gandolfi & Moor (1987). Embryos had to complete a minimum of three cell cycles to be classified as viable.

Radio-immunoassays

Antibody titres, at embryo recovery, and serum E_2 levels were determined by radio-immunoassay (RIA) as described by Butcher *et al.* (1974). The RIA had an extraction efficiency of 93% and a sensitivity (twice the standard deviation of the blank sample) of 2.4 pg/ml. The inter- and intra-assay coefficients of variation were 17% and 9%, respectively. Commencing at 16 h after sponge withdrawal, and continuing for a further 12 h, blood samples (10 ml) were collected by jugular venepuncture from the ewes at 2-hourly intervals for the determination of the preovulatory E_2 peak.

Statistical analyses

Data for the time to onset of oestrus, total ovarian response, number of ovulations, yield of viable embryos and the preovulatory E_2 peak were analysed by analysis of variance (ANOVA). Data for responses expressed in proportions, such as fertility, were analysed by logistic analysis. All data were analysed using Genstat (Version 5.1.3, 1988, Lawes Agricultural Trust, Rothamstead Experimental Station, U.K.).

Results

Owing to problems initially experienced with immunisation, the culture system and embryo recovery, the only data used from the first block of ewes was that of the ovulatory responses. The means of the four treatments, together with the main effects of immunisation against E_2 and hCG treatment, are presented in Tables 1 and 2.

Table 1 Effects of immunisation against E_2 and of hCG treatment on the ovulatory response (mean ± s.e.m.) of superovulated ewes

Treatment		No. of ovulations/ ewe	Total ovarian response[1]
Immunisation	hCG injection		
None (nIMM)	None	16.6 ± 1.9	19.8 ± 2.0
None (nIMM)	1500IU	12.7 ± 1.9	18.8 ± 2.0
Against E_2 (IMM)	None	15.9 ± 2.0	20.1 ± 2.3
Against E_2 (IMM)	1500IU	14.9 ± 2.0	18.9 ± 2.1
None (nIMM)		14.6 ± 1.3	19.3 ± 2.1
Against E_2 (IMM)		15.4 ± 1.4	19.5 ± 1.6
	None	16.3 ± 1.4	20.0 ± 1.5
	1500hCG	13.7 ± 1.4	18.8 ± 1.5

[1] total ovarian response = number of ovulations + number of large (> 5 mm in diameter) unovulated follicles

Table 2 Effects of immunisation against E_2 and hCG treatment on the embryo recovery rate and the fertility of superovulated ewes (mean ± s.e.m.)

Treatment		Recovery rate[1] (%)	Fertility rate[2] (%)
Immunisation	hCG injection		
None (nIMM)	None	64.8 ± 4.2	76.8 ± 4.5
None (nIMM)	1500IU	70.5 ± 4.5	81.9 ± 4.4
Against E_2 (IMM)	None	72.3 ± 5.0	87.0 ± 4.3
Against E_2 (IMM)	1500IU	63.0 ± 4.6	90.6 ± 3.4*
None (nIMM)	–	67.8 ± 3.1	79.3 ± 3.2[a]
Against E_2 (IMM)	–	67.5 ± 3.4	88.8 ± 2.8[b]
–	None	68.1 ± 3.2	81.5 ± 3.2
–	1500IU	67.2 ± 3.2	85.9 ± 2.8

[1] recovery rate = (no. of embryos recovered)/(no. of ovulations)

[2] fertility rate = (no. of fertile embryos)/(no. of recovered embryos)

[a,b] main effect values of a factor with different superscripts differ significantly ($p < 0.05$)

* treatment mean significantly ($p < 0.05$) different from control within column

Antibody titres

Anti-E_2 titres were very low and extremely variable with very few titres capable of binding more than 50% of titriated E_2. Thus, antibody levels were defined as the dilution of antiserum which bound 40% of the titriated E_2. Using this expression, reciprocal antibody titres varied widely from 3 to 741 (mean = 217 ± 251).

Oestrus

Of the 40 ewes that had intravaginal sponges inserted for oestrus synchronisation, two (5%) lost their sponges and were thus excluded from the experiment. Only one ewe (immunised) did not exhibit oestrus. On average, the mean time to onset of oestrus was 2.4 h later (not significant) in immunised ewes (mean = 24.4 ± 1.2), than in ewes not immunised against E_2.

