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Comparison of the effects of pretreatment with Veramix sponge (medroxyprogesterone acetate) or CIDR (natural progesterone) in combination with an injection of estradiol-17β on ovarian activity, endocrine profiles, and embryo yields in cyclic ewes superovulated in the multiple-dose Folltropin-V (porcine FSH) regimen

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ABSTRACT

Follicular wave status at the beginning of exogenous FSH administration is an important contributor to variability in superovulatory responses in ruminants. Studies in ewes have shown a decrease in the number of ovulations when superovulation is initiated in the presence of ostensibly ovulatory-sized ovarian follicles. Hormonal ablation of large antral follicles with the progestin–estradiol (E_2-17β) treatment significantly reduces this variability in superovulated anestrous ewes, but the effects of the treatment in cycling ewes have not yet been assessed. Sixteen Rideau Arcott × Polled Dorset ewes (November–December) received either medroxyprogesterone acetate (MAP)–releasing intravaginal sponges (60 mg) or controlled internal drug release (CIDR) devices (containing 300 mg of natural progesterone) for 14 days (Days 0–14), with a single intramuscular injection of 350 μg of E_2-17β on Day 6. The superovulatory treatment consisted of six injections of porcine FSH (Folltropin-V) given twice daily, followed by a bolus GnRH injection (50 μg intramuscular) on Day 15. There were no differences ($P < 0.05$) in the ovulatory responses and embryo yields between the two groups of ewes. In both subsets of animals, the next follicular wave emerged ~2.5 days after an E_2-17β injection ($P > 0.05$). A decline in maximum follicle size after an E_2-17β injection was more abrupt in CIDR- compared with MAP-treated animals, and the ewes pretreated with exogenous progesterone had significantly more 3-mm follicles at the start of the superovulatory treatment. The metabolic clearance rate of exogenous E_2-17β appeared to be greater in MAP-treated ewes, but circulating concentrations of porcine FSH failed to increase significantly after each

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Folltropin-V injection in CIDR-treated animals. The CIDR-treated ewes exceeded ($P < 0.05$) their MAP-treated counterparts in serum E_2-17β concentrations during superovulation. In spite of differences in antral follicle numbers and endocrine profiles between MAP- and CIDR-treated cyclic ewes receiving E_2-17β before ovarian superstimulation, there were no differences in superovulatory responses.

1. Introduction

Assisted reproductive technologies are widely used in the modern agricultural industry to improve livestock genetics and enhance reproductive efficiency [1–4]. In sheep, hormonal ovarian stimulation is used in multiple ovulation and embryo transfer (MOET) programs to maximize the number of viable embryos per cycle of treatment [4]. Multiple ovulation and embryo transfer typically entails an initial progestin pretreatment to synchronize estrus and ovulations after hormonal ovarian stimulation, combined with the administration of supraphysiological doses of exogenous gonadotropins to induce the growth and ovulation of multiple antral follicles, and subsequent surgical embryo recovery from the reproductive tract of donor animals [4]. The outcome of superovulatory treatments in ewes is highly variable as ovarian responses and embryo yields are dependent on several intrinsic and extrinsic factors [5–10]. Extensive research has resulted in improved ovarian stimulation protocols [7,11–15]; however, in spite of numerous technical advancements, the variable responses still continue to limit the use of superovulation in commercial and research-allied applications.

Ovine antral follicles develop in a wave-like manner. A rise in circulating FSH concentrations triggers the simultaneous emergence of one to four small antral follicles (follicular wave) that grow to an ostensibly ovulatory size before regression or ovulation [16–18]. Three to four waves of follicular growth typically occur during the ewe's estrous cycle, with an interwave period of 3 to 5 days [16–20]. The rhythmic emergence of follicular waves continues throughout the seasonal anestrus in ewes [21]. The ovulatory response and total number of transferable embryos after superovulation are strongly affected by the size and number of antral follicles present in the ovary at the outset of the superovulatory treatment [3,5,6]. Differences in follicular wave status at the beginning of exogenous FSH administration appear to be one of the major contributors to the variability in superovulatory responses in sheep [3,5,6,8,22,23]. Several authors postulated that large ovarian follicles exerted a dominant effect in sheep by reducing FSH availability *via* estradiol and inhibin A secretion, thereby suppressing the growth of smaller, gonadotropin-dependent antral follicles [5,22–25]. Studies have shown a decrease in the number of ovulations in ewes when superovulation was begun in the presence of ovulatory-sized ovarian follicles [3,5,22,23]. A potential codominant effect of the two largest antral follicles (F1 and F2) has also been suggested as the physiological status of F1 and F2 at the outset of the superovulatory regimen in cyclic ewes has been shown to affect the ovulation rate and embryo recovery [25]. In contrast to these observations, more recent investigations into the growth dynamics of the two largest follicles detected at the start of superovulation in seasonally anovular ewes showed no difference in superovulatory outcomes regardless of follicular size or stage of the lifespan [6]. Follicular dominance in sheep remains controversial due mainly to the inconsistent evidence present in the available literature [18].

Progestin–estradiol combination treatment synchronizes follicular wave emergence in cattle and significantly reduces the variability associated with superovulatory treatments initiated at random stages of follicular wave development [26,27]. Few studies have evaluated the effects of both exogenous steroids on follicular wave kinetics in sheep. In anestrous ewes, the progestin–estradiol treatment enhanced the regression of large antral follicles and caused synchronous wave re-emergence approximately 5 days after estradiol-17β (E_2-17β) administration [27,28]. The synchronization of follicular waves with medroxyprogesterone acetate (MAP) and E_2-17β before superovulation reduced the variability in superovulatory responses without compromising embryo yields in seasonally anovular ewes [7,9]. Medroxyprogesterone acetate–releasing intravaginal sponges are made of a polyurethane material containing 60 mg of synthetic progestin and are inserted near the os cervix. Before this study could be performed in cyclic ewes, MAP-sponges were discontinued. Controlled internal drug release (CIDR) devices are an alternative progestin source commonly used for estrus synchronization in cattle. The CIDR is an elastic rubber insert containing 300 mg of natural progesterone (P_4) and sits in the midvagina. Controlled internal drug release devices have only recently been approved for commercial use in sheep; however, their usefulness in cattle and goats offers a promising alternative for the follicular wave synchronization with the progestin–estradiol treatment in ewes [29].

No attempt has been made to date to compare the outcomes of superovulatory treatments in cycling ewes after the MAP sponge or CIDR priming in combination with E_2-17β injections. It would be of value to assess an exogenous progestin alternative that may be used to further investigate the effect of follicular wave synchronization pretreatments on superovulatory yields in sheep. Determining an effective synchronization pretreatment and the associated ovarian and hormonal functions in cyclic ewes may also offer significant advantages to MOET programs; improved understanding as well as subsequent control and manipulation of these variables may aid in the development of an optimal superovulatory approach that will accurately and reliably produce consistent results in sheep and other species of veterinary interest. Hence, the main objective of the present study was to determine and

compare the ovarian and endocrine activity, and embryo yields in superovulated ewes pretreated with either MAP-releasing vaginal sponges or CIDRs and E_2-17β during the breeding season.

2. Materials and methods

The methodologies outlined for this study are based on well-established and validated techniques [7,9]. All experimental procedures were carried out in accordance with the Canadian Council on Animal Care guidelines (http://www.ccac.ca/en_/standards/guidelines), with formal approval from the local animal care committee at the University of Guelph (AUP #04R115).

