CONTROL OF OVULATION RATE IN BEEF CATTLE

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1. SUMMARY

Under intensive production systems, the greatest potential for effecting increases in production and economic efficiency in the beef cow herd lies in the possibility of increasing the frequency of twin births. Embryo transfer is technically a successful method of inducing twin births in cattle. While an embryo transfer approach is too costly to allow commercial twinning, it has been used to show that ovulation rate and not uterine capacity is the limiting factor in increasing the reproductive rate of the cow. While ovulation of one or more viable oocytes is central to normal reproduction, knowledge of the control of ovulation and of folliculogenesis on which ovulation depends, is limited. In spite of the fact that many follicles are subjected to the same endogenous hormonal environment and theoretically should all be capable of ovulating, only a tiny proportion do. While gonadotrophic hormones play a central role in ovarian follicle development and ovulation, their action at the ovarian level seems to be controlled by intra-ovarian factors. This intra-ovarian control of ovulation is thought to be exerted partly by the hormone inhibin and partly by other, as yet, unidentified compounds in follicular fluid. This project focused on identification and isolation of ovarian compounds involved in the control of ovulation rate, followed by immunisation against these compounds in order to study the effect on ovulation and the twin calving rates. The main results are summarised here and detailed results have been published in the papers listed at the end of this report.

Isolation of ovulation controlling compounds in cattle

Isolation of granulosa cell inhibitory factor

A novel compound with a number of major biological effects, implicated in ovulation control, has been isolated from cattle ovarian follicles.

- At very low concentrations it markedly inhibits granulosa cell proliferation *in vitro*.
- It selectively inhibits the proliferation of granulosa cells from small and medium sized follicles but has no effect on cells from large follicles.
- Its effect is specific to ovarian follicles.

This is a novel ovarian compound, previously unidentified and now termed "granulosa cell inhibitory factor" or GCIF. Based on elution position on the gel filtration columns, it is probable that the molecular weight of GCIF is <5 kDa.

The evidence from this study suggests that the GCIF described here may well be one of the specific inhibitor(s) produced by the dominant follicle and involved in the inhibition of subordinate follicles. This hypothesis is supported by the susceptibility of granulosa cells from small and medium follicles to inhibition by GCIF. Evidence that production of GCIF is greatest in large dominant follicles lends even more support to this hypothesis.

Isolation of inhibin

A partly purified inhibin preparation was isolated from steroid free follicular fluid by sequential chromatography.

- The biological activity of the inhibin preparation was measured by an *in vitro* bioassay by determining its capacity to inhibit FSH secretion by rat pituitary cells in culture.

- Following partial purification the biological activity of the inhibin preparation was increased by 200-fold compared with starting follicular fluid and inhibited FSH secretion by 60% in the *in vitro* bioassay.

- Electrophoresis revealed that the inhibin preparation had an apparent molecular mass of 68kDa.

Immunisation against GCIF or inhibin and ovulation rate

GCIF and ovulation rate

Sheep were used as a model in the immunisation studies as the supply of partly purified inhibin was insufficient to immunise cattle.

- Ovulation rate over 8 boosts was increased in GCIF immunised sheep.

- The overall mean was 2.75 ± 0.15 ovulations for the GCIF immunised and 1.85 ± 0.12 for control immunised sheep.

- Immunisation against GCIF increased the average ovulation rate by 36% with the maximum increase (140%) recorded following the third boost.

- This increase in ovulation rate was the result of a greater number of sheep having 3 ovulations rather than a smaller number having very high ovulation rates.

Inhibin peptides and ovulation rate

Inhibin peptides from the amino acid sequence of inhibin were identified by computer analysis as likely immunological epitopes and were used in immunisation studies.

- Immunisation of cattle against inhibin peptides generated an immune response to inhibin and resulted in up to 80% of heifers in one of the immunised groups responding with twin-ovulations.

- The increase in ovulation rate was mainly confined to twin ovulations and to the cycle following each boost. Ten of the 15 immunised (67%) and 4 of 5 control (80%) heifers conceived to first service while 60% of heifers in one of the peptide immunised group gave birth to twin calves.

2. INTRODUCTION

The combination of single calving and a nine-month gestation length inherently limits output and profit margins of beef cow enterprises. In this context the greatest potential for effecting higher biological and economic efficiencies lies in the possibility of increasing the frequency of twin births. In earlier twin embryo transfer studies, carried out at the Teagasc, Belclare Research Centre we showed that it is the ovulation rate and not uterine capacity that limits the frequency of twin births in cattle. In animal research institutions world-wide several approaches including, genetic selection, embryo transfer, hormone administration and controlling the ovulation rate have been followed in attempts to increase the incidence of multiple births in cattle. In the project outlined in this report the approach taken was based on attempts to control ovulation rate. While ovulation of one or more viable oocytes is central to normal reproduction, knowledge of the control of ovulation and of folliculogenesis on which ovulation depends, is limited. In spite of the fact that many follicles are subjected to the same endogenous hormonal environment and theoretically should all be capable of ovulating, only a tiny proportion do. While gonadotrophic hormones play a central role in ovarian follicle development and ovulation, their action at the ovarian level seems to be controlled by intra-ovarian factors. This intra-ovarian control of ovulation is thought to be exerted partly by the hormone inhibin and partly by other, as yet, unidentified compounds in follicular fluid. This project focused on identification and isolation of ovarian compounds involved in the control of ovulation rate, followed by immunisation against these compounds in order to study the effect on ovulation and the twin calving rates. The main results are summarised here and detailed results have been published in the papers listed at the end of this report.

Objective

The overall objective of the project was to increase ovulation and twin calving rates in cattle and this was addressed in a series of studies, the results of which are summarised in this report. Detailed and more comprehensive results have been published in the series of papers listed at the end of the report.