Ovulatory responses

Contrary to expectation, hCG did not reduce the proportion of unovulated large follicles (> 5 mm in diameter) in immunised ewes and unexpectedly increased ($p < 0.01$) this proportion in non-immunised animals (Figure 1A). The consequence of this significant interaction ($p < 0.05$) was for hCG to have a negative effect (not significant) on the number of ovulations, with ewes that did not receive hCG having, on average, nearly three more ovulations per ewe than ewes treated with hCG (Table 1).

Embryo recovery and fertilisation rates

Neither immunisation against E_2 nor hCG treatment had an effect on the embryo recovery rate (Table 2). The mean recovery and fertility rates were 68.0 ± 8.2% and 83 ± 9.0%, respectively.

Immunisation against E_2 had a positive ($p < 0.05$) effect of 9.5 ± 3.4% on the fertility rate, with the IMM combined with 1500 IU hCG (Treatment 4) having the highest rate (Table 2). This positive effect of immunisation occurred in spite of the increased proportion ($p < 0.05$) of unfertilised oocytes (Figure 1B) and the reduced ($p < 0.05$) proportion of two-cell embryos obtained from ewes not receiving hCG (Figure 1C).

hCG also increased ($p < 0.05$) the proportion of unfertilised oocytes recovered from nIMM ewes, but had the opposite effect (not significant) in IMM ewes (Figure 1B). The response to hCG, in terms of two-cell embryos, would be expected to mirror that seen for the unfertilised oocytes. However, this did not occur (Figure 1C), whereas the proportion of four-cell embryos followed the expected trend (Figure 1D).

The proportion of embryos recovered in the most advanced developmental stage (four-cell stage) was increased by both immunisation against E_2 ($p < 0.01$) and by hCG injection ($p < 0.01$). Both treatments which included hCG administration were associated with significantly ($p < 0.01$) higher proportions of four-cell embryos recovered than the control treatment (Figure 1D).

Embryo development in culture

Treatment with hCG increased ($p < 0.05$) the proportion of embryos that were arrested at the < eight-cell stage after six days of culture (Table 3). Neither hCG nor immunisation had any significant effect on the proportion of embryos arrested in the 8- to 16-cell stage. Immunisation against E_2 increased ($p < 0.01$) the proportion of embryos that developed beyond the 16-cell stage, but this positive effect did not extend to the blastocyst stage (Table 3). In fact, immunisation, without the addition of hCG (Treatment 3), decreased ($p < 0.05$) the proportion of embryos reaching this stage, when compared to the control treatment.

Embryo viability

The proportion of embryos, viable after culture, was decreased ($p < 0.05$) by hCG treatment, with Treatment 4 (IMM plus 1500 IU hCG) resulting in a lower ($p < 0.05$) proportion of viable embryos than the control treatment (Table 4). If the number of viable embryos is expressed as a percentage of the number of embryos recovered, hCG treatment had no negative main effect. Neither immunisation against E_2 nor hCG treatment had an effect on the mean yield of viable embryos, with the overall average being 7.1 ± 4.3