2.1. Donor ewes

Sixteen clinically healthy, sexually mature Rideau Arcott × Polled Dorset ewes were obtained during the breeding season (November–December) from the research herd housed at the Ponsonby research station near Guelph, Ontario, Canada (latitude, 43°33′N). All ewes in this study were of a similar age and weight, with a documented reproductive history and confirmed fertility (Table 1). No animal had previously been used for superovulation or as an embryo recipient in an MOET program nor was subjected to any abdominal surgery. They were provided with a maintenance diet of alfalfa hay, water, and cobalt iodized salt licks *ad libitum* and kept in an indoor–outdoor pen separate from other sheep. The ewes were randomly assigned to one of the two treatment groups: (1) MAP–releasing intravaginal sponge (60 mg, Veramix; Pharmacia and Upjohn Animal Health, Orangeville, Ontario, Canada) or (2) CIDR (300 mg of natural P_4; Eazi-Breed CIDR; Pfizer Ltd., Mt. Eden, Auckland, New Zealand), with both groups receiving a single intramuscular (i.m.) injection of E_2-17β (350 μg/ewe; Long Wing International, Oakville, Ontario, Canada) dissolved in 1 mL of sesame oil. This treatment protocol was based on the successful follicular wave synchronization achieved in the same herd during the seasonal anestrus (May–June [7,9]).

2.2. Superovulatory protocol and breeding soundness evaluation of rams

The animals received either an MAP sponge (n = 8) or CIDR (n = 8; Day 0) for 14 days in combination with a single i.m. injection of E_2-17β administered on Day 6 [7,9]. The superovulatory treatment commenced on the afternoon of Day 12 and consisted of six i.m. injections of porcine FSH (pFSH; Folltropin-V; Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada; 2.5 mL × 1 and 1.25 mL × 5) given at approximately 8 AM and 4 PM until the morning of Day 15. All animals received an i.m. injection of 500 IU of eCG (Folligon; Intervet Canada Ltd., Whitby, Ontario, Canada) concurrently with the first pFSH injection. As in our previous study [6], separate vials and batches of gonadotropins (Folltropin-V and Folligon) were pooled to prepare sufficient quantities of both drugs to treat all 16 ewes; this was done to avoid the effects of potential interbatch variations in drug bioactivity [30]. A bolus i.m. injection of 50 μg of GnRH (Cystorelin; Merial Canada Inc., Baie-d'Urfe, Québec, Canada) was administered to all ewes on the afternoon of Day 15, and they were then placed in a fenced paddock with four adult Rideau Arcott × Polled Dorset rams for 36 hours.

All rams used in this study underwent routine breeding soundness evaluation 3 weeks before breeding. Three main

Table 1
Characteristics of cyclic Rideau Arcott × Polled Dorset ewes pretreated with a single dose of estradiol-17β and controlled internal drug release (CIDR) devices or medroxyprogesterone acetate (MAP)–releasing intravaginal sponges and then superovulated in a multiple-dose porcine FSH regimen.

Group	Variable						
	Age (y)	Weight (kg)	Breed (% RA)[a]	No. of times bred	No. of times lambed	Average no. of lambs/lambing[b]	Postpartum period (days)
CIDR treated (n = 8)	7	82	75	6	4	2.5	162
	4	72	81.3	3	3	1.7	159
	5	90	62.5	5	3	1.3	390
	4[c]	89	81.3	2	1	2	886
	2[d]	79	81.3	2	1	2	221
	7	83	93.8	7	5	2.2	707
	4[d]	80	81.3	2	2	3	532
	4	100	56.3	2	1	1	748
Mean	4.6 ± 0.6	84.4 ± 3.0	76.6 ± 4.2	3.6 ± 0.7	2.5 ± 0.5	2.0 ± 0.2	475 ± 101
MAP treated (n = 8)	7	84	63.8	5	4	2.2	809
	4	79.5	56.3	2	2	1.5	634
	4[c]	72.5	81.3	4	2	1.5	886
	4	83.5	68.8	4	2	2	280
	2[d]	74	37.5	1	2	2	221
	8[d]	73.5	87.5	8	4	2.8	315
	6	94	62.5	5	5	2.2	158
	4	86.5	56.3	1	1	2	745
Mean	4.9 ± 0.7	80.9 ± 2.6	64.9 ± 5.5	3.7 ± 0.8	2.7 ± 0.5	2.0 ± 0.1	506 ± 103

[a] RA: Rideau Arcott genotype.
[b] Number of live births.
[c] Two ewes with uterine inclusion cysts detected on the day of embryo recovery.
[d] Animals with an ovulatory-sized antral follicle(s) at the start of the superovulatory treatment.

parameters were analyzed: (1) scrotal circumference; (2) sperm motility: gross motility or mass activity (1: very good [rapid swirling], 2: good [slower swirling], 3: fair [generalized oscillation], and 4: poor [sporadic oscillation]) and percentage of progressive motility; and (3) sperm morphology [31]. The scrotal hair was clipped for more accurate measurements of scrotal circumference and semen was collected by electroejaculation. Sperm motility was evaluated by a trained individual immediately after collection using a phase-contrast microscope under × 400 image magnification on a slide prewarmed to 37 °C to 38 °C. Sperm morphology was assessed in fixed semen smears stained with eosin–nigrosin under a light microspore (× 1000 magnification). In addition, the rams were fitted with crayon-marking harnesses and observations were conducted every 12 hours to monitor their mounting activity or libido during mating after the superovulatory treatment of ewes. Testosterone levels were also measured in blood samples collected from the rams [32]. The primary testosterone antibody was obtained from Dr R. Etches (Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada); this antiserum cross-reacts with dihydrotestosterone (60%) and androstenedione (15.7%). Duplicate serum samples (25 μL) were assayed directly; the range of standards was from 10 to 1000 pg/mL, the assay detection limit was 10 pg/mL, and the intra-assay coefficients of variation were less than 10%.

2.3. Ovarian ultrasonography

Transrectal ultrasonography of ovaries was conducted daily from the day of E_2-17β injection until 1 day after the first Folltropin-V injection, and it used a stiffened, 7.5-MHz linear-array transducer connected to a portable B-mode scanner (HS-2000; Honda Electronics Ltd., Toyohashi, Japan). One experienced operator performed all examinations. All follicles ≥2 mm in diameter were measured to the nearest 1 mm using internal electronic calipers, and the number, diameter, and relative positions of all detected follicles were recorded on ovarian charts.

2.4. Assessment of ovulation and embryo collection

Laparotomy was conducted 7 days after a GnRH injection at which time the number of all luteal structures was also noted. All animals were deprived of food and water 24 hours before surgery. Surgical procedures were performed under general anesthesia induced with xylazine (6 mg i.m., Rompun; Bayer Animal Health, Etobicoke, Ontario, Canada) and ketamine (10 mg/kg intravenous; Bioniche). The reproductive tract was exposed, and all visible corpora lutea (CL; projections from the surface of the ovary characterized by the presence of the ovulatory stigma [33]) were enumerated. The oviducts and uterine horns were flushed with medium (PBS + 1% BSA + penicillin + streptomycin; approximately 100 mL/ewe) to collect embryos. The flushing was performed using a 3 1/5 French Tomcat catheter (14 cm in length) inserted into the oviduct approximately 1 cm from the uterotubal junction, and a pediatric French catheter (silicone elastomer coated, size 10) inserted into the uterine horn at the bifurcation of the uterus and kept in place by an inflatable balloon (maximum diameter: 35 mm). Immediate morphologic evaluation of all embryos was carried out using a stereomicroscope at a magnification of × 40 or × 80. Embryos developing to the blastocyst or morula stage were graded as follows: grade 1: excellent, 2: good/fair, 3: poor, and 4: degenerated; ([34]); grades 1 to 3 were considered transferable quality embryos. After surgery, all ewes were given an i.m. injection of a luteolytic dose of $PgF_{2\alpha}$ (15 mg, Lutalyse; Pfizer Animal Health, Kirkland, Québec, Canada).