3. ISOLATION OF OVULATION CONTROLLING COMPOUNDS IN CATTLE

For the isolation of ovulation controlling compounds follicular fluid was aspirated from cattle ovarian follicles, charcoal treated to remove steroids and a low (<10kDa) or high (>10kDa molecular weight fraction prepared by ultrafiltration. The objective of this study was to isolate compound(s) from cattle follicular fluid that are involved in ovulation control. The results of a number of experiments are summarised below.

Experiment 1: *Purification and in vitro testing of low molecular weight fractions isolated from cattle ovarian follicles.*

The low molecular weight fraction of bovine follicular fluid was run on a series of gel filtration columns to separate it into different molecular weight fractions. The different fractions were tested for biological activity using a standard test which involved measuring the effect of different concentrations of each fraction on the proliferation of ovarian follicle granulosa cells. Gel filtration of the prepared bovine follicular fluid by Sephadex G-25 chromotography resulted in a consistent elution profile comprising seven distinct fractions. Similar elution profiles were also found for follicular fluid isolated from the follicles of sheep, and horses (Fig. 1). The elution volumes, fraction volumes and peptide equivalent content of all seven fractions isolated from bovine follicular fluid are shown in Table 1.

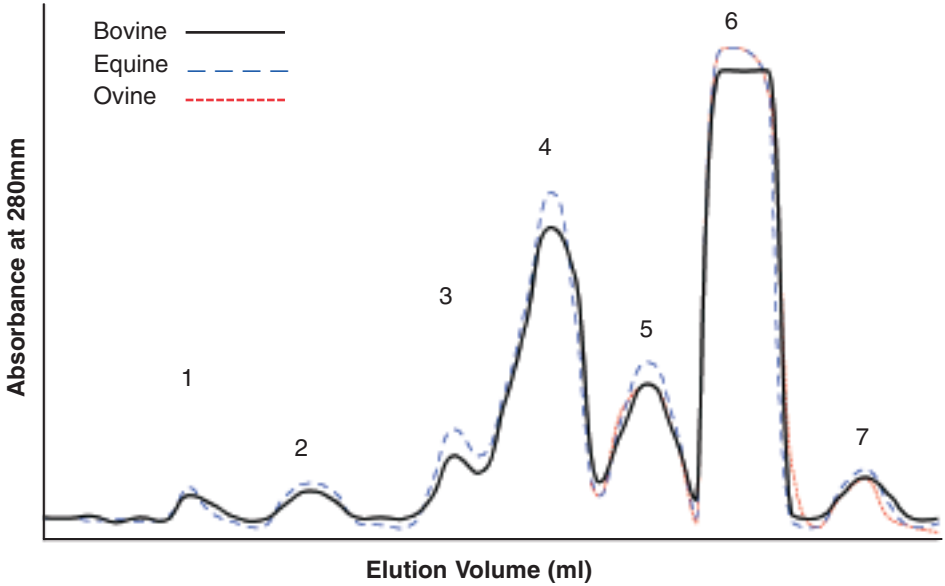

Fig. 1. The chromatographic elution profile of a low molecular weight fraction of bovine, equine and ovine follicular fluid.

Table 1. The recovery of peptide equivalent material from 10 mg of the LMW fraction of bovine follicular fluid following fractionation on a Sephadex G-25 column.

Peak No.	Elution volume (ml)	Peak volume (ml)	Peptide equivalent (mg)
1	33.80	7.00	0.14
2	67.60	8.60	0.26
3	77.80	11.00	0.40
4	85.40	9.00	1.07
5	96.60	12.20	0.47
6	113.60	21.00	4.04
7	136.00	18.60	0.81

The biological effect of each of these fractions, measured in terms of their effect on granulosa cell proliferation *in vitro* is shown in Fig 2. Only Fractions 3 and 4 had a significant effect on granulosa cell proliferation. Fraction 3 stimulated ($P<0.01$) and fraction 4 significantly inhibited ($P<0.01$) cell proliferation in each case. Further tests showed that the inhibitory effect of Fraction 4 on cell proliferation was not due to either a toxicity effect or to inhibition of cell attachment.

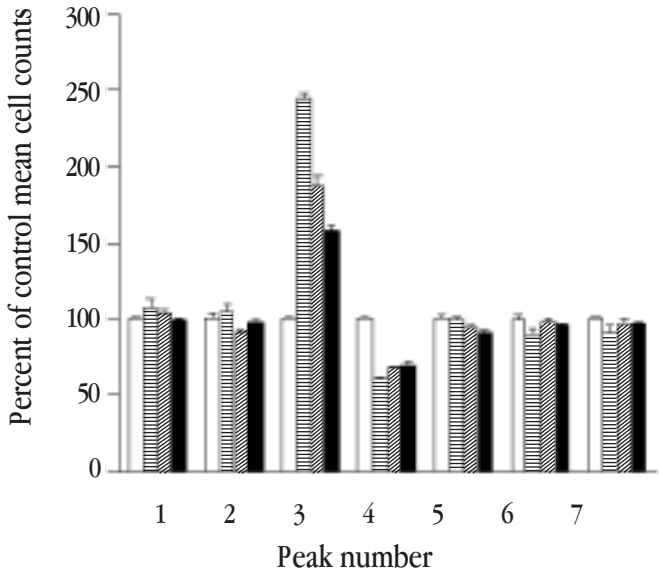

Fig. 2. Effect of various peaks from the G-25 chromatographic separation of the low molecular weight fraction of follicular fluid on granulosa cell proliferation as measured by haemocytometric cell counting. Peptide equivalent concentrations of the various peaks are shown as follows:- 0 ng ml^{-1}, □ ; 10 ng ml^{-1}, ▤ ; 100 ng ml^{-1}, ▨ ; 1000 ng ml^{-1}, ■ .

Values are means±SEM based on the following numbers of replicates:-

4 replicates for peaks 1, 2, 3, 5 and 7; 14 replicates for peak 4 and 8 replicates for peak 6. Control mean cell numbers for the various peaks varied from 44-68 x 104 ml^{-1}.