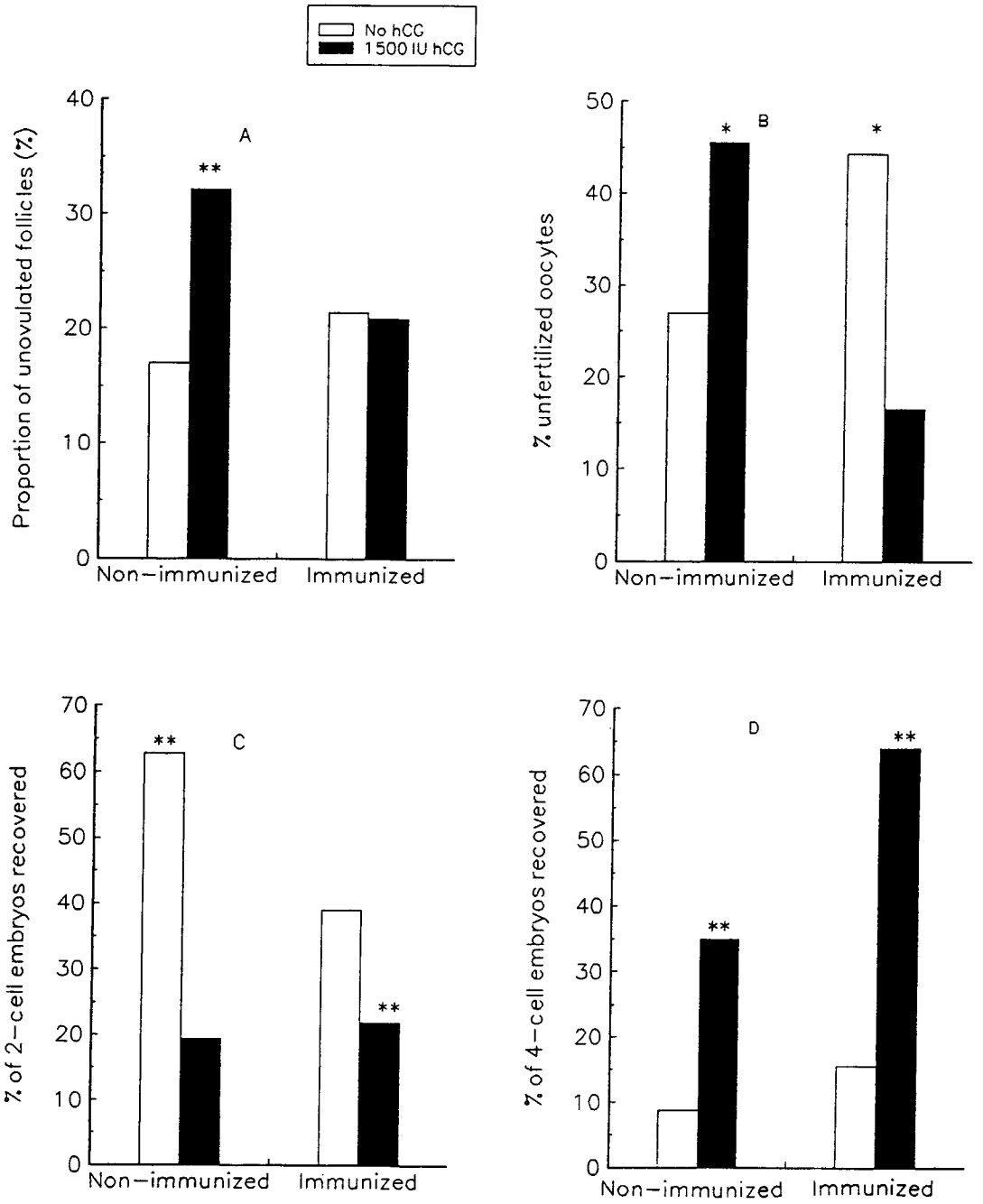

Figure 1 Occurrence of unovulated large follicles (A), unfertilised oocytes (B), and two- (C) or four-cell embryos (D) in superovulated ewes following immunisation against oestradiol-17ß and hCG administration. Treatments significantly different from Control treatment (non-immunized, no hCG) are indicated by * ($p < 0.05$) and ** ($p < 0.01$)

per ewe (Table 4).

Preovulatory E_2 peaks

Immunisation against E_2 was found to increase ($p < 0.05$) the preovulatory E_2 peak nearly three-fold and also the preovulatory E_2 peak, corrected for the total ovarian response (Table 5).

Discussion

The hypothesis that the immunisation of superovulated ewes against E_2 would improve their fertility was supported by the results obtained in this investigation. High preovulatory levels of E_2 in superovulated ewes do appear to play some part in the reduced fertility observed in such ewes. However, it is unclear what aspect of these preovulatory levels is detrimental to fertility. The question remains as to whether the magnitude, duration or some other characteristic of the preovulatory E_2 detrimentally affects fertility.

