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Ovarian and endocrine determinants of superovulatory responses in anestrous ewes

P.M. Bartlewski *, B.D. Alexander [1], W.A. King

Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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Abstract

Ovarian follicular status and secretory function at the outset of the superovulatory treatment in cyclic ewes are closely related to the resulting ovulation rate and embryo yields. The objective of this study was to examine if the size and developmental pattern of the two largest follicles, antral follicle numbers (follicles ≥2 mm in diameter) and circulating estradiol-17β (E_2-17β) concentrations at the onset of gonadotropin stimulation are related to superovulatory responses in anestrous ewes. Twenty-four anestrous (May–June) Rideau Arcott ewes were treated with medroxyprogesterone acetate (MAP) intravaginal sponges (60 mg) for 14 days. The superovulatory regimen consisted of six i.m. injections of pFSH (Folltropin®-V) administered twice daily (1× 2.5 ml and 5× 1.25 ml), from 48 h before to 12 h after sponge removal, followed by a bolus injection of GnRH (50 μg i.m.). A single i.m. dose of 500 IU eCG was given concurrently with the 1st pFSH injection. Jugular blood samples were collected and the number of ovarian antral follicles ≥2 mm in diameter recorded, using transrectal ovarian ultrasonography, at the time of the 1st and the 2nd pFSH injection. The size and developmental status (i.e., growing, static or regressing) of the two largest follicles had no influence on ovarian responses and embryo yields. The numbers of medium-sized follicles (4 mm in diameter) at the time of the 2nd pFSH injection were positively correlated to the numbers of luteal structures and the numbers of viable embryos (both $r = 0.44$, $P < 0.05$). Serum E_2-17β concentrations at the time of the 1st and the 2nd pFSH dose were positively correlated to the numbers of non-viable embryos ($r = 0.41$, $P < 0.05$ and $r = 0.60$, $P < 0.01$, respectively). It can be concluded that the number of gonadotropin-responsive small antral follicles and follicular estrogenicity appear to be major determinants of the ovarian response and embryo quality in superovulated anestrous ewes.

Keywords: Sheep; Anestrous; Superovulation; FSH; Estradiol; Ultrasonography; Ovarian response

1. Introduction

Hormonal ovarian stimulation in sheep is used mainly in multiple ovulation and embryo transfer (MOET) programs (Gordon, 1997; Cognie et al., 2003; Gonzalez-Bulnes et al., 2004). The sheep is also a good animal model for the study and amelioration of a broad range of assisted reproductive technologies (ART's), of which superovulation is an extensively used technique (Cognie et al., 2003). In spite of the numerous advancements in hormonal follicle manipulation techniques in sheep, the variation in superovulatory ovarian response continues to be one of the most frustrating problems limiting the profitability of commercial MOET operations and the application of superovulation in research.

* Corresponding author. Tel.: +1 519 824 4120x53330; fax: +1 519 767 1450.
E-mail address: pmbart@uoguelph.ca (P.M. Bartlewski).
[1] Present address: Department of Farm Animal Production and Health, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka.

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Thus, the development of a simple and inexpensive test for predicting the outcome of superovulatory treatments in individual ewes would have significant financial and practical advantages.

The ovarian response to superovulation in ewes is dependent upon many inherent and external factors, including season, breed, age, plane of nutrition, gonadotropin products and doses used, type of insemination, and the interval between successive treatments (Gordon, 1997; Cognie et al., 2003; Gonzalez-Bulnes et al., 2004; Shipley et al., 2007). However, even when these sources of variation among the treated donor animals have been minimized or eliminated, the superovulatory response remains highly variable, especially in seasonally anestrous ewes. From earlier endoscopic and ultrasonographic studies performed in cyclic ewes, it is plausible that ovarian follicular status at the onset of the superovulatory treatment may impinge on the superovulation performance (Cognie et al., 2003; Gonzalez-Bulnes et al., 2004). Several reports have shown a decrease in the number of ovulations when superovulation was initiated in the presence of a large ($\geq$6 mm) ovarian follicle(s) (Rubianes et al., 1995, 1997; Cognie et al., 2003; Gonzalez-Bulnes et al., 2004). A recent study by Veiga-Lopez et al. (2006) has shown the physiological status of the two largest follicles, detected by ultrasonography during the first 12 h of a superovulatory treatment, to affect the ovulation rate and embryo recovery in ewes. The ovarian response and embryo output after superovulation were also enhanced when a large number of small (2–3 mm in diameter) antral follicles were present, in the absence of large ovarian follicles, at the onset of gonadotropin stimulation (Cognie et al., 2003). The total number of small antral follicles and serum inhibin A concentrations, at the onset of the superovulatory treatment, were also positively correlated with the number of corpora lutea (CL's) and the total number of recovered embryos (Gonzalez-Bulnes et al., 2002). Inhibin A is a peptide hormone produced by ovarian antral follicles, which suppresses FSH secretion from the pituitary and directly enhances the process of follicular granulosa and theca cell differentiation and steroidogenesis (McNeilly and Baird, 1989; Campbell et al., 1991). To the best of the team's knowledge, there has been no comprehensive study of antral follicular populations, growth patterns and endocrine function in relation to the outcome of superovulatory procedures in seasonally anestrous sheep.