Experiment 2: ***The effect of inhibitory Fraction 4 on proliferation of granulosa cells from small (<2 mm), medium (2-10 mm) or large follicles (10-20 mm).***

Fraction 4 inhibited the proliferation of granulosa cells from small ($P < 0.01$) and medium-sized ($P < 0.01$) follicles but had no effect on granulosa cells from large follicles (Fig. 3).

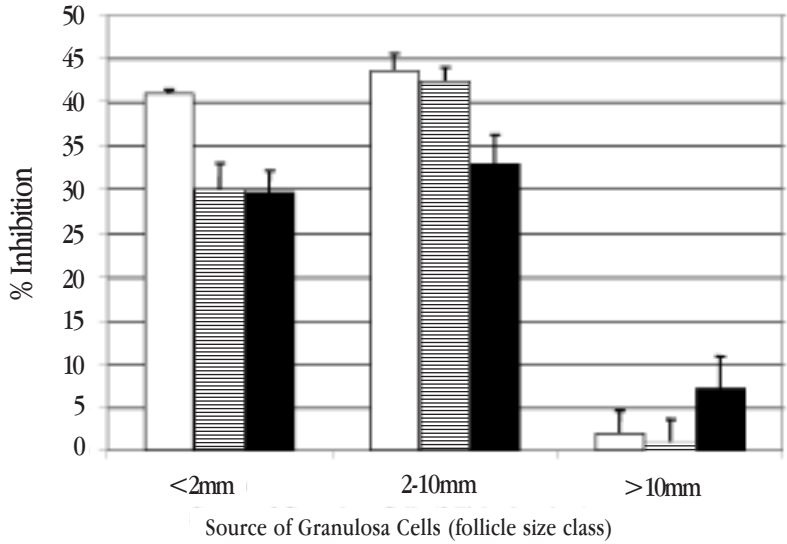

Fig.3. Effects of peak 4 isolated from a population of mixed follicles on the proliferation of granulosa cells at 0.01 ng/ml, (□); 0.10ng/ml (▤) and 1.0ng/ml, (■) monitored by [3^H]thymidine incorporation into acid-precipitable material. Values are expressed as a percentage of control ± sem.

The specific nature of the effect on ovarian follicle cell proliferation was then established by demonstrating its failure to affect proliferation of the cells of other organ tissues.

These results demonstrate clearly that bovine follicular fluid contains a low molecular weight fraction (Fraction 4) with the following major biological effects:- (1) at very low concentrations it markedly inhibits granulosa cell

proliferation *in vitro*, (2) it selectively inhibits the proliferation of granulosa cells from small and medium sized follicles but has no effect on cells from large follicles, (3) its effect is specific to ovarian follicles. This is a novel ovarian fraction, previously unidentified and now termed "granulosa cell inhibitory factor" or GCIF. Based on elution position on the Sephadex gel filtration columns, it is probable that the molecular weight of GCIF is <5 kDa.

The evidence from this study suggests that the GCIF described here may well be one of the specific inhibitor(s) produced by the dominant follicle and involved in the inhibition of subordinate follicles. This hypothesis is supported by the susceptibility of granulosa cells from small and medium follicles to inhibition by GCIF. Evidence that production of GCIF is greatest in large dominant follicles lends even more support to this hypothesis.

It is interesting to speculate that a very useful property of a factor produced by a dominant follicle to suppress competing follicles would be the ability to inhibit the granulosa cells of the competing follicle on both ovaries at very low concentrations coupled with an inability to inhibit granulosa cells at high concentrations such as might be found in the dominant follicle producing it.

Experiment 3: *Purification and in vitro testing of inhibin isolated from cattle ovarian follicles.*

Inhibin was isolated from the high molecular weight ($>$10kDa) fraction of steroid free follicular fluid by sequential chromatography on affinity (Red Sepharose) and ion exchange columns (Mono Q). The activity of inhibin in this preparation was increased 207-fold over starting material with an overall recovery of activity of 3% (Table 2). The biological activity of the inhibin preparation was measured by an *in vitro* bioassay by determining its capacity to specifically inhibit FSH secretion by rat pituitary cells in culture without affecting LH secretion. This inhibin preparation was effective in suppressing FSH secretion by 60% with no effect on LH concentrations. (Table 3). These data indicate that inhibin is present at very low concentrations in the bovine follicle as approximately 1 mg of this active fraction was recovered from a total of 50 ml (3000mg) of bovine follicular fluid. Electrophoresis of the preparation was carried out and revealed the presence of one protein which migrated with an apparent molecular mass of 68kDa. This inhibin preparation was used for the immunisation studies.

Table 2. Relative specific activity (RSA) and recovery of inhibin from bovine follicular fluid following chromatography on Red Sepharose and Mono Q at pH 7.9 and pH 9.2.

Purification Step	RSA	(Overall)	Recovery (%)	(Overall)
Follicular Fluid	1	(1)	100	(100)
Red Sepharose	8-12	(8-12)	28-37	(28-37)
Mono Q @ pH 7.9	2.5-3.7	(20-46)	28-35	(8-13)
Mono Q @ pH 9.2	4.5	(207)*	24	(3.2)

* The most active fraction had a relative specific activity (RSA) of 335.

Table 3. Mean production of FSH and LH by 100,000 rat pituitary cells after 72 hr in culture in the presence or absence of 2µg of inhibin purified from bovine follicular fluid.

Treatment	FSH (ng/ml)	LH (ng/ml)
Control	31.7 ± 5.72	11.2 ± 1.27
Inhibin	14.1 ± 3.89 *	12.0 ± 1.21

* Significantly different from control ($P<0.05$)

4. IMMUNISATION AGAINST GCIF OR INHIBIN AND OVULATION RATE

Immunisation against GCIF and ovulation rate

The purpose of this experiment was to determine whether active immunisation against GCIF derived from bovine follicular fluid could elicit an immune response in sheep and affect ovulation rate.