The increased fertility rate of IMM ewes did not result in an increased yield of viable embryos per ewe (Table 4). This indicates that either the improvement of about 10% in fertility was insufficient to improve the yield of viable embryos significantly, or the viability of embryos recovered from IMM ewes was lower than that of embryos recovered from nIMM ewes. The former possibility is unlikely because a very strong correlation between the fertility rate and the mean yield of

Table 3 The proportion of cultured embryos at various cleavage stages after six days of culture (mean ± s.e.m.) as influenced by immunisation and hCG administration

Treatment		Cleavage stage after culture (%)			
Immunisation	hCG injection	< 8 cells[1]	8 to 16 cells	>16 cells[2]	Blastocysts
None	None	6.1 ± 2.9	30.6 ± 5.7	45.1 ± 6.2	18.7 ± 5.0
None	1500IU	21.0 ± 5.4*	32.9 ± 6.2	36.7 ± 6.3	9.7 ± 3.8
Against E_2	None	9.0 ± 3.8	25.5 ± 5.9	58.6 ± 6.7	6.2 ± 3.5*
Against E_2	1500IU	13.5 ± 4.3	19.3 ± 4.9	56.8 ± 6.2	11.9 ± 4.2
None	–	13.5 ± 3.1	31.7 ± 4.2	40.9 ± 4.4[a]	14.2 ± 3.1
Against E_2	–	11.2 ± 2.9	22.4 ± 3.9	57.7 ± 4.6[b]	9.1 ± 2.8
–	None	7.4 ± 2.4[a]	28.3 ± 4.2	51.4 ± 4.6	12.9 ± 3.2
–	1500IU	17.5 ± 3.5[b]	26.6 ± 4.0	46.0 ± 4.4	10.7 ± 2.8

[1] main effect values of a factor with different superscripts differ significantly ($p < 0.05$)

[2] main effect values of a factor with different superscripts differ significantly ($p < 0.01$)

* treatment mean significantly ($p < 0.05$) different from control within column

Table 4 Embryo viability (at least three cell divisions) and the yield of viable embryos of superovulated ewes (mean ± s.e.m.)

Treatment		% Fertilised embryos viable[1]	% Recovered embryos viable	Yield of viable embryos
Immunisation	hCG injection			
None	None	90.3 ± 3.8	69.1 ± 5.0	7.3 ± 1.6
None	1500IU	84.8 ± 4.7	69.5 ± 5.4	6.1 ± 1.6
Against E_2	None	88.4 ± 4.5	76.7 ± 5.5	8.0 ± 1.7
Against E_2	1500IU	75.7 ± 5.5*	69.1 ± 5.5	7.0 ± 1.7
None	–	87.7 ± 3.0	69.3 ± 3.7	6.7 ± 1.1
Against E_2	–	82.1 ± 3.6	72.9 ± 3.9	7.5 ± 1.3
–	None	89.5 ± 2.9[a]	72.7 ± 3.8	7.6 ± 1.2
–	1500IU	80.6 ± 3.6[b]	69.3 ± 3.9	6.5 ± 1.2

[1] main effect values of a factor with different superscripts differ significantly ($p < 0.05$)

* treatment mean significantly ($p < 0.05$) different from control within column

Table 5 E_2 peak and E_2 peak corrected for total ovarian (TOR) response in non-immunised and immunised superovulated ewes (means ± s.e.m.)

	Non-immunised	Immunised	p
Peak E_2 (pg/ml)	14.0 ± 7.3	39.9 ± 6.4	< 0.05
Peak E_2 corrected for TOR[1] (pg/ml)	0.7 ± 0.4	2.1 ± 0.4	< 0.05

[1] total ovarian response (TOR) = no. of ovulations + no. of large (> 5 mm in diameter) unovulated follicles