2.5. Hormone assays

Blood samples were drawn from all ewes daily (from the day of E_2-17β administration until the first pFSH injection and for 7 days after GnRH injections) and every 8 hours throughout the superovulatory treatment. Samples (10 mL) were obtained by jugular venipuncture into vacutainers (Becton Dickinson, Rutherford, NJ, USA), allowed to clot at room temperature for 18 to 24 hours, and then centrifuged at 1500 × *g* for 10 minutes. Harvested serum was stored at −20 °C until assayed using validated radioimmunoassays ([12,35–37]) for concentrations of ovine FSH (oFSH), E_2-17β, and P_4 by the laboratory facility at the University of Saskatchewan, Saskatoon, Saskatchewan, Canada. The ranges of standards, sensitivity of the assays, and intra-assay or interassay coefficients of variation are respectively listed: (1) oFSH: 0.06 to 16.0 ng/mL (oFSH-SIAFP-RP-2); 0.1 ng/mL; 4.8% or 6.0%; and 6.4% or 8.6%, for reference sera with a mean concentration of 1.53 or 5.10 ng/mL; (2) E_2-17β: 1.0–50 pg/mL; 1.0 pg/mL; 6.5% or 8.4%, for reference sera with a mean concentration of 8.2 or 22.1 pg/mL; (3) P_4: 0.03 to 10.0 ng/mL; 0.03 ng/mL; 6.2% or 7.8%; and 8.3% or 13.3%, for reference sera with a mean concentration of 0.24 or 1.20 ng/mL. Serum concentrations of pFSH were determined using a porcine assay with sensitivity of 0.13 ng/mL and concentrations of a standard (USDA-pFSH-B-1) ranging from 0.13 to 64.0 ng/mL; all samples were analyzed in a single assay with the intra-assay coefficients of variation of 5.5% or 5.0% for the reference sera with a mean concentration of 0.69 or 4.28 ng/mL [7,9]. The cross-reactivity of the gonadotropins with the heterospecific antibody was determined by measuring FSH concentrations from the ovine and porcine reference sera inserted in both assay systems. The estimated cross-reactivity of anti-pFSH with oFSH was 8.9%, and the anti-oFSH has been found to bind 51.2% of pFSH. Therefore, the net concentrations of endogenous (oFSH) and exogenous FSH (pFSH) during the treatment with pFSH were then calculated by subtracting the cross-reactivity from all serum FSH concentrations obtained between 8 and 72 hours after the first pFSH injection [7,9]. Equine chorionic gonadotropin did not cross-react in the present FSH assays.

2.6. Statistical analyses

Ovarian responses were combined for both ovaries of each ewe and analyzed on a per animal basis. The following time intervals were noted for each animal: (1) between E_2-17β injection and regression of all follicles ≥5 mm in diameter present on the day of the injection to 2 to 3 mm;

Table 2
Age, breed, reproductive history, and a summary of breeding soundness evaluation scores for the four Rideau Arcott × Polled Dorset rams.

Variable	Ram no.			
	1	2	3	4
Age (y)	4	3	3	4
Breed (% Rideau Arcott)	57	44	57	82
No. of exposed breeding groups	2	2	3	2
Ewes exposed/group	5	12.5	5	5
% Pregnancy testing[a]	50	92	73.33	50
Total lambs sired	14	22	20	14
Scrotal circumference (cm)	35	34	27	32.5
Semen volume (mL)	0.5	0.5	3	0.6
Testosterone concentrations (ng/mL)	1.47	1.93	2.58	1.46
Sperm gross motility	2	1	1	1
Sperm progressive motility (%)	50	50	60	55
% Normal sperm	98	98	91	95
% Abnormal heads	0	0	0	0
% Abnormal midpieces	2	1	0	2
% Abnormal tails	0	1	7	3
% Loose heads	0	0	2	0
% Proximal droplets	0	0	0	0
% Distal droplets	0	0	0	0

[a] Percentage of pregnant ewes based on a single ultrasonographic examination (transabdominal) performed between Days 50 and 55 of gestation.

(2) between E_2-17β injection and the next oFSH peak; and (3) between E_2-17β injection and the emergence of the next follicular wave. A follicular wave was regarded as a follicle or group of follicles emerging within a 24-hour period that grew from 2 to 3 to ≥5 mm in diameter. Daily numbers of follicles in four different size classes (2, 3, 4, and ≥5 mm in diameter) and the size of the largest follicle (or maximum follicle diameter) were also noted. Peaks in oFSH concentrations in individual ewes were identified with the cycle-detection computer program [38]. The peak amplitude was defined as the difference between the peak concentration and the nadir preceding the peak concentration. Basal oFSH concentrations were calculated by averaging the lowest points (nadirs) between peaks. The mean duration of complete fluctuations (nadir-to-peak-to-nadir) was also determined for each animal. Ovarian follicular data and hormone concentrations were analyzed by two-way repeated measures ANOVA (Sigmastat 3.0 for Windows, 2003; Systat Software Inc., Richmond, CA, USA) with Fischer's protected least significant difference as a post-ANOVA test for comparisons of individual means. All serial data were initially assessed for outliers using the Dixon *Q* test. Data were transformed by natural logarithm or square root before further analysis when the normality or equal variance tests were significant. Single time point observations were compared between the two groups of ewes by Student *t* test, and the variability in ovarian responses and embryo yields was compared using the standard deviation criteria or *F* (variance ratio) tests [39]. For the comparisons between two data sets with non-homogenous variances, the data were analyzed by the Mann–Whitney rank sum test. Proportions were compared by a χ^2-test (Brandt-Snedecor formula [40]). Differences were considered statistically significant at $P < 0.05$. All results are given as the mean ± standard error of the mean.

3. Results

3.1. General results and superovulatory responses

All rams used in this experiment were confirmed to produce ejaculates of a 0.5-mL minimal volume with 50% or

Table 3
Superovulatory responses after a multiple-dose porcine FSH treatment of cycling Rideau Arcott × Polled Dorset ewes pretreated with a single dose of estradiol-17β and controlled internal drug release (CIDR) devices or medroxyprogesterone acetate (MAP)–releasing intravaginal sponges.