The GCIF was isolated, purified and tested for granulosa cell inhibitory activity as described above and was then conjugated to human serum albumin for immunisation studies. Because of the small amount of follicular fluid and consequently the minute amounts of GCIF available following the chromatographic isolation and purification procedures, sheep were used as the model. Ewes of Cambridge and Belclare ancestry were immunised against a GCIF-HSA conjugate. Control ewes were immunised against either HSA alone or a GnRH-HSA conjugate. A total of eight booster injections were given at intervals of 6-10 weeks after the primary immunisation over a period of two breeding seasons. Blood samples were taken before primary injection and at regular intervals for measurement of antibody titre. Antibody titres were measured using a solid phase enzymeimmunoassay. Because the GCIF fraction is not a pure preparation, it was not possible to measure the antibody titres specifically raised against GCIF. Antibody titres against GnRH, however, were measured in all ewes as an index of the efficiency of the conjugation and immunisation procedures with a small peptide. Ovulation rate in all ewes was measured by mid-ventral laparoscopy during the luteal phase of each relevant oestrous cycle.

All sheep immunised against GnRH attained antibody titres greater than 1 in 60,000 by the end of the experiment (1 in 75,500 $\pm$ 6,028 over all measurements taken). This indicates that the peptide conjugation and immunisation procedures used were efficient.

The effects of immunisation against GCIF on ovulation rate and follicle numbers are shown in Fig. 4 and Tables 4 and 5. With the exception of the first boost, ovulation rate over 8 boosts was increased in the GCIF immunised sheep (Fig.4 (a) and Table 4 ($P < 0.01$).

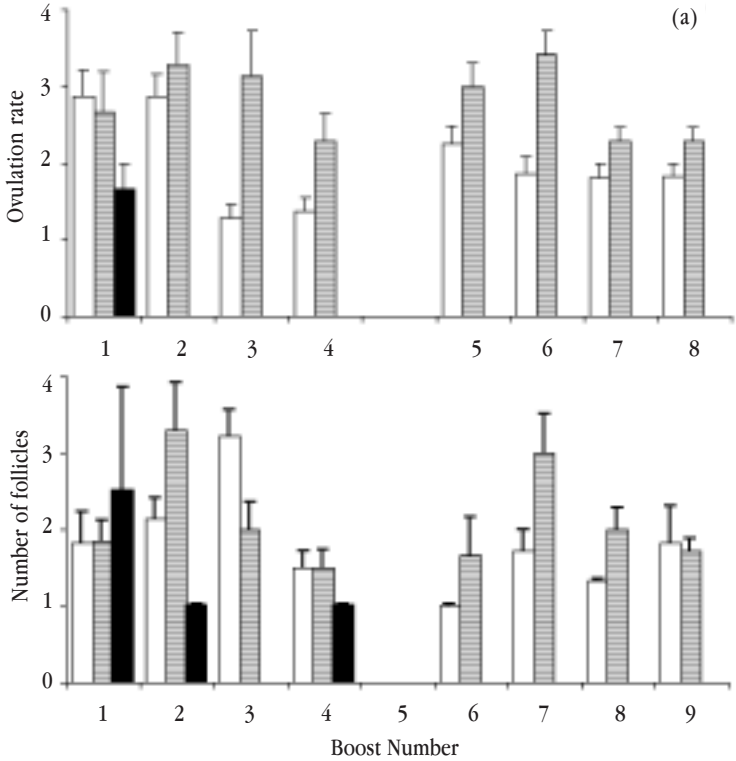

Fig.4 Effect of immunization of sheep with human serum albumin (HSA) alone (□), granulosa cell inhibitory factor GCIF -HSA (▤) or GnRH-HSA (■) over two breeding seasons on (a) ovulation rate and (b) number of follicles with a diameter >3mm per animal. Sheep immunized with GnRH were boosted only once. They did not ovulate after the first boost and had no follicles >3mm diameter after the first breeding season. Values are mean±s.e.m. based on all animals and 7 to 8 replicates. The overall ovulation rate was significantly different from that of HSA immunized sheep ($p<0.01$). The number of follicles for GCIF-and HAS-immunized sheep were not significantly different ($P>0.05$)

The overall mean was 2.75±0.15 ovulations for the GCIF immunised and 1.85±0.12 for HSA immunised sheep. When only animals that had ovulated were included in the analysis, immunisation against GCIF increased the average ovulation rate by 36% (2.80±0.14 vs 2.05±0.13). The maximum increase in ovulation rate (140%) was recorded following boost 3 in GCIF immunised animals. When all animals were included in the analysis, immunisation against GCIF increased ovulation rate by an average of 48%, with a maximum increase of more than 180% following boost 3.

Table 4. The frequency distribution of the ovulation rate for human serum albumin (HSA) and granulosa cell inhibitory factor (GCIF) immunized ewes

		Ovulation rate class (%)						
Immunization treatment	Cycles	0	1	2	3	4	5	6
HSA	64	6(9)	15(23)	30(47)	8(13)	5(8)	-	-
GCIF-HSA	56	1(2)	4(7)	23(41)	15(27)	9(16)	3(5)	1(2)

The HSA and GCIF-HSA immunisation treatments were significantly different from each other (chi-squared test, $P<0.01$)

A frequency distribution table for ovulation rate (Table 4) shows that 50% of the cycles of GCIF immunised sheep resulted in 3 or more ovulations compared to only 21% of HSA immunised sheep. This increase in ovulation rate was the result of a greater number of sheep having 3 ovulations rather than a smaller number having very high ovulation rates.