viable embryos has been observed (Mullins *et al.*, 1988). Statistical analysis of the percentage of recovered embryos that were classed as viable, that is, underwent three cell cycles during culture, did not indicate any significant difference in viability between embryos recovered from IMM and nIMM ewes. However, the latter possibility is supported by the finding that embryos derived from IMM ewes tended to be less likely to develop to the blastocyst stage after six days of culture than embryos from nIMM ewes. This was despite the observation that the proportion of embryos from IMM ewes in the preceding stage (< 16 cells) was higher ($p < 0.01$) than the proportion of embryos from nIMM ewes. In addition, embryos from IMM ewes were at a more advanced stage of development at the start of culture (the percentage of 4-cell embryos recovered was higher ($p < 0.01$) for IMM ewes than nIMM ewes), yet this did not translate into more of these embryos reaching the more advanced development stages. This suggests that immunisation against E_2 had a negative effect on embryo viability. Numerous studies have shown increased embryonic mortality rates in androstenedione IMM ewes (Smith *et al.*, 1985; Boland *et al.*, 1986; Hanrahan & Quirke, 1987; Philipon & Terqui, 1987), but these have usually been associated with excessively high antibody titres. No studies have yet shown that E_2 immunisation affects embryo mortality rates, but the high pre-ovulatory E_2 peaks (Table 5) might have had a negative effect on the oviductal and uterine environments, as suggested by Stone *et al.* (1977) and Armstrong (1993).

In the light of the finding that active immunisation against E_2 improved ovarian responses (Boland *et al.*, 1985), it could be argued that the low anti-E_2 titres (present study) were ineffective in increasing the ovarian response. The same immunisation protocol, using HSA as a protein conjugate instead of BSA, as used in this investigation, resulted in reciprocal antibody titres between 100 and 1000 (Boland *et al.*, 1985). These titres bound 50% of the labelled E_2 whereas setting the level at 40% (present study) still gave titres lower than that reported by Boland *et al.* (1985). This disappointingly low antibody response confirms the statement of Webb *et al.* (1984) that BSA is a weak carrier protein.

Two findings of this investigation do not support the argument that the low antibody titres were largely ineffective. Firstly, IMM ewes tended to have a delayed onset of oestrus relative to their nIMM counterparts. This has been observed in other immunisation studies (Pathiraja, 1982; Scaramuzzi & Hoskinson, 1984; Eppleston *et al.*, 1988). Secondly, the most convincing evidence is that of the considerably higher mean preovulatory E_2 peak of IMM ewes relative to their nIMM counterparts. Elevated steroid levels in ewes immunised against steroids is a classical sign that immunisation has created an effective antibody response to, at least partially, negate the effects of the steroid they were immunised against. This phenomenon has been observed in numerous immunisation studies (Fairclough *et al.*, 1976; Rawlings *et al.*, 1978; Scaramuzzi *et al.*, 1980; Scaramuzzi *et al.*, 1981; Crighton *et al.*, 1991).

Overall, the preovulatory E_2 peaks were generally much lower than the results of Jabbour & Evans (1991) who used an identical superovulation protocol. Their study detected preovulatory E_2 peaks in the order of 162 ± 27 pg/ml, a peak nearly 14 times higher than the peak of 12 pg/ml observed in unstimulated ewes (Karsch *et al.*, 1978). This difference

seems unduly large considering that Guilbault *et al.* (1992) observed only a two-fold increase in the preovulatory E_2 peak of cattle superovulated with FSH and in ewes 1500IU PMSG yielded E_2 levels similar to those recorded here (Martemucci *et al.*, 1995). Unfortunately, most studies concerned with the comparison of the endocrinological states of superovulated versus unstimulated animals, have been carried out on cattle.

The failure of hCG treatment to improve ovulation rates also requires explanation. Although it has been shown that the interval between oestrus and the preovulatory LH surge, in non-superovulated ewes, is about 6–7 h (Land *et al.*, 1973), it would appear that hCG treatment 6 h after the onset of oestrus in this investigation was too early, particularly in nIMM ewes. This might account for the depressed ovulation rate and the larger ($p < 0.01$) percentage of unovulated follicles in nIMM ewes treated with hCG than nIMM ewes not receiving hCG. If indeed this was the case, then perhaps a large percentage of the follicles could not ovulate because they were insufficiently mature, with the granulosa cells not yet having developed sufficient LH receptors to respond to the artificial LH surge (Baird & McNeilly, 1981). In addition, Murphy *et al.* (1984) postulated that the provision of exogenous LH before the natural preovulatory LH surge could also inhibit some follicles from responding to the later natural LH surge. This would be due to the exogenous LH causing down-regulation of the LH receptors on the theca and/or granulosa cells. The higher ($p < 0.01$) proportion of four-cell embryos recovered from nIMM ewes treated with hCG compared to nIMM ewes not receiving hCG, provides further evidence that ovulation time was advanced when nIMM ewes were treated with hCG.