Group	Variable							
	No. of CL	No. of blastocysts (class 1 to 3)	No. of morulae (class 1 to 3)	Total number of transferrable embryos	Number of degenerated embryos (class 4)	Number of unfertilized oocytes	Recovery rate (%)[a]	Viability rate (%)[b]
CIDR treated (n = 8)	27	11	4	15	1	0	59.3	93.75
	9	2	4	6	0	0	66.7	100
	20	0	0	0	0	11	55	0
	5[c]	0	0	0	0	0	0	—
	10[d]	0	0	0	0	5	50	0
	12	1	0	1	3	2	50	16.7
	21[d]	12	2	14	0	0	66.7	100
	9	4	1	5	0	0	55.6	100
Mean	14.1 ± 2.7	3.7 ± 1.8	1.4 ± 0.6	5.1 ± 2.2	0.50 ± 0.39	2.2 ± 1.4	50.4 ± 7.5*	58.6 ± 18.9
MAP treated (n = 8)	16	6	5	11	2	0	81.2	84.6
	15	5	1	6	3	2	73.3	54.4
	12[c]	0	0	0	0	8	66.7	0
	8	5	1	4	0	0	75	100
	9[d]	1	0	1	0	6	77.8	14.3
	10[d]	3	5	8	0	0	80	100
	6	2	0	2	0	0	33.3	100
	12	6	5	11	0	0	91.7	100
Mean	11.0 ± 1.2	3.5 ± 0.8	2.1 ± 0.8	5.4 ± 1.5	0.62 ± 0.42	2.0 ± 1.1	72.4 ± 6.1**	69.3 ± 14.7

[a] (Total number of embryos + number of unfertilized eggs)/no. of corpora lutea (CL) × 100.
[b] Number of transferrable embryos/(total number embryos + number of unfertilized eggs) × 100.
[c] Two ewes with uterine inclusion cysts detected on the day of embryo recovery; *,**$P < 0.05$.
[d] Animals with an ovulatory-sized antral follicle(s) at the start of the superovulatory treatment.

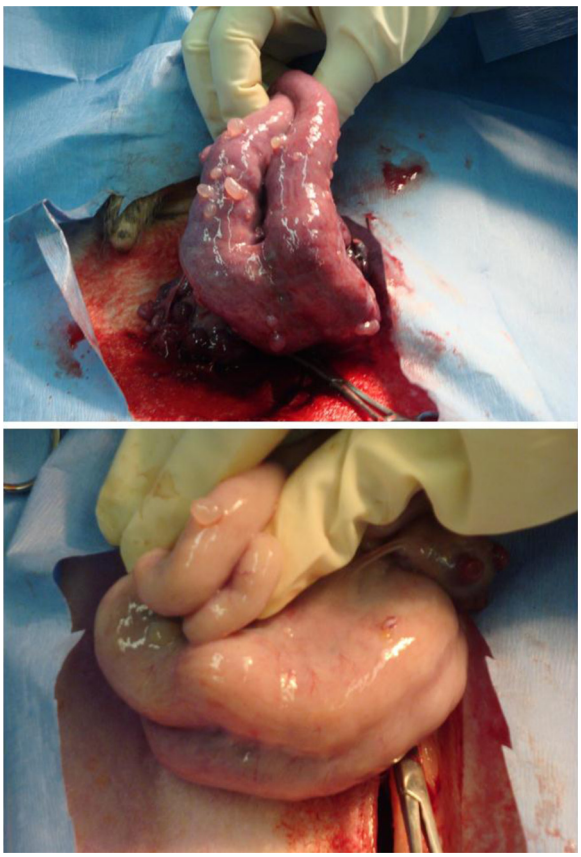

Fig. 1. Serosal inclusion cysts observed on the day of surgical embryo recovery in one superovulated ewe pretreated with a controlled internal drug release device (upper panel) and one ewe primed with a medroxyprogesterone acetate–soaked intravaginal sponge (lower panel).

greater sperm progressive motility and 91% or greater morphologically normal spermatozoa (Table 2). All ewes showed signs of behavioral estrus at the time of GnRH injection and had CL on the day of surgical embryo recovery. The ovarian responses and embryo yields in the ewes of the present study are summarized in Table 3. Three of eight flushings in CIDR-treated animals (37.5%) and one of eight collections in MAP-treated ewes (12.5%) contained no viable embryos ($P > 0.05$). Unfertilized eggs were found in 37.5% (3 of 8) of CIDR-treated and in 37.5% of MAP-treated ewes; two CIDR-treated ewes and one MAP-treated animal that had unfertilized eggs produced no viable embryos. There were no significant differences between the two groups of ewes for any of the superovulatory responses analyzed, including the analysis of variability, except for the embryo recovery rate that was greater ($P < 0.05$) in MAP-treated animals than in CIDR-primed animals by 13% (Table 3).

Uterine serosal inclusion cysts were observed on the day of embryo recovery in one ewe pretreated with a CIDR device and one ewe that had received an MAP-releasing intravaginal sponge (Fig. 1); the more severely affected ewe was pretreated with a CIDR. Both animals had CL (five and 12 CL in a CIDR- and MAP-treated ewe, respectively), but none of them yielded any embryos and eight unfertilized eggs were collected from a CIDR-treated ewe. Both ewes were 4 years of age, multiparous, the 81.3% Rideau Arcott ewes, and had lambed for the last time approximately 2.5 years before the present experiment (they have not been bred since the last lambing.)

3.2. Antral follicle numbers and wave emergence

On the day of an E_2-17β injection, ovulatory-sized antral follicles (≥5 mm in diameter) were detected ultrasonographically in 7 of 8 (87.5%) CIDR-treated ewes and in 6 of 8 (75%) ewes receiving MAP-containing sponges ($P > 0.05$). The mean number of follicles ≥5 mm in size per ewe was 1.5 ± 0.3 and 1.0 ± 0.4 ($P > 0.05$), and all of them regressed to 2-3 mm in diameter in 2.3 ± 0.6 days (range: 1–5 days) and 3.5 ± 1.1 days (range: 1–7 days; CIDR- and MAP-treated ewes, respectively; $P > 0.05$). All but one sheep had either one or two emerging follicular waves during the 5 days after estradiol treatment (1.4 ± 0.3 and 1.6 ± 0.2 waves/ewe in CIDR- and MAP-treated ewes, respectively); one CIDR-treated ewe had no follicular waves during that period. The interval from an E_2-17β injection to emergence of the first follicular wave was 2.5 ± 1.5 and 2.5 ± 0.8 days (range: 0–6 in both subsets of ewes) in CIDR- and MAP-treated ewes, respectively ($P > 0.05$). In animals with two follicular waves, the second wave emerged on Day 5.2 ± 0.4 (range: 4–6 days) and 5.6 ± 0.2 (range: 5–6 days) after an E_2-17β injection in CIDR- ($n = 4$) and MAP-treated ($n = 5$) ewes, respectively ($P > 0.05$).

Changes in daily numbers of follicles in different size classes from the day of an E_2-17β injection to 1 day after an application of the first Folltropin-V dose are shown in Figure 2. In CIDR-treated ewes, mean daily numbers of 2-mm follicles decreased ($P < 0.05$) from 1 to 3 days after an E_2-17β injection and from 2 days before to 1 day after the first Folltropin-V injection. In MAP-treated ewes, the number of 2-mm follicles rose ($P < 0.05$) 1 day after an E_2-17β injection followed by a decline ($P < 0.05$) 24 hours later. In both groups of ewes, mean daily numbers of 3- and 4-mm follicles increased ($P < 0.05$) from 1 day before to 1 day after the beginning of the superovulatory treatment. The number of 3-mm follicles was greater ($P < 0.05$) in CIDR-treated ewes than in MAP-treated ewes on the day of and 1 day after the beginning of Folltropin-V injections, and the MAP-treated ewes exceeded ($P < 0.05$) their CIDR-treated counterparts in the mean number of 4-mm follicles 2 days after an E_2-17β injection. There were no differences ($P > 0.05$) in mean daily numbers of follicles ≥5 mm in size over time or between the two subsets of ewes studied. Maximum follicle diameter decreased ($P < 0.05$) 1 day after an E_2-17β injection only in CIDR-treated ewes but increased ($P < 0.05$) in both groups 1 day after the first pFSH injection (Fig. 3). Ovulatory-sized antral follicles (≥5 mm in diameter) were detected ultrasonographically in two CIDR-treated and two MAP-treated ewes at the beginning of the superovulatory treatment (Table 3). One of the CIDR-treated animals had no transferable embryos and only one blastocyst was retrieved from an MAP-treated ewe, but the remaining