The data from all cycles with one or more ovulations indicates that immunisation against GCIF did not affect the numbers of cycles with ovulation on both sides. In ovulating sheep that were immunised against HSA, in 32 of 58 cycles (55%) there were no ovulations on one side in contrast to 27 of 55 cycles (49%) with no ovulations on one side in ovulating sheep that were immunised against GCIF ($P>0.05$). In contrast, the data from cycles with ovulations on one side only clearly showed that immunisation with GCIF did increase the numbers of ovulations on that side. Following immunisation against HSA, there were 15 of 32 cycles (47%) with two or more ovulations on the ovulating ovary in contrast to 23 of 27 cycles (85%) with two or more ovulations in sheep that were immunised against GCIF ($P<0.01$).

In contrast with the effects on ovulation, the effects of immunisation against GCIF on follicles were much less marked. While the average number of follicles of 3 mm or greater in diameter present on the ovaries was increased following immunisation against GCIF (Fig. 4(b) and Table 5), this increase was neither consistent nor significant ($P > 0.05$).

Table 5. The frequency distribution of the number of follicles >3mm in diameter for

		Number of follicles class (%)						
Immunization treatment	Cycles	0	1	2	3	4	5	6
HSA	64	17(27)	23(36)	15(23)	9(14)	-	-	-
GCIF-HSA	56	10(18)	15(27)	20(35)	7(12)	2(4)	1(2)	1(2)

The HSA and GCIF-HSA immunisation treatments were not significantly different (chi-squared test, $P > 0.05$)

Immunisation against GnRH inhibited ovulation. In the first cycle following the first boost no sheep at any stage had an antibody titre of less that 1 in 60,000. This indicates that the peptide conjugation and immunisation procedures used were efficient in eliciting a good immune response.

Immunisation of sheep against GCIF clearly increased ovulation rate. This increase was maintained as animals were boosted into the second year of the experiment. Because the GCIF fraction is a relatively crude fraction no effort was made to obtain antibody titres against the active factor in immunised animals.

If GCIF is a factor secreted by a dominant follicle or follicles to suppress growth of other follicles, it is interesting to ask the question, whether, in multiple ovulating animals such as the sheep, it acts mainly to inhibit follicles on the same or opposite ovary. The finding that immunisation against GCIF does not increase the number of sheep ovulating from both ovaries but does increase the number of ovulations on the single ovulating ovary indicates that GCIF is only active in the ovary in which it is produced and does not affect the opposite ovary. This failure to affect the opposite ovary in the sheep may indicate that GCIF is

restricted to the ovary that produces it but it is more likely that it is due to the fact that the concentration of GCIF is reduced greatly by dilution in the systemic blood system. The failure to affect the opposite ovary is an interesting finding; it would be even more interesting to see if this situation is also the case for mono-ovulating species such as the cow. These results also indicate clearly that the immunisation effect on ovulation is not due to some non-specific effect exerted on the secretion of hypothalamic or pituitary hormones but to a local ovarian effect.

This report provides further evidence that a low molecular weight factor in follicular fluid, GCIF, which inhibits granulosa cell proliferation in vitro also inhibits large follicle growth and ovulation *in vivo* and also indicates that it may be possible to control ovulation rate in cattle by immunisation against this factor.

Immunisation against inhibin and ovulation rate

The purpose of this experiment was to determine whether active immunisation with a partly purified inhibin preparation derived from cattle follicular fluid would elicit an immune response to inhibin and affect ovulation rate. Because the amount of partly purified inhibin available for immunisation studies, prepubertal lambs were used as a model to determine the effects of immunisation on the immune response to inhibin and on ovulation rate.

The partly purified inhibin was used directly for immunisation. Blood samples were taken at relevant intervals to measure antibody titres. Following the first booster injection, vasectomized rams fitted with harnesses and crayons were used to check for standing estrus in order to establish the time of onset of puberty and estrous cycle length. Ovulation rate was determined at mid-cycle by direct laparoscopic observation.

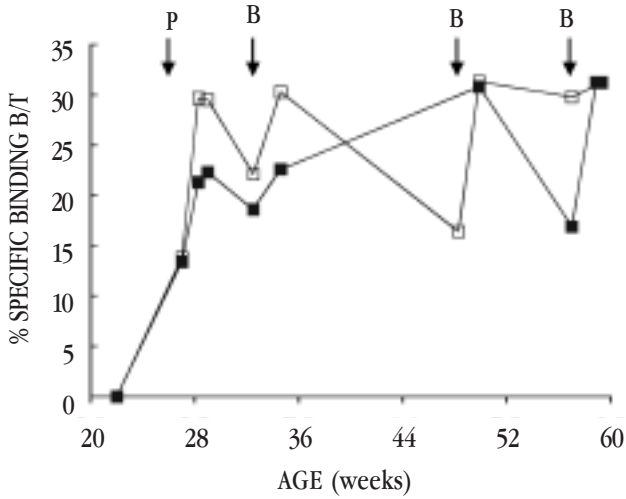

Figure 5 Percentage binding of radioiodinated 68kDa inhibin by serum from ewe lambs immunized with a partly purified preparation of bovine follicular fluid. Arrows indicate time of immunization.

The antigen binding capacity of the serum from the immunised lambs was increased ($P<0.002$) relative to their pre- immune serum (Fig.5). Radioactive binding by pre-immune sera was less than 0.5%. The number of ovulations measured for each of the first three cycles of the season starting approximately one week after the final boost was 1, 7, and 4 for one of the lambs and 1, 2 and 6 for the other.

Table 6. Number of corpora lutea (CL) of control (C) and inhibin immunized (I) Suffolk x Galway ewe lambs at different ages and mean± s.e.m. of the within-lamb ovulation rate.

		No. of CL at different ages (weeks)							Ovulation
Group	N	28	30	31	32	33	38	42	Rate
C	9	3	1	7	1	6	10	2	1.15±0.09
		(2)	(1)	(5)	(1)	(6)	(9)	(2)	
I	8	2	5	2	2	4	17	7	1.95±0.28*
		(1)	(2)	(2)	(2)	(3)	(6)	(4)	

Values in parentheses are the number of lambs that ovulated.
*Significantly higher than for Group C lambs, $P<0.05$.