The proportion of four-cell embryos recovered in IMM ewes was also increased ($p < 0.01$) by hCG treatment. This suggests that ovulation also occurred earlier in IMM ewes treated with hCG than in IMM ewes not receiving hCG. Strangely, hCG treatment did not increase the percentage of unovulated follicles in IMM ewes, as it did in nIMM ewes. This suggests that the follicles of IMM ewes were at a more advanced stage of maturity than the follicles of nIMM ewes and, hence, they were able to respond to the artificial LH surge. Indeed, Scaramuzzi & Hoskinson (1984) have found that a larger proportion of follicles from androstenedione immunised ewes were in the final maturation stages than the follicles from non-immunised ewes.

Treatment with hCG did not appear to be conducive to normal embryo development. A larger ($p < 0.05$) proportion of embryos from ewes treated with hCG were arrested in the earliest cleavage stages (< eight cells) than embryos from ewes not treated with hCG. In addition, when embryo viability was expressed as a percentage of the number of fertile embryos recovered, hCG treatment had a negative main effect ($p < 0.05$) on embryo viability. This small, yet significant difference, did not translate into hCG treatment having a negative main effect on the proportion of recovered embryos that were viable or the yield of viable embryos. Nevertheless, ewes treated with hCG tended to produce one less viable embryo than ewes not treated with hCG.

Finally, both the superovulation protocol and the co-culture system used in this investigation proved successful. The superovulation protocol involving the use of FSHp and PMSG, in combination, produced a high ovulation rate (mean = 15 ± 5). This was much higher than the rates of 2–7 achieved with PMSG superovulation in other South African studies (van Zyl *et al.*, 1987; Barry *et al.*, 1988). The mean embryo recovery rate of 68% was very acceptable, considering that rates of 50% (D.G. Shaw, unpublished data), 47% (Eppleston *et al.*, 1988) and 51% (Burgman, 1993) are ordinarily achieved in ewes superovulated with PMSG. In addition, the fact that only one ewe (2.5% of the treated ewes) did not respond to this superovulation protocol, demonstrates the advantage of this protocol over a pure FSH protocol where a larger percentage of ewes fail to respond (Eppleston *et al.*, 1984).

References

ARMSTRONG, D.T., 1993. Recent advances in superovulation of cattle. *Theriogenology* 39, 7.

ARMSTRONG, D.T., PFITZNER, A.P., WARNES, G.M., RALPH, M.M. & SEAMARK, R.F., 1983. Endocrine responses of goats after induction of superovulation with PMSG and FSH. *J. Reprod. Fert.* 67, 395.

BAIRD, D.T. & MCNEILLY, A.S., 1981. Gonadotrophic control of follicular development and function during the oestrous cycle of the ewe. *J. Reprod. Fert. Suppl.* 30, 119.

BARRY, D.M., VAN NIEKERK, C.H., COETZER, W.A. & ROBERTSON, M.S., 1988. Superovulatory response and time of ovulation in sheep treated with FSH-p or PMSG followed by GnRH or hCG. *Theriogenology* 29, 217 (Abstr.).

BAINBRIDGE, D.R.J., CHAPPLE, D., LOUDON, A.S.I. & JABBOUR, H.N., 1995. Immunoneutralization with monoclonal antibody attenuates the superstimulatory effects of PMSG on endocrine and ovarian responses in red deer (*Cervus elaphus*). *Theriogenology* 43, 133.

BOLAND, M.P., MURRAY, J.D., HOSKINSON, R.M., HAZELTON, I.G., SUTTON, R. & NANCARROW, C.D., 1985. Ovarian response to PMSG treatment in ewes immunized against oestradiol-17β. *Austr. J. Biol. Sci.* 38, 339.