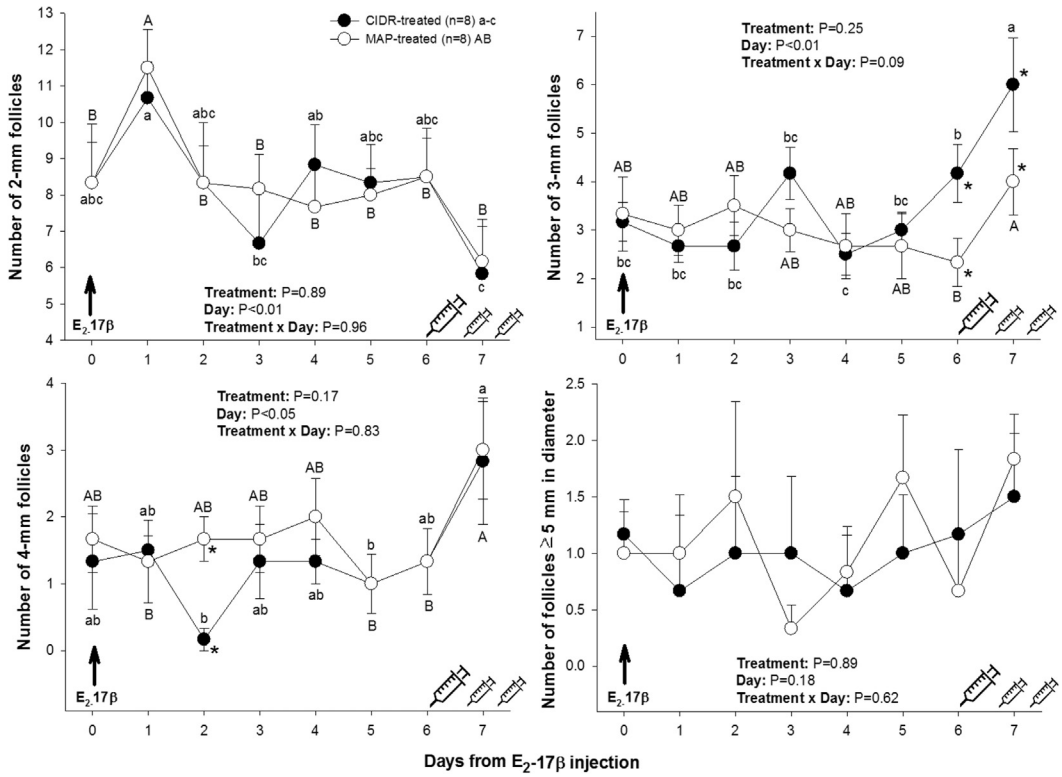

Fig. 2. Mean (±standard error of the mean) daily numbers of ovarian antral follicles in different size classes (2-, 3-, 4-, and ≥5-mm in diameter) recorded in ultrasonographically monitored Rideau Arcott × Polled Dorset ewes (November–December) from 6 days before to 1 day after the beginning of the superovulatory treatment. A single intramuscular injection of estradiol-17β (E_2-17β; 350 μg/ewe) was given on Day 0. The remaining symbols along the X-axis indicate approximate times of gonadotropin injections (large syringe: 2.5 mL of Folltropin-V + 500 IU of eCG; small syringes: 1.25 mL of Folltropin-V). Asterisks denote significant differences between controlled internal drug release (CIDR) device–treated (●) and medroxyprogesterone (MAP)–treated (○) ewes, and different letters (a–c, AB) denote the differences ($P < 0.05$) within both groups over time.

two animals had an above average number of class 1 to 3 embryos.

3.3. Hormonal profiles

In the ewes fitted with CIDRs, mean serum concentrations of P_4 declined ($P < 0.05$) from 1 to 4 days after an E_2-17β injection and again 24 hours after CIDR removal (Fig. 4). In MAP-treated animals, serum P_4 concentrations also declined from 1 to 4 days after an E_2-17β injection and then remained relatively stable ($P > 0.05$) until the end of the superovulatory protocol (i.e., time of GnRH injection). Serum P_4 concentrations were greater ($P < 0.05$) in CIDR-treated ewes than in MAP-treated ewes 2 to 3 days after an E_2-17β injection as well as 0 to 8 hours and 32 to 48 hours after the first Folltropin-V injection.

There was a significant main effect of time for daily serum concentrations of oFSH during the 5 days after an E_2-17β injection, but the post-ANOVA test failed to reveal any significant differences within either of the two groups of ewes studied (Fig. 5). Circulating oFSH concentrations declined ($P < 0.05$) between 8 and 16 hours after the beginning of the superovulatory treatment in MAP-treated ewes and increased ($P < 0.05$) 8 hours after the last gonadotropin injection in CIDR-treated ewes. Endogenous FSH secretion was greater ($P < 0.05$) in MAP-treated ewes at 8 hours and but it was greater ($P < 0.05$) in CIDR-treated animals at 72 hours after the first pFSH injection. During the 6-day period after an E_2-17β injection, the number of peaks, mean peak concentration and amplitude, and duration of fluctuations for oFSH concentrations, as determined by the cycle-detection computer program, did not differ ($P > 0.05$) between CIDR- and MAP-treated ewes (Table 4).

Peripheral concentrations of E_2-17β increased ($P < 0.05$) in both subsets of ewes 1 day after an E_2-17β injection and then declined ($P < 0.05$) to basal (pretreatment) levels (Fig. 6). In MAP-treated animals, they did not vary significantly during the entire superovulatory treatment, but in CIDR-treated ewes, they rose ($P < 0.05$) from 24 to 48 hours after an application of the first Folltropin-V dose. Serum E_2-17β concentrations were higher ($P < 0.05$) in CIDR-treated ewes than in MAP-treated ewes 1 day after administration of E_2-17β as well as between 48 and 64 hours after the first Folltropin-V injection.

During the superovulatory treatment in MAP-treated ewes (time 0 hours = first Folltropin-V injection), mean circulating concentrations of pFSH increased ($P < 0.05$) 8 hours after Folltropin-V doses administered

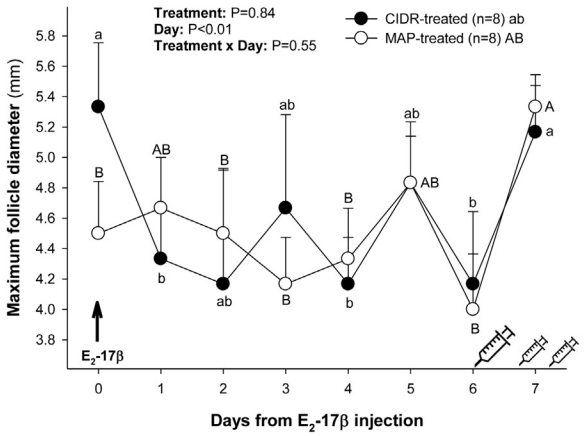

Fig. 3. Maximum follicle diameter (mean ± standard error of the mean) determined in ultrasonographically monitored Rideau Arcott × Polled Dorset ewes (November–December) from 6 days before to 1 day after the beginning of the superovulatory treatment. A single intramuscular injection of estradiol-17β (E_2-17β; 350 μg/ewe) was given on Day 0 to the controlled internal drug release (CIDR) device–treated (●) and medroxyprogesterone (MAP)–treated (○) ewes. The remaining symbols along the X-axis indicate approximate times of gonadotropin injections (large syringe: 2.5 mL of Folltropin-V + 500 IU of eCG; small syringes: 1.25 mL of Folltropin-V). Different letters (ab, AB) denote significant differences within the groups over time.

at 16, 40, and 64 hours (Fig. 7). Serum levels of pFSH in this group of animals were greater ($P < 0.05$) at 24, 32, 48, 56, and 72 hours than the nadirs at times 16 and 40 hours. In CIDR-treated animals, there were only numerical increments in circulating pFSH concentrations following successive Folltropin-V injections and the only significant differences were between nadirs at time 16 h and 40 h, and a peak observed 8 h after the last pFSH dose (time 72 h). An increase (numerical or statistically significant) in mean serum pFSH concentrations occurred 8 hours after each Folltropin-V injection in all animals under study except after the fifth Folltropin-V injection given at the time of sponge withdrawal in MAP-treated ewes (time 48 hours).