The objective of this study was thus to employ transrectal ovarian ultrasonography and serum estradiol RIA to examine if the: (i) size and developmental patterns of the two largest antral follicles; (ii) number of follicles $\geq$2 mm in diameter; and (iii) circulating estradiol-17β (E_2-17β) concentrations (determined at the time of the first two injections of the superovulatory FSH treatment), were related to the ovarian responses and embryo yields in anestrous ewes. The existence of such relationships would provide a basis for devising an ultrasound-based or hormonal test to reliably predict superovulatory yields in seasonally anovular ewes.

2. Materials and methods

2.1. Animals and experimental procedures

All experimental procedures were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the local animal care committee. Twenty-four clinically healthy sexually mature Rideau Arcott ewes (aged between 5 and 7 years, mean body weight of 79 ± 3 kg) were used in the present study during mid-anestrous (May–June). Animals used in this study had lambed two to six times, with the mean number of lambs born per ewe (live births) being 2.7 ± 0.2. No animal had previously been used for superovulation or as an embryo recipient in a MOET program. Animals were kept outdoors, with access to indoor facilities, under natural photoperiod conditions and temperature, at the sheep research station in Ponsonby near Guelph, Ontario, Canada (latitude: 43°33′ N). Ewes were daily fed maintenance diets of alfalfa pellets and hay, with water and cobalt iodized salt licks being available *ad libitum*. All ewes were treated with intravaginal sponges containing medroxyprogesterone acetate (MAP; 60 mg; Veramix®, Pharmacia and Upjohn Animal Health, Orangeville, Ontario, Canada; day 0) for 14 days. The superovulatory treatment consisted of six i.m. injections of pFSH (Folltropin®-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada; 1× 2.5 ml and 5× 1.25 ml), given at 12-h intervals, from the afternoon of day 12, until the morning of day 15. Concurrently with the 1st pFSH injection, all ewes received an i.m. injection of 500 IU eCG (Folligon®, Intervet Canada Ltd., Whitby, Ontario, Canada). In order to avoid the effects of inter-batch variations in drug bioactivity (Braileanu et al., 1998), separate batches of both gonadotropins (Folltropin®-V and Folligon®) were pooled to prepare sufficient quantities to treat all 24 animals. The progestagen sponges were removed concurrently with the 5th pFSH injection. On the afternoon of day 15, all animals received a single injection of 50 μg GnRH i.m. (Cystorelin®, Merial Canada Inc., Baie d'Urfe, PQ, Canada) to induce a synchronous preovulatory LH surge and were placed in a fenced paddock with four adult fertile Rideau Arcott rams for a period of 36 h.

2.2. Ultrasonographic technique

Transrectal ultrasonography of the ovaries was performed in all animals just prior to each of the first two pFSH treatments (times 0 and 12 h), with aid of a stiffened, 7.5-MHz linear-array transducer connected to a portable B-mode scan-

ner (HS-2000, Honda Electronics Ltd., Toyohashi, Japan), as described by Bartlewski et al. (2007). One experienced operator performed all the examinations. Ovarian follicles were measured using internal electronic calipers, and the number, diameter and topographic location of all antral follicles ≥2 mm were sketched on ovarian charts. The mean diameter (average of two dimensions—vertical and horizontal) of the two largest follicles (F1 and F2) were also recorded from the still images.