BOLAND, M.P., MURRAY, J.D., SCARAMUZZI, R.J., NANCARROW, C.D., HOSKINSON, R.M., SUTTON, R. & HAZELTON, I.G., 1986. Embryonic mortality in steroid immunized sheep. In: Embryonic Mortality in Farm Animals,. (eds) J.M. Sreenan & M.G. Diskin, p. 171. Martinus Nijhoff Publishers, Dordrecht.

BURGMAN, J.M., 1993. Effect of delayed sponge withdrawal on the superovulatory response to pregnant mare serum gonadotrophin and on embryo recovery in sheep. *S. Afr. J. Anim. Sci.* 23, 111.

BUTCHER R.L., COLLINS, W.E. & FUGO, N.W., 1974. Plasma concentrations of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4-day cycle of the rat. *Endocrinology* 94, 1704.

CALLESEN, H., GREVE, T. & HYTTEL, P., 1987. Premature ovulations in superovulated cattle. *Theriogenology* 28, 155.

CRIGHTON, J.S., LISHMAN, A.W. & HUNDLEY, M., 1991. Role of dihydrotestosterone in the control of sexual behaviour in castrated male sheep. *J. Reprod. Fert.* 75, 9.

CROKER, K.P., ROBINSON, T.J. & SHELTON, J.N., 1975. The effect of oestrogen administered during the progestational phase of the cycle on transport of spermatozoa in ewes. *J. Reprod. Fert.* 44, 11.

DE LOOS, F., BEVERS, M.M., DIELEMAN, S.J. & KRUIP, T.A.M., 1991. Follicular and oocyte maturation in cows treated for superovulation. *Theriogenology* 35, 357.

EPPLESTON J., BILTON, R.J. & MOORE, N.W., 1984. Effects of

FSH dose and treatment regime on ovulatory response in sheep. *Proc. Austr. Soc. Reprod. Biol.* 16, 68.

EPPLESTON, J., NANCARROW, C.D., BATTYE, K., HOSKINSON, R.M. & ROBERTS, E.M., 1988. Effect of anti-oestradiol-17 beta antibodies on the reproductive performance of ewes superovulated with PMSG. *Austr. J. Biol. Sci.* 41, 441.

EVANS, G. & ARMSTRONG, D.T., 1984. Reduction of sperm transport in ewes by superovulation treatments. *J. Reprod. Fert.* 70, 47.

FAIRCLOUGH, R.J., SMITH, J.F. & PETERSON, A.J., 1976. Passive immunization against oestradiol-17ß and its effect on luteolysis, oestrus and ovulation in the ewe. *J. Reprod. Fert.* 48, 169.

GANDOLFI, F. & MOOR, R.M., 1987. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *J. Reprod. Fert.* 81, 23.

GUILBAULT, L.A., LUSSIER, J.G. & GRASSO, F., 1992. Interrelationships of hormonal and ovarian responses in superovulated heifers pretreated with FSH-p at the beginning of the estrous cycle. *Theriogenology* 37, 1029.

HANRAHAN, J.P. & QUIRKE, J.F., 1987. The effect of immunisation against steroids on ovulation rate in the ewe. In: Follicular Growth and Ovulation Rate in Farm Animals, p. 151. Martinus Nijhoff Publishers, Dordrecht.

HAWK, H.W., 1988. Gamete transport in the superovulated cow. *Theriogenology* 29, 125.

HAWK, H.W. & CONLEY, H.H., 1975. Involvement of the cervix in sperm transport failures in the reproductive tract of the ewe. *Biol. Reprod.* 13, 322.

HAWK, H.W., COOPER, B.S. & CONLEY, H.H., 1987. Inhibition of sperm transport and fertilization in superovulating ewes. *Theriogenology* 28, 139.

HURN, B.A.L. & CHANTLER, S.M., 1980. Production of reagent antibodies. *Methods Enzymol.* 70, 104.

JABBOUR, H.N. & EVANS, G., 1991. Ovarian and endocrine responses of Merino ewes to treatment with PMSG and/or FSH-P. *Anim. Reprod. Sci.* 26, 93.

KARSCH, F., LEGAN, S., RYAN, D. & FOSTER, D., 1978. The feedback effect of ovarian steroids on gonadotrophin secretion. In: Control of ovulation, p. 29. (Eds) D.B. Crighton, G.R. Foxcroft, N.B. Haynes & G.E. Lamming. Butterworths, London.