4. Discussion

To the best of the authors' knowledge, this is the first study comparing superovulatory outcomes in cyclic ewes pretreated with either synthetic progestin or natural P_4 in combination with a single dose of E_2-17β. In anestrous ewes, the MAP–E_2-17β treatment significantly reduced the variability in ovarian responses and embryo yields, without affecting the number of transferrable embryos [7]. A degree of variation in superovulatory responses of both groups of ewes in this study was similar to that in anestrous sheep [7]. However, the embryo recovery rate was significantly (by 22%) lower in cyclic ewes pretreated with CIDRs as compared with MAP-treated animals. Elevated estrogen concentrations are believed to alter gamete and early embryo transport through the genital tract and thereby decrease the embryo recovery rate [41]. The CIDR-treated ewes exceeded their MAP-treated counterparts in serum concentrations of E_2-17β in the second half of the pFSH superovulatory protocol, but we did not measure circulating estrogen concentrations during the 7 days before embryo flushing.

The variability in superovulatory responses can be, at least in part, explained by the ewe-related characteristics. In this study, 4 of 10 ewes with an average of two or less lambs per lambing produced no transferrable embryos and seven of these donor sheep had less than the average number of class 1 to 3 embryos, indicating that previous lambing success may be predictive of superovulatory responses. Body weight appeared to have an effect on unfertilized egg numbers, as a large proportion of ewes (4 of 6) that had unfertilized oocytes were lighter than average. Interestingly, Shorten et al. [42] have shown that mean

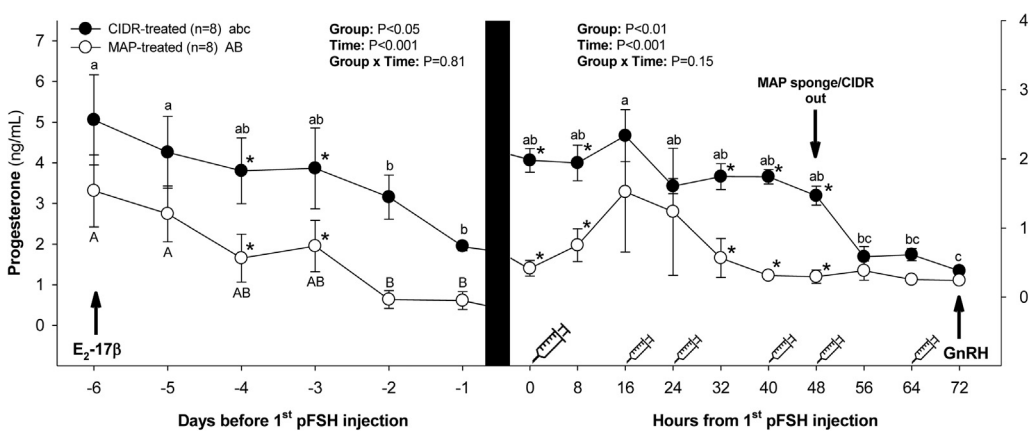

Fig. 4. Total serum concentrations (mean ± standard error of the mean) of progesterone during the 6 days after an estradiol-17β injection (350 μg/ewe) and from 0 to 72 hours from the first porcine FSH (pFSH) dose administered to cycling Rideau Arcott × Polled Dorset ewes that had been pretreated with medroxyprogesterone acetate (MAP)–releasing sponges (○) or natural progesterone from controlled internal drug release (CIDR) devices (●). The symbols along the X-axis indicate approximate times of gonadotropin injections (large syringe: 2.5 mL of Folltropin-V + 500 IU of eCG; small syringes: 1.25 mL of Folltropin-V). Asterisks denote significant differences between the two groups, and different letters (a-c, AB) denote the differences ($P < 0.05$) within both groups over time. GnRH (50 μg/ewe intramuscular).

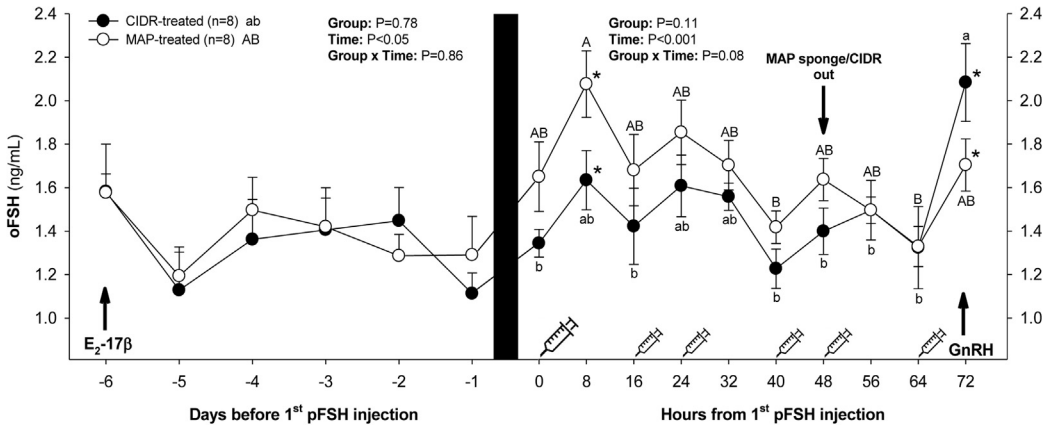

Fig. 5. Serum concentrations (mean ± standard error of the mean) of ovine FSH (oFSH) during the 6 days after an estradiol-17β injection (350 μg/ewe) and from 0 to 72 hours from the first porcine FSH (pFSH) dose administered to cycling Rideau Arcott × Polled Dorset ewes that had been pretreated with medroxyprogesterone acetate (MAP)-releasing sponges (○) or natural progesterone from controlled internal drug release (CIDR) devices (●). The symbols along the X-axis indicate approximate times of gonadotropin injections (large syringe: 2.5 mL of Folltropin-V + 500 IU of eCG; small syringes: 1.25 mL of Folltropin-V). Asterisks denote significant differences between the two groups, and different letters (ab, AB) denote the differences ($P < 0.05$) within both groups over time. GnRH (50 μg/ewe intramuscular).

litter size in sheep peaks at the attainment of certain body mass (~85 kg in various meat breeds) before declining with further increase in weight. A relatively small sample size precluded our being able to find significant associations between weight and superovulatory yields in the ewes of the present study. Future investigations into the efficacy of superovulatory protocols in sheep would benefit from increasing the sample size and including characteristics such as live body weight and condition scores of animals.