Immunisation increased ovulation rate in the Suffolk cross lambs by 70% ($P<0.05$) (Table 6). Ovulation rate in the immunised Finn x Dorset Horn ewe lambs was 3.38 ± 0.76. This was 47% higher than the ovulation rate of 2.29 ± 0.16 recorded over two cycles for mature ewes ($n=12$) from the same flock measured over the same time period (Table 7). There was no effect of immunisation on the age at puberty or on estrous cycle length in either breed type (Table 8).

Table 7. Number of corpora lutea (CL) for inhibin immunized Finn x Dorset Horn ewe lambs at different ages and mean±s.e.m. of the within-lamb ovulation rate.

		No. of CL at different ages (weeks)						
Group	N	32	33	35	36	41	45	Ovulation Rate
I	4	4 (2)	1 (1)	4 (2)	2 (2)	16 (3)	17 (3)	3.38±0.76

Values in parentheses are the number of lambs that ovulated.

Table 8. Effect of immunisation of prepuberal lambs on the onset of puberty and oestrous cycle length (mean±SEM)

Breed Type	Status	N	Onset of Puberty		Cycle (Days)
			Date*	Age (Days)	
Suffolk x Galway	Immunised	8	344±10	224±10	15.3±0.4 (33)
	Control	9	334±07	214±07	15.5±0.3 (44)
Finn x Dorset Horn	Immunised	4	327±06	235±05	15.8±0.5 (21)
	Control**	89	321±03	252±03	17.8±0.3 (109)

* Date is referenced to January 1st = Day 1. ** After Quirke (1978)
Numbers in parentheses are the number of estrous cycles.

There was no effect of immunisation on the number of Suffolk x Galway lambs showing estrus or on the number ovulating. However, one inhibin immunised lamb failed to ovulate despite showing estrous activity on two occasions. A frequency distribution for ovulation rate is shown in Table 9 for all breeds.

Table 9. Frequency distribution of ovulation rate for inhibin immunized (I) and control (C) Suffolk x Galway (SxG) and Finn x Dorset Horn (FxDH) ewe lambs.

Group	No. of cycles	Ovulation Rate Class						
		1	2	3	4	5	6	9
I, SxG	20	9	6	4	-	-	1	-
		(45)	(30)	(20)			(5)	
C, SxG	26	23	2	1	-	-	-	-
		(88)	(8)	(4)				
I, FxDH	13	3	4	2	1	1	-	2
		(23)	(31)	(15)	(8)	(8)		(15)
C, FxDH	24	3	13	6	2	-	-	-
		(13)	(54)	(25)	(8)			

Values in parentheses are percentages.

This study shows that active immunisation of prepuberal ewe lambs against a partly purified preparation of bovine follicular fluid raised antibodies capable of binding to a homogenous preparation of bovine inhibin. Active immunisation increased ovulation rate without affecting the onset of puberty or estrous cycle length. Even though FSH secretion was not measured, these results suggest that, immunisation against inhibin increases ovulation rate in sheep, possibly by acting to increase FSH secretion.

5. OVULATION RATE AND FERTILITY IN CATTLE IMMUNISED AGAINST INHIBIN PEPTIDES

As the amount of inhibin activity that could be purified from bovine follicular fluid was sufficient only for immunisation of sheep another approach was used for cattle immunisation.

In this approach the amino acid sequence of the bovine inhibin alpha-subunit was first analyzed using a computer programme that combined algorithms for the prediction of hydrophylicity/hydrophobicity and secondary structure and that enabled the rapid identification of likely immunological epitopes. From this, three peptide sequences (Fig. 6) were identified as potential antigenic sites. The natural sequences were modified during synthesis by the addition of either tyrosyl or cystenyl residues, linked through a glycine spacer where appropriate, in order to facilitate iodination and conjugation respectively. The three peptides were synthesized with a purity of greater than 90%. Each peptide was

P1: bI-α-[YG](18-30):[YG]QRPPEEPAAHADC(NH2)

P2: bI-α-(63-72)[GY]:CGLSPPQDLP[GY](NH2)

P3: bI-α-[CG](107-122):[CG]HVRTTSDGGYSFKYEM(NH2)

Fig. 6. *Sequences of the synthetic peptides derived from the bovine α-inhibin subunit.*

conjugated to human serum albumin (HSA) and conalbumin. Twenty nulliparous Hereford cross heifers, approximately 18 months of age and 367±7 kg (mean±sem) in weight were randomly allotted in equal numbers to three treatment groups and a control group. Three booster injections were administered at intervals of 11 weeks. Blood samples were taken at relevant intervals to measure anti-peptide antibodies. Five days after each boost, heifers had their oestrous cycles synchronized. Ultrasonography was carried out between Days 6 and 10 on all heifers during each oestrous cycle to determine the number of ovulations that had occurred. At the first oestrus and the first repeat

oestrus only, following boost 3, heifers were mated. The number of fetuses present at 45 days after mating was counted by ultrasonography and at parturition the number of calves born was recorded.

Antibodies were generated to each of the peptides with titres reaching a maximum between 7 and 21 days following a boost (Fig 7). The highest individual peptide titres recorded within groups P1, P2 and P3 were 316,000, 284,000 and 59,000 respectively. Peptide titres measured in the HSA immunised control heifers and in the pre-immune sera were less than 100.