2.3. Assessment of ovarian responses and embryo collection

Surgical embryo recovery was performed 7 days after the GnRH injection under general anaesthesia induced with xylazine (Rompun®, Bayer Animal Health, Ethobicoke, Ontario, Canada; 0.2 mg/kg i.m.) and ketamine (Bioniche, Bellville, Ontario, Canada; 5 mg/kg i.v.). All animals were deprived of food and water 24 h before surgery. The reproductive tract was exposed and all luteal structures counted. Embryos were recovered by flushing both oviducts and uterine horns (flushing medium: PBS + 1% bovine serum albumin + penicillin and streptomycin; ~70 ml per ewe) with a 3 1/5 French Tomcat catheter (14 cm in length) inserted into the oviduct approximately 2 cm from the utero-tubal junction, and a paediatric French catheter (silicone elastomer coated, size 10) inserted into the uterine horn at the bifurcation of the uterus. Embryos were evaluated morphologically, using a stereomicroscope (magnification 80×). Embryos that developed to the morula or blastocyst stage at the time of flushing were graded on a scale from 1 to 4 (1: excellent, 2: good/fair, 3: poor and 4: degenerated) (Lindner and Wright, 1993; Rubianes et al., 1995). Embryo classes of 1–3 were collectively regarded as viable embryos. Following surgery, all ewes were treated with an i.m. injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse®, Upjohn, Orangeville, Ontario, Canada; 15 mg).

2.4. Hormone assays

Blood samples (10 ml) were collected by jugular venepuncture into vacutainers (Becton Dickinson, Rutherford, NJ, USA) before each ultrasonographic examination. The blood was allowed to clot overnight at room temperature. After removal of the blood clots and centrifugation at 1500 × *g* for 10 min, the serum was harvested and stored at −20 °C until assayed for serum E_2-17β concentration (Rawlings et al., 1984). The range of standards was from 1.0 to 50 pg/ml, and the sensitivity of the assay was 1.0 pg/ml. For the reference sera with a mean estradiol-17β concentration of 8.2 or 22.0 pg/ml, the intra- and interassay CV's were 7.2% or 6.6% and 8.2% or 8.9%, respectively.

2.5. Statistical analyses

The following follicular and endocrine variables were determined for each ewe: (i) number of small (2–3 mm), medium (4 mm) and large (≥5 mm in diameter) antral follicles; (ii) size of the two largest follicles (F1 and F2) (Veiga-Lopez et al., 2006); (iii) developmental pattern of F1 and F2 (i.e., growing, static or regressing) follicles; and (iv) serum E_2-17β concentrations. The largest antral follicles were regarded as growing or regressing if the difference in mean follicular diameters recorded at 0 and 12 h was ≥1 mm. The ovarian responses and embryo recovery data were compared between animals differing in the growth patterns of F1 and F2 follicles. As F2 follicles that were regressing 12 h after the onset of pFSH treatment were detected in only two animals, the comparisons were restricted to ewes with growing and non-growing F2 follicles (Table 3). Correlation analyses (Pearson Product Moment) were performed on antral follicle numbers/diameters and circulating E_2-17β concentrations at 0 and 12 h and superovulatory responses. All statistical tests were performed using SimgaStat®3.0 for Windows® (Systat Software Inc., Richmond, CA, USA). Ovarian responses and embryo yields for different subsets of ewes were analyzed using a one-way ANOVA. For all data sets with non-homogenous variances, the data were submitted to the Kruskal–Wallis one-way ANOVA on ranks (comparisons between three groups) or the Mann–Whitney rank sum test (comparisons between two groups). All data are expressed as the mean ± S.E. unless otherwise stated. Statistical significance was set at $P < 0.05$.

3. Results

3.1. General results

The superovulatory response and antral follicular status in the ewes of the present study are summarized in Table 1. All ewes showed signs of behavioral estrus within 12 h of GnRH injection. Of the ewes, 79% (19/24) had luteal structures detected at laparotomy 7 days post-treatment, with 67% of the animals (16/24) possessing ≥3 luteal structures (superovulated ewes; Fig. 1). A mean of 4.1 embryos and 2.5 viable embryos (grades 1–3) were obtained from a donor ewe (Table 1). Approximately 33% (9/24) of all collections contained no embryos and nearly 46% (11/24) yielded no viable embryos. In 79% of the ewes (19/24), less than the average number of transferable quality embryos were collected. Significant positive correlations were recorded between the numbers of detected luteal structures and recovered embryos ($r = 0.76$; $P < 0.001$). The coefficients of correlation and *P*-values for viable and non-viable embryos were $r = 0.44$ ($P < 0.05$) and $r = 0.78$ ($P < 0.001$), respectively.