Serosal inclusion cysts were detected on the surface of uterine horns in one MAP-primed and one CIDR-treated ewe. Both animals had not lambed for nearly 2.5 years. In carnivores, the inclusion cysts typically develop immediately after parturition, during uterine involution [43,44]. They are likely the result of rapid myometrial contractions, when the portions of the mesothelium are folded onto itself and trapped [43,44]. However, serosal inclusion cysts are typically found in aged pluriparous individuals and are usually localized to certain areas rather than disseminated [43]. The alternatives include anomalous lymphatics and parasitic cysts (cysticercosis; [43,45]). If there were cysts on the mesometrium, a lymphatic anomaly would be a possibility, and a parasitic invasion can be ruled out as the animals have been maintained in a pathogen-free unit under the strict zoosanitary conditions. Uterine serosal inclusion cysts are extremely rare in sheep. The effects of this pathologic condition on fertility are largely unknown, yet current literature suggests that serosal cysts are clinically benign (as the endometrium remains unaffected) and do not interfere with normal reproduction [44]. The reason behind impaired superovulatory responses in the two ewes with these cystic changes remains unclear.

After an injection of E_2-17β, there was a transient rise in the number of 2-mm antral follicles (statistically significant in MAP-treated ewes) followed by a decline 1 to 2 days later in all ewes studied. Previously, a rise in small antral follicle numbers has been observed in cycling ewes during the periovulatory period and it was suggested that it might be a consequence of low circulating concentrations of luteal P_4 and/or an increase in serum levels of follicular estradiol [7]. An exogenous source of progestagens was provided in the present study and so an increase in the numbers of 2-mm follicles appears to be due mainly to elevated estrogen concentrations; it occurred immediately after the E_2-17β injection and was followed by a decline once the estrogen has been metabolized. This observation may have an important practical implication for ameliorating the superovulatory protocols currently used. The number of small antral follicles is, in most instances, representative of a follicular population potentially responsive to FSH [5], and hence, a positive correlation exists between the

Table 4
Characteristics of fluctuations in mean daily ovine FSH (oFSH) concentrations (mean ± standard error of the mean) in cyclic Rideau Arcott × Polled Dorset ewes treated for 14 days with medroxyprogesterone acetate (MAP)–releasing intravaginal sponges or controlled internal drug release (CIDR) devices and a single intramuscular injection of estradiol-17β given 6 days after sponge or CIDR insertion.

Variable	Group	
	CIDR treated (n = 8)	MAP treated (n = 8)
Number of FSH peaks/6 days	1.9 ± 0.2	1.9 ± 0.2
oFSH peak concentration (ng/mL)	1.67 ± 0.14	1.69 ± 0.09
oFSH peak amplitude (ng/mL)	0.65 ± 0.18	0.58 ± 0.09
Basal oFSH concentration (ng/mL)	1.09 ± 0.09	1.08 ± 0.06
Duration of oFSH fluctuations (days)	3.3 ± 0.5	3.3 ± 0.3

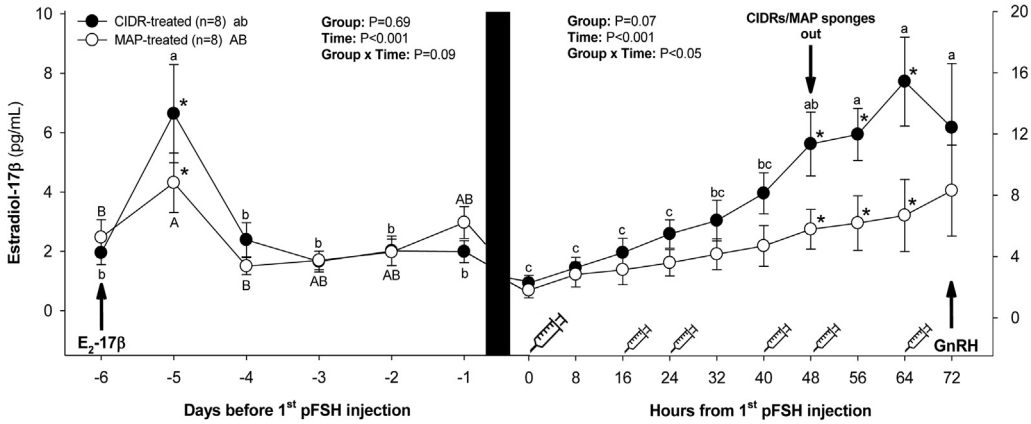

Fig. 6. Serum concentrations (mean ± standard error of the mean) of estradiol-17β (E_2-17β) during the 6 days after an estradiol-17β injection (350 μg/ewe) and from 0 to 72 hours from the first porcine FSH (pFSH) dose administered to cycling Rideau Arcott × Polled Dorset ewes that had been pretreated with medroxyprogesterone acetate (MAP)-releasing sponges (○) or natural progesterone from controlled internal drug release (CIDR) devices (●). The symbols along the X-axis indicate approximate times of gonadotropin injections (large syringe: 2.5 mL of Folltropin-V + 500 IU of eCG; small syringes: 1.25 mL of Folltropin-V). Asterisks denote significant differences between the two groups, and different letters (a-c, AB) denote the differences ($P < 0.05$) within both groups over time. GnRH (50 μg/ewe intramuscular).

number of small ovarian follicles detected at the beginning of the superovulatory treatment and the ensuing ovulation rate and embryo yield in cyclic sheep [6]. Therefore, a single E_2-17β injection just before the first gonadotropin dose could be incorporated into ovarian stimulation protocols.

On the first 2 days of the superovulatory treatment, the CIDR-primed ewes exceeded their MAP-treated counterparts in the mean daily numbers of 3-mm follicles. This difference did not impinge on the superovulatory responses seen, suggesting that an average of two more 3-mm follicles per donor ewe is not large enough to result in significant shifts in ovulation rates and embryo production in superstimulated ewes. This increase in the number of 3-mm follicles followed the withdrawal of progestin-releasing intravaginal devices. Previous research has demonstrated that intermediate metabolism and clearance of the synthetic P_4 take longer than those of natural P_4 [46]. Because progestagens tend to decrease small follicle numbers in cycling ewes, it can be inferred that lower numbers of 3-mm follicles in the present study were due to residual effects of MAP on antral follicle growth.

There was a significant decrease in the number of medium (4-mm) follicles 48 hours after an E_2-17β injection in ewes pretreated with CIDRs. This is likely due to elevated levels of P_4 in CIDR-treated ewes in combination with a significant peak in serum concentrations of E_2-17β observed 24 hours after treatment. Combined actions of elevated P_4 and estrogen concentrations suppress follicular wave development in ewes by truncating the peaks in circulating FSH concentrations [27,28]. However, none of the parameters of FSH secretion differed significantly between CIDR- and MAP-treated ewes before the beginning of the superovulatory treatment; coinciding with a peak in estradiol concentrations, all ewes experienced only a transient numeric drop in circulating FSH levels. This would suggest that the effects of exogenous and endogenous steroids on the number of 4-mm follicles in CIDR-treated ewes were direct (i.e., independent of FSH influences). Alternatively, the effects of steroids could be mediated, at least in part, by changes in pulsatile LH secretion [47]. The latter is, however, unlikely because serum estradiol concentrations did not vary between the two groups of ewes or over time during the 4 days before the superovulatory treatment, suggesting a lack of differences in mean and basal LH concentrations or pulse frequency.