LAND, R.B., PELLETIER, J., THIMONIER, J. & MAULEON, P., 1973. A quantitative study of genetic differences in the incidence of oestrus, ovulation and plasma LH concentration in the sheep. *J. Endocrinol.* 58, 305.

MARTEMUCCI, G., D'ALESSANDRO, A., TOTEDA, F., FACCIOLONGA, A.M. & GAMBACORTA, M., 1995. Embryo production and endocrine response in ewes superovulated with PMSG, with or without monoclonal anti-PMSG administered at different times. *Theriogenology* 44, 691.

MOOR, R.M., OSBORN, J.C. & CROSBY, I.M., 1985. Gonadotrophin-induced abnormalities in sheep oocytes after superovulation. *J. Reprod. Fert.* 74, 167.

MULLINS, S., MACDONNEL, H.F. & GORDON, I., 1988. Effects of superovulation treatments on plasma progesterone, oestradiol and luteinizing hormone profiles and on embryo production in heifers following artificial insemination. *11 th Int. Congr. Anim. Reprod. Art. Insem.* 2, brief communications.

MURPHY, B.D., MAPLETOFT, R.J., MANNS, J. & HUMPHREY, W.D., 1984. Variability in gonadotrophin preparations as a factor in the superovulation response. *Theriogenology* 21, 117.

PATHIRAJA, N., 1982. Physiological basis of genetic variation in ovulation rate. Ph.D. Thesis, University of Edinburgh.

PHILIPON, P. & TERQUI, M., 1987. Increase of prolificacy in sheep immunized against androstenedione. In: Follicular Growth and Ovulation Rate in Farm Animals, p. 129. Martinus Nijhoff Publishers, Dordrecht.

RAWLINGS, N.C., KENNEDY, S.W. & HENRICKS, D.M., 1978. Effects of active immunization of the cyclic ewe against oestradiol-17ß. *J. Endocrinol.* 76, 11.

SCARAMUZZI, R.J., BAIRD, D.T., CLARKE, I.J., MARTENESZ, N.D. & VAN LOOK, P.F.A., 1980. Ovarian morphology and the concentration of steroids during the oestrous cycle of sheep actively immunized against androstenedione. *J. Reprod. Fert.* 58, 27.

SCARAMUZZI, R.J., BAIRD, D.T., MARTENSZ, N.D., TURNBULL, K.E. & VAN LOOK, P.F.A., 1981. Ovarian function in the ewe after active immunization against testosterone. *J. Reprod. Fert.* 61, 1.

SCARAMUZZI, R. J. & HOSKINSON, R.M., 1984. Active immunization against steroid hormones for increasing fecundity. In: Immunological Aspects of Reproduction in Mammals, p. 475. (Ed.) D.B. Crighton. Butterworths Scientific, London.

SMITH, J.F., MACLEAN, K.S., MCGOWAN, L.T. & POTTS, I.R., 1985. Effect of ewe live weight on the level of response to steroid immunisation. *Proc. N. Z. Soc. Anim. Prod.* 45, 184.

STONE, S.L., RICHARDSON, L.L., HAMNER, C.E. & OLIPHANT, G., 1977. Partial characterization of hormone mediated inhibition of embryo development in rabbit oviduct fluid. *Biol. Reprod.* 16, 647.

VAN ZYL, J.G.E., STEYN, D.F., VAN NIEKERK, W.A., COERTZE, R.J. & GROENEVELD, H.T., 1987. Some aspects of superovulation and fertilisation of Dorper sheep. *J. S. Afr. Vet. Assoc.* 58, 3.

WEBB, R., LAND, R.B., PATHIRAJA, N. & MORRIS, B.A., 1984. Passive immunization against steroid hormones in the female. In: Immunological Aspects of Reproduction in Mammals, p. 475. (Ed.) D.B. Crighton. Butterworths Scientific, London.

WHYMAN, D. & MOORE, R.W., 1980. Effects of PMSG and the prostaglandin F-2 analogue, cloprostenol, on superovulation, fertilization and egg transport in the ewe. *J. Reprod. Fert.* 60, 267.