3.2. Relationships of the size and growth patterns of the two largest follicles present at the onset of pFSH treatment to superovulatory responses

Neither the mean follicular diameter of F1 and F2 follicles nor the difference in follicular size between F1 and

Table 1
The mean (±S.E.) superovulatory responses and ovarian antral follicular status at the time of the first two pFSH injections (times 0 and 12 h) in ewes

Variable	Mean ± S.E.	Range
Number of luteal structures	9.9 ± 2.4	0–40
Total number of recovered embryos	4.1 ± 1.2	0–24
Recovery rate (%)	46.6 ± 7.8	0–100
Number of embryos (grades 1–3)	2.5 ± 1.0	0–21
Number of embryos (grade 4)	1.6 ± 0.6	0–12
Viability rate (%)	65.6 ± 10.5	0–100

	Time			
	0 h	12 h	0 h	12 h
F1 diameter (mm)	4.8 ± 0.2	5.5 ± 0.2	3.7–6.1	4.2–7.0
F2 diameter (mm)	3.7 ± 0.1	4.7 ± 0.2	3.1–5.0	3.4–5.9
E_2-17β concentrations (pg/ml)	2.7 ± 0.3	7.9 ± 1.2	1.0–4.8	1.7–27.5
Number of 2- and 3-mm follicles	7.2 ± 0.3	7.3 ± 0.3	5–10	4–9
Number of 4-mm follicles	1.8 ± 0.2	3.2 ± 0.2	0–4	1–5
Number of follicles ≥5 mm	0.8 ± 0.2	2.0 ± 0.3	0–4	0–6

F2 groups at the time of the 1st two doses of pFSH was significantly correlated with superovulatory responses. The grouping of animals based on the growth patterns of F1/F2 follicles is set out in Table 2. There were no differences in any of the superovulatory outcomes between anestrous ewes differing in the developmental status of the two largest follicles detected at the onset of ovarian stimulation (Table 3).

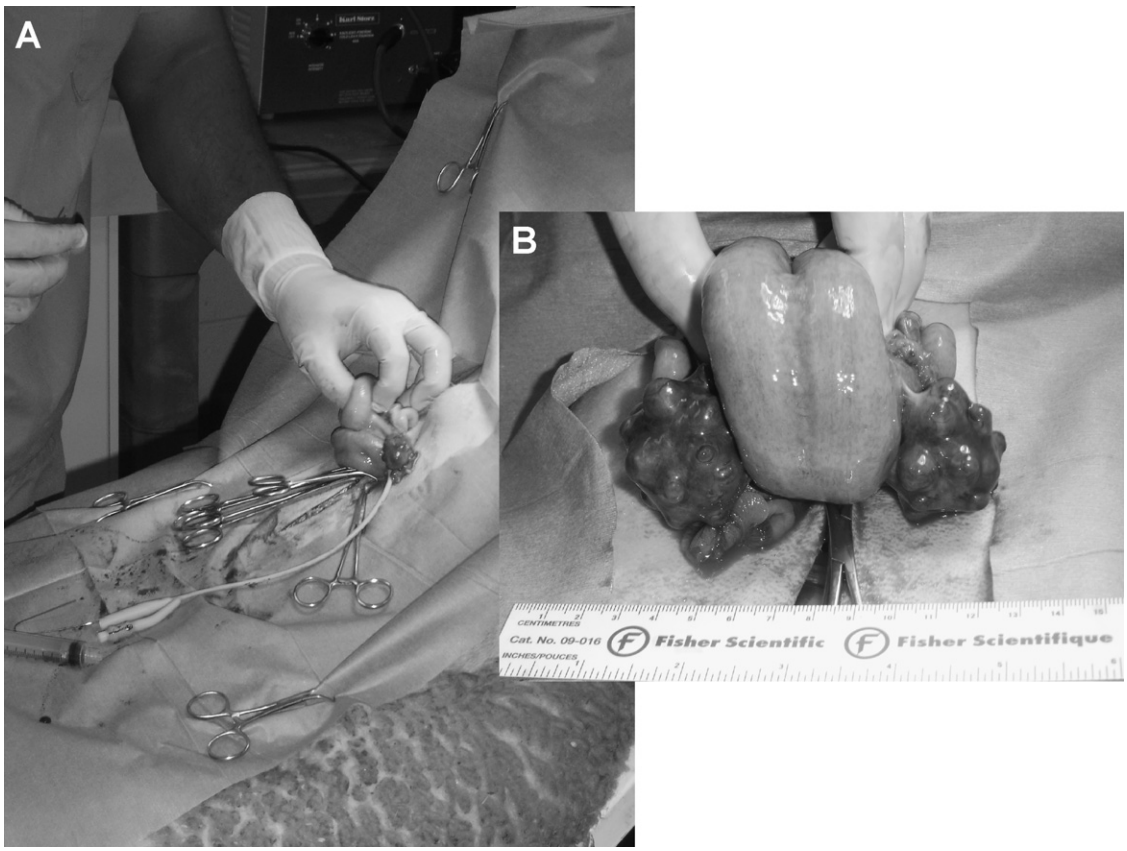

Fig. 1. (A) Embryo collection using a paediatric French catheter inserted into the uterine horn and (B) enumeration of luteal structures at laparotomy 7 days after a bolus injection of GnRH in superovulated anestrous Rideau Arcott ewes.