In seasonally anovular ewes, the interval between an E_2-17β injection and regression of all ovulatory-sized follicles to 2-3 mm in diameter averaged 2.6 days and the next follicular wave emerged approximately 5.5 days after injection [7]. In the present study, it took an average of 2.3 days in CIDR-treated ewes and 3.5 days in MAP-treated ewes for all large antral follicles to regress, but the next wave emerged 2.5 days after the E_2-17β treatment in both groups of ewes. Clearly, a degree of suppression of FSH release and follicular wave emergence by the combined progestin−E_2-17β treatment is considerably lower in cycling than in seasonally anovular ewes. Moreover, none of the characteristics of oFSH secretion during the 6 days after the administration of E_2-17β differed between CIDR- and MAP-treated ewes in this study and so all variations in large antral follicle growth patterns appear to be governed mainly by differing serum concentrations of endogenous and exogenous steroids, their direct effects on the ovary or on the metabolism and bioavailability of other reproductive hormones.

Generally, circulating P_4 concentrations declined from 6 days before until the end of the superovulatory treatment because of regression of CL in all ewes studied. During the superovulatory treatment and before CIDR or MAP sponge withdrawal, serum P_4 concentrations remained higher in CIDR-treated animals except for a period from 16 to 24 hours after the start of the treatment. During that period, there was a transient albeit nonsignificant rise in circulating P_4 concentrations in both groups of animals and

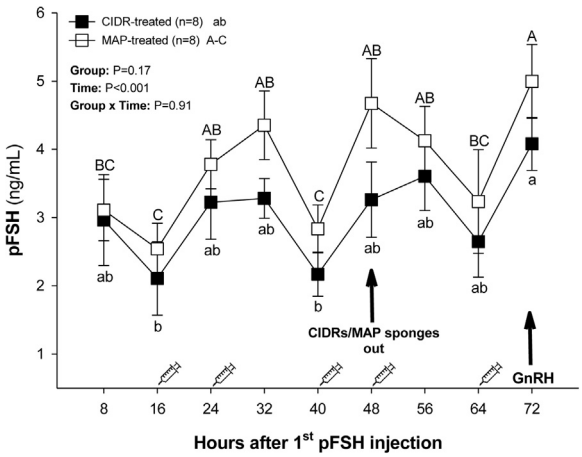

Fig. 7. Circulating concentrations (mean ± standard error of the mean) of porcine FSH (pFSH) from 8 to 72 hours after the first superovulatory dose of Folltropin-V in cycling Rideau Arcott × Polled Dorset ewes pretreated with a single injection of estradiol-17β and medroxyprogesterone acetate (MAP)–releasing sponges (□) or natural progesterone from controlled internal drug release (CIDR) devices (■). The remaining symbols along the X-axis indicate approximate times of gonadotropin injections (1.25 mL of Folltropin-V). Within each group of ewes, means denoted by different letters (ab, A–C) are different ($P < 0.05$). GnRH (50 μg/ewe intramuscular).

increased variation in serum P_4 levels, particularly in MAP-treated ewes; it was likely an effect of exogenous gonadotropins on the steroidogenic activity of CL still existing in some ewes. Even though eCG and pFSH cannot reverse the process of luteolysis in sheep [48], they may have caused a brief and slight deviation in P_4 secretion by "old" CL, with the MAP-treated ewes displaying somewhat more pronounced and extended response.

While analyzing endogenous FSH profiles, it is important to note that mean serum concentrations of oFSH were greater in MAP-treated ewes 8 hours after the beginning of the superovulatory protocol but were greater in ewes pre-treated with CIDRs at the time of GnRH injection. These differences and the times at which they occurred are difficult to explain. It is possible that inhibin production by antral follicles at these two times differed significantly between CIDR- and MAP-treated ewes, but the specific reason for such a difference remains to be elucidated.

Greater serum concentrations of estradiol in the CIDR-treated compared with MAP-treated ewes 24 hours after an E_2-17β injection and from 48 to 64 hours after the start of the superovulatory treatment can be attributed to the differential effects of both progestins on estradiol metabolism, LH pulse frequency, or a number and estrogenicity of ovarian antral follicles. Progestins exert suppressive effects on LH secretion and LH directly stimulates follicular estradiol synthesis, which leads us to suggest that combined suppressive effects of MAP and endogenous P_4 on LH release as well as on antral follicle growth and endocrine function are greater than those of P_4 in CIDR-treated ewes. Although this difference in progestin potency can explain the difference in serum levels of E_2-17β during the superovulatory treatment, a greater spike in estrogen levels after a bolus injection of E_2-17β is most likely due to the varying effects of both progestins on estradiol biotransformation and clearance rates.

Porcine FSH levels fluctuated throughout the observed time period after the first Folltropin-V injection. The levels rose after each Folltropin-V dose, resulting in peak FSH levels sufficient to stimulate follicular growth. Over time, FSH concentrations gradually declined as exogenous FSH was metabolized and excreted [49,50]. However, these recurrent increases in pFSH concentrations were significant only in MAP-treated animals, suggesting the existence of a difference in FSH absorption and clearance rates between MAP- and CIDR-treated animals. In addition, the fifth pFSH injection given 48 hours after the beginning of superovulation was followed by a decline in serum pFSH concentrations in MAP-treated ewes. A similar phenomenon was observed in seasonally anestrus ewes undergoing the multiple pFSH superovulatory treatment [9]. The fifth pFSH injection coincided with the removal of MAP-releasing sponges, and it was suggested that a lack of a peak in pFSH levels after this injection could be explained by an increased metabolic clearance rate of pFSH due to unopposed effects of high concentrations of estradiol [9]. Interestingly, a similar tendency could be observed for endogenous (ovine) FSH concentrations after the removal of vaginal pessaries. Medroxyprogesterone acetate from the Veramix sponges is absorbed differently than the natural P_4 contained in the CIDR [51]. Additionally, removing the CIDR is invariable associated with an abrupt removal of P_4 source, whereas the physical process of removing the MAP sponge includes a squeezing motion, which deposits progestin in the vagina and extends its residual hormone effects. All these observations are indicative of the differences in intermediate metabolism of FSH between MAP- and CIDR-primed ewes but also indicate that a degree of changes in serum levels of pFSH observed in this study did not affect the superovulatory outcome.

In summary, the administration of a single dose of E_2-17β to MAP- or CIDR-treated ewes in the breeding season failed to suppress and resynchronize follicular wave emergence as it did in anestrous ewes. It remains to be determined if a lack of inhibitory effects of the E_2-17β–progestin treatment on FSH secretion and follicular wave kinetics in cyclic ewes is due to an inadequate dose of estrogen used or photoperiod-driven neurophysiological differences in the functioning of the ewes' hypothalamo–pituitary axis. In general, the outcome of the superovulatory treatment started 6 days after E_2-17β injection did not vary between MAP- and CIDR-treated ewes in this study even though an application of both progestins was associated with dissimilar direct effects on antral follicle populations. The only difference observed was a diminished embryo recovery rate in a subset of ewes that had received CIDRs. There exist differences in metabolic clearance rates of estrogens and FSH between CIDR- and MAP-treated ewes, but these varying influences of exogenous progestins did not alter the ovulatory responses and embryo yields. Detection of such differences allowed us to eliminate certain intrinsic factors that may determine the superovulatory responses in ewes (e.g., the clearance rate of

pFSH or moderate differences in small follicle numbers at the outset of the superovulatory protocol), but further studies on the neuroendocrine control of FSH secretion and FSH dependency of antral follicular growth in the ewe and other mammalian species are required to optimize superovulatory treatments currently used.

Acknowledgments

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