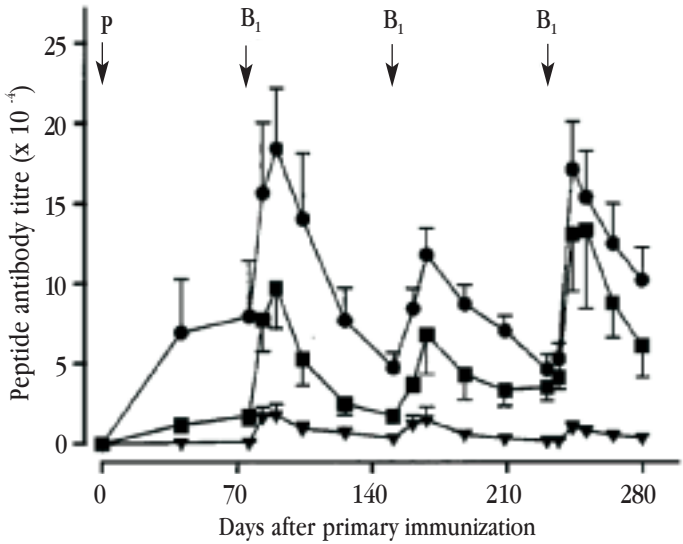

Fig. 7. Peptide antibody titres (means±SEM) in serum from heifers immunized against inhibin peptides (■) P1, (●)P2, and (▼) P3. Heifers were injected at intervals of 11 weeks (arrows). P: primary immunization;B1-B3: consecutive booster injections.

All peptide immunised heifers generated antibodies which bound to inhibin, with binding reaching a maximum between 7 and 21 days after a boost (Fig 8). The highest individual [^{125}I] *M*r 32,000 bovine inhibin binding recorded was 35%, 42% and 13% for P1, P2, and P3 respectively. Inhibin binding in HSA only immunised heifers and in the pre-immune sera was less than 2%.

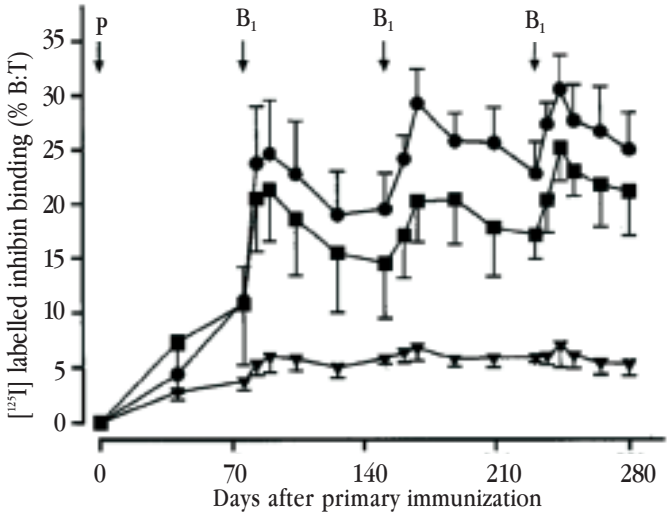

Fig. 8. Percentage binding (means±SEM) of 125I-labelled 32kDa bovine inhibin by serum from heifers immunized against inhibin peptides (■) P1, (●)P2, and (▼) P3. Heifers were injected at intervals of 11 weeks (arrows). P: primary immunization;B1-B3: consecutive booster injections.

Boost number did not affect ($P>0.05$) the percentage of radiolabeled inhibin bound at peak titre. There were significant differences between the peptide immunised groups in the percentage of radiolabelled inhibin bound at peak titre (Table 10) with P2 exhibiting the highest binding. Peptide and inhibin titres were correlated for groups P1 ($r=0.46$, $df=68$, $P<0.01$) and P2 ($r=0.74$, $df=68$, $P<0.01$) only.

Table 10. Specific peak inhibin binding (%) following immunization of heifers with inhibin peptides.

Group	Inhibin Binding (%)	
P1	21.5[a]	16.5-27.1
	(15)	4.6-35.0
P2	27.5[b]	22.0-33.5
	(15)	6.4-42.2
P3	5.5[c]	2.9 - 8.8
	(15)	1.1-13.4

Values are mean followed by 95% confidence limits of the mean of three boosts with numbers of observations in parentheses and the range.
b>a $P<0.05$; a,b>c $P<0.01$.

The proportion of heifers showing oestrus was not affected (P>0.05) by peptide immunisation. Oestrous cycle length (least square mean±s.e.m) for groups P1, P2, P3 and the control group was 19.8 ± 0.5, 20.3 ± 0.5, 19.2 ± 0.5 and 20.6 ± 0.5 days, respectively (P>0.05).

The individual animal ovulation response is detailed in Table 12. Peptide immunisation increased (P<0.05) the ovulation rate overall, however, there was no difference between peptide groups (P>0.05). In the control group the ovulation rate was 1.0 at each cycle throughout the immunisation period. Ovulation rate was increased in 1/5, 5/5 and 4/5 of the heifers and in 6/38 (16%), 13/37 (35%) and 6/33 (18%) of the ovulatory cycles measured following immunisation against P1, P2 and P3 respectively. Of the 25 multiple ovulatory cycles recorded, 19 (76%) were twin ovulatory. Boost number did not affect the ovulation rate (P>0.05), however, there was a significant (P<0.05) effect of cycle number on ovulation rate and also a significant (P<0.005) treatment by cycle interaction (Table 12). One heifer (No. 13, P3) became anovulatory for a period of 60-70 days following boost 2. Ovulation rate in the first oestrous cycle following a boost and inhibin binding at peak titre were correlated (r=0.56, df=13, P<0.05) for group P2 only.