Table 2
The grouping of individual anestrous Rideau Arcott ewes based on the growth patterns of the two largest antral follicles (F1 and F2) at the time of the first two pFSH injections

F2 developmental status	F1 developmental status		
	Growing	Static	Regressing
Growing	7 ewes	6 ewes	5 ewes
Static	–	2 ewes	2 ewes
Regressing	2 ewes	–	–

3.3. Correlations between antral follicle numbers/estradiol-17β concentrations at the beginning of pFSH treatment and superovulatory responses

The number of medium-sized follicles (4 mm in diameter) at the time of the 2nd pFSH injection (time 12 h) was positively correlated to the number of luteal structures ($r = 0.44$; $P < 0.05$), the total number of embryos ($r = 0.45$; $P < 0.05$), and the number of viable embryos ($r = 0.44$; $P < 0.05$). Serum E_2-17β concentrations at the time of the 1st pFSH injection (time 0 h) were negatively correlated to embryo viability rates ($r = -0.56$; $P < 0.05$) and positively correlated to the numbers of non-viable embryos (grade 4) in individual ewes ($r = 0.41$; $P < 0.05$). Serum estradiol-17β concentrations at the time of the 2nd pFSH injection were positively correlated to the numbers of luteal structures ($r = 0.75$; $P < 0.001$), the total numbers of recovered embryos ($r = 0.55$; $P < 0.01$), and the numbers of non-viable (grade 4) embryos ($r = 0.60$; $P < 0.01$).

4. Discussion

When superovulation treatment was initiated in the presence of a large antral follicle(s), it was less successful when compared to the treatment that began in the absence of such follicles in anestrous ewes (Rubianes et al., 1995). However, other studies failed to observe any follicular dominance in ewes superovulated during anestrus (Gonzalez-Bulnes et al., 2003; Bartlewski et al., 2007). A recent study by Veiga-Lopez et al. (2006) showed the superovulatory yields in cyclic ewes to be related to the size and physiological status of the two largest follicles detected at the onset of the FSH treatment (F1 and F2 follicles). The size of F1 follicles was negatively correlated with embryo recovery and viability rates. Although the diameter of F2 follicles was inversely related to embryo viability, the presence of regressing F2 follicles in the donor ewes was associated with higher ovulatory responses and embryo recovery rates, suggesting the existence of follicular co-dominance during superovulatory treatments in sheep. The present comparisons of ovarian responses and embryo yields between animals differing in the developmental status of the two largest follicles clearly demonstrate the absence of follicular dominance or co-dominance in seasonally anestrous sheep.

Several studies in cyclic ewes have shown the presence of large growing follicles at the beginning of the superovulatory FSH treatment to exert a negative effect on embryo production and viability. However, it did not negatively affect the number of antral follicles ovu-

Table 3
Mean (±S.E.) ovulatory responses and embryo yields in anestrous Rideau Arcott ewes differing in the developmental status of the two largest follicles (F1 and F2) at the time of the first two pFSH doses of the superovulatory regimen

Variable	F1 developmental status			
	Growing ($n = 9$)	Static ($n = 8$)	Regressing ($n = 7$)	Static or regressing ($n = 15$)
Number of luteal structures	8.5 ± 3.4	12.9 ± 5.5	9.2 ± 3.6	10.9 ± 3.2
Total number of recovered embryos	2.3 ± 1.0	5.5 ± 2.0	4.4 ± 2.7	4.9 ± 1.7
Recovery rate (%)	35.1 ± 12.2	48.0 ± 11.1	51.7 ± 15.6	49.7 ± 9.3
Number of embryos (grades 1–3)	1.0 ± 0.4	2.7 ± 1.4	3.6 ± 2.3	3.2 ± 1.4
Number of embryos (grade 4)	1.3 ± 1.1	2.7 ± 1.6	0.9 ± 0.5	1.8 ± 0.8
Viability rate (%)	66.7 ± 23.6	59.4 ± 16.9	73.5 ± 18.8	65.3 ± 12.2

Variable	F2 developmental status	
	Growing ($n = 18$)	Static or regressing ($n = 6$)
Number of luteal structures	9.7 ± 3.0	12.0 ± 4.5
Total number of recovered embryos	3.8 ± 1.2	5.7 ± 3.7
Recovery rate (%)	49.4 ± 9.0	40.5 ± 16.1
Number of embryos (grades 1–3)	1.8 ± 0.8	4.8 ± 3.3
Number of embryos (grade 4)	1.9 ± 0.9	0.8 ± 0.5
Viability rate (%)	56.5 ± 14.3	85.8 ± 6.7

lating as a result of treatment (Cognie et al., 2003; Gonzalez-Bulnes et al., 2003; Veiga-Lopez et al., 2006). The presence of an ovulatory-sized antral follicle(s) was associated with an earlier preovulatory LH surge, which in turn was related to greater numbers of non-viable embryos (Veiga-Lopez et al., 2006). Delayed or truncated preovulatory LH surges may also negatively affect the ovulatory responses in individual animals (Lopez-Alonso et al., 2005). In the present experiment, ovulations were synchronized to reduce the variation in the timing of the LH surge, but this did not reduce the variation in embryo production and quality in the treated donor ewes.

A negative correlation was determined between serum E_2-17β concentrations at the time of the 1st pFSH injection and the embryo viability rate. At 12 h after the administration of the 1st pFSH dose, serum estradiol concentrations were strongly and positively correlated with the total number of luteal structures and the number of non-transferrable (grade 4) embryos. Lower viability rates of *in vivo* produced embryos are typically observed in ewes with greater ovulatory responses, possibly due to the recruitment and ovulation of large numbers of immature antral follicles containing less viable oocytes (Cognie et al., 2003; Veiga-Lopez et al., 2006). However, detrimental effects of elevated estrogen levels on the growth and maturation of large antral follicles and/or oocyte quality cannot be ruled out. Supraphysiological concentrations of serum estradiol in progestin-treated ewes enhanced the regression of large follicles and blocked the emergence of new antral follicles (Barrett et al., 2006). Nevertheless, high circulating estradiol concentrations, particularly in blood samples obtained 12 h after the onset of the FSH treatment, appear to be a reliable indicator of the impending decline in embryo quality in superovulated anestrous ewes. The reason for the variable follicular estrogenicity in response to pFSH injections in anestrous ewes remains unknown. There remains tremendous variation between individual anestrous ewes in the degree of follicular responsiveness to gonadotropic stimuli (Bartlewski et al., 2001, 2004). It is feasible to speculate that previously described differences in antral follicular development and endocrine function between ewes going anestrous early or late, may lead to variable responses after ovarian superstimulation (Bartlewski et al., 2000; Huchkowsky et al., 2002). An inverse relationship between the time of onset of seasonal anestrous and the number and size of antral follicles developing during anestrous in sheep has been observed (Bartlewski et al., 2000), but early anestrous ewes tend to have higher serum estradiol levels (Huchkowsky et al., 2002).

Enhanced production of transferable quality embryos has been reported in cycling ewes with large numbers of small antral follicles (2–3 mm in diameter) (Cognie et al., 2003). In the present study, the numbers of small antral follicles at the beginning of the superovulatory treatment were not correlated to superovulatory responses, but the number of medium-sized (4 mm in diameter) antral follicles 12 h after the 1st pFSH injection was correlated with the number of luteal structures and viable embryos. Due to the fact that the number of gonadotropin-responsive follicles in anestrous ewes (as indicated by the number of follicles attaining 4 mm in size after the first superovulatory pFSH dose) was less than the total number of small antral follicles detected at time 0 h (Table 1), it would appear that, in spite of the acquisition of gonadotropin receptors, only a proportion of small antral follicles may utilize exogenous FSH for further growth culminating in ovulation (Scaramuzzi et al., 1993).

A considerable amount of literature has been generated on the determinants of superovulatory responses in sheep. From this it is evident that an accurate and reliable control of multiple ovulations and consistent embryo viability has yet to be achieved (Cognie et al., 2003). The present observations indicate that both the number of gonadotropin-responsive antral follicles and follicular estrogenicity may be primary determinants of the ovulatory response and embryo quality in anestrous ewes. The results of this study may provide a basis for developing a practical ultrasound- and/or RIA-based test for the prediction of the superovulatory outcome in anestrous sheep.

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