Table 11. Number of ovulations in successive oestrous cycles following each of three booster injections for inhibin peptide immunised and control groups

		Booster injection number									
		1				2				3	
		Cycle number									
Group	Heifer number	1	2	3	4	1	2	3	4	1	2
P1	1	1	1	1	-[$]	1	-	1	-	1	-
	2	2	1	4	-	1	3	2	-	3	4
	3	1	1	1	-	1	1	1	-	1	-
	4	1	1	1	1	1	1	1	-	1	-
	5	1	1	1	-	1	1	1	1	1	1
P2	6	2	1	1	1	2	1	1	-	3	2
	7	1	1	1	1	2	1	1	-	1	-
	8	0[§]	1	1	-	1	1	2	1	2	-
	9	3	1	1	-	2	1	1	-	2	-
	10	1	0	1	-	2	2	1	-	2	-
P3	11	2	1	1	-	2	1	1	-	1	1
	12	2	-	-	1	2	1	1	1	1	-
	13	0	1	1	1	0	0	0	-	2	-
	14	1	1	1	-	1	1	1	-	2	1
	15	1	1	1	-	0	1	1	-	1	-
Control	16	1	1	1	-	1	0	1	-	1	-
	17	1	1	1	-	1	1	1	1	1	-
	18	1	1	1	1	1	1	1	-	1	1
	19	1	1	1	1	1	1	1	-	1	-
	20	1	1	1	-	1	1	1	-	1	-

[$] No observations were made. [§] Anovulation

Data on pregnancy, calving and twin-calving rates are presented in Table 13. Nine of the 15 peptide immunised heifers and 4 of the 5 control heifers conceived to first service, as determined by ultrasonography at Day 45. The remaining 7 heifers conceived to the second service. The overall conception rate in peptide immunised heifers for first and second services combined, was 9/11 (82%) and 6/9 (67%) for single and multiple ovulating animals, respectively. Of the 6 heifers

with multiple pregnancies at Day 45, 5 had 2 ovulations and 4 of these produced twin calves at term. The remaining heifer had 4 ovulations and had 4 foeti at Day 45, all of which were subsequently lost. Two of the 9 peptide-immunised, single-ovulating heifers, pregnant at Day 45, subsequently lost pregnancies. All 5 single-ovulating control heifers pregnant at Day 45 subsequently calved.

Table 12. Conception and calving data following breeding after booster injection three

Group	Heifer number	Number of corpora lutea	Number of fetuses at 45 days	Calves born
P1	1	1	1	1
	2	4(R)	4	0
	3	1	1	1
	4	1	1	1
	5	1(R)	1	1
P2	6	2(R)	2	2
	7	1	1	1
	8	2	2	2
	9	2	2	2
	10	2	2	0
P3	11	1(R)	1	0
	12	1	1	1
	13	2	2	2
	14	1(R)	1	0
	15	1	1	1
Control	16	1	1	1
	17	1	1	1
	18	1(R)	1	1
	19	1	1	1
	20	1	1	1

R: Observation from first repeat oestrus.

This study demonstrates that active immunisation of heifers against synthetic

peptide sequences of the a-subunit of bovine inhibin can increase ovulation rate and twin-calving rate. The three inhibin peptides used for immunisation differed in the ability of the antisera generated to bind to inhibin. Binding was highest for P2, followed by P1 while binding for P3 was 5-fold lower than P2. The relative magnitude of the immune response was similar between groups.

Immunisation did not affect either the proportion of heifers showing oestrus or oestrous cycle length. The highest ovulation rate increase was recorded in group P2 with all heifers responding with an increased ovulation rate at some time. At each of the first oestrous cycles following the second and third boosts, 4/5 (80%) heifers responded with an increased ovulation rate in P2. A total of 11/22 (50%) ovulatory cycles with multiple ovulations were recorded in group P2 following the second and third boosts of which 10 (91%) were twin ovulatory. The incidence of twin ovulations decreased in subsequent cycles even though inhibin binding was still relatively high, indicating that physiological compensation was occurring as free antibody titres decreased.

Immunisation against the peptides had no apparent effect on conception or calving rates. A slightly greater number of the immunised heifers, both single and multiple ovulating, lost pregnancies after Day 45. The small numbers of heifers involved in this study does not allow a firm conclusion to be drawn regarding the effects of immunisation on pregnancy rate, clearly, however, the production of 4 sets of twin calves from 6 twin ovulatory cycles suggests that peptide immunisation is a successful approach to the induction of twinning in cattle. This is the first report of an increase in calving rate following immunisation against inhibin.

6. DISCUSSION AND CONCLUSIONS

Ovulation rate is controlled partly by the negative feedback effects of steroids hormones, at the level of the pituitary to inhibit FSH secretion and partly by locally produced ovarian factors acting in an autocrine or paracrine manner to affect follicular growth. Immunomodulation of the negative feedback effect of steroids has been successful in increasing ovulation rate in sheep but not in cattle. This report describes how immunisation against locally produced ovarian factors was successfully used as an alternative approach to increasing ovulation rate in cattle.

One of the ovarian factors (GCIF), was isolated in this study and shown to be present in cattle ovarian follicles. This factor produced by large follicles acts in a paracrine fashion to inhibit the growth of the smaller or subordinate follicles. Its effect is local and is limited to the ovary producing it and could thus be considered as one of the factors involved in the regulation of ovulation rate in cattle. Immunisation of sheep against GCIF resulted in a controlled increase in ovulation rate without inducing a superovulatory response. Sufficient quantities of GCIF were not available for cattle immunisation, however, and this will have to await its chemical characterisation and synthesis.

Inhibin, also present in small quantities, was isolated from cattle follicular fluid. Sufficient quantities were recovered to enable immunisation of sheep. In order to immunise cattle, inhibin peptides derived from the amino acid sequence of bovine inhibin were synthesized. One of these peptides, which generated the highest immune response consistently increased the number of twin ovulation in up to 80% of heifers following a booster immunisation, with a very low incidence of 3 ovulations. Subsequently, following breeding, three heifers from this peptide immunised group gave birth to twin calves. This is the first time that an increase in ovulation rate in cattle following immunisation using this approach has resulted in an increase in the number of calves born.

Overall, these studies demonstrates that immunomodulation of farm animal fertility by immunisation against locally produced ovarian factors has potential, not only to increase ovulation rate but also the number of calves born.

7. PUBLICATIONS ARISING FROM THIS REPORT

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